Spatial and temporal distribution of growth factors and their receptors in diabetic retinopathy

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TABLE OF CONTENTS

Title

Declaration

Acknowledgements

Table of Contents 1

List of Figures 5

List of Tables 10

Abstract 11

1. Introduction 13

1.1 General introduction 13

1.2 Diabetes and its complications 13

1.3 Structure and function of the non-pathological retina 14

1.3.1 The neuronal system of the retina 15

1.3.2 The glial system of the retina 18

1.4 Vascular supply to retina 19

1.5 Diabetic retinopathy 22

1.6 Modulators of the microvascular complications of diabetic retinopathy 25

1.6.1 Introduction 25

1.6.2 Angiogenic modulators 25

1.6.2.1 The VEGF family 26

1.6.2.2 The VEGF/VEGFR system and diabetic retinopathy 29

1.6.2.3 The angiopoietin family 31

1.6.2.4 The angiopoietin family and ocular neovascularization 34

1.6.2.5 TNF-α 35

1.6.2.6 TNF-α and ocular angiogenesis 37

1.6.3 Inhibitors of angiogenesis 38

1.6.3.1 PEDF 38

1.6.3.2 PEDF and ocular angiogenesis 41

1.7 The caveolae system 43

1.7.1 Introduction 43

1.7.2 Systemic expression of caveolae and caveolins 45

1.7.3 Expression of caveolae and caveolins within the eye 46

1.8 Aims 48

2. **Materials and methods** 49
2.1 Materials 49
2.1.1 Chemical reagents and antibodies 49
2.1.2 Human tissue 49
2.2 Methods 49
2.2.1 Clinical assessment of donor eyes 49
2.2.2 Categorisation of donor eyes 49
2.2.2.1 Non-diabetic eyes 49
2.2.2.2 Diabetic with no overt retinopathy 50
2.2.2.3 Diabetic with intra-retinal changes but no evidence of PDR 50
2.2.2.4 Diabetic with preretinal PDR 50
2.2.2.5 Diabetic with scatter laser photocoagulation but no evidence of residual PDR 50
2.2.3 Dissection of donor eyes 50
2.2.4 Fibrovascular membranes 51
2.2.5 Preparation of retinal tissue for wax Sectioning 51
2.2.6 Preparation of fibrovascular membranes for wax sectioning 51
2.2.7 Haematoxylin and eosin staining of wax Sections 52
2.2.8 Immunohistochemical studies 52
2.2.8.1 Selection of sections for immunostaining 52
2.2.8.2 General protocol for immunostaining of sections 53
2.2.8.3 Assessment of immunostaining 55
2.2.8.4 Statistical analysis 55

3. **Histological categorization of non-diabetic and diabetic human retinas and fibrovascular membranes** 56
3.1 Introduction 56
3.2 Haematoxylin and eosin staining of non-diabetic retinas 56
3.3 Haematoxylin and eosin staining of diabetic retinas 59
3.4 Haematoxylin and eosin staining of fibrovascular membranes 68
3.5 Discussion 70

4. Expression of pro-angiogenic growth factors and receptors in the normal and diabetic human retina 73
4.1 Introduction 73
4.2 Control staining 73
4.3 Immunolocalisation of pro-angiogenic growth factors 75
4.3.1 VEGF-A_{165} and VEGF-C immunostaining of retinal sections and fibrovascular membranes 75
4.3.2 VEGF receptor immunostaining of retinal sections and fibrovascular membranes 92
4.3.3 Angiopoietin immunostaining of retinal sections and fibrovascular membranes 117
4.3.4 Tie-2 immunostaining of retinal sections and fibrovascular Membranes 134
4.3.5 TNF-α Immunostaining of retinal sections and Fibrovascular Membranes 143
4.4 Discussion 152

5. Expression of the anti-angiogenic growth factor PEDF in normal and diabetic human retina 170
5.1 Introduction 170
5.2 Control staining 170
5.3 PEDF immunostaining of retinal sections 170
5.4 Discussion 172

6. Expression of caveolin-1, -2, and -3 in the normal and diabetic human retina 175
6.1 Introduction 175
6.2 Control staining 175
6.3 Immunolocalisation of caveolin-1, -2, and -3 175
6.3.1 Caveolin-1 immunostaining of retinal sections and fibrovascular membranes 175
6.3.2 Caveolin-2 immunostaining of retinal sections 178
6.3.3 Caveolin-3 immunostaining of retinal sections 178
6.4 Discussion 186

7 General Discussion 190
7.1 Microvascular complications and diabetic retinopathy 190
7.2 Growth factor expression during diabetic retinopathy 190
7.3 The expression of PEDF in diabetic retinopathy 193
7.4 The expression of caveolins in diabetic retinopathy 194

7.5 Future work 195

Abbreviations 197

Appendices I 201

Appendices II 203

References 205

Publications 302

List of figures:

1.1 Structure of the Eye 15

1.2 Structure of the Retina 16

1.3 Structure of a Capillary 20

3.1 Photomicrographs of Transverse Sections Showing H and E staining of non-diabetic retinas 58

3.2 Photomicrographs of transverse sections Showing H and E staining of unlasered diabetic retinas with no obvious microvascular abnormalities 64

3.3 Photomicrographs of transverse sections showing H and E staining of diabetic retinas with NPDR 65

3.4 Photomicrographs of transverse sections showing H and E staining of diabetic retinas with PDR 66

3.5 Photomicrographs of transverse sections showing H and E staining of lasered retinas 67

3.6 Photomicrographs of transverse sections showing H and E staining of fibrovascular membranes 69

4.1 Photomicrographs of transverse sections showing negative control staining for VEGF-A_{165}, VEGF-C, VEGFR-1, VEGFR-2, VEGFR-3, Ang-1, Ang-2, Tie-2, TNF-α 74

4.2 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-A in non-diabetic retinas 80

4.3 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-A in unlasered diabetic retinas with no obvious microvascular abnormalities 81

4.4 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-A in unlasered diabetic retinas with NPDR 82

4.5 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-A in unlasered diabetic retinas with PDR 83

4.6 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-A in lasered diabetic retinas 84

4.7 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-A in fibrovascular membranes 85

4.8 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-C in non-diabetic retinas 86

4.9 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-C in unlasered diabetic retinas with no obvious microvascular abnormalities 87

4.10 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-C in unlasered diabetic retinas with NPDR 88
4.11 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-C in unlasered diabetic retinas with PDR 89
4.12 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-C in lasered diabetic retinas 90
4.13 Photomicrographs of transverse sections showing the immunolocalisation of VEGF-C in fibrovascular membranes 91
4.14 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-1 in non-diabetic retinas 97
4.15 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-1 in unlasered diabetic retinas with no obvious microvascular abnormalities 98
4.16 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-1 in unlasered diabetic retinas with NPDR 99
4.17 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-1 in unlasered diabetic retinas with PDR 100
4.18 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-1 in lasered diabetic retinas 101
4.19 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-1 in fibrovascular membranes 102
4.20 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-2 in non-diabetic retinas 104
4.21 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-2 in unlasered diabetic retinas with no obvious microvascular abnormalities 105
4.22 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-2 in unlasered diabetic retinas with NPDR 106
4.23 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-2 in unlasered diabetic retinas with PDR 107
4.24 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-2 in lasered diabetic retinas 108
4.25 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-2 in fibrovascular membranes 109
4.26 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-3 in non-diabetic retinas 111

4.27 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-3 in unlasered diabetic retinas with no obvious microvascular abnormalities 112
4.28 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-3 in unlasered diabetic retinas with NPDR 113
4.29 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-3 in unlasered diabetic retinas with PDR 114
4.30 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-3 in lasered diabetic retinas 115
4.31 Photomicrographs of transverse sections showing the immunolocalisation of VEGFR-3 in fibrovascular membranes 116
4.32 Photomicrographs of transverse sections showing the immunolocalisation of Ang-1 in non-diabetic retinas 121
4.33 Photomicrographs of transverse sections showing the immunolocalisation of Ang-1 in unlasered diabetic retinas with no obvious microvascular abnormalities 122
4.34 Photomicrographs of transverse sections showing the immunolocalisation of Ang-1 in unlasered diabetic retinas with NPDR 123
4.35 Photomicrographs of transverse sections showing the immunolocalisation of Ang-1 in unlasered diabetic retinas with PDR 124
4.36 Photomicrographs of transverse sections showing the immunolocalisation of Ang-1 in lasered diabetic retinas 125
4.37 Photomicrographs of transverse sections showing the immunolocalisation of Ang-1 in fibrovascular membranes 126
4.38 Photomicrographs of transverse sections showing the immunolocalisation of Ang-2 in non-diabetic retinas 128
4.39 Photomicrographs of transverse sections showing the immunolocalisation of Ang-2 in unlasered diabetic retinas with no obvious microvascular abnormalities 129
4.40 Photomicrographs of transverse sections showing the immunolocalisation of Ang-2 in unlasered diabetic retinas with NPDR 130
4.41 Photomicrographs of transverse sections showing the immunolocalisation of Ang-2 in unlasered diabetic retinas with PDR 131
4.42 Photomicrographs of transverse sections showing the immunolocalisation of Ang-2 in lasered diabetic retinas 132
4.43 Photomicrographs of transverse sections showing the immunolocalisation of Ang-2 in fibrovascular membranes 133

4.44 Photomicrographs of transverse sections showing the immunolocalisation of Tie-2 in non-diabetic retinas 137
4.45 Photomicrographs of transverse sections showing the immunolocalisation of Tie-2 in unlasered diabetic retinas with no obvious microvascular abnormalities 138
4.46 Photomicrographs of transverse sections showing the immunolocalisation of Tie-2 in unlasered diabetic retinas with NPDR 139
4.47 Photomicrographs of transverse sections showing the immunolocalisation of Tie-2 in unlasered diabetic retinas with PDR 140
4.48 Photomicrographs of transverse sections showing the immunolocalisation of Tie-2 in lasered diabetic retinas 141
4.49 Photomicrographs of transverse sections showing the immunolocalisation of Tie-2 in fibrovascular membranes 142
4.50 Photomicrographs of transverse sections showing the immunolocalisation of TNF-α in non-diabetic retinas 146
4.51 Photomicrographs of transverse sections showing the immunolocalisation of TNF-α in unlasered diabetic retinas with no obvious microvascular abnormalities 147
4.52 Photomicrographs of transverse sections showing the immunolocalisation of TNF-α in unlasered diabetic retinas with NPDR 148
4.53 Photomicrographs of transverse sections showing the immunolocalisation of TNF-α in unlasered diabetic retinas with PDR 149
4.54 Photomicrographs of transverse sections showing the immunolocalisation of TNF-α in lasered diabetic retinas 150
4.55 Photomicrographs of transverse sections showing the immunolocalisation of TNF-α in fibrovascular membranes 151
5.1 Photomicrographs of transverse sections showing the immunolocalisation of PEDF 171
6.1 Photomicrographs of transverse sections showing the immunolocalisation of caveolin-1in non-diabetic retinas and negative control retina 179
6.2 Photomicrographs of transverse sections showing the immunolocalisation of caveolin-1in unlasered diabetic retinas with no obvious microvascular abnormalities 180
6.3 Photomicrographs of transverse sections showing the immunolocalisation of caveolin-1in unlasered diabetic retinas with NPDR 181
6.4 Photomicrographs of transverse sections showing the immunolocalisation of caveolin-1 in unlasered diabetic retinas with PDR 182

6.5 Photomicrographs of transverse sections showing the immunolocalisation of caveolin-1 in lasered diabetic retinas 183

6.6 Photomicrographs of transverse sections showing the immunolocalisation of caveolin-1in fibrovascular membranes 184

6.7 Photomicrographs of transverse sections showing the immunolocalisation of caveolin-2 and caveolin-3 185

List of tables:

2.1 The pre-treatments, blocking agents and secondary antibodies used in the immunostaining for growth factors, and their receptors, PEDF, TNF-α, caveolin-1, -2, and -3 54

3.1 Non-diabetic retinas 57

3.2 Unlasered diabetic retinas with no obvious microvascular abnormalities 60

3.3 Unlasered diabetic retinas with obvious microvascular abnormalities 61

3.4 Diabetic retinas with PDR 62

3.5 Lasered retinas without any obvious microvascular abnormalities 63

3.6 Fibrovascular membranes 68

4.1 Mean intensity of $VEGF_{165}$ Immunostaining 78

4.2 Mean intensity of VEGF-C Immunostaining 79

4.3 Mean intensity of VEGFR-1 Immunostaining 96

4.4 Mean intensity of VEGFR-2 Immunostaining 103

4.5 Mean intensity of VEGFR-3 Immunostaining 110

4.6 Mean intensity of Ang-1 Immunostaining 120

4.7 Mean intensity of Ang-2 Immunostaining 127

4.8 Mean intensity of Tie-2 Immunostaining 136

4.9 Mean intensity of TNF-α immunostaining 145

6.1 Mean intensity of Caveolin-1 immunostaining 177

ABSTRACT

PURPOSE

To determine the distribution of vascular endothelial growth factor (VEGF) isoforms, angiopoietins (Ang-1 and -2) and their receptors, tumour necrosis factor alpha (TNF-α), pigment epithelium-derived factor (PEDF), and caveolin family members (Cav-1, -2, and -3) in non-diabetic retinas and diabetic retinas at different stages of diabetic retinopathy.

METHODS

Human eyes, obtained at post-mortem, were divided into those without diabetes and those with diabetes. Diabetic retinas were examined by microscopy and categorised as either non-lasered with no obvious features of retinopathy, non-lasered with intraretinal changes (microaneurysms, exudates etc.) but no evidence of proliferative diabetic retinopathy (PDR), diabetic with proliferative retinopathy, and those which had received scatter laser photocoagulation therapy and who no longer had evidence of PDR. Immunohistochemistry was used to determine the localisation of growth factors and caveolins in diabetic and control retinas, as well as in excised PDR membranes.

RESULTS

There appeared to be both temporal and spatial changes in the staining pattern for each growth factor in diabetic retina which correlated with the stage of disease progression. Apart from Ang-2 and PEDF, immunostaining was raised in diabetic retina as compared to non-diabetic retina. Immunostaining was apparent in endothelial cells and the perivascular cells of the vessels. Immunostaining was also apparent within specific retinal layers for VEGF-A, VEGF-C, VEGFR's, Ang-2, Tie-2, TNF-α, PEDF, and caveolin 1, -2, and -3.

CONCLUSION

These data suggest a role for both angiogenic factors and anti-angiogenic factors and the caveolins in the pathogenesis of diabetic retinopathy, possibly by acting synergistically to mediate a wide range of cellular responses culminating in the formation of a fibrovascular membrane. Therapeutic intervention to the VEGF and Tie-2 receptor, and possibly stimulation of the PEDF signalling, pathways may prove useful for the treatment of PDR.

CHAPTER 1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Diabetes mellitus is a metabolic disorder characterised by hyperglycaemia and alterations in fat and protein metabolism and is associated with a specific set of long term microvascular and neurological complications (Nathan, 1996). World-wide it is estimated that over 2.5 million people in the 25-65 year age group are blind due to diabetes, which makes it the fourth leading cause of blindness and an increasing problem in developing nations (Foster, 1988). Typical ocular complications of diabetes include diabetic retinopathy, iris neovascularization, glaucoma, cataract and microvascular abnormalities of the optic nerve (Infeld *et al.*, 1998). The most frequent complication is diabetic retinopathy in which retinal ischaemia, occurring as a consequence of widespread capillary non-perfusion, results in the production of vasoproliferative substances and the development of neovascularization (Infeld *et al.*, 1998). Signs of retinopathy are not usually manifest before duration of 15 to 20 years, at which time the prevalence can approach 80% to 100% in insulin-dependent diabetics (Frank, 1986).

1.2 DIABETES MELLITUS AND ITS COMPLICATIONS

Diabetes mellitus is associated with typical patterns of long term vascular complications which vary with the organ involved. The microvascular kidney disease (Olgemoller *et al.*, 1993) is characterised by thickening of the capillary basement membranes and increased deposition of extracellular matrix components, while loss of microvessels with subsequent neovascularization is predominant in the eye and peripheral nerves (Pfeiffer *et al.*, 1995). Macrovascular disease is characterised by accelerated atherosclerosis. These complications are dependent on long term hyperglycaemia. Specific biochemical pathways linking hyperglycaemia to microvascular changes have been proposed: the polyol pathway (Greene *et al.*, 1987), non-enzymatic glycation of proteins (Brownlee *et al.*, 1988), glucose autooxidation and oxidative stress (Hunt *et al.*, 1990), hyperglycaemic pseudohypoxia (Williamson *et al.*, 1993), and enhanced activation of protein kinase C by synthesis of diacetyl glycerol (Lee *et al.*, 1989; DeRubertis and Craven, 1994). These pathways are not mutually exclusive (Larkins and Dunlop, 1992; Pfeiffer and Schatz, 1992). They may be linked to alterations in the synthesis of growth factors particularly since atherosclerosis and angiogenesis are associated with increased proliferation of endothelial cells.

Diabetic retinopathy may be classified as nonproliferative (NPDR) or proliferative (PDR) [Neely *et al.*, 1998]. NPDR may be graded as mild, moderate, or severe. The level of

severity correlates with the probability of progression to PDR (Neely *et al.*, 1998). NPDR is characterised by structural abnormalities of the retinal vessels (primarily capillaries but also venules and arterioles), varying degrees of retinal nonperfusion, retinal oedema, lipid exudates, and intraretinal haemorrhages. An important pathological event is the loss of capillary pericytes which are modified smooth muscle cells, which support the vascular endothelium of the retina (Kohner, 1993). This appears to be the initial event in NPDR. PDR may include any of the changes present in nonproliferative disease with additional findings of optic disc, retinal or iris neovascularization (Neely *et al.*, 1998).

Both NPDR and PDR may cause visual loss with the major vision-threatening complications being macula oedema, macula ischaemia, neovascularization with preretinal or vitreous haemorrhage, traction retinal detachment, and neovascular glaucoma (Neely *et al.*, 1998). Before considering the pathogenesis of diabetic retinopathy in detail it is important to have a basic knowledge of retinal anatomy and physiology.

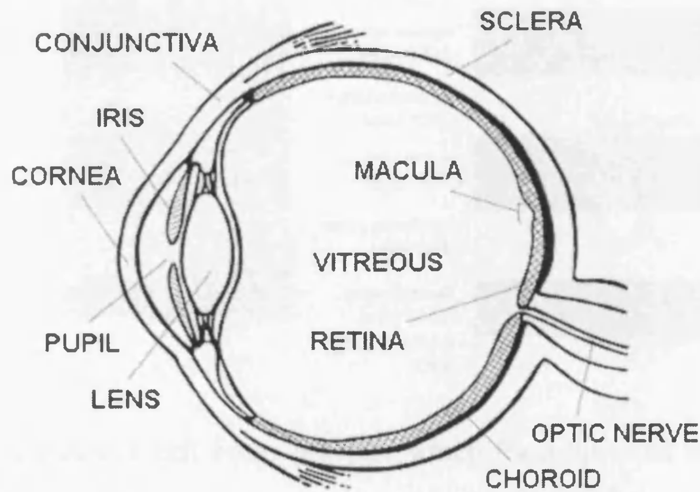
1.3 STRUCTURE AND FUNCTION OF THE NON-PATHOLOGICAL RETINA

The retina lines the posterior two-thirds of the eyeball (figure 1.1) and is separated from the sclera by the retinal pigment epithelium and the choroid (Naumann *et al.*, 1986). The retina is firmly attached to the underlying pigment epithelium at the optic nerve head and the ora serrata.

Light rays, scattered from objects in the outside world, enter the eye and hit the retina. Some processing of images then occurs in the retina, from which electrical impulses are then transmitted through the optic nerve to the primary and secondary visual centres of the brain.

The eye is normally directed so that the image of an object falls upon the fovea centralis the central portion of the macula, a depression in the retina situated about a millimetre or so to the lateral side of the posterior pole of the eye formed by lateral displacement of the cells of the inner retinal layers (Emslie-Smith *et al.*, 1988). It serves the function of central and colour vision and is therefore susceptible to many diseases of the retina such as diabetic retinopathy.

Figure 1.1 Structure of the Eye (Google Images)



At the microscopic level, the retina is a highly organised structure, consisting of alternate layers of cell bodies and synaptic processes. A single common pathway, mediated by the retinal ganglion cells, carries information from the retina to the brain (Miller, 1994). The retina can be classified into three systems, the neuronal system, the glial system and the vascular system (Blanks, 1994). These three systems are located within the 9 layers of the retina (figure 1.2) and are described below.

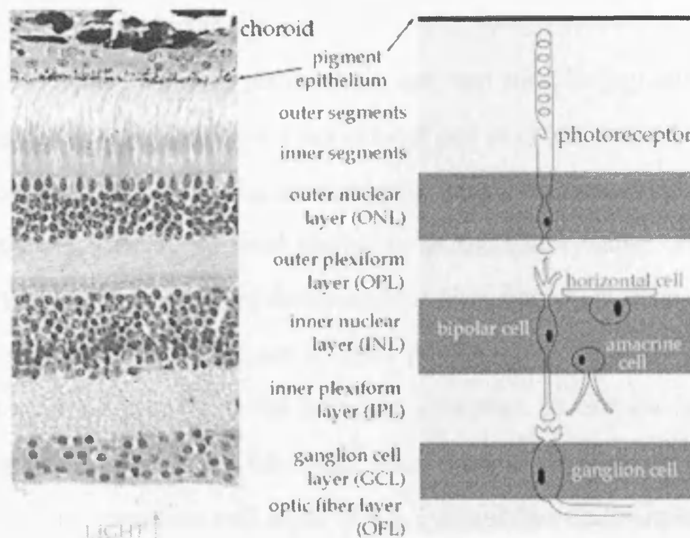
1.3.1 The Neuronal System of the Retina

The neuronal system traverses the entire thickness of the retina and consists of photoreceptor cells, intermediate neurones and ganglion cells (Naumann *et al.*, 1986).

The photosensitive cells, the rods and cones, lie in the outermost layer of the retina next to the pigment epithelium and serve the function of light perception (Emslie-Smith *et al.*, 1988). Light rays pass through the ganglion cells and inner retinal layers to reach the photoreceptor cells, where light is transformed into an electrochemical event (Blanks, 1994).

Each photoreceptor consists of an outer segment and an inner segment, both of which lie external to the external limiting membrane (ELM). Each photoreceptor also

Figure 1.2 Structure and organization of the retina (Google Images)



contains a cell body, together which form the outer nuclear layer (ONL). The processes of both rods and cones interact with the axons of the bipolar cells and horizontal cells in the outer plexiform layer (Naumann *et al*., 1986). The rods are thin, cylindrical structures which are primarily responsible for peripheral vision and vision in low illumination (scotopic vision). The cones, which in histological sections are distinguished by their broader flask-shaped profiles, are primarily responsible for phototopic vision and for highly discriminatory central and colour vision.

The cell bodies of four cells can be distinguished in the inner nuclear layer (INL): the most numerous of these are the bipolar cells. Their processes synapse outwardly with the photoreceptors, forming the middle limiting "membrane," and inwardly with the dendrites of the ganglion cells (Naumann *et al*., 1986).

Three types of bipolar cells have been distinguished morphologically. One morphologic type exclusively relates to rods, and two types relate exclusively to cones. The two cone-related types of bipolar cells make different kinds of synaptic connections with the cones, have axonal terminals that end in different parts of the inner plexiform layer, and appear to be involved in the generation of either ON or OFF responses to light in the retina. Rod-related bipolar cells extend their dendrites into invaginations of the rod terminals. The dendritic ends of the rod bipolar cells are considerably larger than those of the cone bipolar cells and allow a single rod bipolar cell to contact as many as 45 rod terminals.

The horizontal cells are located at the outer region of the INL. The horizontal cells (and amacrine cells which are discussed below) provide numerous "horizontal" neural

interconnections between groups or fields of retinal sensory neurones. In the retina, the processes of horizontal cells (and amacrine cells) modulate and transform visual information that is conveyed to the brain.

In the primate retina there are two morphologically distinct types of horizontal cells (Blanks, 1994). The *type I horizontal cell* is characterised by stout dendrites that contact only cones, and a single, long axon ending with a terminus that contacts only rods. These dendritic terminals form the lateral elements of the rod synapse. The *type II horizontal cell* contacts only cones with its slim dendritic branches and short axon.

Amacrine cells are located primarily in the inner portion of the inner nuclear layer. They extend processes to adjacent amacrine or bipolar cells, and their axons synapse with ganglion cells within the inner plexiform layer. Displaced amacrine cells are also present within the ganglion cell layer (GCL). Amacrine cells are more diversified and numerous than horizontal cells.

Until recently, amacrine cells have been classified according to the degree of stratification of their processes, e.g., as *unstratified, bistratified,* or *multistratified* if many branches of the main processes ramify in one, two, or more levels, respectively, or as *diffuse* if the many branches ramify without stratification. In the last several years, classification of amacrine cells, based on their neurotransmitters, has changed dramatically because of the finding that there may be as many as 30 different types. It has been shown that amacrine cells with differently shaped dendritic trees can be matched with particular neurotransmitters

Interplexiform cells were recognised only 15 years ago as a distinct class of neurones in the vertebrate retina. This interneurone has a cell body located at the innermost border of the INL, but its processes extend into both the inner and outer plexiform layers.

Nasally, the GCL consists of a single row of cells, which are separated by the processes of the Müller fibres (Naumann *et al.*, 1986). They are closely grouped near the optic nerve but are widely separated in the periphery: they are only occasionally seen at the region of the ora serrata. Temporally from the nerve, the ganglion cells become multilayered, increasing to six to eight layers of cells in the macula region.

The axons of ganglion cells converge to form the nerve fibre layer and exit the eye as the optic nerve. Ganglion cells and to a lesser extent bipolar cells, can have quite extensively spreading dendrites so that each ganglion cell may be influenced by the activity of a large number of rods and cones. Further lateral interactions are mediated by the horizontal and amacrine cells. In all regions of the retina, except the central fovea, the ganglion cells can be seen to be connected, through intermediate bipolar cells, to rods as well as cones.

1.3.2 The Glial System of the Retina

The glial system of the retina consists of Müller Cells, astrocytes and microglia. Müller cells are the most prominent of all retinal cells and they stretch from the internal limiting membrane (ILM) to beyond the ELM (Naumann *et al.*, 1986). The Müller cell bodies lie in the inner two thirds of the INL, among the cell bodies of the amacrine and bipolar cells. Müller cell radial fibres are prominent in the inner retina, where the main ascending processes are thick and relatively straight. They pass directly through the inner plexiform layer, among the ganglion cells, and between the nerve fibre bundles where they terminate internally on the outer surface of the ILM by formation of basal foot processes. Many delicate horizontal processes extend laterally from these radial fibres, which are particularly prominent as the horizontal fibres of the nerve fibre, inner and outer plexiform layers. In the nuclear layers, these lateral processes form a honeycomb around the various cell bodies. Müller cell processes intervene between most smaller vascular elements and neuronal processes. In the outer retina the ELM is formed by a network of cell junctions between Müller cell processes and photoreceptors .

In contrast to most other elements of the retina, which possess either a photoreceptive or neurotransmission function, the Müller cell provides structural support and contributes to the metabolism of the sensory retina. The cytoplasm of Müller cells has a high content of glial fibrils, glycogen granules, and abundant smooth endoplasmic reticulum. It also contains abundant lactic acid dehydrogenase suggesting they have an important role in carbohydrate metabolism in the retina. Müller cells also may be active in the degradation of synaptic transmitters such as Gamma amino butyric acid (GABA) and in regulating extracellular levels of glutamine released during neuronal activity. Another important function of Müller cells concerns the maintenance of potassium homeostasis in the retina. Neuronal activity is associated with the release of potassium into the extracellular space. Potassium is removed from the outer retina, probably by an active pump located in the Müller cell villi, and leaves these cells most likely with the aid of an active pump located in the endfoot membrane.

Fibrous astrocytes, typical of those found elsewhere in the central nervous system (CNS), are common in the nerve fibre layer of the retina where their number is proportional to the thickness of the layer. There are 2 types of atrocytes present in the retina, elongate astrocytes and stellate astrocytes. Elongate astrocytes have multiple long slim processes that traverse the retina within the nerve fibre bundles. They have not been observed to contact blood vessels. Stellate astrocytes have many slender processes that cross the nerve fibres; some of these processes contact nearby blood vessels with a bulbous endfoot. Astrocytes are

found occasionally at other locations in the inner half of the retina, but with much less frequency than the nerve fibre layer. Blood vessels never directly contact neuronal processes; intervening processes may be astrocytic but are most commonly derived from Müller cells.

Microglia contain an elongate nucleus and a slender cell body with two or more thick basal processes from which multiple coarse, short secondary processes may branch. The entire cell is covered with blunt spines from which slender hair-like extensions may continue 10-25 microns from the cell. The cells tend to be flat and are most common in the inner plexiform layer but they also occur in the external plexiform layer. Microglia are mesodermal in origin and are thought to arise from pericytes of blood vessels. As in the brain, they are amoeboid, migrate freely, are phagocytic, and become activated under pathological conditions of the retina.

The retinal pigment epithelium (RPE) is composed of a single-layered cuboidal or cylindrical epithelium containing pigment granules, a nucleus, mitochondria, and other cell organelles that mediate the active metabolic, fluid exchange, and phagocytic functions of the cells. (Naumann *et al.*, 1986). Because the sensory-pigment epithelium attachment is firm only at the ora serrata and optic nerve, the retina is susceptible to pathological separation from the underlying pigment epithelium.

1.4 VASCULAR SUPPLY TO THE RETINA

The primary arterial supply to the eye is the ophthalmic artery, which is the first branch of the internal carotid artery. The ophthalmic artery enters the orbit with the optic nerve through the optic canal and then divides into two major subdivisions: (1) the retinal system, in particular the central retinal artery, which supplies the inner one-half of the retina; and (2) the ciliary system, which supplies the uvea as well as the outer half of the sensory retina and optic nerve (Naumann *et al.*, 1986).

The secondary and tertiary branches of the central retinal artery occurring on the optic nerve head, as well as more peripherally in the fundus, form the arterioles and the primary blood supply to the four quadrants of the fundus. In a small percentage of cases a cilioretinal artery is present. This vessel originates from the short posterior ciliary vessels (the circle of Zinn) and enters the superficial aspect of the retina at the margin of the optic nerve. The four primary fundus arterioles lie superficially in the nerve fibre layer. The arterioles can easily be distinguished from the venules- not only clinically by their colour differences and smaller diameter but also histologically by evaluation of the vessel wall thickness. The terminal

fundus arterioles bend sharply and dip almost vertically into the retina, forming a rich capillary network. The capillaries extend as deeply as the outer aspect of the INL, sometimes extending just into the outer plexiform layer to the dividing line formed by the middle limiting "membrane".

The central fovea is almost totally devoid of blood vessels; however, the parafoveal and perifoveal zones or the more peripheral macula zones are richly vascularized by three arcades of capillaries within the inner half of the retina. Most of the extramacula and extrapapillary fundus is supported by two layers of capillaries; peripherally, this is reduced to a scanty single layer as the ora serrata is approached.

The branches of the retinal vein run alongside the arteries, the retinal vein passing out of the eye in the optic nerve (Emslie-Smith *et al.*, 1988). A separate choroidal system of vessels lies between the pigment epithelium and the sclera and supplies the outer layers of the retina.

The retinal capillaries consist of two distinct cell types: the endothelial cell (EC) and the pericyte (PC) (Naumann *et al.*, 1986). ECs line the lumen of the capillary and in turn are encircled by their basement membranes (BM) [figure 1.3].

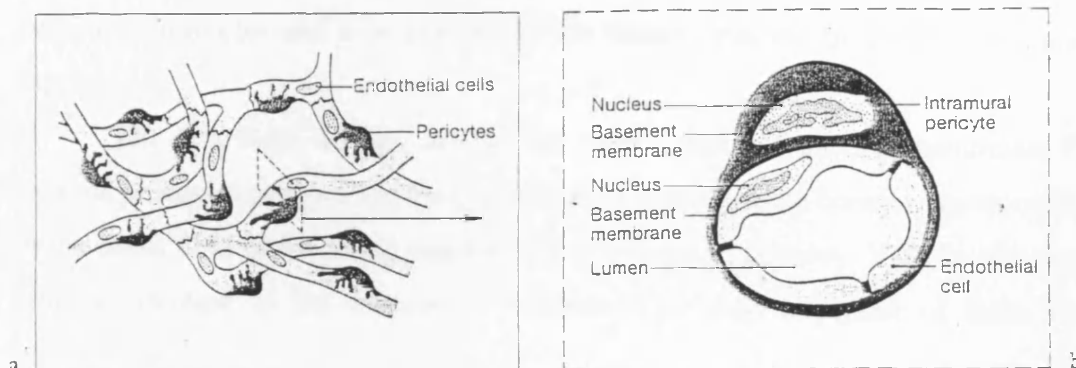


Figure 1.3 Normal Retinal Capillaries. a. Flat Preparation. b. Cross Section Showing The Nonfenestrated Endothelial Lining. (Copied from Naumann *et al.*, 1986)

The ECs of a normal retinal capillary are closely bound together about the lumen by intercellular junctions of the zonula occludens type (tight junctions). These inter-cellular bridges render the vascular channels nonfenestrated and thus prohibit a free flow of fluids from the vascular lumen into the retinal interstitium outside the capillary. These junctions, in

association with the astrocyte and Müller cell foot processes that encircle the retinal capillaries, probably contribute to the blood-retina barrier. Newly formed vessels (e.g. neovascularization in diabetic retinopathy) characteristically leak dye after fluorescein angiography, perhaps because of qualitatively and/or quantitatively incomplete intercellular tight junctions.

In addition to regulating vascular permeability ECs have a number of other functions. They are responsible for preserving vascular integrity with respect to maintaining uninterrupted blood flow by inducing platelet aggregation to plug injured sites and by triggering the extrinsic pathway of clotting. They are involved in the inhibition of intravascular thrombosis and lysis of established clots. They participate in the control of vascular tone, both vasodilator and vasoconstrictor substances are produced within the endothelium, e.g. nitric oxide (NO) [Garner, 1994] being a potent mediator of the first of these functions and endothelin-1 an equally effective mediator of the second.

ECs also have an inflammatory role in terms of leukocyte binding through the expression of specific adhesion molecules. The endothelium also can express class II major histocompatibility complex antigens and function as an antigen-presenting tissue in immunological reactions. It is a source of BM constituents and other extracellular proteins, such as fibronectin and a source of growth factors involved in angiogenesis and wound repair.

The PCs form a "cap" around the outer aspects of the EC membrane. Each cell extends processes that envelop the capillary and are sandwiched between layers of BM; in the retina about 85% of the lining endothelium is covered in this way. Various diseases cause a relative decrease in the number of pericytes. The most important of these is diabetic retinopathy.

The basic component of vascular BM is type IV collagen, which acts as a structural backbone and binds to other membrane components; it also has an inhibitory effect on EC proliferation, such that BM dissolution is obligatory before new vessel formation can take place. Laminin is another major constituent relating primarily to cell attachment, whereas fibronectin has a wider range of binding capacity. Proteoglycans, with heparin sulphate proteoglycan being the major one present and, due to the presence of their anionic charges, are crucial to the selective barrier function of the membranes.

The retinal vessels are also surrounded by perivascular glial elements, consisting of astrocytic foot processes and Müller cell processes (Naumann *et al.*, 1986).

1.5 DIABETIC RETINOPATHY

Angiogenesis, the formation of new capillary blood vessels by sprouting from existing microvessels to promote neovascularization, plays a major role in the evolvement of a vascular supply in the normal processes of ovulation, placental development, and wound healing, as well as variously clinically significant pathological processes such as tumour growth and diabetic retinopathy (Ferrara, 1995a; Ferrara *et al.*, 1995b). In contrast, the development of new vessels from blood islands, i.e., committed stem cells, in early embryogenesis is termed vasculogenesis. Although different in many respects, vasculogenesis shows some similarities to angiogenesis. However, vasculogenesis does not seem to contribute to repair and disease in postembryonic life (Battegay, 1995). In some of these processes the formation of new vessels contributes to necessary reparative processes involving tissue remodelling or preservation of vitally important ischaemic tissue. In other situations, such as in diabetic retinopathy, angiogenesis can significantly add to tissue destruction and promote disease (Battegay, 1995).

Many of the most sight-threatening ophthalmic disorders affecting patients throughout the world involve intraocular neovascularization as their major destructive component (Klein and Moorehead, 1970). Such disorders include not only the leading cause of blindness among infants (retinopathy of prematurity) [ROP], the leading cause of blindness among working age individuals (diabetic retinopathy), and the leading cause of blindness among the elderly (age-related macula degeneration), but other conditions such as central retinal vein occlusion, rubeosis iridis, sickle cell retinopathy, radiation retinopathy etc. (Klein and Klein, 1985).

Angiogenesis begins with the degradation of the parent vessel's BM followed by vascular EC migration outside the vessel wall, which results in vascular sprouts. The ECs of these sprouts proliferate, form lumina, and eventually generate new BM and recruit PCs (Folkman and Haudenschild, 1980).

Extracellular proteolysis is believed to be an essential component of the angiogenic process. Various elements of the angiogenic process may be mediated by extracellular proteolysis including degradation of the basement membrane of the parent vessel and invasion of the interstitial extracellular matrix by migrating ECs. Plasminogen activators (PAs) are key mediators of these processes and PA activity must be balanced by physiological inhibitors such as EC-derived Plasminogen activator inhibitor-1 (PAI-1) for normal capillary morphogenesis (Moscatelli and Rifkin, 1988; Pepper and Montesano, 1990).

During the stage of proliferative retinopathy, active proliferation of new vessels occurs on the retinal surface (Aiello, 1996). Although these vessels rarely cause visual loss, they are fragile and prone to bleed. As a result vitreous haemorrhage is a major component of visual loss during this period. Proliferating vessels are typically accompanied by a fibrous component containing glial tissue which can contract and may lead to traction on the retina. This traction can distort the retina, induce vitreous haemorrhage, or cause retinal detachment. The fibrovascular component often becomes prevalent as the retinopathy progresses. Fibrovascular proliferation in diabetic retinopathy typically proceeds from the disk along the major temporal vascular arcades to encircle the macula with increasing traction. This arrangement has led to the term *wolf-jaw* fibrovascular proliferation as the scar tissue slowly "bites down" on the macula. In the most severe final stages of diabetic retinopathy, this fibrous tissue creates a complete retinal detachment, contracting the retina into a funnel-shape. Untreated, these complications can lead to severe or total irreversible visual loss. The final stage of diabetic retinopathy is quiescence, which occurs both spontaneously over time or following laser photocoagulation. The final visual status of a person with diabetes is determined by whether this quiescence is reached without destruction of visually critical structures in the eye. Laser panretinal photocoagulation seems to accelerate this progression to quiescence therefore, more commonly resulting in inactive disease before the development of sight-threatening complications.

In severe cases, proliferation of new blood vessels may also occur in the anterior portion of the eye, especially on the iris and the anterior chamber angle. If these vessels obstruct the aqueous fluid outflow facilities of the eye, they can lead to neovascular glaucoma which is a severe sight-threatening disorder (Aiello, 1996).

PC loss is an early event in the pathogenesis of diabetic retinopathy. PCs are known to produce an inhibitor of EC growth which appears to be mediated through secretion of activated transforming growth factor-β (TGF-β). When the contact between ECs and PCs is lost, through PC dropout, ECs are free to proliferate (Orlidge and D'Amore, 1987).

Thickening of the extracellular matrix and alteration in its chemical composition is also probably of extreme importance. In diabetes, the documented changes in chemical composition of the BM include increased nonenzymatic glycosylation of collagen, decreased levels of heparan sulphate proteoglycans, and variable reports on changes in fibronectin and laminin. (Williamson *et al.*, 1988). Such changes might alter cell-cell contact and perhaps

allow cellular invasion and breakdown of the BM, one of the earliest features of angiogenesis.

The development of intraocular neovascularization is often correlated with areas of retinal capillary nonperfusion. During retinal development, the growing vessels are noted to invade areas of nonvascularized retina (Michaelson, 1948). In most of the ischaemic retinopathies, neovascularization of the retina or iris is preceded by increasing retinal capillary nonperfusion (Aiello, 1997). Often the neovascularization itself is located at the borders of perfused and nonperfused retina. Therapies which involve destruction of ischaemic retina such as laser photocoagulation or cryotherapy, often result in regression and quiescence of intraocular neovascularization (Aiello, 1997).

These observations suggest that the development and progression of neovascularization may be mediated by factors whose activity is induced by the onset of retinal ischaemia. As early as 1948, Michaelson used these clinical observations as support for the initial growth factor hypothesis concerning regulation of the developing retinal vasculature. This theory, known as the "Michaelson" hypothesis, was later refined by Ashton (1957). It was postulated that ischaemia of the retina produces a factor or factors capable of stimulating the growth of new vessels. To account for the clinical observations noted above, such a factor should be secreted and freely diffusible (accounting for neovascularization of adjacent retinal tissue or distant neovascularization of the iris), should be mitogenic for ECs (to account for proliferation of vessels), should be induced by retinal hypoxia, should have receptors located on retinal ECs (to permit stimulation of this cell type by the molecule), should be increased during periods of active intraocular neovascularization and diminished when neovascularization becomes quiescent either due to natural progression of the disease or successsful therapy.

It has now become widely accepted that growth factor release in retinopathy occurs as a consequence of hypoxia (Pfeiffer and Schatz, 1995). Hypoxia causes profound biochemical changes in cells and multiple systems within cells are effected by hypoxia. It is not yet known in detail how cells sense hypoxia, or how this translates into the secretion of angiogenic molecules. Possible mechanisms of hypoxia-sensing are similar to those of additional oxygen-responsive genes such as erythropoeitin (EPO) [Fisher *et al*, 1992; Goldberg *et al.*, 1994; Minchenko *et al.*, 1994a], endothelin-1, interleukin-1α, ornithine decarboxylase (Goldberg *et al.*, 1994), and glucose transporter (Loike *et al.*, 1994). Erythropoeitin has recently been shown to induce angiogenesis (Anagnostou *et al.*, 1994).

Similar to erythropoeitin, hypoxia-induced VEGF gene expression depends on a haem-containing protein (Fisher *et al.*, 1992; Goldberg *et al.*, 1994; Minchenko *et al.*, 1994a). Furthermore, induction of VEGF in response to hypoxia is due to transcriptional activation (Galis *et al.*, 1994).

1.6 MODULATORS OF THE MICROVASCULAR COMPLICATIONS OF DIABETIC RETINOPATHY

1.6.1 INTRODUCTION

Proliferation of retinal blood vessels is one of the most striking features of advanced diabetic retinopathy. This feature has led to the conclusion that the normal balance of growth factors, which usually serves to keep angiogenesis in check, is disturbed in diabetic retinopathy (Sharp, 1995) and in ocular disorders such as ROP, leading to a devastating effect in the retina. PDR is one of the few examples of human pathological conditions in which abnormal growth of new vessels is one of the primary features. In normal ocular tissue, angiogenic homeostasis is controlled by the balance between angiogenic stimulators and angiogenic inhibitors

1.6.2 Angiogenic Modulators

Growth factors can be defined as multifunctional signals or mediators which modify cell growth or proliferation, alone or in concert, by binding to specific cell surface receptors (Sporn *et al.*, 1990). Their biological effects on cells include cell adhesion, migration, survival, differentiation, extracellullar matrix secretion, protease and protease inhibitor release, production of other growth activities, and angiogenesis. (Wiedemann, 1992).

A feature of growth factors is that, in most instances, they act locally within the tissues in which they are synthesised. This local action of growth factors has led to the concepts of 'paracrine' and 'autocrine' action. Paracrine action occurs when a growth factor is secreted by a cell and interacts with responsive cells in the immediate vicinity and autocrine action occurs when a growth factor is expressed, and acts upon, the same cells (McKay, 1993).

One of the earliest candidates for a growth factor involved in retinal neovascularization was growth hormone (GH), based on clinical observations (Lundbaek *et al.*, 1970), but latterly it has been thought that the retina itself produces the factors responsible for new vessel growth (Glaser *et al.*, 1980). However, many years elapsed before techniques became available which permitted the *in vitro* demonstration of cell growth-

promoting activity in soluble extracts of retinal tissue (Glaser *et al.*, 1980). Since then, research in this area has progressed to the point where many growth factors and other cytokines with angiogenesis-modulating effects have been described. Some of these factors are stimulatory and some are inhibitory for one or more of the stages of the angiogenic response; others have no effect or have not been tested for a particular activity.

Numerous investigations, in an attempt to identify the factors involved in the neovascular response, have led to the characterisation of several molecules including insulin-like growth factor (IGF) and basic fibroblast growth factor (bFGF). Both of these molecules have shown some association with intraocular neovascularization (Montesano *et al.*, 1986), however, neither fulfils all of the criteria suggested by the Michaelson hypothesis. Although clearly an endothelial mitogen found in the eye (Klein *et al.*, 1970), bFGF lacks a signal sequence necessary for secretion (Abraham *et al.*, 1986). In addition, bFGF has not been consistently associated with active neovascularization in animal or human studies. IGF is a growth-promoting peptide with multiple biological effects (Le Roith and Roberts, 1993). IGF-1 is capable of inducing neovascularization, however, only at concentrations thought to be 10,000 times higher than the levels found in clinical disease (Grant *et al.*, 1993) Therefore, although both FGF and IGF probably play a role in the overall angiogenic response, each has characteristics making them unlikely to be the primary ocular angiogenic growth factor.

Vascular endothelial growth factor (VEGF) appears to be the most promising as the major angiogenic factor of diabetic retinopathy. Even though other growth factors have been implicated in the process of intraocular vascular proliferation, these factors do not show a consistent increase as would be expected if they played a significant causative role (Hannehan *et al.*, 1991; Meyer-Schwickerath *et al.*, 1993).

The following sections will discuss in detail some of the growth factor families thought to be present in retinal neovascularization

1.6.2.1 The VEGF family

Several members of the VEGF family have been identified. VEGF-A was initially identified (Senger *et al.*, 1983; Connolly *et al.*, 1989; Ferrara *et al.*, 1989; Levy *et al.*, 1989; Plouët *et al* 1989; Conn *et al.*, 1990). At least six different isoforms of VEGF-A polypeptides of different sizes (121,145,165,183,189 and 206 amino acid residues) are known to exist. These isoforms have distinct but overlapping functions in angiogenesis due to their differential binding to heparin sulphate (Leung *et al.*, 1989; Houck *et al.*, 1991; Tisher *et al.*,

1991; Claffey *et al.*, 1992; Ferrara *et al.*, 2003; Tammela *et al.*, 2005). Since the discovery of VEGF-A 5 other family members have been identified. VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral) and VEGF-F (snake) [Lyttle DJ *et al.*, 1994; Joukov *et al.*, 1996; Rak *et al.*, 1995; Schmidt *et al.*, 1997; Achen *et al.*, 1998; Wise *et al.*, 1999; Sheta *et al.*, 2000; Veikkola *et al.*, 2000; Shibuya, 2003; Suto *et al.*, 2005]. Structurally, the VEGFs are related to the PDGF family of growth factors, with intrachain and interchain disulphide bonds between eight cysteine residues in conserved positions (Keck *et al.*, 1989; Leung *et al.*, 1989; Tischer *et al.*, 1989; Claffey *et al.*, 1992). The crystal structure of VEGF-A consists of two monomers that are organized in an anti-parallel fashion to form a dimer, with the receptor-binding sites located at each pole of the dimer

The VEGF receptors, VEGFR-1, VEGFR-2, AND VEGFR-3 belong to the receptor tyrosine kinase (RTK) gene family (Ullrich and Schlessinger, 1990). Each is a type III RTK containing seven extracellular immunoglobulin-like (IgL) domains (Shibuya *et al.*, 1990; Matthews *et al.*, 1991; Terman *et al.*, 1991). They also consist of a transmembrane domain, a juxtamembrane domain, a kinase domain interrupted by a 69 amino acid residue long insert, and a C-terminal tail (Shibuya *et al.*, 1990; Matthews *et al.*, 1991; Terman *et al.*, 1991; de Vries *et al.*, 1992).

High expression of VEGF and its receptors is tightly controlled and restricted to developing microvessels and angioblasts during angiogenesis and vasculogenesis in developing mouse embryos. (Breier *et al.*, 1992; Peters *et al.*, 1993; Quinn *et al.*, 1993; Yamaguchi *et al.*, 1993).

In adults VEGF and its receptors are expressed in healthy organs with generally non-proliferating vessels such as lung, kidney, adrenal gland, liver, stomach, and heart. (Ladoux and Frelin, 1993a; Olofsson *et al.*, 1996) and in organs undergoing vascular remodelling such as in the female reproductive system. (Philips *et al.*, 1990; Galland *et al.*, 1992; Charnock Jones *et al.*, 1993; Shweiki *et al.*, 1993).

In tumours which contain hypoxic regions, VEGF is produced and secreted by blood vessels and tumour cells in the vicinity of tumours and acts on tumour ECs which express VEGF receptors. (Senger *et al.*, 1986; Weindel *et al.*, 1992; Brown *et al.*, 1993a; Brown *et al.*, 1993b; Shweiki *et al.*, 1993; Weindel *et al.*, 1994; Dvorak *et al.*, 1995; Hatva *et al.*, 1995; Leung *et al.*, 1997; Yancopoulos *et al.*, 2000; Padro *et al.*, 2002; Pallares *et al.*, 2006).

VEGF and its receptors are also increased in other pathological disorders characterised by hypoxia, microvascular permeability and neovascularization such as wound

healing (Peters *et al.*, 1993; Nissen *et al.*, 1998), psoriasis (Detmar *et al.*, 1994), and rheumatoid arthritis (Fava *et al.*, 1994; Koch *et al.*, 1994).

During later stages of development and in adult tissues, VEGFR-3 mRNA becomes restricted to developing lymphatic vessels and high endothelial venules (Fournier *et al.*, 1995; Kaipainen *et al.*, 1995; Lymboissaki *et al.*, 1998).

VEGF has been detected in neuronal cells, cardiac myocytes and cornea which are not directly associated with neovascularization (Breier *et al.*, 1992; Plate *et al.*, 1992; Ladoux and Frelin, 1993a).

VEGF and its receptors initiate signalling pathways that play a pivotal role during embryonic development and pathological angiogenesis (Breier, 2000; Yancopulas *et al.*, 2000; Bates and Harper, 2002; Carmeliet and Storkebaum, 2002; Sun *et al.*, 2003; Crol *et al.*, 2004). Heterozygous mutations inactivating the VEGF gene and the VEGFR-2 and VEGFR-1 genes in mice result in embryonic lethality and dramatic defects in angiogenesis and haematopoiesis (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Dumont *et al.*, 1998).

VEGF activates a broad spectrum of biological responses in endothelial cells, including cell proliferation, migration, survival, differentiation and permeability to macromolecules (Senger *et al.*, 1983; Senger *et al.*, 1990; Ladoux *et al.*, 1993a; Waltenberger *et al.*, 1994; Rahimi *et al.*, 2000; Suarez and Ballmer-Hofer, 2001; Yilmaz *et al.*, 2003; Fu and Shen, 2004).

VEGFR-1 transmits only weak mitogenic signals in ECs, however VEGFR-1 may stimulate biological responses in ECs potentially by heterodimerizing with VEGFR-2 (Kanno *et al.*, 2000; Rahimi *et al.*, 2000; Autiero *et al.*, 2003). VEGFR-1 activation in some non-endothelial cells, such as monocytes, is reported to stimulate cell migration and proliferation (Athanassiades *et al.*, 1998). Recently VEGFR-1 was found to play a critical role in recruiting stem cell-differentiated endothelial cells into newly formed blood vessels (Eriksson *et al.*, 2002; Carmeliet, 2003).

VEGFR-2 binds VEGF-A, VEGF-C, and VEGF-D (Wise *et al.*, 1999; Zachary and Gliki, 2001). VEGFR-2 expression is low in quiescent endothelium (Ortega *et al.*, 1997), however, the VEGFR-2 signalling pathway is crucial in bringing about the effects of VEGFs in actively proliferating tissue, including vasodilatation, endothelial cell migration and proliferation (Dejana, 1996; Esser *et al.*, 1998; Kroll and Waltenberger, 1998; Neufield *et al.*, 1999; Rahimi *et al.*, 1999; Yang *et al.*, 1999; Yancopoulos *et al.*, 2000; Venkiteswaran *et al.*, 2002; Calera *et al.*, 2004).

VEGFR-3 binds VEGF-C and VEGF-D (Cao *et al.*, 1998; Marconcini *et al.*, 1999; Makinen *et al.*, 2001; Stacker *et al.*, 2001). VEGFR-3 is believed to play a critical role in the development of the embryonic vascular system but was originally believed to be restricted postnatally to the ECs of lymphatic vessels and specialized fenestrated capillaries (Dumont *et al.*, 1998; Karkkainen *et al.*, 2000; Karkkainen *et al.*, 2001). However, VEGFR-3 may also play a role in adult vascular endothelium. VEGFR-3 expression has been demonstrated to be associated with vascular endothelial cells in tumours and to play a role in vascular tubular morphogenesis (Valtola *et al.*, 1999; Witmer *et al.*, 2001; Clarijs *et al.*, 2002; Persaud *et al.*, 2004). VEGF-D is mitogenic for endothelial cells and given that VEGF-D can activate VEGFR-3, it could be involved in the regulation and/or differentiation of lymphatic and blood vessel endothelium just like VEGF-C (Jeltsch *et al.*, 1997).

1.6.2.2 The VEGF/VEGFR System and Diabetic Retinopathy

VEGF has been postulated to be one of the major retinal angiogenic factors for the following reasons. First, VEGF is known to promote increased vascular permeability and extravasation of plasma proteins (Keck *et al.*, 1989; Ferrara *et al* 1992; Senger *et al.*, 1993) resulting in the formation of an extravascular fibrin gel, a substrate for ECs (Dvorak *et al.*, 1995). Fibrin deposition in diabetic retinas has been demonstrated (Murata *et al.*, 1995).

Second, the production of VEGF is enhanced by ischaemia (Ferrara *et al.*, 1992; Shweiki *et al.*, 1993; Senger *et al.*, 1993; Minchenko *et al.*, 1994b; Dvorak *et al.*, 1995) and ocular pathological angiogenesis is almost always associated with the occurrence of capillary nonperfusion and regions of retinal ischaemia. The expression of VEGF, VEGFR-1 and VEGFR-2 occurs in numerous ocular cell types including RPE cells (Adamis *et al.*, 1993; Aiello *et al.*, 1995; Guerrin *et al.*, 1995; Lutty *et al.*, 1996; Ohno- Schwesinger *et al.*, 2000; Matsui *et al.* 2001; Grossniklaus *et al.*, 2002), PCs (Aiello *et al.*, 1995; Lutty *et al.*, 1996; Takagi *et al.*, 1996b; Darland *et al.*, 2003), ganglion cells (Pierce *et al.*, 1995; Dorey *et al.*, 1996; Murata *et al.*, 1996), glial cells (Lutty *et al.*, 1996; Pe'er *et al.*, 1996; Sueishi *et al.*, 1996), choriocapillary endothelium (Lutty *et al.*, 1996; Amin *et al.*, 1997), and microvascular ECs (Murata *et al.*, 1995; Stone 1995; Dorey *et al.*, 1996; Lutty *et al.*, 1996; Pe'er *et al.*, 1996; Sueishi *et al.*, 1996; Amin *et al.*, 1997) and their production is dramatically increased in these cells when oxygen levels decrease (Adamis *et al.*, 1993; Aiello *et al.*, 1995; Shima *et al.*, 1995; Sueishi *et al.*, 1996).

Third, the number of receptors for VEGF in retinal ECs is higher than that of ECs in other tissues such as aorta, and cultures of BRECs can proliferate and migrate upon the addition of recombinant VEGF - two crucial steps of retinal angiogenesis (Simorre-Pinatel *et*

al., 1994; Enaida *et al.*, 1998; Rajah *et al.*, 2002) and it increases in response to hypoxia (Thieme *et al.*, 1995). The higher level of VEGF receptor expression in retinal endothelial cells suggests that the retinal microcirculation may be more sensitive to the effects of VEGF and this may contribute to the pathogenesis of diabetic retinopathy.

Fourth, VEGF is diffusible, because it has the signal sequence needed for secretion and is water soluble (Ferrara *et al.*, 1992; Senger *et al.*, 1993). Other candidate growth factors such as the FGF's lack a classic signal peptide for secretion from the cell (Abraham *et al.*, 1986; Jaye *et al.*, 1986).

VEGF is angiogenic *in vivo* and its expression, along with expression of VEGFR-1 and VEGFR-2 is temporally and spatially associated with the onset of ischaemia-induced intraocular neovascularization in several animal models (Miller *et al.*, 1994; Pe'er *et al.*, 1995; Pierce *et al.*, 1995; Stone *et al.*, 1996; Aiello *et al.*, 1997; Luna *et al.*, 1997; Ozaki *et al.*, 1997; Segawa *et al.*, 1998; Suzuma *et al.*, 1998; Alikacem *et al.*, 2000; Wong *et al*, 2001; Tolentino *et al.*, 2002). In diabetic rats increased levels of VEGFR-1 and VEGFR-2 were expressed in vascular and non-vascular structures of the inner retinas, indicating that the VEGF/VEGFR system is upregulated in early diabetic retinopathy before neovascularization has occurred (Hammes *et al.*, 1998). Inhibition of VEGF reduces the formation and development of new vessels (Adamis *et al.*, 1994; Aiello *et al.*, 1995; Adamis *et al.*, 1996; Qaum *et al.*, 2001; Tolentino *et al.*, 2002).Increased expression of VEGF in the retina is sufficient to cause retinal and subretinal neovascularization (Okamoto *et al.*, 1997; Tobe *et al.*, 1998; Kinnunen *et al.*, 2006) and signalling through VEGF receptors is necessary for retinal neovascularization (Adamis *et al.*, 1996; Robinson *et al.*, 1996; Tolentino *et al.*, 1996; Ozaki *et al.*, 2000; Ferrara *et al.*2003). Elevations of VEGF levels have been reported in the ocular fluid of eyes with active proliferative retinopathy as compared with non-diabetic patients, patients with nonproliferative diabetic retinopathy, and quiescent PDR (Adamis *et al.*, 1994; Aiello *et al.*, 1994; Malecaze *et al.*, 1994; Kosano *et al.*, 1999; Mitamura *et al.*, 2002; Ogata *et al.*, 2002ab; Funatsu *et al.*, 2003; Funatsu *et al.*, 2004). Vitreous fluid containing measurable VEGF stimulated the growth of retinal ECs cells *in vitro*. This stimulation was inhibited by VEGF-neutralising antibodies (Aiello *et al.*, 1994). Plasma levels of VEGF have also been shown to be higher in PDR patients than in non-diabetic controls (Lip *et al.*, 2000).

In developing retinas VEGF expression has been shown to be temporally and spatially correlated with retinal vasculogenesis. VEGF mRNA and proteins are localised in the developing vessels and the angioblasts, i.e. endothelial precursors and to the nerve fibre and

the GCL where the inner vascular network develops, and also in the INL where the outer vascular network develops (Stone, 1995; Dorey *et al.*, 1996; Murata *et al.*, 1996). Once vasculogenesis becomes inactive, VEGF mRNA expression is markedly decreased.

Immunohistochemical studies of rat and human retina have demonstrated that in diabetic retinas, VEGF immunoreactivity is markedly increased in the vascular endothelium and blood vessels walls and is observed in all layers of the retina (Murata *et al.*, 1995; Lutty *et al.*, 1996; Pe'er *et al.*, 1996; Sueishi *et al.*, 1996; Amin *et al.*, 1997; Kunz Mathews *et al.*, 1997;; Ishihama *et al.*, 2001; Famigletti *et al.*, 2003).

In situ hybridisation of sections from whole globes which were enucleated at the time of ongoing neovascularization demonstrated that proliferation of vascular elements in PDR and neovascularization of the retina and/or iris secondary to central retinal vein occlusion, retinal detachment, and intraocular tumours were always accompanied by induction of retinal VEGF expression (Pe'er *et al.*, 1995). In each case, expression of VEGF was induced only in a particular layer of the retina (either the ONL, the INL, or the GCL), matching the zones affected by impaired perfusion.

VEGF, VEGFR-2 and VEGFR-1 have also been detected in samples of neovascular membranes from diabetic patients at vitrectomy (Malecaze *et al.*, 1994; Chen *et al.*, 1997; Armstrong *et al.*, 1998a).

1.6.2.3 The Angiopoietin Family

Four members of the angiopoietin family have been identified. Ang-1 and Ang-2 were initially isolated (Davies *et al.*, 1996; Maisonpierre *et al.*, 1997) followed by Ang-3 and Ang-4, which are probably interspecies homologs (Kim *et al.*, 1999a; Valenzuela *et al.*, 1999). Angiopoietins contain an amino-terminal angiopoietin-specific domain followed by a coiled-coil domain, a linker peptide and a carboxy-terminal fibrinogen homology domain (Davies *et al.*, 1996; Maisonpierre *et al.*, 1997; Valenzuela *et al.*, 1999). The fibrinogen homology domain is responsible for receptor binding, the coiled-coil domain is required for dimerization of angiopioietin monomers, and the short amino-terminal region forms ring-like structures that cluster dimers into variable sized multimers necessary for receptor activation (Procopio *et al.*, 1999; Davies *et al.*, 2003; Cho *et al.*, 2004a). Ang-1, Ang-2 and Ang-4 bind to the receptor tyrosine kinase Tie-2. Tie-2 contains two IgL domains, flanking three EGF-like domains followed by three fibronectin type-III repeats in the extracellular region, a single hydrophobic transmembrane region and a split catalytic domain in the cytoplasmic

region (Partanen *et al.*, 1992; Dumont *et al.*, 1993; Iwama *et al.*, 1993; Sato *et al.*, 1993; Schnurch *et al.*, 1993).

Tie-2 expression appears to be mostly restricted to cells of the vascular system during embryogenesis (Sato *et al.*, 1993; Korhonen *et al.*, 1994). Its expression has also been shown to be localised to the endothelium of new vessels undergoing angiogenesis (Wong *et al.*, 1997). Tie-2 is also expressed in certain primitive haematopoietic stem cells (HSCs) and B lymphocytes but not in other lineage-committed cells.

Tie-2 and Ang-1 are co-expressed in quiescent arteries, veins and capillaries, in a wide range of adult tissues (Suri et al., 1996; Maisonpierre *et al.*, 1997; Tsurumi *et al.*, 1997; Stratmann *et al.*, 1998; Audero *et al.*, 2001; Nourhaghighi *et al.*, 2003). Ang-1 is constitutively secreted by periendothelial cells (PCs/SMC's) in these quiescent vessels and in cultured SMC's (Mandriata *et al.*, 1998: Stratmann *et al.*, 1998; Tanaka *et al.*, 1999; Kim *et al.*, 2000a; Loughna and Sato., 2001). This suggests a role for Tie-2 in the maintenance of quiescent vessels at all levels of the vasculature. Ang-1 mRNA expression has also been reported in neurons (Stratmann *et al.*, 1998; Acker *et al.*, 2001; Audero *et al.*, 2001; Hashimoto *et al.*, 2001).

Ang-2 is found only at sites of vascular remodelling including the ovary, placenta, and uterus and is selectively expressed in ECs and in some instances smooth muscle cells (Maisonpierre *et al.*, 1997; Gale *et al.*, 2002). Ang-2 and Tie-2 expression are abundantly expressed in both tumour cells and in ECS of sprouting microvessels of solid tumours (Stratmann *et al.*, 1998; Bunone *et al.*, 1999; Holash *et al.*, 1999a; Takahama *et al.*, 1999; Tanaka *et al.*, 1999; Brown *et al.*, 2000; Etoh *et al.*, 2001; Yu *et al.*, 2001; Nakayama *et al.*, 2004). In contrast to Ang-2, the expression of Ang-1 is not high in most tumours (Hayes *et al.*, 2000).

Tie-2 and Ang-1 appear to play a major role at later stages of vascular development, i.e., during vascular maturation, maintenance of integrity and remodelling, unlike other angiogenic growth factors, such as VEGF, which function during the earliest stages of vascular development. (Keck *et al.*, 1989; Dumont *et al.*, 1994; Sato *et al.*, 1995; Suri *et al.*, 1996; Witzenbichler *et al.*, 1998). Tie-2 has been shown to recruit peri-endothelial cells (Dumont *et al.*, 1994; Sato *et al.*, 1995; Hanahan, 1997). Ang-1 secretion by periendothelial cells in normal quiescent vessels was shown to stabilise vessels by maintaining contacts between ECs and periendothelial cells (Stratmann *et al.*, 1998; Tanaka *et al.*, 1999). However, more recently Uemura *et al.*, 2002 showed that vessel stabalisation by Ang-1 occurred in the absence of pericyte recruitment.

In Ecs Ang-1 induces tube formation, sprouting, adherence, EC differentiation, and survival (Witzenbichler *et al.*, 1998a; Koblizek *et al.*, 1998; Hayes *et al.*, 1999; Kwak *et al.*, 1999; Papapetropoulos *et al.*, 1999; Kim *et al.*, 2000b; Kwak *et al.*, 2000) but not migration (Witzenbichler *et al.*, 1998; Ferrara *et al.*, 1989). Ang-1 also decreases permeability, changes the distribution and activation of junctional adhesion molecules, and stabilizes cell-cell junctions (Gamble *et al.*, 2000; Kim *et al.*, 2000b; Iizasa *et al.*, 2002; Wang *et al.*, 2004; Baffert *et al.*, 2006). Ang-1 also has anti-inflammatory functions (Gamble *et al.*, 2000; Kim *et al.*, 2001a; 2002b; Joussen *et al.*, 2002a; Pizurki *et al.*, 2003; Lemieux *et al.*, 2005).

Ang-2 has been shown to be a natural antagonist of Ang-1 by competitive binding to Tie-2 without stimulating autophosphorylation of the receptor and is thought to play an earlier role at sites of vessel invasion (Maisonpierre *et al.*, 1997). Overexpression of Ang-2 produces vascular defects similar to those in Ang-1 or Tie-2 deficient mice (Keck *et al* 1989; Ferrara *et al.*, 1991; Sato *et al.*, 1995; Suri *et al.*, 1996; Korpelainen *et al.*, 1999). In tumours, Ang-2 expression exceeds Ang-1 expression and is specifically associated with small capillaries with few peri-endothelial support cells (Stratmann *et al.*, 1998; Tanaka *et al.*, 1999). Inhibition of Ang-1 by Ang-2 promotes SMC/PC drop-off leading to loosening of the contacts between endothelial and peri-endothelial cells (Hanahan, 1997) which has been shown to be a requirement for rendering and maintaining ECs accessible to angiogenic inducers such as VEGF (Asahara *et al.*, 1998; Korff *et al.*, 2001; Lobov *et al.*, 2002; Visconti *et al.*, 2002; Oshima *et al.*, 2004; Oshima *et al.*, 2005; Scharpfenecker *et al.*, 2005). Down regulation of Ang-2 in quiescent ECs may permit physical interaction of ECs with peri-endothelial cells, leading to inhibition of endothelial proliferation and maturation of the vessel wall. However, in certain in vitro assays Ang-2 was shown to induce-Tie-2 phosphorylation and MMP-9 expression, migration and sprouting, and tube formation of ECs (Maisonpierre *et al.*, 1997; Kim *et al.*, 2000c; Korff *et al.*, 2001; Teichert-Kuliszweska *et al.*, 2001; Mochizuki *et al.*, 2002; Das *et al.*, 2003). This suggests that Ang-2 can act as an agonist depending on cell type and experimental context. Also, overexpression of Ang-2 in cancer cells in mice promotes growth and vascularization of tumours (Tanaka *et al.*, 1999; Ahmad *et al.*, 2001). In contrast, pharmacological inhibition specific to Ang-2 suppresses angiogenesis and growth of tumours (Oliner *et al.*, 2004) and inhibits neovascularization in the rat cornea (White *et al.*, 2003; Oliner *et al.*, 2004).

1.6.2.4 The Angiopoietin Family and Ocular Neovascularization

Ang-2 has been shown to be upregulated in the retinas of diabetic rats, preceding the onset of pericyte loss suggesting that upregulation of Ang-2 plays a critical role in the loss of pericytes in diabetic retina. (Hammes *et al.*2004).

Ang-1 was shown to suppress the development of diabetic retinopathy and reduced both vascular endothelial injury and blood-retinal barrier breakdown (Holash *et al.*, 1999a; b; Joussen *et al.*, 2002a). This is consistent with the findings of Thurston *et al.*, 1999 who showed that addition of Ang-1 was able to prevent diabetes-related vascular leakage and leukocyte adhesion and EC death. These effects were mediated in part via down-regulation of VEGF and VEGF has been shown to regulate vascular permeability through downregulation of the tight junction proteins occludin and zonula occluden 1 (Antonetti *et al.*, 1998; Antonetti *et al.*, 1999). In Ang-1-deficient mice ECs are poorly associated with the underlying matrix and do not properly recruit endothelial supporting cells (Suri *et al.*, 1996). Leukocyte adhesion is thought to trigger the disorganization of the EC zonula adherence and tight junctions (Del Maschio *et al.*, 1996; Bolton *et al.*, 1998). They therefore speculated that Ang-1 not only prevents leakage by stabilization of the endothelial-pericyte interaction, but also via inhibition of leukocyte adhesion. The protective role of Ang-1 in the mature vasculature therefore appears to act at several levels: a maturation signal during the development of vessels; as an anti-inflammatory and anti-permeability agent that prevents leukocyte adherence and damage to the EC layer; and in the maintenance of the blood-retina barrier.

Adult mice with induced expression of Ang-1 ubiquitously, or specifically in the retina, appeared normal and had no identifiable changes in retinal or choroidal blood vessels or in retinal function as assessed by electroretinography (Nambu *et al.*, 2004). This inhibition of ocular neovascularization is interesting because overexpression of Ang-1 in skin stimulates neovascularization. Ang-1 also significantly reduced VEGF-induced retinal vascular permeability.

Ang-2 has been shown to be up-regulated in the retina during development of the deep retinal capillaries by angiogenesis and during pathologic angiogenesis in diabetic retina (Adamis *et al.*, 1996; Hackett *et al.*, 2000; Lim *et al.*, 2005; Patel *et al.*, 2005). In human epiretinal membranes obtained from eyes with ischaemic retinal disorders, substantial upregulation of Ang-2 and tie-2 was found than in those from eyes with non-ischaemic diseases, whereas expression of Ang-1 was consistent in all membranes. Both Ang-1 and Ang-2 promoted tube-forming activity and enhanced the effects of VEGF in cultured BRECs

(Takagi *et al.*, 2003). Lim *et al.*, 2005 showed that Ang-2 is raised in diabetes. In the absence of VEGF, Ang-2 induces vessel regression but facilitates EC migration and proliferation in concert with VEGF (Etoh *et al.*, 2002; Lobov *et al.*, 2002; Oshima *et al.*, 2005). Therefore, selective up-regulation of VEGF and Ang-2 may lead to aberrant proliferation of leaky, friable vessels, which is characteristic off diabetic eye disease.

Ang-1 and Ang-2 have also been shown to be co-localised with VEGF in human choroidal neovascular membranes (Otani *et al.*, 1999; Hangai *et al.*, 2001). VEGF was shown to upregulate Ang-1 mRNA and protein levels in RPE cells by increasing mRNA stability. This suggests that VEGF may selectively modulate Ang expression during choroidal neovascularization (CNV).

1.6.2.5 TNF-α

Tumour necrosis factor alpha (TNF-α) was discovered as a serum protein released after systemic treatment of rodents with "bacilli Calmette-Guérin" and lipopolysaccaharide (Carswell *et al.*, 1975). It was shown to beTNF belongs to a large family of structurally related proteins called the "TNF Ligand Superfamily". The actions of these factors are diverse involving inflammation, apoptosis, cell proliferation and the stimulation of various aspects within the immune system (Pfeiffer, 2003; Goetz *et al.*, 2004).

TNF-α is a 26-kDa transmembrane protein containing a C terminus that is external to the cell and cytoplasmic domain. In mammals, TNF can be released from the membrane by a protease of the metalloproteinase/disintegrin/cysteine-rich family called TACE (TNF-alpha converting enzyme), to produce a 17-kDa soluble protein (Black *et al.*, 1997a,b; Blobel, 1997; Ware, 1998). The overall structure of TNF is described as a 'β-jellyroll' in which eight antiparallel β-strands form a sandwich 3D structure (Grass, 1996; Ware *et al.*, 1998; Idriss and Naismith, 2000). The TNF-α gene contains four exons and three introns. The average size of mammalian TNF-α is 234 amino acids (Idriss and Naismith, 2000).

TNF-α is produced mainly from monocyte-macrophages but it has also been detected in astroyctes, microglia, smooth muscle cells, endothelial cells, neutrophils, and fibroblasts. (Frangogiannis *et al.*, 1998; Meldrum, 1998; Meldrum *et al.*, 1998). High levels of TNF-α in fluids and serum have been associated with inflammatory processes such as rheumatoid arthritis, Crohns disease, and multiple sclerosis (Brennan and Feldman, 1996; Rink and Kirchner, 1996). In addition, TNF-α is overexpressed in multiple myeloma cell lines and several reports have been associated with the detection of abnormally high levels of TNF-α protein in the blood of cancer patients with a wide range of tumour types (Partanen *et al.*,

1995; Leek *et al.,* 1998; Karayiannakis *et al.,* 2001; Yoshida *et al.,* 2002). Within groups of patients with the same tumour type higher levels of TNF-α were correlated with advanced tumour stage, greater paraneoplastic complications and shorter survival time.

Upregulation of TNF-α and TNF-α receptor I mRNA and protein in glaucomatous retina has been demonstrated in the inner retinal layers predominantly in glial cells and ganglion cells and it was suggested that TNF-α-mediated cell death, through binding to TNFR-I, is involved in the neurodegeneration process of glaucoma (Tezel *et al.,* 2001).

The biological responses to TNF-α are mediated by two types of TNF receptors, which can be differentiated by their molecular weight of ~ 55kDa (TNFR1) and 75 kDa (TNFRII). After binding to its receptors intracellular signal transduction pathways activated by TNF-α can be grouped into three general categories. TNF-mediated apoptotic cell death, which is mediated through a caspase cascade (Leong and Karsan, 2000; Petak and Houghton, 2001), TNF-α induced mitogenic, or TNF-α-induced inflammatory responses. TNF-α acts as an inflammatory factor by promoting leukocyte adhesion to vascular endothelium both in vitro and in vivo. Leukocyte adhesion is mediated by the complex interplay of adhesion receptors on both leukocytes and ECs. TNF-α induces the transcription of the leukocyte adhesion molecules ICAM1, VCAM, E-selectin, IL-6, IL-8, and cyclooxygenase (COX)-2, among others (Herz and Gerard, 1993; Rogers *et al.,* 1996; Ridley *et al.,* 1997; Bergstrom *et al.,* 2000; Dunford *et al.,* 2001; Gustin *et al.,* 2004) by activating transcription factors like Activator Protein one (AP-1) and nuclear factor kappa B (NF-κB) [Leong and Karsan, 2000; Wajant and Scheurich, 2001; Hoefen and Berk, 2002; Paria *et al.,* 2003; Viemann *et al.,* 2004; Trickler *et al.,* 2005; Wang *et al.,* 2006). Zhou *et al.,* 2004 showed that TNF-α activates NFκB, PI3K, and MAPK with concomitant downstream expression of Bcl-2. Bcl-2 is an anti-apoptotic factor whose expression is regulated in response to hypoxia.

TNF-α has also been shown to alter vascular tone (Hollenberg *et al.,* 1991; Luckman *et al.,* 1998) and increase microvascular permeability in endothelial monolayers (Maruo *et al.,* 1992; Brown and Robbins, 1999; Desai *et al.,* 2002; Sedgwick *et al.,* Vandamme *et al.,* 2003; Kerkar *et al.,* 2006). In addition, TNF-α has also been shown to up-regulate the expression of growth factors such as PDGF, VEGF, and Ang-2 (Kim *et al.,* 2000d; Yang *et al.,* 2003; Wang *et al.,* 2006) and to alter the expression of VEGFR-2 (Stadelmann and Lassmann., 2000).

Although originally identified as an endotoxin-induced protein which causes necrosis of tumours (Carswell *et al.,* 1975), TNF-α has recently been shown to mediate tumour progression by causing the proliferation, invasion and metastasis of tumour cells (Balkwill,

2002). When TNF-α is produced by tumours and tumour-associated macrophages or stromal cells at physiological levels, it promotes tumour growth and additional macrophage recruitment, stimulating the release of angiogenic and growth factors from infiltrating cells.

TNF-α has also been shown to modulate neuronal functions (Cunningham *et al.*, 1996; Pan *et al.*, 1997), and to stimulate glial proliferation (Barna *et al.*, 1990; Selmaj *et al.*, 1990; Dopp *et al.*, 1997), and glial activation (Aloisi *et al.*, 1992; Merrill, 1992; Panek *et al.*, 1994; Romero *et al.*, 1996; Munoz-Fernandez and Fresno, 1998).

1.6.2.6 TNF-α and Ocular Angiogenesis

TNF-α overexpresssion in diabetes is thought to contribute to several complications in diabetes, including retinopathy, nephropathy, neuropathy, and diabetes-enhanced periodontal disease (Nishimura *et al.*, 2003; Satoh *et al.*, 2003; Siragy *et al.*, 2003; Gonzalez-Clemente *et al.* 2005; Krady *et al.*, 2005). This may result from the effects of hyperglycaemia and advanced glycation end-products (AGES) as has been shown in HUVECs (Rashid *et al.*, 2004). Diabetic retinopathy has an underlying inflammatory component, involving leukocyte recruitment and adhesion to the retinal vasculature and up-regulation of inflammatory genes (Jousson, 2002). Jousson, 2002 showed that leukocyte adhesion in diabetic rat retinas was elevated two-fold more than levels in non-diabetic animals and treatment with the TNF-α receptor inhibitor eternacept reduced leukocyte adhesion in retinal vessels of treated vs. diabetic controls. Retinal neovascularization was also shown to be abrogated by other selective TNF-α blockers (Zhu *et al.*, 2006).

TNF-α has also been shown to be the predominant pro-inflammatory cytokine observed within the extracellular matrix and luminal and abluminal surface of infiltrating vessels in PDR membranes and has also been demonstrated in PVR membranes (Limb *et al.*, 1996; Armstrong *et al.*, 1998a). Increased levels of TNF-α and its receptors TNFR-I and II have also been detected in the vitreous of eyes with PVR, rhegmatogenous retinal detachment (RRD), and PDR compared with control eyes (Limb, 2001).

Retinal ischaemia has been shown to upregulate TNF, TNFR1 and TNFR2 (Fontaine, 2002; Lahat *et al.* ,2003). TNF-α mRNA was increased in mouse models of retinal neovascularization. (Majka, 2002; Yoshida *et al.*, 2004) and was shown to be expressed by Müller glial cells, in the inner nuclear layer and outer nuclear layer. Yoshida et al., 2004 showed that the TNF-α expression level was enhanced in macrophages/microglia 4 days after hypoxia.

Zhao *et al.*, 2006 showed that TNF-α upregulated VEGF-C and VEGFR-2 expression in retinal ECs. Flow cytometry results showed that VEGF-C prevented EC apoptosis induced by TNF-α and that the anti-apoptotic effect was mainly via VEGFR-2.

TNF-α has also been shown to colocalize with Ang-1, Ang-2 and VEGF in human CNV specimens (Hangai *et al.*, 2006). It was shown to induce the sequential upregulation of Ang-2 and then Ang-1 and VEGF mRNA and protein expression in choroidal microvascular ECs in vitro.

TNF-α has also been shown to produce an angiogenic response in mouse cornea assays which was dramatically increased when Ang-1 or Ang-2 were added suggesting that Tie-2 signalling synergistically amplifies and participates in TNF-α-mediated angiogenesis (Nakoa *et al.*, 2003; Chen *et al.*, 2004).

1.6.3 Inhibitors of Angiogenesis

Numerous inhibitors of angiogenesis have been reported to counteract the effects of growth factors and endogenous angiogenic inhibitors are believed to be essential for maintaining the homeostasis of angiogenesis in the retina (Raymond, 1982). Extracts of cornea and vitreous contain substances that inhibit the proliferation and angiogenesis of endothelial cells.

1.6.3.1 PEDF

Among various molecules with antiangiogenic properties which have been reported to counteract the effects of growth factors, pigment epithelium-derived factor (PEDF) appears to play a prominent role (Mori *et al.*, 2001; Stellmach *et al.*, 2001). Recent studies suggest that the induction of angiogenesis in the eye requires not only an elevation of VEGF but also a decrease in PEDF (Tombran-Tink and Johnson, 1989).

PEDF is a glycoprotein of 50-kDa and a member of the serine protease inhibitors (serpine) superfamily related through their highly conserved folded conformation (Steele *et al.*, 1993; Becerra *et al.*, 1995). PEDF binds to heparin and other glycosaminoglycans in the ECM through lysine residues at a novel binding site for members of the serine family (Simonovic *et al.*, 2001). It was first isolated based on its ability to convert dividing retinoblastoma cells into differentiated neurones, and was therefore characterized as a neurotropic factor (Tombran-Tink and Johnson, 1989; Tombran-Tink *et al.*, 1991). Later, it was shown that besides its neurotropic functions, PEDF is a potent inhibitor of angiogenesis in the eye (Dawson *et al.*, 1999), where it inhibits stimulatory activity of several

proangiogenic factors. Subsequent workers also demonstrated that PEDF is associated with both the cell cycle and senescence (Pignolo *et al.,* 1995; Palmieri *et al.,* 1999; Tresini *et al.,* 1999).

The human PEDF gene spans approximately 16kb and contains eight relatively small exons and seven introns with the largest intron (intron1) approximately 4 kb in length (Tombran-Tink *et al.,* 1996). The human PEDF gene contains an open reading frame encoding a 418-amino-acid protein with a hydrophobic signal that is characteristic of secreted proteins (Steel *et al.,* 1993).

Whether or not PEDF exerts its actions trough a classical transmembrane receptor remains an open question. High affinity PEDF-binding sites and proteins have been shown to be present in plasma membranes of the retina, retinoblastoma, CNS, pericytes and ECs, consistent with a cell surface PEDF receptor protein (Wu *et al*., 1995; Alberdi *et al.,* 1999; Aymerich *et al.,* 2001; Bilak *et al.,* 2002; Filleur *et al.,* 2005). However, little is known about the identity of the receptor and molecular mechanism(s) by which PEDF functions to regulate neuronal and EC behaviour. Notari *et al.,* 2006 found a novel RPE gene, which they termed PEDF-R. They demonstrated the expression of PEDF-R in the retina and showed it had a binding affinity for PEDF.

PEDF mRNA is found in many human foetal and adult tissues and the protein is secreted in primary cultures of various cell types including osteoblasts (Tombran-Tink *et al.,* 1996, 2004; Tombran-Tink and Barnstable. 2004) . It is expressed not only in the retina, but also in the vitreous and aqueous humours, in association with fibroblasts, in ciliary epithelium, in cultured RPE, as well as in the adult human brain, the spinal cord, pineal gland, skeletal muscle, bone, heart, placenta liver, teeth, bone and cartilage matrix and human plasma. (Wu *et al.,* 1995; Tombran-Tink *et al.,* 1996 ; Bilak *et al.,* 1999; Karakousis *et al.,* 2001; Behling *et al.,* 2002; Kunci *et al.,* 2002; Peterson *et al.,* 2003; Tombran-Tink and Barnstable, 2003).

PEDF is among the most potent known natural antiangiogenic factors and it is even more active than angiostatin, thrombospondin-1, and endostatin (Dawson *et al.,* 1999). It plays a role in preventing neovascularization by excluding vessels from invading the retina, vitreous, and cornea (Dawson *et al.,* 1999; Stellmach *et al.,* 2001). PEDF inhibits endothelial cell migration towards many angiogenic factors including platelet-derived growth factor (PDGF), VEGF, IL-8, fibroblast growth factor (FGF), and lysophosphatidic acid (Dawson *et al.,* 1999). PEDF has been shown to inhibit VEGF-induced vascular permeability in mice (Liu *et al.,* 2004) and the inhibitory effect of PEDF on VEGF-induced angiogenesis was

shown to result from the enhanced γ-secretase-dependent cleavage of the C terminus of VEGFR-1, which in turn inhibited VEGFR-2-induced angiogenesis (Cai *et al.,* 2006). In addition, PEDF was also able to regulate the phosphorylation of VEGFR-1, which itself can regulate VEGFR-2 signalling. This identifies two novel pathways by which PEDF inhibits VEGF-induced angiogenesis: regulated intramembrane proteolysis and inhibition of VEGFR-1 phosphorylation.

The antiangiogenic effects of PEDF are associated with induction of endothelial cell apoptosis/cell death (Becerra, 2006). PEDF discriminates between ECs forming new vessels (cells that it destroys) and those that are part of pre-existing vessels (cells that it does not harm) by making use of the same Fas ligand–Fas receptor system that the immune system uses to apoptopically eliminate unwanted lymphocytes (Volpert *et al.,* 2002). In apoptosis, Fas ligand and its receptor, Fas/CD95 are tightly linked to the cell death cascade. When ECs are treated with PEDF Fas ligand expression increases and the Fas/FasL transduction cascade becomes activated which has been shown to lead to cell death of ECs (Volpert *et al.,* 2002). However, it is still unclear whether other pathways are involved in PEDF signalling of apoptopic events since PEDF can still inhibit neovascularization of mice lacking either Fas or FasL (Barreiro *et al.,* 2003). Chen *et al.,* 2006 provide strong evidence that PEDF induces death of ECs through activation of both receptor-mediated and mitochondria-mediated pathways of caspase activation and that activation of these pathways is p38-dependent.

Overexpression of PEDF has been shown to inhibit tumour growth and progression (Crawford *et al.,* 2001; Abe *et al.,* 2004; Matsumoto *et al.,* 2004). The inhibition of tumour growth was shown to occur via PEDF's anti-proliferative and anti-apoptopic effects and it was shown to suppress VEGF expression in osteoscarcoma cells. Its transcription was shown to decrease with increasing grade of glioma tumour and it was absent in the most aggressive ones (Guan *et al.,* 2003).

PEDF also has neurotrophic, neuronotrophic, nueroprotective and gliastatic properties and acts in neuronal survival and differentiation of photoreceptor and retina cells as well as neurons of the central nervous system (Araki *et al.,* 1994; Cayouette *et al.,* 1999; Cao *et al.,* 2001; Jablonski *et al.,* 2001; Bilak *et al.,* 2002; Kunci *et al.,* 2002).

1.6.3.2 PEDF and Ocular Angiogenesis

Several groups have suggested that shifts in the balance between pro-angiogenic factors such as VEGF and anti-angiogenic factors such as PEDF may be responsible for the pathology seen in choroidal and inner-retinal neovascularization (Gao, 2001; Ogata, 2001a; Ohno-Matsui, 2001; Gao et al., 2002; Gao and Ma, 2002; Ogata, 2002abc). To initiate angiogenesis, the balance between the positive and negative regulators is likely to be shifted such that angiogenic factors are up-regulated or inhibitory factors are decreased.

In foetal and adult human eye tissue, PEDF is expressed by the cornea, ciliary body, the RPE and cells of the inner and outer retina and ganglion cell layer (Karakoisis, 2001; Behling, 2002; Ogata *et al.,* 2002bc; Eichler *et al.,* 2004;). It is thought that PEDF secreted by these cells accumulates in avascular spaces of the eye such as the aqueous and vitreous humour and the interphotoreceptor matrix where it acts as a major inhibitor of angiogenesis (Karakousis, 2001; Behling, 2002).

PEDF is reported to inhibit retinal endothelial cell growth, migration and suppress ischaemia-induced retinal neovascularization (Mori 2001; Stellmach *et al.,* 2001; Duh *et al.,* 2002; Stellmach *et al.,* 2002; Yamagishi *et al.,* 2002, 2003; Duh *et al.,* 2003). In diabetic patients the levels of PEDF has been reported to be lower in the vitreous of patients with PDR (where new vessels are actively forming) than in healthy individuals or in diabetic patients whose vessels are quiescent (Ogata *et al.,* 2001a; 2002a,b,c; Colombo, 2002; Holekamp *et al.,* 2002). Retinal neovascularization, which was induced by hypoxia, was shown to stimulate a reduction in retinal PEDF and increases in VEGF protein and mRNA levels (Gao *et al.,* 2002; Eichler *et al.,* 2004).

AAV-mediated intraocular PEDF gene transfer has been found to increase cell survival after ischaemia-reperfusion injury of the rat retina and cause vessel regression in established neovascularization (Mori, 2001; Stellmach, 2001; Auricchio, 2002; Duh, 2002; Mori *et al.,* 2002ab; Raisler, 2002; Takita, 2003).

PEDF mRNA and protein have shown to be significantly upregulated in RPE cells and the retina after photocoagulation therapy (Ogata, 2001b; Schmidt-Erfurth *et al.,* 2002; Hattenbach *et al* 2005). Choroidal neovascular tissues, induced experimentally by panretinal laser photocoagulation, contain significant amounts of PEDF mRNA and protein (Ogata *et al.,* 2002c; Martin *et al.,* 2002). The expression of PEDF in these membranes is specifically localized to the proliferating RPE cells that cover the membranes. One reason that may account for the success of panretinal treatment is reducing neovascularization may be, in part, due to an upregulation of PEDF in proliferating RPE cells and its inhibitory effects on

VEGF-induced vessel outgrowth (Ogata *et al.,* 2001b). Matsuoka *et al.,* 2004 showed that PEDF and VEGF were strongly expressed in the vascular ECs and RPE cells in the CNVMs where numerous new vessels were prominent (Clinically active CNVMs). On the other hand, immunoreactivity for PEDF and VEGF was weak in the new vessels where fibrosis was prominent (clinically quiescent CNVMs). However, the RPE cells were still positive for PEDF and VEGF.

It has been suggested that PEDF prevents retinal endothelial cells from responding to ischaemic signals by an apoptopic mechanism (Bouck *et al.,* 2002). Addition of PEDF to the endothelial cultures markedly increased the number of apoptopic cells and apoptopic endothelial cells were more common in retina of hyperoxic animals treated with PEDF than those not receiving PEDF. Mori, 2002c showed that increased expression of PEDF causes regression of ocular neovascularization by promoting apoptosis of cells within neovascular lesions by use of AAV-mediated gene transfer of PEDF in a murine model of choroidal neovascularization. In the cornea, this apoptosis can be shown to be essential because PEDF fails to inhibit angiogenesis as the caspase enzymes required for apoptosis are prevented from functioning (Volpert, 2001).

Zhang *et al.,* 2005 hypothesized that the inhibition of retinal NV and permeability by PEDF is mediated by its anti-inflammatory activity. They showed that retinal and plasma PEDF levels were drastically decreased in rats with endotoxin-induced uveitis (EIU), which suggests that PEDF is a negative acute-phase protein. Intravitreal injection of PEDF significantly reduced vascular hyperpermeablity in rat models of diabetes and oxygen-induced retinopathy, correlating with decreased levels of retinal inflammatory factors, including VEGF, VEGFR-2, MCP-1, TNF-α, and ICAM-1. In cultured retinal capillary endothelial cells, PEDF significantly decreased TNF-α and ICAM-1 expression under hypoxia. Moreover, down-regulation of PEDF expression by siRNA resulted in significant increases of VEGF and TNF-α secretion in retinal Müller cells. These findings suggest that PEDF is a novel endogenous anti-inflammatory factor in the eye. The decrease of ocular PEDF levels may contribute to inflammation and vascular leakage in the eye.

Yamagishi *et al.,* 2006 showed that PEDF could inhibit the AGE-induced retinal vascular hyperpermeability and the mechanism by which it might achieve this beneficial effect. AGEs decreased retinal PEDF levels in rats. Treatment with PEDF inhibited the AGE-elicited VEGF-mediated permeability by down-regulating mRNA levels of p22phox and gp91phox, membrane components of NADPH oxidase, and subsequently decreasing retinal levels of an oxidative stress marker. PEDF also inhibited the AGE-induced vascular

hyperpermeablity evaluated by transendothelial electrical distance by suppressing VEGF expression. PEDF decreased reactive oxygen species (ROS) generation in AGE-exposed endothelial cells by suppressing NADPH oxidase activity via down-regulation of mRNA\levels of p22PHOX and gp91PHOX. This led to blockade of the AGE-elicited RAS activation and NF-κB-dependent VEGF gene induction in ECs. These results indicate that the central mechanism for PEDF inhibition of the AGE signalling to vascular permeability is by suppression of NADPH oxidase-mediated ROS generation and subsequent VEGF expression.

Gao, 2002 showed that intravitreal injection of plasminogen kringle 5 (K5), a potent inhibitor of ischaemia-induced retinal neovascularization (Zhang, 2001) downregulates VEGF, and upregulates PEDF in a dose-dependent manner in cultured retinal vascular cells and in the rat retina. Retinal RNA levels of VEGF and PEDF were also changed by K5.

1.7 THE CAVEOLAE SYSTEM

1.7.1 Introduction

Caveolae (or 'little caves') are 50-100 nm plasma membrane invaginations and were originally identified in the 1950's by electron microscopy (Palade, 1953, Yamada, 1955). They have a very unique lipid composition enriched in cholesterol and sphingolipids (Razani *et al.*, 2002. In many cell types, caveolae occur singularly or in chains or grape-like clusters. (Predescu *et al.*, 1994).

Caveolin (cav), a 21-24 kDa integral membrane protein, is a major protein component of caveolae and is necessary for the formation of invaginated caveolae (Rothberg, 1992; Van Deurs, 2003). Multiple members of the caveolin gene family have been identified (cav-1, cav-2, and -3) that differ in molecular structure and in tissue distribution (Razani *et al.*, 2002). Cav-1 has two isoforms: cav-1α and cav-1β which are translated from a different mRNA. Cav-2 has also been found to have multiple isoforms (α,β,γ) that were discovered in adipocytes (Fra, 1999; Fra, 2000). Among the three caveolins, cav-1 and -3 show high homology (Tang *et al.*, 1996) and either is sufficient for the formation of caveolae invaginations (Fra *et al.*, 1995; Li *et al.*, 1998; Park *et al.*, 2002). Cav-2 requires the presence of cav-1. It has to heterologomerize with cav-1, otherwise it remains trapped within the Golgi complex and is also degraded by cav-1 (Monier, 1995; Sargiocomeo, 1995; Scherer, 1997).

Caveolin is composed of cytoplasmic N and C termini and a central intramembrane domain (Carman *et al.*, 1999). The N-terminal region contains the caveolin scaffolding domain, which is essential for both the formation of caveolin oligomers and the interaction of caveolin with a range of other proteins (van Deurs, 2003). The membrane-spanning domain

(MS) forms a hairpin-like loop into the membrane but does not penetrate it. In addition, caveolin is attached to the lipid bilayer by three palmitoyl anchors in the C-terminal region.

Cav-1 null mice are viable but they lack cav-1 protein expression and plasmalemmal caveolae suggesting that cav-1 plays a vital role in caveolae biogenesis (Razani et al., 2001; Razani and Lisanti, 2001). Also analysis of cultured fibroblasts from cav-1 null embryos showed a loss of cav-2 protein expression. Therefore cav-1 expression is required to stabilize the cav-2 protein product.

Caveolae play a role in the internalization and transendothelial trafficking of solutes (Predescu *et al.,* 1994). Caveolar release from the plasma membrane is an important mode of endocytosis in ECs (Predescue *et al.,* 1993), and is the first step in migration of vesicles to the basal membrane (Schnitzer *et al.,* 1996; Oh *et al.,* 1998; Niles *et al.,* 1999; Minshall *et al.,* 2000). The vesicles detach from the plasmalemmal shuttle to the basal membrane where they fuse and release their contents, a process termed transcytosis (Predescu *et al.,* 1994; Ghitescu *et al.,* 1996; Milici *et al.,* 1997; Minshall *et al.,* 2000; Vogel *et al.,* 2001). Caveolae-mediated transcytosis is an important mechanism of transendothelial transport of albumin and delivery of albumin-conjugated nutrients, fatty acids, and hormones across the endothelial barrier (Anderson, 1998).

Caveolin-1 contains a so-called scaffolding domain that is thought to concentrate signalling molecules in caveolae and which binds to and inhibits the activity of several signalling molecules. This allows cav-1 to organize proteins in caveolae through protein-protein interactions, enabling finely tuned regulation of physiological responses (for example, Ca^{2+} entry and eNOS activation) [Drab, 2001].

Many signalling molecules present in caveolae play a role in adhesion, migration, and invasion. They include G-proteins, tyrosine kinases (src, Fyn), receptors for TGF-β type II, insulin, IGF-1, EGF, PDGF and VEGF, Angiopoietin, eNOS, and some components of the MAPK pathway. (Li *et al.,* 1995; Liu *et al.,* 1996; Mineo *et al.*, 1996; Liu *et al.,* 1997; Yamamoto *et al.,* 1998; Feng *et al.*, 1999a; Puyraimond 2001; Razani *et al* 2002; Huo *et al.,* 2003; Yoon *et al.*, 2003; Schwartz *et al.,*2005).

A large variety of in vitro and in vivo studies have implicated caveolae and caveolin in the pathogenesis of cancer, atherosclerosis and vasoproliferative diseases, cardiachypertrophy and heart failure, degenerative muscle dystrophies and diabetes mellitus (Schwencke *et al.,* 2006). Several studies have suggested that cav-1 plays a central role in angiogenesis (Liu *et al.,* 1999; Griffoni *et al.,* 2000;; Brouet *et al.,* 2001; Woodman *et al.,* 2003; Sonveaux *et al.,* 2004). However, it appears that a bimodal type of regulation seems to

characterise the role of cav-1 in angiogenesis. Cav-1 has been shown to mediate cell transformation, proliferation and capillary tubule formation (Kim *et al.*, 2002b; Griffoni, 2000). Increased expression of cav-1 and microvessel density was shown to correlate with metastasis and poor prognosis in renal cell carcinoma (Joo *et al.*, 2004) Conversely, other workers have shown that Cav-1 inhibits cellular proliferation (Hulit *et al.*, 2000; Razani *et al.*, 2000; Galbiati *et al.*, 2001) and prevents cell transformation (Engelman *et al.*, 1997; Galbiati *et al.*, 1998) and promotes cell-cycle arrest as well as senescence (Galbiati *et al.*, 2001; Volonte *et al.*, 2002). Caveolin-1 has also been shown to function as a tumour suppressor protein in a large variety of cellular settings (Quest *et al.*, 2004; Williams and Lisanti, 2005).

Endothelial-specific overexpression of cav-1 was shown to impair VEGF microvascular permeability and angiogenesis and inhibited the VEGFR-2-mediated angiogenic signalling cascade (Liu *et al.*, 1999). These defects were associated with negative regulation of the PI-3K/Akt/eNOS signalling module, consistent with the established inhibitory action of cav-1 on eNOS (Bucci *et al.*, 2000) and PI-3K activity (Zundel *et al.*, 2000).

Liu *et al.*, 2002 showed that cav-1 is down-regulated by endothelial growth factors (VEGF, PDGF, bFGF, HGF) that stimulate the initial proliferative stage of angiogenesis. This is consistent with the above studies showing that cav-1 is a negative regulator of cell proliferation and cell cycle progression. They also showed that cav-1 expression stimulated EC differentiation and tubule formation.

1.7.2 Systemic Expression of Caveolae and Caveolins

Caveolae can occur at different surface densities in different cell types and they exist most abundantly in terminally differentiated cells including endothelium, adipocytes, type I pneumocytes, fibroblasts, and smooth muscle cells (Kogo *et al.*, 2006) Caveolae and cav-1 are particularly abundant in ECs and comprise 95% of cell surface vesicles and ~15% of EC volume (Predescu *et al.*, 1993). There is a marked variation in caveolar surface density within a given cellular type, which is best exemplified in the case of the different types of endothelium (Simionescu *et al.*, 1974) or mesothelia (Von Ruhland *et al.*, 2004). In the endothelium, the largest population of caveolae occurs in the continuous type, while their numbers are much lower in the fenestrated to occasional caveolae in the endothelium of the discontinuous type (Simionescu *et al.*, 1974).

Cav-1 and cav-2 are co-expressed in most cell types (van Deurs *et al.,* 2002). Cav-1 and cav-2 are usually co-expressed and assembled into hetero-oligomers in the ER and Golgi complex (Scheiffele *et al.,* 1998) and these hetero-oligomeric complexes eventually reach the plasma membrane for caveolae biogenesis (Scherer *et al.,* 1997; Li *et al.,* 1998; Das *et al.,* 1999; Mora *et al.,* 1999; Parolini *et al.,* 1999; Lahtinen *et al.,* 2003; Sowa *et al.,* 2003). Cav-1 is expressed by ECs and pericytes in brain microvessels and in astrocytes surrounding microvessels where it may be involved in blood-brain barrier functioning and also supports coordinated activities between these cells (Virgintino, 2002).

Cav-3 (Way *et al.,* 1996) is expressed in skeletal muscle and myocardium where it is essential for the formation of caveolae (Hagiwara *et al.,* 2000; Galbiati *et al.,* 2001). It was also shown to be expressed in smooth muscle cells (Song *et al.,* 1996), astrocytes (Camero, 1997; Ikezu, 1998), chondrocytes (Nishiyama *et al.,* 1999; Schwab *et al.,* 1999) and sinus ECs (Uehara *et al.,* 2002).

1.7.3 Expression of Caveolae and Caveolins within the Eye

In the eye caveolae were shown to be associated with retinal ECS (Feng *et al.,* 1999b; Kim *et al.,* 2006) and cav-1 has been localized to the RPE (Bridges, 2001). In addition, ROS-derived detergent-resistant membranes (DRMS), and photoreceptor synaptic ribbons (Kachi 2001; Elliot *et al.,* 2003; Martin *et al.,* 2005) were shown to be enriched in cav-1 where it was suggested that it may participate in phototransduction (Seno *et al.,* 2001). Caveolae, cav-1 and cav-2 have also been demonstrated in the lens (Lo and Zhang, 1989; Lo *et al.,* 1998, Lo *et al.,* 2003; Lin *et al.,* 2003; Rujoi *et al.,* 2003; Lo *et al.,* 2004; Sexton *et al.,* 2004). It was shown to co-immunprecipitate with PKCγ and connexins 46 and 50 and was suggested to protect against oxidative stress which can trigger cataractogenesis. Sexton *et al.,* 2004 also suggested that in the lens cav-1 may play possible roles in cholesterol trafficking, cell to cell communication and signal transduction. Cav-3 expression was shown in the papillary sphincter smooth muscle cells but not the ciliary muscle and papillary dilator muscle (Kogo *et al.,* 2006). Cav-1 was detected in the ciliary smooth muscle cells and the papillary sphincter muscle cell, but not in the papillary dilator and ciliary muscle. The localisation of caveolin at the cell membrane of corneal epithelium has previously been reported in the context of wound healing (Amino *et al.,* 1997).

Western blot analysis showed that cav-1 and -2 are present in the rat retina (Kim *et al.,* 2006). Immnohistochemistry indicated that cav-1 was expressed in the majority of the retinal layers, including the GCL, IPL, OPL, and vascular endothelial cells of the retina. Cav-

2 was primarily immunostained in the vessels, but in a few other elements as well. This was the first demonstration of caveolin differential expression in the retina of rats, and suggests that caveolin plays an important role in signal transduction in glial cells and neuronal cells. Russelakis-Carneiro *et al.,* 2004 demonstrated cav-1 expression in the neuronal cell bodies of the retina and in axons of the optic nerve in mice.

Stitt, 2000 showed that AGES bind to receptors in caveolin-rich membrane fractions and are internalized within caveolae organelles in retinal microvascular ECs. In a human retinal endothelial cell line, ECV 304, exposure to VEGF, and other growth factors resulted in a reduced endothelial cell expression of cav-1 which was mediated by a negative feedback mechanism through the VEGFR-2 receptor and subsequent downstream p4/44 MAP kinase pathway (Liu *et al.,* 1999). It can therefore be proposed that cav-1 protein contributes in a unique manner to the angiogenic process in PDR, i.e. growth factors stimulate relaxation of cav-1's action as a 'molecular brake' allowing growth-factor-induced endothelial cell proliferation.

1.8 AIMS

The hypothesis of this study is that spatial and temporal differences in angiogenic growth factors and associated mediators are critical in the progressive stages that result in diabetic retinopathy. Although it has long been established that VEGF is a primary candidate in the pathology of diabetic retinopathy, limited information is available on the exact cellular location of VEGF, VEGF-C and the VEGF receptors in diabetic retina. Furthermore the role, if any, of the angiopoietins and PEDF in diabetic retinopathy needs to be further investigated. Caveolae play an important role in the compartmentalization of growth factor signalling molecules. Consequently, the following studies were undertaken to define these issues.

1) To examine diabetic retinas and categorize the tissue, by clinical and histological examination, into groups relating to the different stages of diabetic retinopathy

2) To perform immunohistochemistry on non-diabetic retinas, diabetic retinas and fibrovascular membranes for:

- a) VEGF and VEGF-C;
- b) The receptors which bind VEGF and VEGF-C;
- c) Ang-1 and Ang-2;
- d) Tie-2, the receptor for Ang-1 and Ang-2.
- e) TNF-α
- f) PEDF
- g) Caveolins-1, -2, and -3

3) Examine the immunostained retinas and fibrovascular membranes by light microscopy and evaluate the intensity of staining semi-quantitatively.

CHAPTER 2. MATERIALS AND METHODS

2.1 MATERIALS AND SOLUTIONS

2.1.1 Chemical Reagents and Solutions

All chemical reagents and antibodies are listed in appendix I together with their source. The constituents of solutions are provided in appendix II.

2.1.2 Human Tissue

Fifty Nine human eyes enucleated and fixed in 10% Neutral buffered formalin (NBF- see Appendix II) within 12 hr post-mortem, were obtained from the National Disease Research Interchange (NDRI), Philadelphia, USA. All eyes, unless stated, were normal and had no known pathological disease.

2.2 METHODS

2.2.1 Clinical assessment of donor eyes

The anterior segment was removed and examination of the posterior segment was performed by Professor David McLeod, a Consultant Ophthalmologist at the Manchester Royal Eye Hospital, using a Zeiss Stemi SV8 zoom dissecting microscope with Schott light source a) to note overt features of retinopathy (e.g. the presence of pre-retinal membranes, cotton wool spots, microaneurysms etc.) and b) to determine the extent of any scatter photocoagulation.

2.2.2 Categorisation of donor eyes

Donor eyes were divided into those with no obvious signs of diabetic abnormalities and those with diabetes. Based on the medical records available and on clinical assessment by stereomicroscopy the diabetic eyes were further divided into four groups. They were categorised as either diabetic with no overt retinopathy, diabetic with intra-retinal changes but no evidence of PDR, diabetic with active proliferative retinopathy or diabetic with scatter laser photocoagulation but no evidence of residual PDR.

2.2.2.1 Non-diabetic eyes

This group contained fourteen human eyes with no known ophthalmic disease, no history of diabetes and no abnormalities on stereomicroscopy. Donors ranged in age from 20 to 92 years (mean 56 years).

2.2.2.2 Diabetic with no overt retinopathy

This group contained twelve human eyes from diabetic donors with no clinical history and no overt macroscopic features of retinopathy or retinal photocoagulation. Donors ranged in age from 44 to 89 years (mean 74 years). A complete medical history was unavailable for all donors but, in those where medical histories were known, the duration of diabetes was between 6 and 25 years.

2.2.2.3 Diabetic with intra-retinal changes but no evidence of PDR

This group contained ten human eyes from diabetic donors with intra-retinal changes on stereomicroscopy but no clinical history and no overt macroscopic features of PDR or retinal photocoagulation. Retinas exhibited cotton wool spots and/or obvious microaneurysms or haemorrhages. Donors ranged in age from 55-96 years (mean 71 years). A complete medical history was unavailable for all donors but, in those where medical histories were known the duration of diabetes was between 3 and 21 years.

2.2.2.4 Diabetic with preretinal PDR

This group contained nine human eyes from diabetic donors defined clinically as having PDR and exhibiting preretinal membranes when examined by stereomicroscopy. All eyes had previously received laser photocoagulation. Donors ranged in age from 37-76 years (mean 58 years). Duration of diabetes ranged from 3-18 years (mean 9 years).

2.2.2.5 Diabetic with scatter laser photocoagulation but no evidence of residual PDR

This group contained fourteen human eyes from diabetic donors defined clinically as having had PDR and having received scatter laser photocoagulation (no details were available as to time post laser). No preretinal membranes could be observed when retinas were examined by stereomicroscopy. Donors ranged in age from 41-82 years (mean 63 years). The duration of diabetes ranged from 3 to 35 years (mean 17 years).

2.2.3 Dissection of donor eyes

The posterior segment of each eye was cut in the saggital plane through the centre of the optic nerve head. Cuts were then made perpendicular to this line a) on the horizontal midline on the nasal side and b) at approximately 5mm above and below the midline on the temporal side. A final vertical cut was made parallel to the initial cut and approximately 3mm lateral to the macula. For this study tissue was selected from a portion of retina/choroid/sclera

approximately 3mm lateral to the macula and perpendicular to the horizontal plane (this region was chosen owing to its susceptibility to retinal changes associated with diabetes). Tissue was also selected from an area adjacent to the macula region in one eye where a pre-retinal membrane was observed by stereomicroscopy.

2.2.4 Fibrovascular membranes

Seventeen fibrovascular pre-retinal membranes, which had been excised at vitreous surgery from eyes with PDR, were obtained from the Manchester Royal Eye Hospital. Membranes were fixed in 10% NBF immediately upon removal for a minimum of 12 hr before wax embedding.

2.2.5 Preparation of retinal tissue for wax sectioning

Retinal tissue which had been fixed for a minimum of 24 hr in 10% NBF was dehydrated through a graded series of alcohol concentrations (v/v distilled H_2O) as follows: 50% alcohol for 30 min, 70% alcohol for 1 hr, 90% alcohol for 1 hr, 2 changes of 100% alcohol for 1 hr each. Dehydrated tissue was then immersed in 50% chloroform (v/v alcohol) for 30 min, 100% chloroform for 1 hr followed by 100% chloroform for 30 min and then embedded in wax for 1.5 hr, using a wax embedding system. The wax blocks were trimmed and 200 serial sections of 7μm thickness were cut on a Microm HM330 microtome. Every 50th section was placed into a warm water bath, allowed to flatten at 47-50°C and then transferred onto microscope slides. The slides were dried in an oven at 56°C overnight to allow the sections to adhere to the slides.

2.2.6 Preparation of fibrovascular membranes for wax sectioning

Fibrovascular membranes which had been fixed for a minimum of 12 hr in 10% NBF were dehydrated through a graded series of alcohol concentrations (v/v distilled H_20) as follows: 25% alcohol for 15 min, 33% alcohol for 15 min, 50% alcohol for 15 min, 63% alcohol for 15 min, 70 % alcohol for 30 min, 80% alcohol for 30 min, 90% alcohol for 30 min, 2 changes of 100% alcohol for 30 min each. Dehydrated tissue was then immersed in 25% chloroform (v/v alcohol) for 15 min, 33% chloroform for 15 min, 50% chloroform for 30 min followed by 2 changes of 100% chloroform for 30 min and then immersed in wax for 20 min. The wax blocks were trimmed and serial sections, throughout the whole block, of

5μm thickness were cut. Every 20th section was placed into a warm water bath, allowed to flatten at 47-50°C and then transferred onto microscope slides. The slides were dried in an oven at 56°C overnight to allow the sections to adhere to the slides.

2.2.7 Haematoxylin and eosin staining of wax sections

Sections of retina, and fibrovascular membrane which had been mounted on slides were dewaxed by immersion in two baths of xylene and rehydrated through a graded series of alcohol concentrations (v/v distilled H_2O), 5 min in each of 100% alcohol (twice), 90% alcohol, 70% alcohol and 50% alcohol, followed by immersion in cold running water. After excess alcohol had been removed the slides were submerged in a solution of Harris haematoxylin for 3 min then washed in cold running H_2O until the dye turned blue. Sections were then submerged in eosin for 1 min, briefly washed in cold running H_2O and then dehydrated by immersing them quickly through an alcohol series (the reverse order of that above) followed by two washes with xylene. A coverslip was secured onto each section using Practamount adhesive which was allowed to set by drying overnight in a 60°C oven.

2.2.8 Immunohistochemical studies

2.2.8.1 Selection of sections for immunostaining

Once the sections of retina, and fibrovascular membranes had been stained by haematoxylin and eosin (H and E) they were examined by light microscopy. Non-diabetic retinal sections and diabetic retinal sections showing no overt signs of retinopathy and sections which had overt features of retinopathy, such as pre-retinal membranes, exudates and haemorrhages, were mounted onto 1% Aminopropyltriethoxysilane (APES-see appendix II) coated slides in preparation for immunostaining (sections were selected which were adjacent to the corresponding H and E stained section). Sections of fibrovascular membranes were examined by light microscopy and where blood vessels and a large area of tissue were present adjacent sections were mounted onto 1% Apes coated slides in preparation for immunostaining.

2.2.8.2 General protocol for immunostaining of sections

Sections were dewaxed and rehydrated as previously described (section 2.2.7). Sections were exposed to proteolytic pre-digestion, dependent upon the primary antibody to be used, for between 20 minutes and 30 minutes at 37°C/or in the pressure cooker for 3 minutes (see table 1 and appendix II). Sections were covered for between 20 min and 1 hr at room temperature with the appropriate blocking agent (containing serum from the appropriate secondary antibody host) to block non-specific binding. Sections were washed three times in Tris buffered saline (TBS) [see appendix II]. Primary antibodies were diluted to an appropriate dilution in TBS with 0.2% serum (dilution determined by a dilution series experiment), and added to all the sections, except the negative controls (which received TBS with 0.2% serum) and incubated at 4°C overnight. Slides were washed three times in TBS before addition of the appropriate secondary antibody (biotin conjugated) for 1 hour. After washing three times in TBS, an avidin/biotin complex (ABC) [see appendix II] was applied for 30 min to enhance the antibody signal. After washing the slides three times in TBS a Fast Red substrate or diaminobenzidine (caveolin-1) (see appendix II) for the avidin/biotin complex was filtered and added to the sections to visualise the sites of antibody binding. After washing in running water to quench the substrate reaction slides were counterstained by immersion in a standard solution of Mayers haematoxylin for 10 seconds. Sections were placed in running cold water, air-dried, and coverslips applied using Loctite 358 ultra violet (UV) curing adhesive, which was then sealed by exposure to UV light for 3min.

Primary Ab	**Host**	**Pre-treatment**	**Blocking Agent**	**Secondary Ab**
VEGF165 (1/20)	Goat	0.1% chymotrypsin	10% Milk proteins/10% Rabbit serum	Rabbit anti-goat biotin conjugate
VEGF-C (1/100)	Goat	0.1% Chymotrypsin	10% Milk proteins/10% Rabbit serum	Rabbit anti-goat biotin conjugate
Ang-1 (1/50)	Goat	0.1% Chymotrypsin	Rabbit serum	Rabbit anti-goat biotin conjugate
Ang-2 (1/50)	Goat	0.1% Chymotrypsin	Rabbit serum	Rabbit anti-goat biotin conjugate
VEGFR-1 (1/100)	Rabbit	None	10% Milk proteins/10% Goat serum	Goat anti-rabbit biotin conjugate
VEGFR-2 (1/50)	Rabbit	None	10% Milk proteins/10% Goat serum	Goat anti-rabbit biotin conjugate
VEGFR-3 (1/100)	Rabbit	None	10% Milk proteins/10% Goat serum	Goat anti-rabbit biotin conjugate
Tie-2 (1/50)	Rabbit	0.1% Chymotrypsin	Goat serum	Goat anti-rabbit biotin conjugate
TNF-α (1/25)	Goat	Pressure Cooker	Rabbit serum	Rabbit anti-goat biotin conjugate
PEDF (1/300)	Rabbit	None	Pig serum	Pig anti-rabbit biotin conjugate
Caveolin-1 (1/10)	Mouse	0.2% Triton X-100	Goat serum	Goat anti-mouse biotin conjugate
Caveolin-2 (1/10)	Mouse	0.2% Triton X-100	Goat serum	Goat anti-mouse biotin conjugate
Caveolin-3 (1/10)	Mouse	0.2% Triton X-100	Goat serum	Goat anti-mouse biotin conjugate

Table 2.1 The pre-treatments, blocking agents and secondary antibodies used in the immunostaining for growth factors and their receptors, PEDF, TNF-α, Caveolin-1, -2, and-3

2.2.8.3 Assessment of immunostaining

The degree and pattern of immunostaining both within and between specimens, as observed by standard light microscopy, was assessed by two independent observers (both of which obtained similar results - see appendix V). The intensity of staining was graded qualitatively as background (corresponding to the level of staining seen in the negative controls), weak, moderate, or intense (corresponding to the highest level of immunoreactivity observed). These intensities were recorded as 0, 1, 2, and 3 respectively. For each retinal specimen staining intensity was recorded for photoreceptors, outer retina, inner retina, GCL and retinal vessels. For the fibrovascular membranes staining intensity was recorded for the vessels and the surrounding matrix. An average score was then calculated for each retinal layer within each group.

2.2.8.4 Statistical Analysis

Differences between the retinal layers across tissue catagories was assessed using CHI squared statistical analysis (SPSS 12). Values of less than 0.05 were taken to be significant.

CHAPTER 3 HISTOLOGICAL CATEGORIZATION OF NON-DIABETIC AND DIABETIC HUMAN RETINAS AND FIBROVASCULAR MEMBRANES

3.1 INTRODUCTION

59 retinas were stained with haematoxylin and eosin and examined by light microscopy. Each retina was put into a category depending upon whether they were non-diabetic (see table 3.1) or whether they were diabetic and showed any intraretinal microvascular abnormalities associated with diabetic retinopathy (see tables 3.2 to 3.5). The retinas were categorized as either, non-diabetic, unlasered diabetic retinas with no obvious microvascular abnormalities, unlasered diabetic retina with obvious microvascular abnormalities, diabetic retina with PDR or lasered retinas without any obvious microvascular abnormalities. 17 excised fibrovascular membranes were also stained with haematoxylin and eosin to determine if neovessels were absent or present (see table 3.6).

3.2 Haematoxylin and Eosin Staining of Non-diabetic Retinas

None (14/14) of the non-diabetic retinas showed any microvascular changes associated with NPDR or any neovascular membranes associated with PDR (see figure 3.1). None of the retinas examined showed any indication of having previously received photocoagulation treatment. Donor details and the results from haematoxylin and eosin staining are shown below (see table 3.1).

Table 3.1 Non-diabetic Retinas

Donor Number	Sex	Age	Time Post-Mortem (hours)	Findings On Stereomicroscopy	Findings On H and E
1	M	79	1	No microvascular Abnormalities	No microvascular abnormalities
2	M	88	2	No microvascular Abnormalities	No microvascular abnormalities
3	F	75	3	No microvascular Abnormalities	No microvascular abnormalities
4	M	82	2	No microvascular Abnormalities	No microvascular abnormalities
5	F	63	10.5	No microvascular Abnormalities	No microvascular abnormalities
6	F	20	9	No microvascular Abnormalities	No microvascular abnormalities
7	F	89	1	No microvascular Abnormalities	No microvascular abnormalities
8	F	92	6	No microvascular Abnormalities	No microvascular abnormalities
9	M	49	12	No microvascular Abnormalities	No microvascular abnormalities
10	M	22	5	No microvascular Abnormalities	No microvascular abnormalities
11	M	34	23	No microvascular Abnormalities	No microvascular abnormalities
12	F	57	6	No microvascular Abnormalities	No microvascular abnormalities
13	M	78	4	No microvascular Abnormalities	No microvascular abnormalities
14	F	27	8.25	No microvascular Abnormalities	No microvascular abnormalities

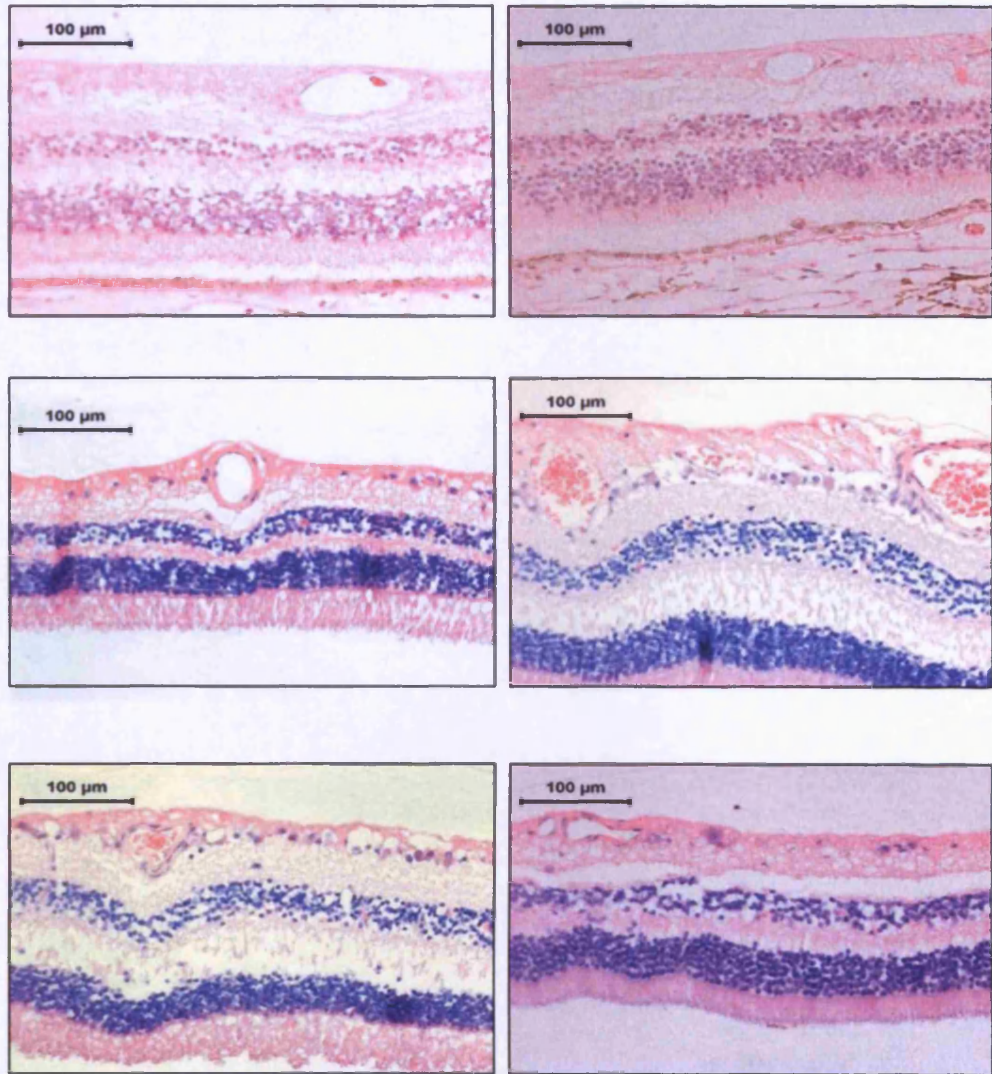


Figure 3.1. H and E Staining of Non-Diabetic Retinas

3.3 Haematoxylin and Eosin Staining of Diabetic Retinas

12/45 of the diabetic retinas examined by light microscopy had no obvious signs of diabetic retinopathy and no indication of having previously received photocoagulation treatment and were placed into the category of unlasered diabetic retinas with no obvious microvascular abnormalities (see table 3.2; figure 3.2). 10/45 of the diabetic retinas examined by light microscopy had microvascular changes associated with NPDR, such as the presence of exudates and gross basement membrane thickening, and were placed into the category of unlasered diabetic retinas with obvious microvascular abnormalities (see table 3.3; figure 3.3). 9/45 of the diabetic retinas had neovascular membranes on their surfaces and were placed into the category of diabetic retinas with PDR (see table 3.4; figure 3.4). 14/45 of the diabetic retinas which had previously had PDR showed evidence of laser treatment but no evidence of NPDR or PDR and were placed into the category of lasered retinas without any obvious microvascular abnormalities (see table 3.5; figure 3.5).

Table 3.2 Unlasered Diabetic Retinas with No Obvious Microvascular Abnormalities

Donor Number	Sex	Age	Time Post-Mortem (hours)	Type Of Diabetes	Duration Of Diabetes (years)	Lasered	Findings On Stereomicroscopy	Findings On H and E
15	M	77	5	Type 2	9	No	No microvascular Abnormalities	No microvascular abnormalities
16	F	82	4	Type2	10	No	No microvascular Abnormalities	No microvascular abnormalities
17	F	89	3	Type 2	6	No	No microvascular Abnormalities	No microvascular abnormalities
18	M	57	11	Type 2	5	No	No microvascular Abnormalities	No microvascular abnormalities
19	M	57	11	Type 2	5	No	No microvascular Abnormalities	No microvascular abnormalities
20	F	76	12	Type 1	8	No	No microvascular Abnormalities	No microvascular abnormalities
21	M	88	2	Type 1	10	No	No microvascular Abnormalities	No microvascular abnormalities
22	M	75	6	Type 2	6	No	No microvascular Abnormalities	No microvascular abnormalities
23	F	82	3	Type 2	6	No	No microvascular Abnormalities	No microvascular abnormalities
24	M	69	3.5	Type 1	5	No	No microvascular Abnormalities	No microvascular Abnormalities
25	F	75	10	Type 1	7	No	No microvascular Abnormalities	No microvascular Abnormalities
26	M	74	3	Type 2	5	No	No microvascular Abnormalities	No microvascular Abnormalities

Table 3.3 Unlasered Diabetic Retinas with Obvious Microvascular Abnormalities

Donor Number	Sex	Age	Time Post-Mortem (hours)	Type Of Diabetes	Duration Of Diabetes (years)	Lasered	Findings On Stereomicroscopy	Findings On H and E
27	F	96	3	Type 1	8	No	Cotton wool Spots	Exudates, basement membrane thickening
28	M	84	11	Type 2	6	No	No microvascular abnormalities	Haemorrhages exudates, basement membrane thickening
29	M	68	5	Type 2	3	No	Cotton wool spots	Basement membrane thickening
30	F	62	4.5	Type 1	15	No	Haemorrhages	Haemorrhages
31	F	62	6	Type 1	21	No	White lesions	Haemorrhages exudates, basement membrane thickening
32	F	48	3	Type 1	10	No	No microvascular Abnormalities	Exudates, basement membrane thickening
33	M	83	2.5	Type 1	30	No	No microvascular abnormalities	Basement membrane thickening
34	M	33	10	Type 1	23	No	No microvascular abnormalities	Basement membrane thickening
35	M	33	10	Type 1	23	No	No microvascular abnormalities	Basement membrane thickening
36	F	50	12	Type 1	40	No	No microvascular Abnormalities	Basement membrane thickening

Table 3.4 Diabetic Retinas with Proliferative Diabetic Retinopathy

Donor Number	Sex	Age	Time Post-Mortem (hours)	Type Of Diabetes	Duration Of Diabetes (years)	Lasered	Findings On Stereomicroscopy	Findings On H and E
37	M	53	2	Type 2	10	No	Pre-retinal vessels	Pre-retinal vessels
38	F	73	5	Type 2	6	No	Pre-retina vessels, microaneurysms	Pre-retinal vessels
39	F	76	8	Type 1	3	No	Haemorrhages, pre-retinal vessels	Pre-retinal vessels
40	M	37	6	Type 1	5	No	Exudates, haemorrhages, pre-retinal Vessels	Pre-retinal vessels
41	F	47	3.25	Type 1	18	No	Oedema, cotton wool spots	Pre-retinal vessels
42	M	55	2.5	Type 1	21	No	Exudates, pre-retinal vessels	Exudates, pre-retinal vessels
43	M	55	2.5	Type 1	21	No	Exudates, pre-retinal vessels	Exudates, pre-retinal vessels
44	M	41	26	Type 1	23	No	Pre-retinal vessels	Exudates, basement membrane thickening, pre-retinal vessels
45	M	81	16.5	Type 1	30	No	No microvascular Abnormalities	Pre-retinal vessels

Table 3.5 Lasered Retinas Without Any Obvious Microvascular Abnormalities

Donor Number	Sex	Age	Time Post-Mortem (hours)	Type Of Diabetes	Duration Of Diabetes (years)	Lasered	Findings On Stereomicroscopy	Findings On H and E
46	M	55	4	Type 2	35	Yes	No microvascular Abnormalities	No microvascular abnormalities
47	M	76	7	Type 1	25	Yes	No microvascular Abnormalities	No microvascular abnormalities
48	M	55	12	Type 1	24	Yes	No microvascular Abnormalities	No microvascular abnormalities
49	F	41	4	Type 1	29	Yes	No microvascular Abnormalities	No microvascular abnormalities
50	F	41	4	Type 1	29	Yes	No microvascular Abnormalities	No microvascular abnormalities
51	F	47	8	Type 1	16	Yes	No microvascular Abnormalities	No microvascular abnormalities
52	F	66	5	Type 2	12	Yes	No microvascular Abnormalities	No microvascular abnormalities
53	F	66	5	Type 2	12	Yes	No microvascular Abnormalities	No microvascular abnormalities
54	F	55	18	Type 1	15	Yes	No microvascular Abnormalities	No microvascular abnormalities
55	M	61	14	Type 2	20	Yes	No microvascular Abnormalities	No microvascular abnormalities
56	M	82	12	Type 1	8	Yes	No microvascular Abnormalities	No microvascular abnormalities
57	F	40	19	Type 2	20	Yes	No microvascular Abnormalities	No microvascular abnormalities
58	M	55	2	Type 1	12	Yes	No microvascular Abnormalities	No microvascular abnormalities
59	M	53	2	Type 2	10	Yes	No microvascular Abnormalities	No microvascular abnormalities

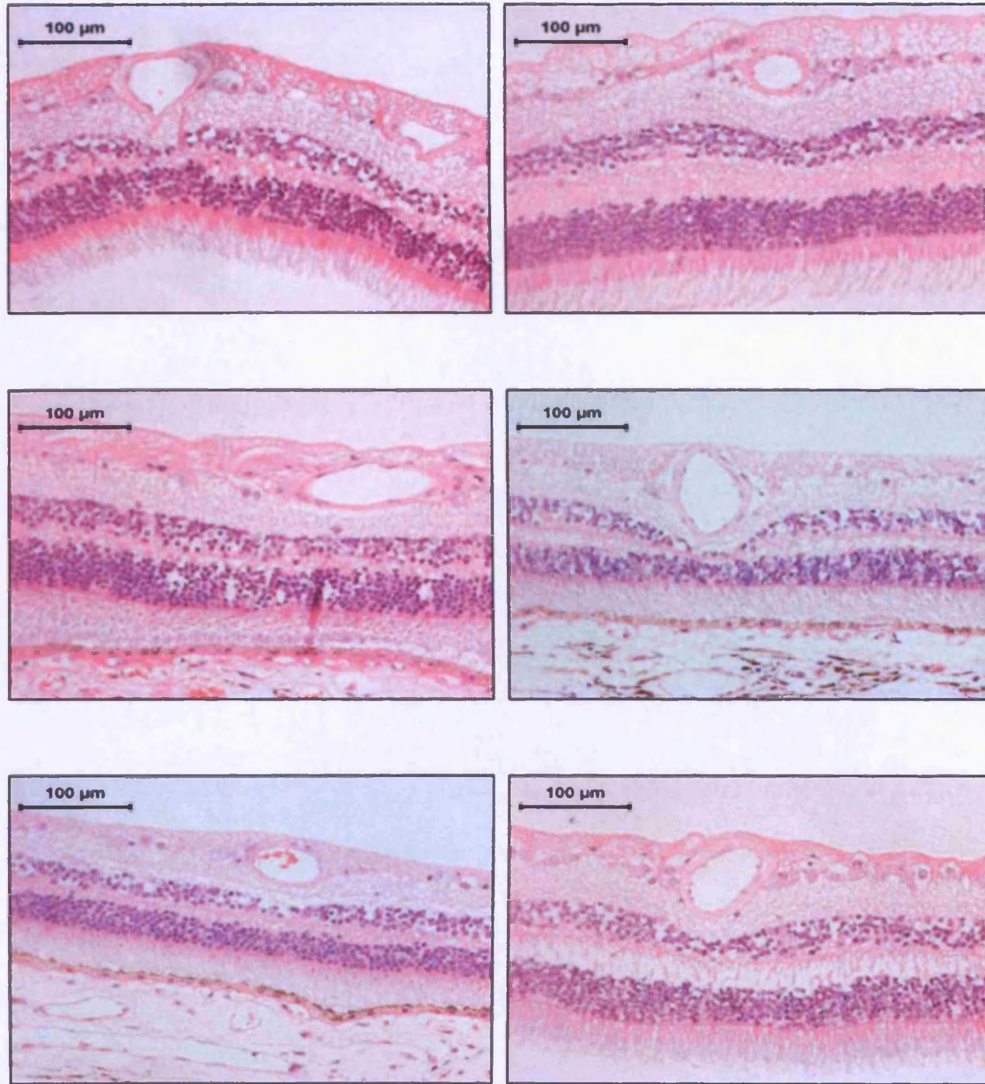


Figure 3.2. H and E staining of Unlasered Diabetic Retinas with No Obvious Microvascular Abnormalities

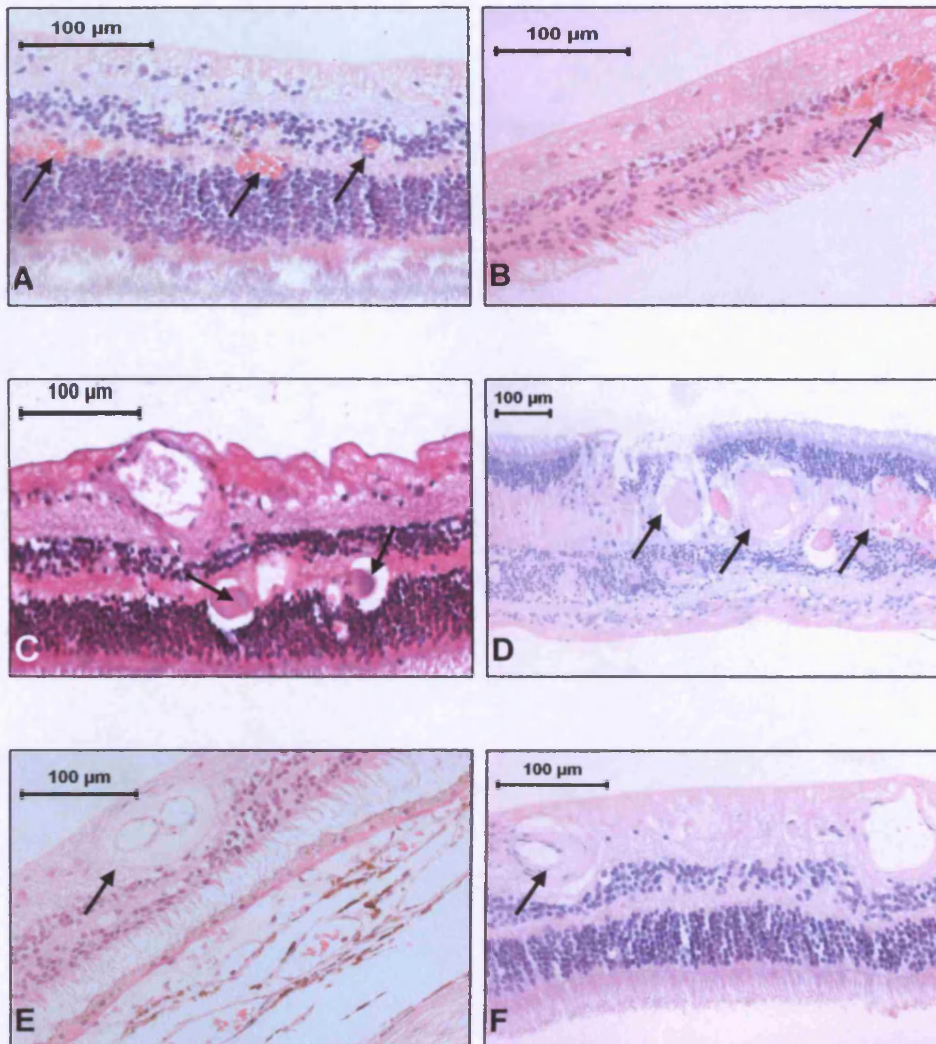


Figure 3.3. H and E Staining of Diabetic Retinas With NPDR
Intraretinal haemorrhages (A, B), exudates (C,D) and BM thickening were observed (E,F). Arrows show location of microvascular abnormalities

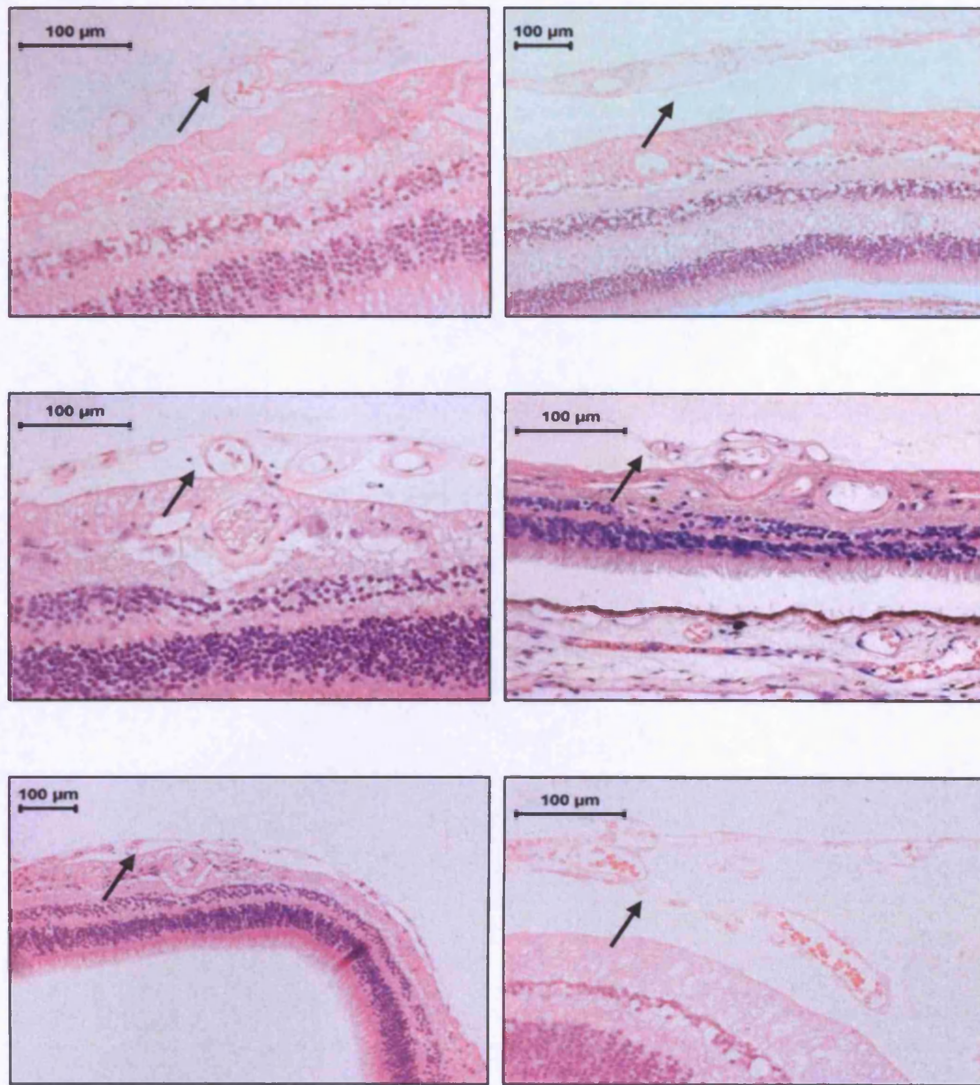


Figure 3.4 H and E Staining of Diabetic Retinas With PDR
Arrows show localisation of preretinal membranes

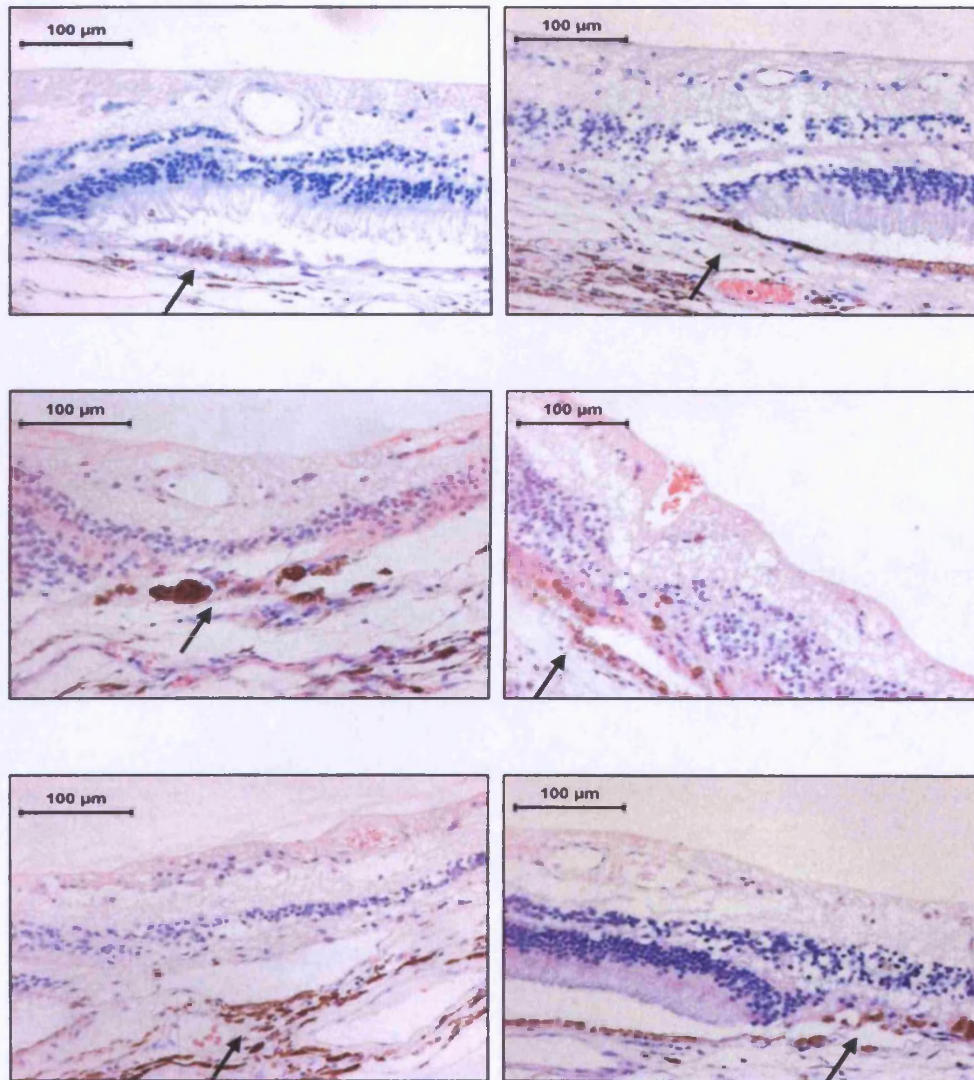


Figure 3.5 H and E Staining of Lasered retinas. Arrows show area of laser burns

3.4 Haematoxylin and Eosin Staining of Fibrovascular Membranes

All (17/17) of the fibrovascular membranes contained blood vessels (see figure 3.6). Donor details are shown below. Medical histories for some donors were not provided (see table 3.6).

Table 3.6 Fibrovascular Membranes

Donor Number	Sex	Age	Neovessels Present
B1a	F	44	Yes
B1b	F	44	Yes
B2a	M	46	Yes
B2b	M	46	Yes
B6	M	27	Yes
B8	M	61	Yes
B9	M	59	Yes
B10	M	23	Yes
B11a	M	36	Yes
B11b	M	36	Yes
B12	M	39	Yes
B13	M	45	Yes
1447	N/A	N/A	Yes
1448	N/A	N/A	Yes
1450	N/A	N/A	Yes
1451	N/A	N/A	Yes
1552	N/A	N/A	Yes
1519	N/A	N/A	Yes
1520	N/A	N/A	Yes
2254	N/A	N/A	Yes
2255	N/A	N/A	Yes
2256	N/A	N/A	Yes
1454	N/A	N/A	Yes

N/A = No Medical Histories Available

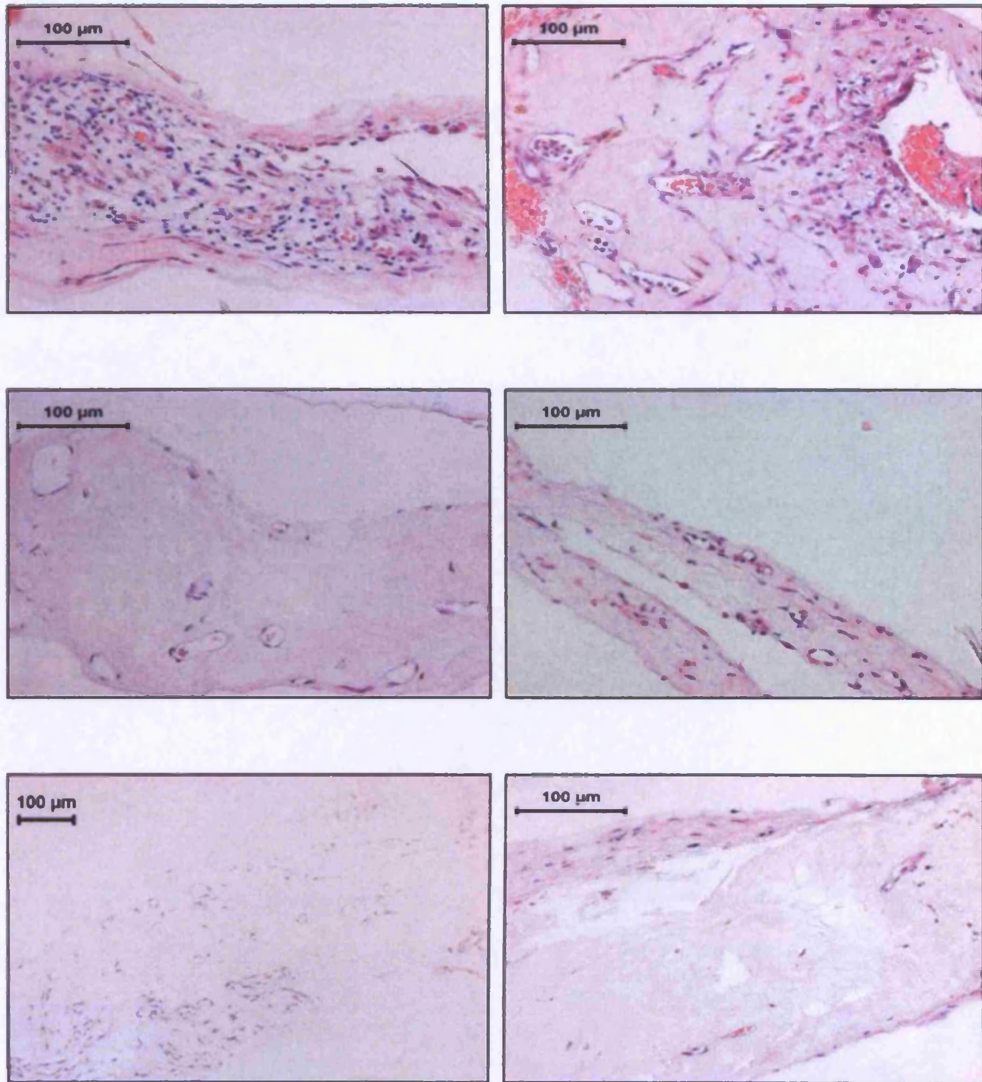


Figure 3.6 H and E Staining of Fibrovascular Membranes

3.5 DISCUSSION

Damage to the retinal microvasculature is a feature of many eye diseases including diabetic retinopathy and some degree of retinopathy occurs in nearly all patients with diabetes of greater than 20 years duration (Klein *et al.*, 1984). Whether retinopathy appears or not depends mainly on the duration of the disease and the degree of metabolic control the patient maintains. Glycaemia-related vascular damage has been hypothesized to be mediated through various biochemical pathways including the hexosamine pathway, the advanced glycation end-product formation pathway, and the diacylglycerol (DAG)-protein kinase C (PKC) pathway (Jawa *et al.*, 2004).

In this study it was important that donors who had previously not been diagnosed with diabetes in their lifetime, be examined for signs of microvascular abnormalities associated with diabetes. This is because it has become increasingly recognized that typical lesions of DR (microaneurysms and haemorrhages) are commonly seen in individuals without clinically diagnosed diabetes (Nguyen and Wong, 2006). However the findings from this study, from stereomicroscopy and from examination of H and E sections, demonstrated that none of the 'non-diabetic' donors showed signs of retinopathy.

I then examined diabetic donors whose medical histories indicated that they had been diagnosed with diabetes but not with DR and whose retinas showed no obvious microvascular abnormalities when viewed by stereomicroscopy. Examination of the H and E sections was consistent with these findings. However, only a small sample of the retina from the macula region was examined by H and E staining as I only stained every 50th section from a total of 200 serial sections. Therefore to get a true picture of what is happening in each retina, it would have been more beneficial to examine every section throughout the macula sample and to take serial sections through all other regions of the retina (the same could apply to the non-diabetic retinas too). Tang *et al.*, 2003 showed that microaneurysms, acellular capillaries and pericyte ghosts were more numerous in the temporal retina than the nasal retina in retinal whole mounts of diabetic patients. However, this does not rule out the presence of microvascular abnormalities in regions other than the macula.

NPDR is characterized by loss of pericytes around capillaries in the retina. This is followed by development of weakness in the capillary wall that leads to capillary aneurysm formation (microaneurysm) and fluid leakage and haemorrhages from capillaries as their walls become more permeable. An increase in the number of microaneurysms is considered a risk sign for progression of retinopathy as well as excessive permeability of the retinal vessels. (Kohner and Sleightholm, 1986; Klein *et al.*, 1995; Ferris *et al.*, 1999). Fluid leakage

can range from microexudates and infiltrating protein or lipid exudates (Davis, 1992; Chew *et al.*, 1996) to the most severe form, macular oedema, which can seriously reduce vision (Ferris and Patz, 1984). Cotton wool spots are also seen which represent stasis of axoplasmic flow due to ischaemia of the nerve fibre layer (Palmberg, 1977; Early Treatment Diabetic Retinopathy Study Research Group, 1991).

In this study I demonstrated that microvascular abnormalities that were present in H and E sections were not always obvious by stereomicroscopy. This emphasises the importance of undertaking H and E staining and the examination of the sections by microscopy. Cotton wool spots and haemorrhages were seen by stereomicroscopy but only basement membrane thickening and exudates could be observed by light microscopy. I also observed haemorrhages with H and E staining.

Histological analysis has also demonstrated the presence of intraretinal haemorrhages, and exudates in several animal models of NPDR (Tolentino *et al.*, 1996; Kim *et al.*, 2004; Kakehashi *et al.*, 2006; Van Eden *et al.*, 2006). Electron, confocal and light microscopy techniques have shown increased BM thickening in numerous diabetic animal models (Fischer *et al.*, 1981; Itabashi *et al.*, 1981; Altshuler and Orney, 1986; Chakrabarti and Sima, 1987; Cuthbertson and Mandel, 1987; Diani *et al.*, 1987; Robinson *et al.*, 1988; Marion and Carlson, 1989; Copeland *et al.*, 1990; Carlson *et al.*, 1997; Miyamura *et al.*, 1999; Hainsworth *et al.*, 2002; Carlson *et al*, 2003; Gardiner *et al.*, 2003; Joussen *et al.*, 2004; Yatoh et al., 2006). BM thickness was shown to increase at longer duration of diabetes compared with age-matched controls (Feit-Leichman *et al.*, 2005). In addition, ultrastructural comparisons of BMs in a variety of tissues from diabetic and normal human subjects have been carried out (Danowski *et al.*, 1972; Kilo *et al.*, 1972; Dunn *et al.*, 1979; Fischer *et al.*, 1979; Jackson *et al.*, 1982; Johnson *et al.*, 1982; Raskin *et al.*, 1983; Sosenko *et al.*, 1984; Feingold *et al.*, 1986, 1989; Osterby, 1990; Osterby *et al.*, 1998, 2001; Bangstad *et al.*, 1999).

PDR typically develops in patients with type 1 diabetes which I have shown in this study as most of the donors with PDR had type 1 diabetes (Klein *et al.,* 1984a,b; Frank, 2004). In all but 2 of the retinas in this study pre-retinal membranes were observed both by stereomicroscopy and on microscopic examination of H and E sections. It is known that pre-retinal neovascularization may be difficult to detect clinically in the fundus periphery (Tolentino *et al.*, 2002). Therefore the examination of H and E sections was important in this study rather than just viewing the retinas by stereomicroscopy. In rat and mouse models of PDR neovascular membranes were seen in retinas when examined using haematoxylin and eosin staining (Lai *et al.*, 2005; Kakehashi *et al.*, 2006). Tolentino *et al.*, 2002 also

demonstrated the presence of preretinal neovascularization histologically using flat-embedded nonhuman primate retinas.

Studies of histopathology and immunohistochemistry demonstrated that epiretinal membranes (ERMs) consist of complex fibrocellular tissue mainly composed of RPE, macrophages, glial cells, fibroblast-like cells and various amounts of extracellular matrix components and vascular elements (Yamamoto *et al.*, 1989; Hai *et al.*, 1998). I showed that neovessels were present in all the fibrovascular membranes stained by H and E. This is consistent with the findings of Tsanou *et al.*, 2005 who demonstrated that all ERMs surgically removed at vitrectomy had microvessels, with some vessels staining positively for Ki67 which is a marker of proliferation.

Panretinal photocoagulation is the treatment of choice for high-risk retinopathy (The Diabetic Retinopathy Study Research Group, 1979; Whiteside and Thompson, 1989; The Early Treatment Diabetic Retinopathy Study Research Group (ETDRS), 1995). The aim of laser photocoagulation is to eliminate areas of ischaemia, induce the regression of new vessels, and close leaking vessels leading to a decrease in exudates and macula oedema (Petrovic and Bhisitkul, 1999; De La Cruz *et al.*, 2004). Laser treatment of clinically significant macular oedema in patients with diabetic retinopathy is beneficial and reduces the overall risk of visual loss by about 50% (ETDRS No 9, 1991). In some cases, however visual acuity deteriorates (Agardh *et al.*, 1993), which could be due to either a rapid progress of oedema with hard exudates and subretinal fibrosis (ETDRS No23, 1997) or subretinal neovascularization membranes (Lewen, 1988; Varley *et al.*, 1988). However in this study none of the lasered retinas showed signs of exudates or neovascular membranes, when examined by stereomicroscopy and on examination of H and E sections, suggesting that the treatment had been successful.

CHAPTER 4 EXPRESSION OF PRO-ANGIOGENIC GROWTH FACTORS AND THEIR RECEPTORS IN THE NORMAL AND DIABETIC RETINA

4.1 INTRODUCTION

Sections were stained to localise and assess the extent of VEGF-A_{165}, VEGF-C and their receptors VEGFR-1, VEGFR-2 and VEGFR-3 presence in the retina and preretinal membranes. Sections were also stained to localise and assess the extent of angiopoietin-1, angiopoietin-2 and their receptor Tie-2 presence in the retina and preretinal membranes. Finally, sections were stained to localise and assess the extent of TNF alpha presence in the retina and preretinal membranes.

Immunostained sections of retina were examined by light microscopy to determine if there was a temporal and spatial relationship between staining intensity and the various pathological changes associated with diabetic retinopathy.

4.2 CONTROL STAINING

To confirm specificity of the immunostaining control sections were processed with 0.2% non-immune serum in place of the primary antibodies. Staining was negative on all the control sections (see figure 4.1).

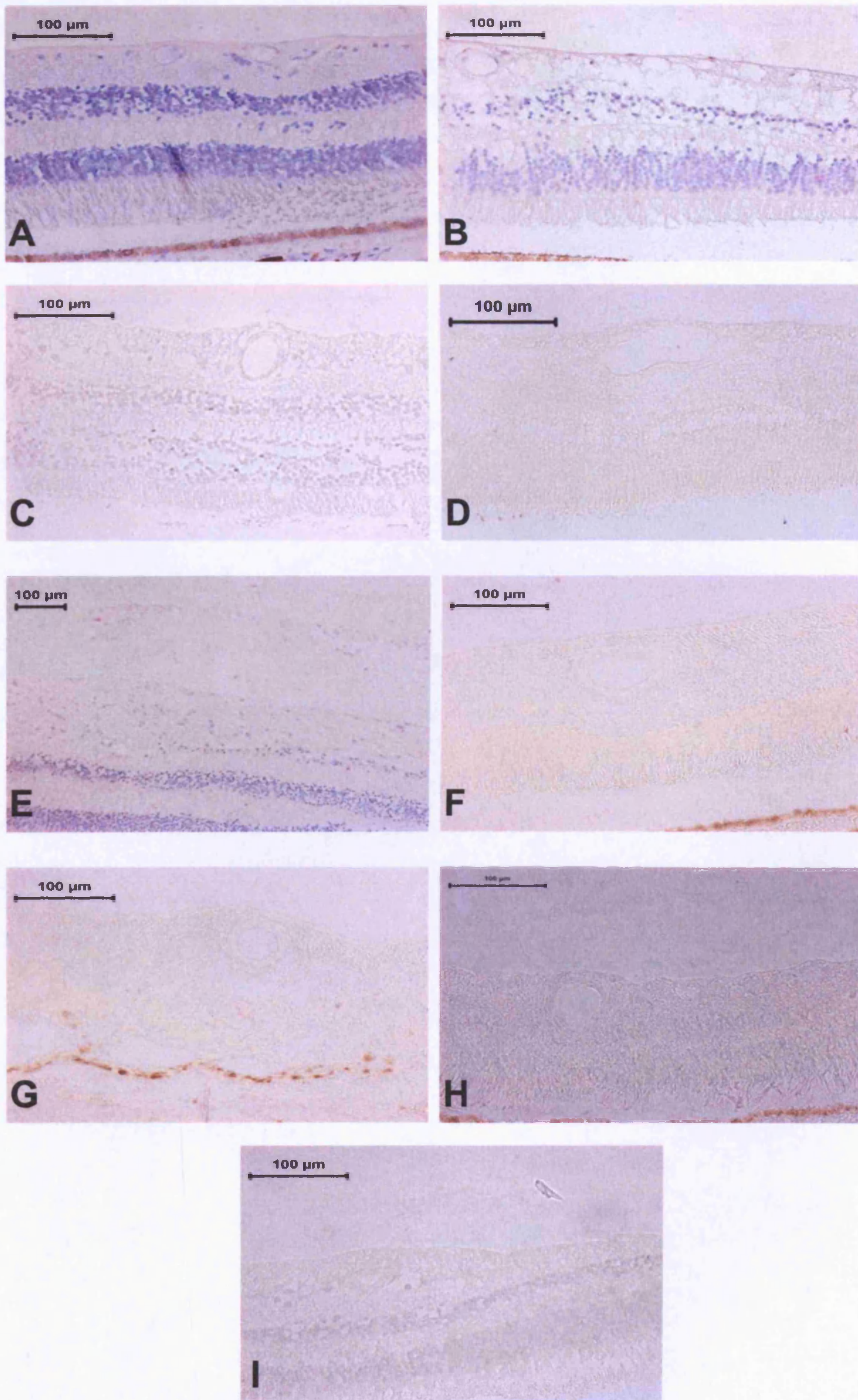


Table 4.1 Transverse Sections Showing Negative Control Staining for VEGF-A_{165} (A), VEGF-C (B), VEGFR-1 (C), VEGFR-2 (D), VEGFR-3 (E), Ang-1 (F), Ang-2 (G), Tie-2 (H), and TNF-α (I).

4.3 IMMUNOLOCALISATION OF PRO-ANGIOGENIC GROWTH FACTORS

4.3.1 VEGF-A_{165}, and VEGF-C immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy, VEGF-A_{165} staining was apparent in most diabetic tissue but generally absent or weak in the non-diabetic tissue. VEGF-A_{165} immunoreactivity was generally confined to endothelial cells and perivascular regions. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 4.1, but this did not correlate with either donor age, post-mortem time, or duration of diabetes. Statistical analysis demonstrated that significant differences were observed within the retinal vessels across the tissue categories ($P = <0.05\%$) but not within the retinal layers.

The average scores and standard deviations for VEGF-A_{165} immunostaining are represented in table 4.1. Although staining for VEGF-A_{165} was generally absent in the non-diabetic retinas, weak staining was associated with the retinal vessels in 7/15 of the specimens. Staining was absent or minimal within the photoreceptors, and the cell bodies in the inner and outer retina and GCL (Fig. 4.2).

In the diabetic retinas with no overt retinopathy VEGF-A_{165} was absent or minimal within the photoreceptors, and the cell bodies in the inner and outer retina and GCL. Staining was raised in the retinal vessels as compared to the non-diabetic retinas and was observed in 12/19 specimens (Fig. 4.3).

In the diabetic retinas showing vascular changes but no evidence of PDR VEGF-A_{165} immunostaining was increased within the retinal vessels and the GCL compared to that observed in normal retinas and diabetic retinas with no overt retinopathy. Staining of the photoreceptors, and in the cell bodies in the outer and inner retina was not significantly elevated above that observed in the normal retinas and the diabetic retinas with no overt retinopathy (Fig. 4.4).

Moderate to intense staining of the retinal vessels for VEGF-A_{165} was observed in all the diabetic retinas (6/6) with active neovascular PDR membranes on their surfaces. Intense staining was also observed within the membranes (Fig. 4.5). Staining was absent within the photoreceptors, within cell bodies in the outer retina, inner retina, and the GCL.

VEGF-A_{165} immunostaining in diabetic retinas that had undergone apparently successful laser therapy (that is, those with no preretinal neovascularization) showed a

similar staining pattern to that observed for non-diabetic retina (Fig. 4.6). Minimal immunoreactivity was observed within the inner retinal vessels. Staining was absent within the photoreceptors, and the cell bodies of the outer retina, and inner retina. Weak staining was also observed within the GCL.

VEGF-A immunoreactivity was moderate to intense within the preretinal vessels of the excised membranes. Weak to moderate staining was observed within the non-vascular components of most (8/11) of the membranes (Fig. 4.7).

The average scores and standard deviations for VEGF-C immunostaining are represented in table 4.2. When examined by light microscopy, VEGF-C staining was apparent in most non-diabetic and diabetic vascular tissue which was confined to retinal endothelial cells and the perivascular region. Increased immunostaining was observed within intra-retinal vessels of diabetic tissue as compared to non-diabetic tissue. Variable staining of the vessels within each retina was observed with some staining positive and some staining negative. Staining was also associated with extravascular regions of the retina. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 4.2, but this did not correlate with either donor age, time post mortem, or duration of diabetes. Statistical analysis demonstrated that significant differences were observed within the GCL across the tissue categories (P = <0.05%) but not within the other retinal layers and the retinal vessels.

In the non-diabetic retinas staining intensity for VEGF-C was generally absent or minimal within the photoreceptors, the cell bodies of the outer retina, the inner retina and the GCL. Weak staining was observed within the retinal vessels of 7/14 non-diabetic retinas (Fig. 4.8).

In the diabetic retinas with no overt retinopathy weak to moderate immunoreactivity for VEGF-C was observed within the GCL (8/12), and the retinal vessels (9/12), which was increased as compared with the non-diabetic retinas. Staining intensity was again absent or minimal within the photoreceptors, and the cell bodies of the outer retina and inner retina (Fig. 4.9).

In the diabetic retinas showing vascular changes but no evidence of PDR moderate to intense immunoreactivity for VEGF-C was demonstrated within the retinal vessels (5/5) which was increased, as compared to the retinas with no overt retinopathy (Fig. 4.10). Staining intensity was again weak to moderate within the GCL and absent or minimal within the photoreceptors and the cell bodies of the outer retina and inner retina.

In the diabetic retinas with active neovascular PDR membranes on their surfaces moderate to intense VEGF-C immunostaining of the retinal vessels was observed in all of the sections (6/6) [Fig. 4.11]. However, only minimal staining was observed within the preretinal vessels (2/6) and weak staining was observed within the extravascular matrix (4/6). Staining intensity was also reduced to minimal levels in the GCL (2/6) as compared to the previous categories of diabetic retinas. Staining intensity was minimal within the photoreceptors, and the cell bodies of the outer retina and inner retina.

In those diabetic retinas which had undergone successful laser therapy, staining intensity for VEGF-C was weak within the retinal vessels. Staining was absent or minimal within the photoreceptors, the cell bodies of the outer retina, the inner retina and the GCL (Fig. 4.12).

VEGF-C immunoreactivity was minimal within the preretinal vessels of the excised membranes. Weak staining was observed within the non-vascular components of most (8/11) of the membranes (Fig. 4.13).

TABLE 4.1. MEAN INTENSITY OF VEGF-A_{165} IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	0 (0)	0 (0)	0.2 (0.1)	0.8 (0.3)	1.0 (0.2)		
No Overt Retinopathy (n=12)	0.1 (0.1)	0 (0)	0.2 (0.2)	0.4 (0.3)	1.2 (0.2)		
Intraretinal Changes (n=10)	0 (0)	0 (0)	0 (0)	1.4 (0.4)	2.2 (0.5)		
PDR (n=9)	0 (0)	0 (0)	0 (0)	0.3 (0.2)	2.5 (0)	2.5	0.6
Laser-No Residual PDR (n=14)	0 (0)	0 (0)	0 (0)	1.1 (0.3)	0.6 (0.3)		
Excised Membranes (n=17)						2.6 (0.3)	1.7 (0.2)

GCL = ganglion cell layer

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

TABLE 4.2 MEAN INTENSITY OF VEGF-C STAINING

Tissue Category	Retinal Layer				Retinal	Membrane	
	Photo-receptors	Outer Retina	Inner Retina	GCL	Vessels	Vessels	Matrix
Non-diabetic (n=14)	0.4 (0.8)	0 (0)	0.1 (0.4)	0.3 (0.4)	1.1 (1.2)		
No Overt Retinopathy (n=12)	0.3 (0.7)	0 (0)	0.1 (0.3)	1.2 (1.0)	1.6 (1.1)		
Intraretinal Changes (n=10)	0.6 (0.9)	0.2 (0.5)	0.6 (0.9)	1.2 (1.1)	2.4 (0.9)		
PDR (n=9)	0.3 (0.5)	0.2 (0.4)	0.3 (0.5)	0.3 (0.5)	2.5 (0.5)	0.7 (1.0)	1.2 (1.0)
Laser-No Residual PDR (n=14)	0.4 (0.6)	0.4 (0.9)	0.3 (0.5)	0.4 (0.5)	0.8 (1.2)		
Excised Membranes (n=17)						0.3 (0.9)	1.1 (0.8)

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

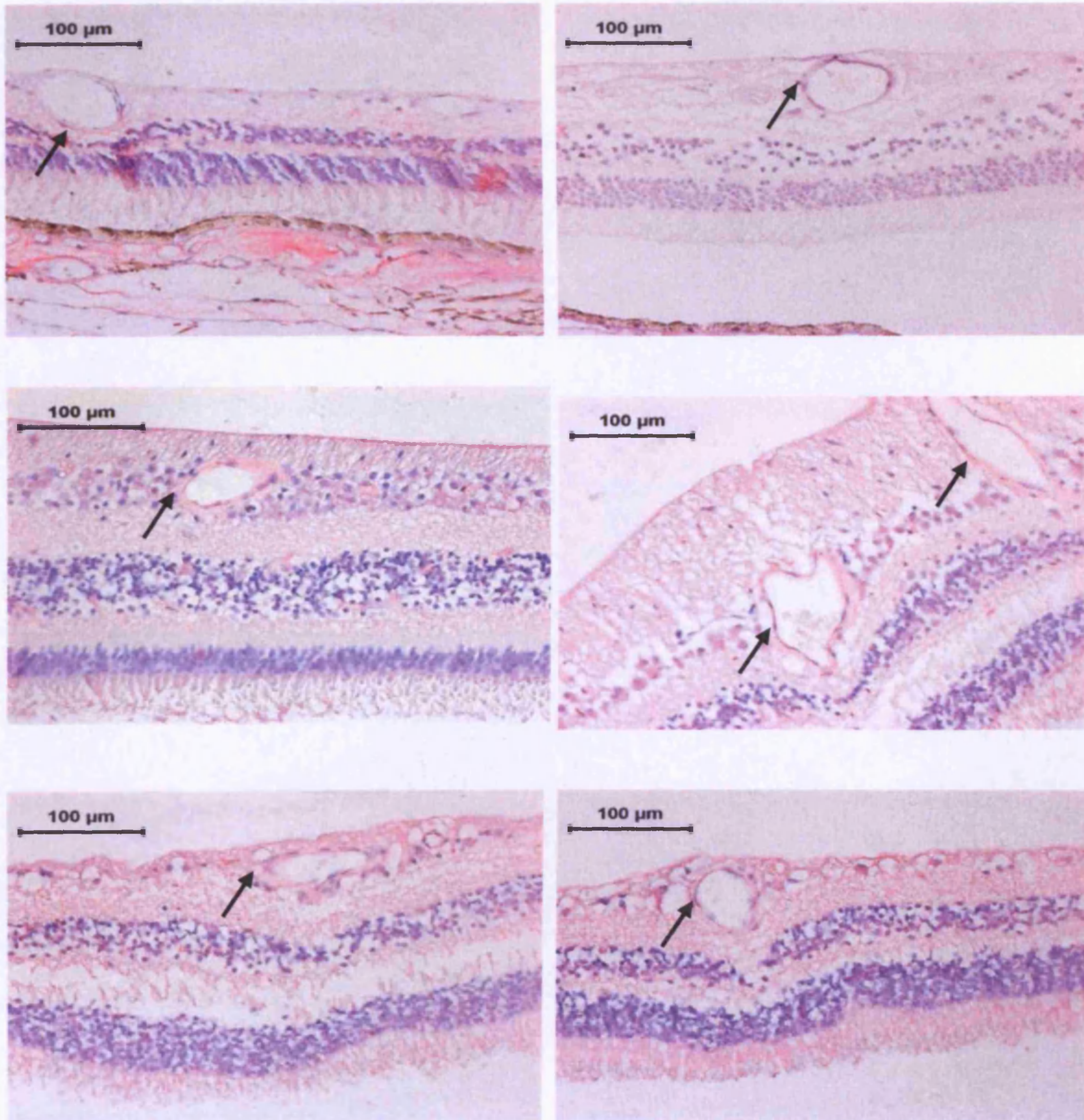


Figure 4.2 Transverse Sections Showing the Immunolocalisation (Arrows) of VEGF-A in Non-Diabetic Retinas

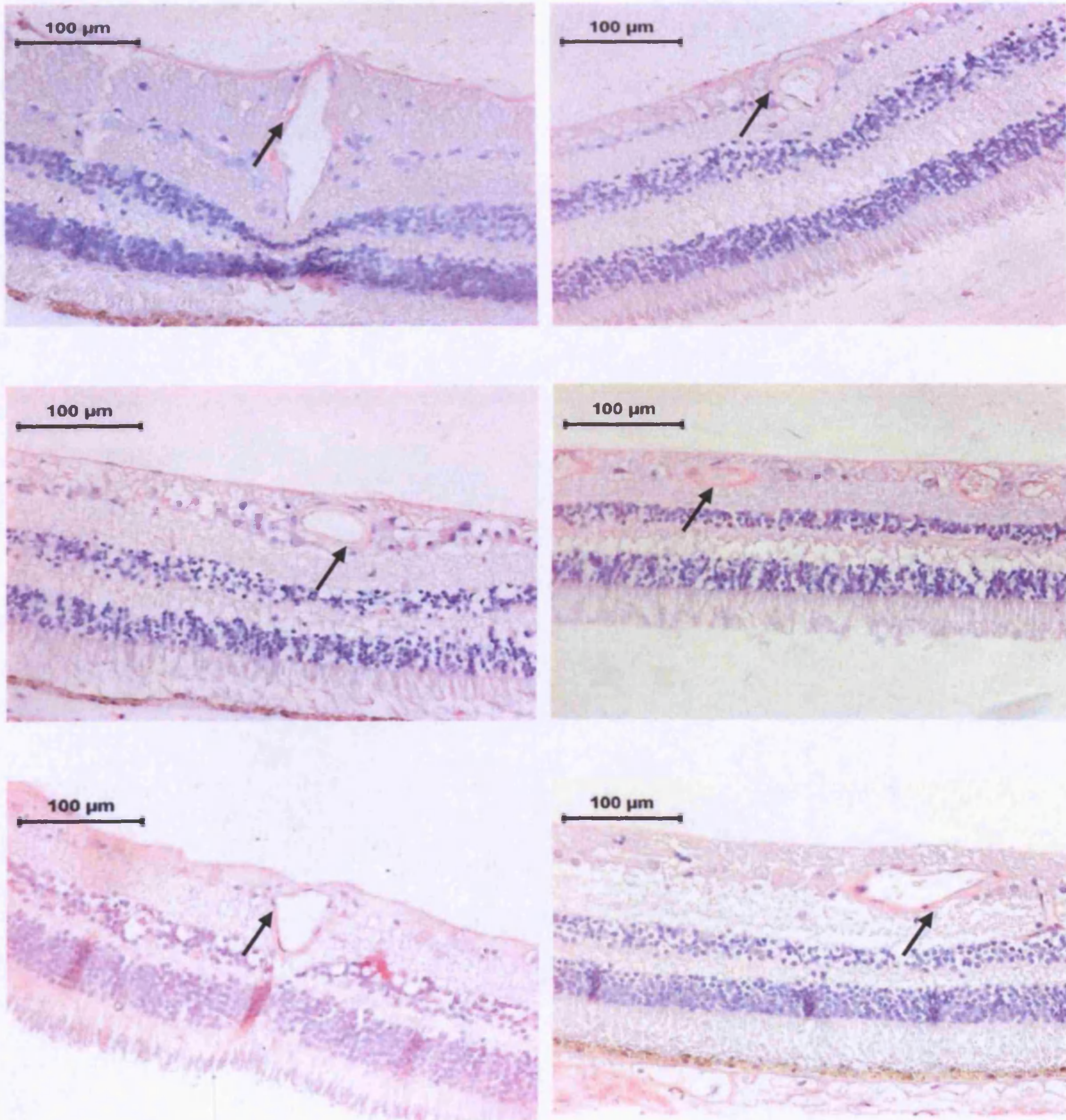


Figure 4.3 Transverse Section Showing the Immunolocalisation of VEGF-A (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.

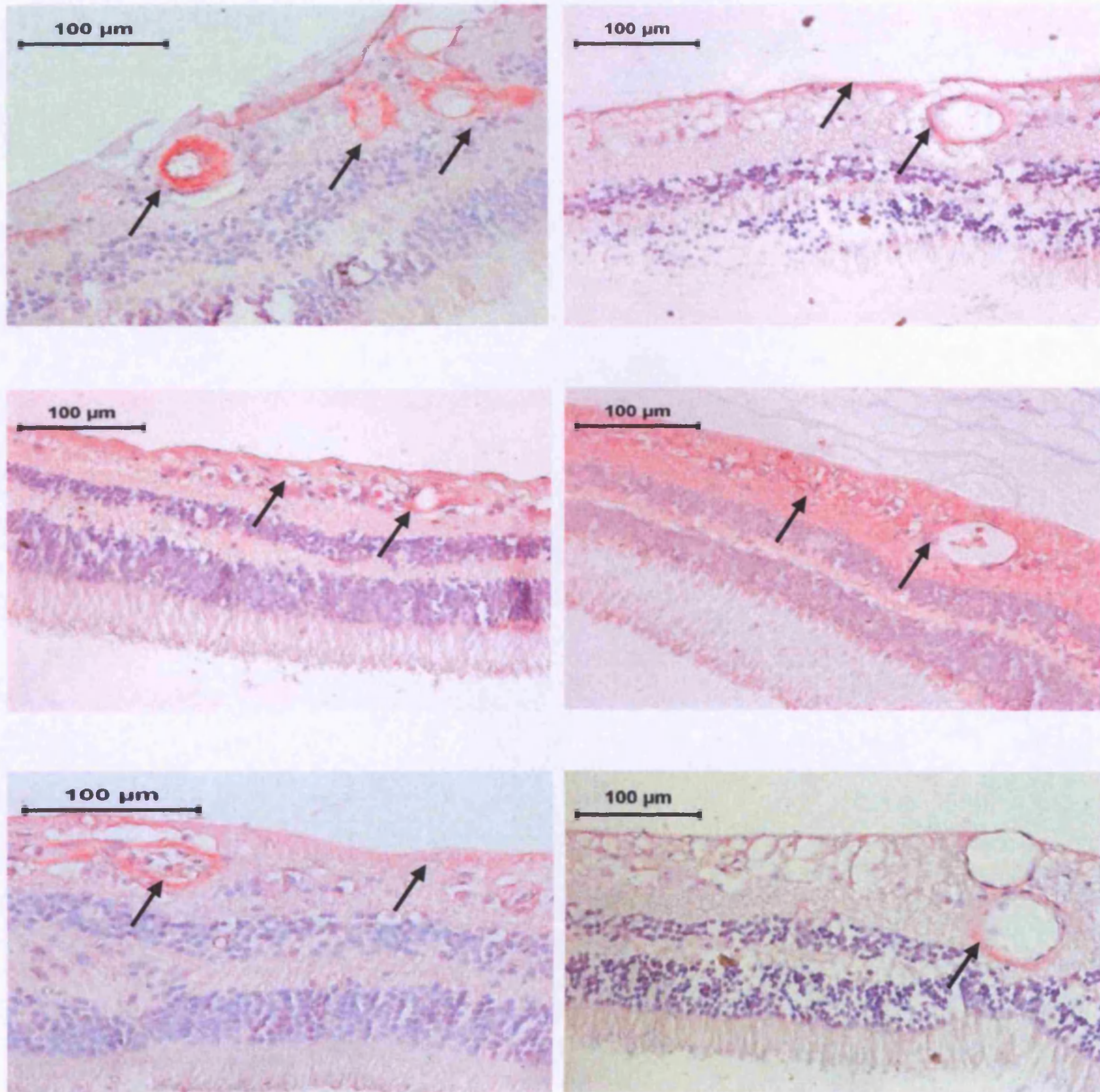


Figure 4.4 Transverse Sections Showing the Immunolocalisation of VEGF-A (Arrows) in Unlasered Diabetic Retinas with NPDR.

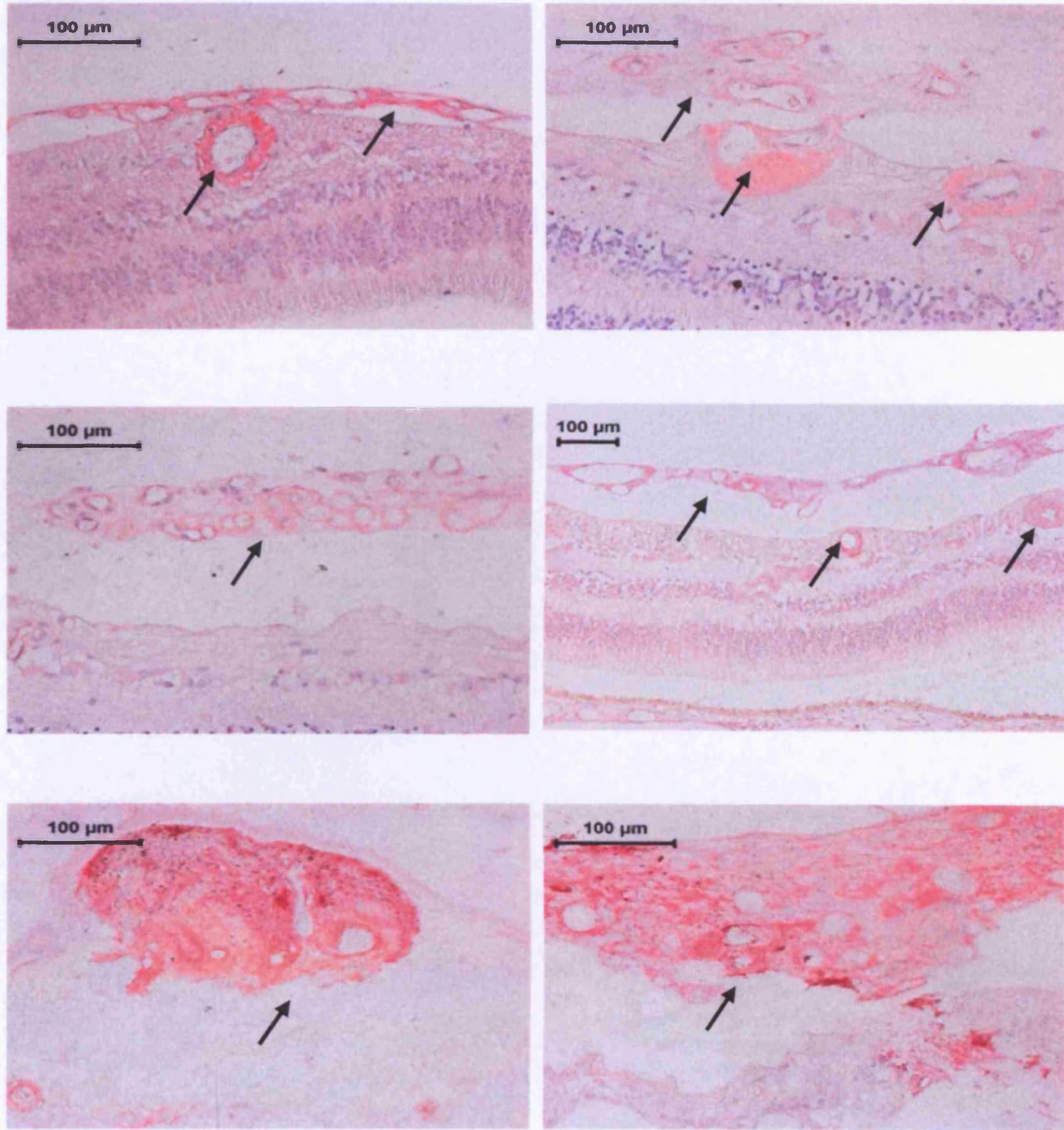


Figure 4.5 Transverse Sections Showing the Immunolocalisation of VEGF-A in Diabetic Retinas with PDR.

Arrows show location of staining in the retinal vessels and the preretinal membranes

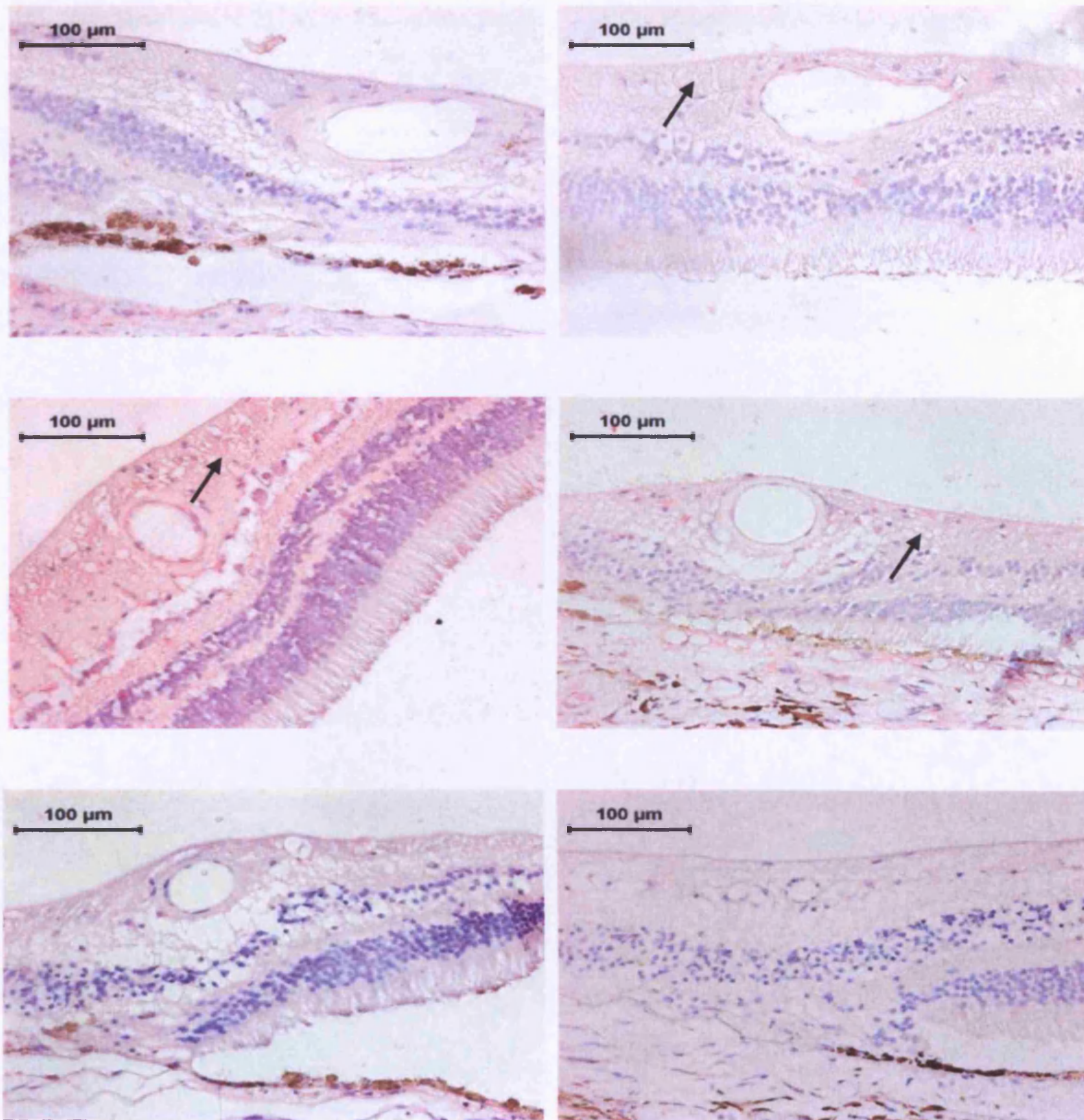


Figure 4.6 Transverse Sections Showing the Immunolocalisation of VEGF-A in Lasered Diabetic Retinas
Weak staining was seen in the GCL of some (arrows) of the retinas

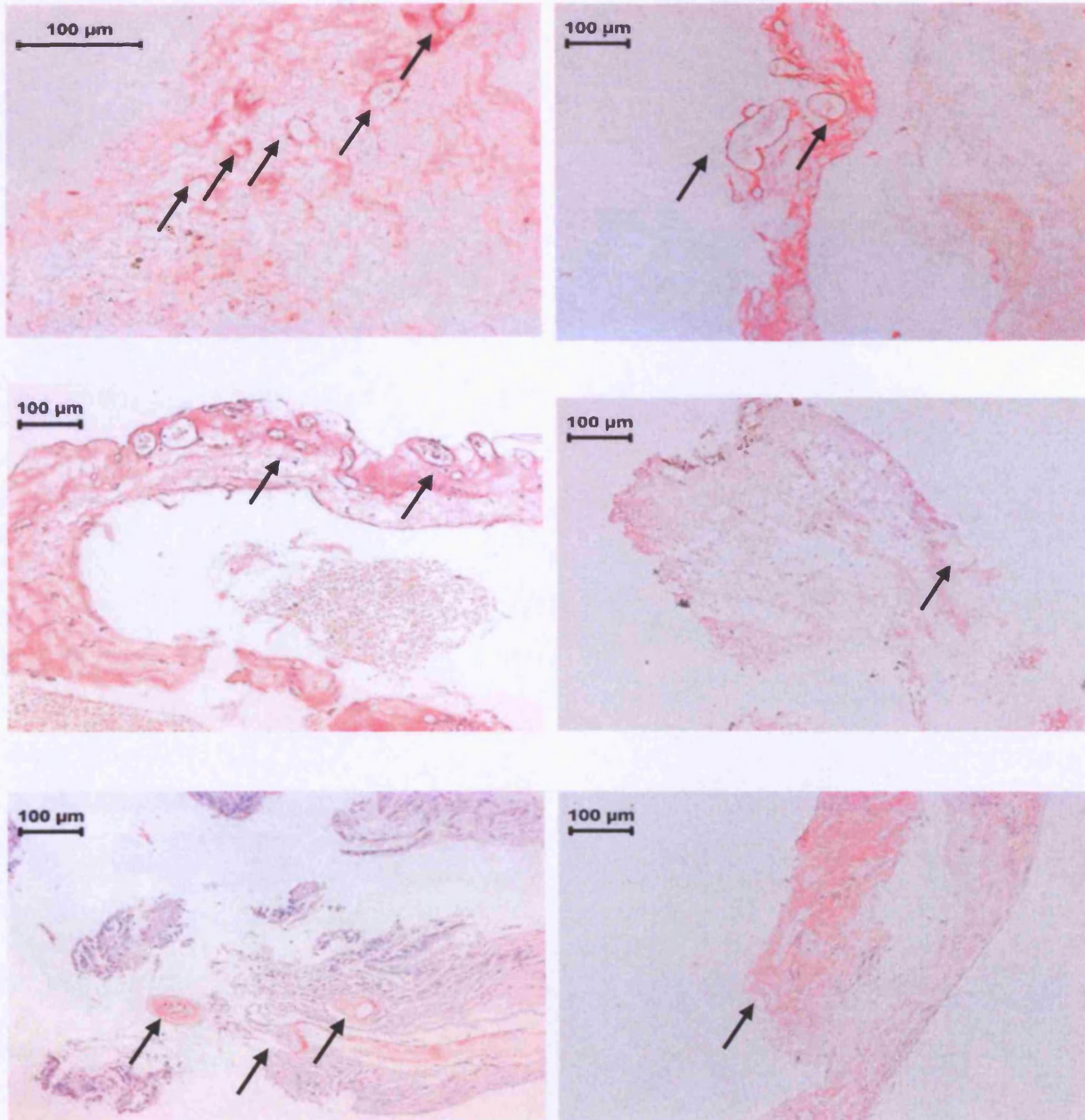


Figure 4.7 Transverse Sections Showing the Immunolocalisation of VEGF-A (Arrows) in Fibrovascular Membranes

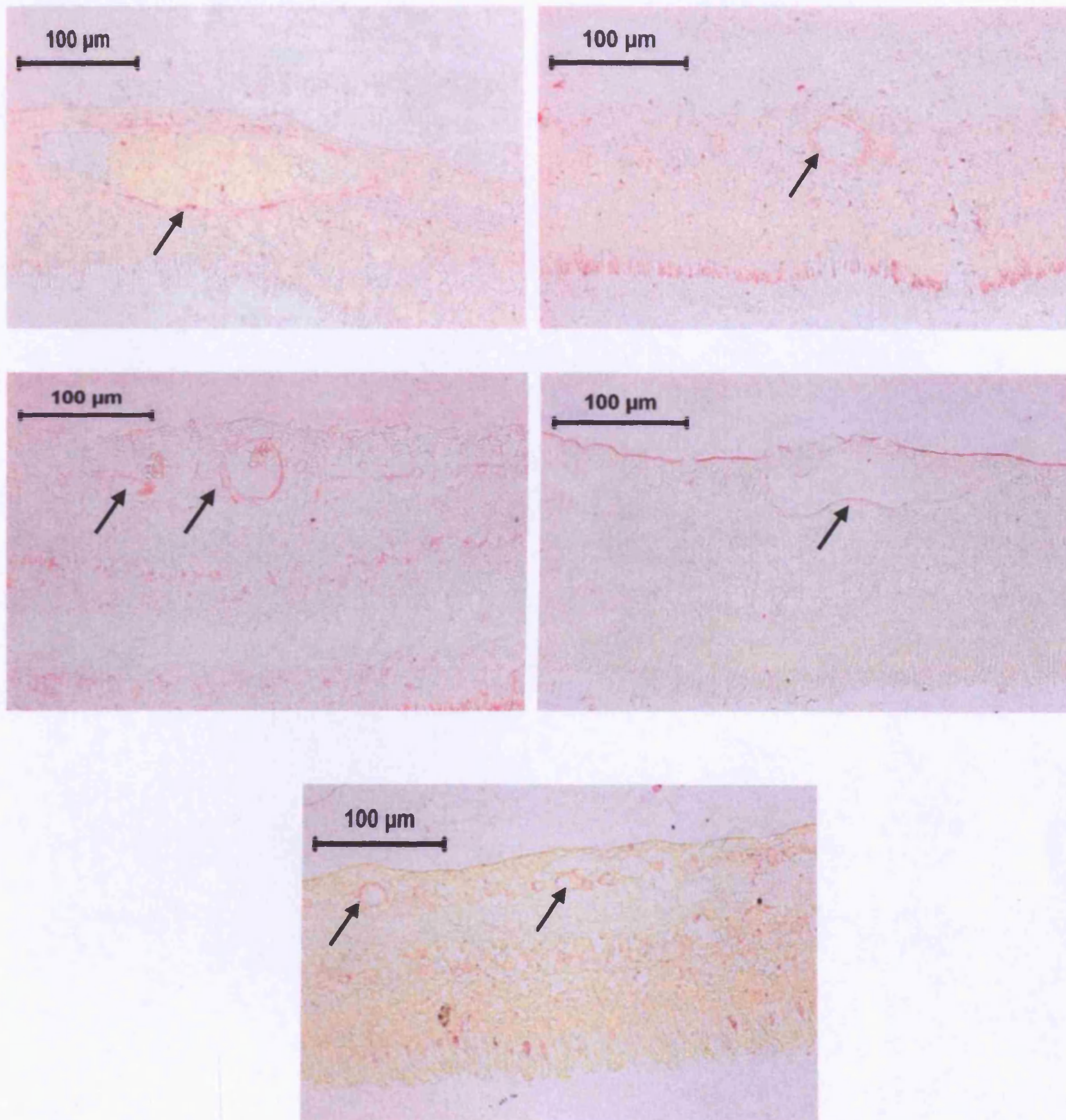


Figure 4.8 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Non-Diabetic Retinas.

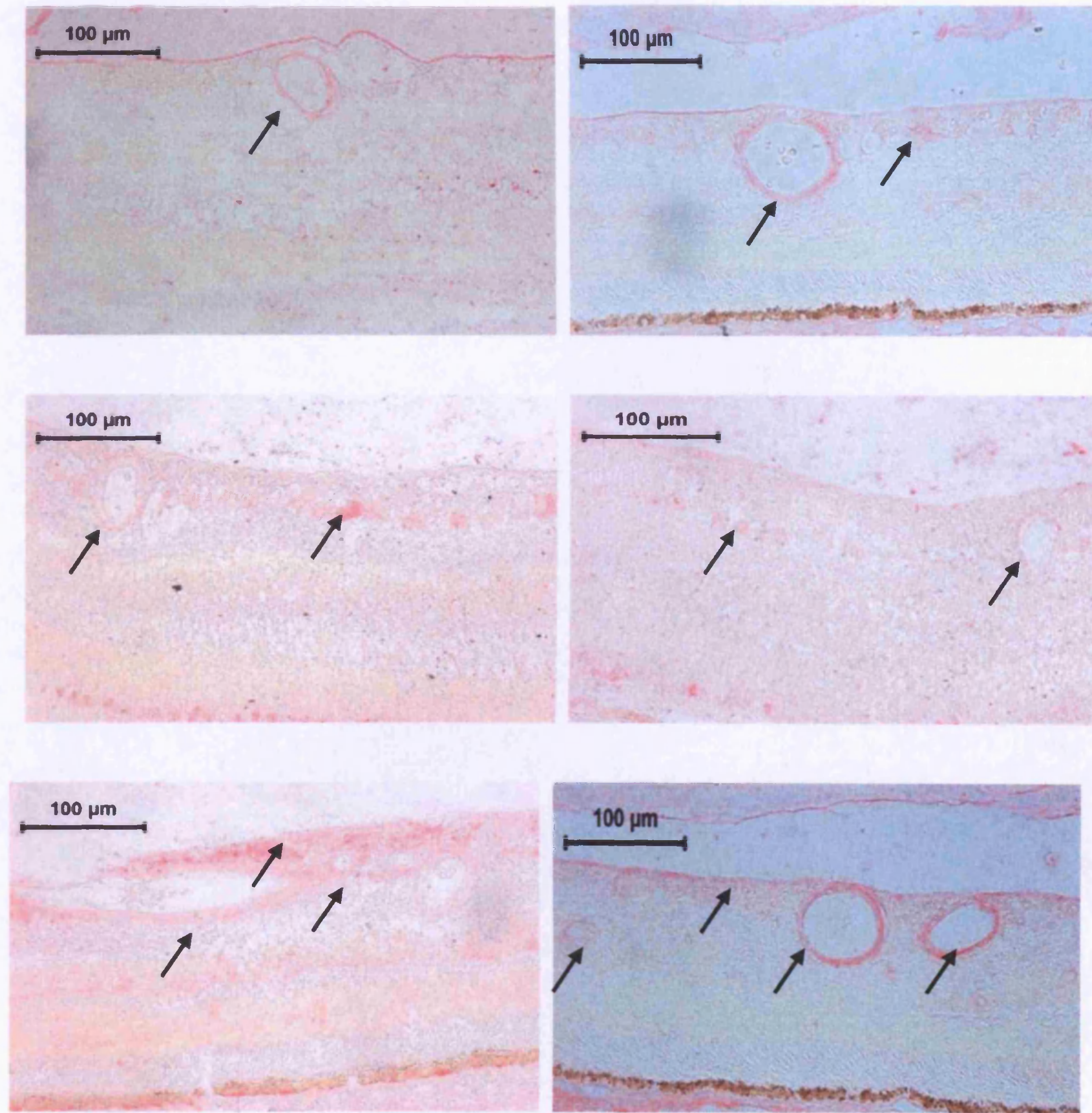


Figure 4.9 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.

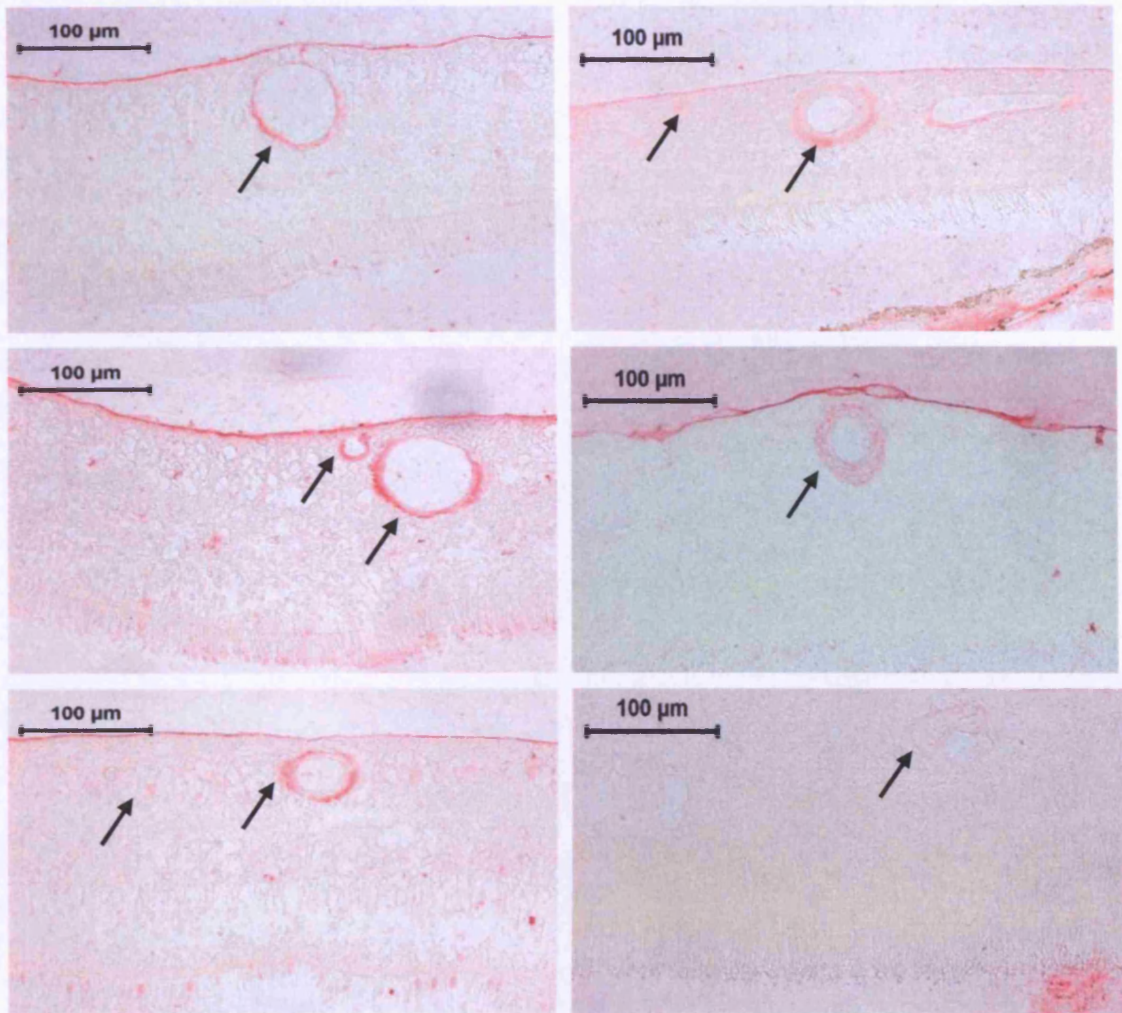


Figure 4.10 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Unlasered Diabetic Retinas with NPDR.

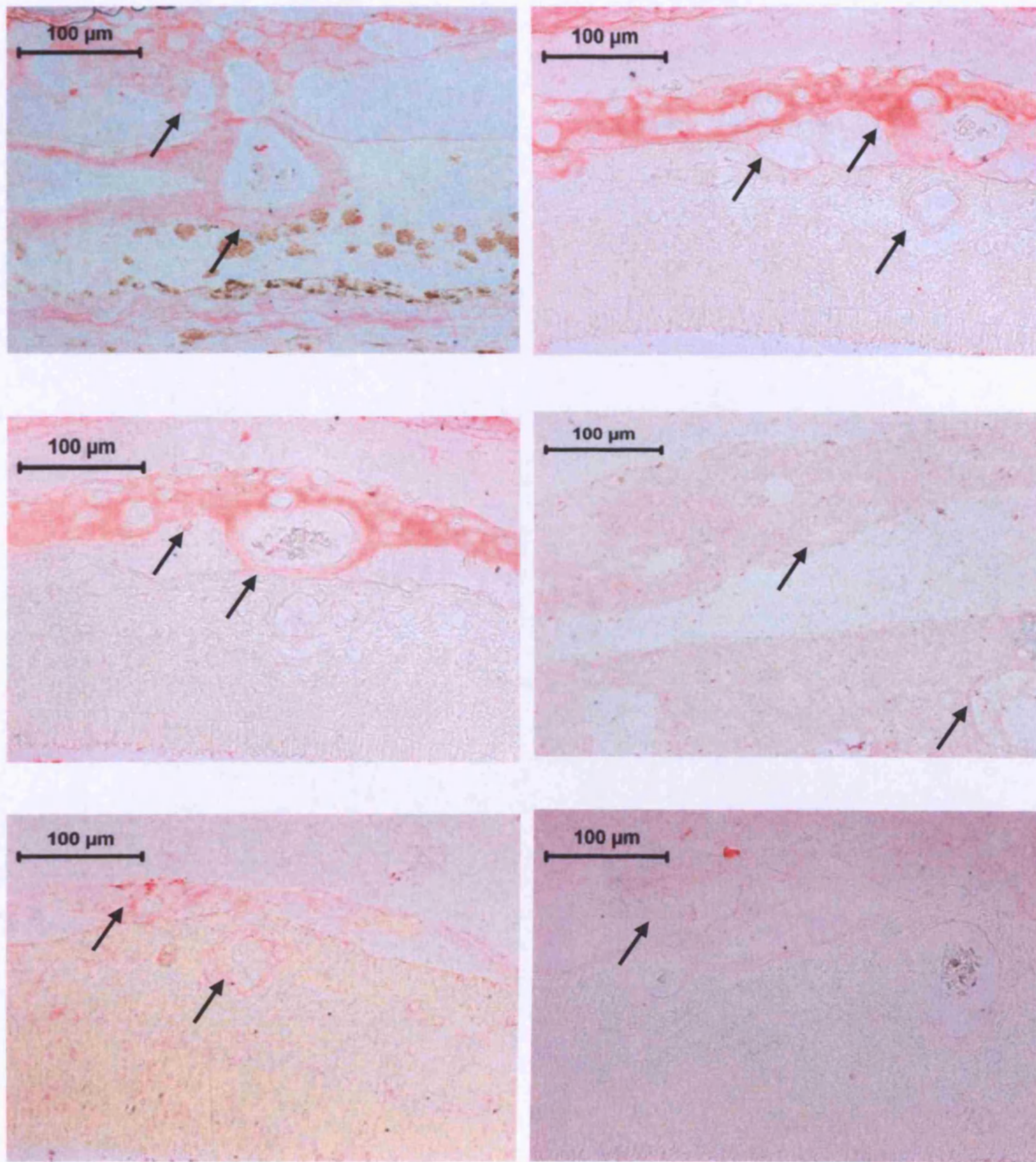


Figure 4.11 Transverse Sections Showing the Immunolocalisation of VEGF-C in Diabetic Retinas with PDR.

Arrows show location of staining in the retinal vessels and the preretinal membranes

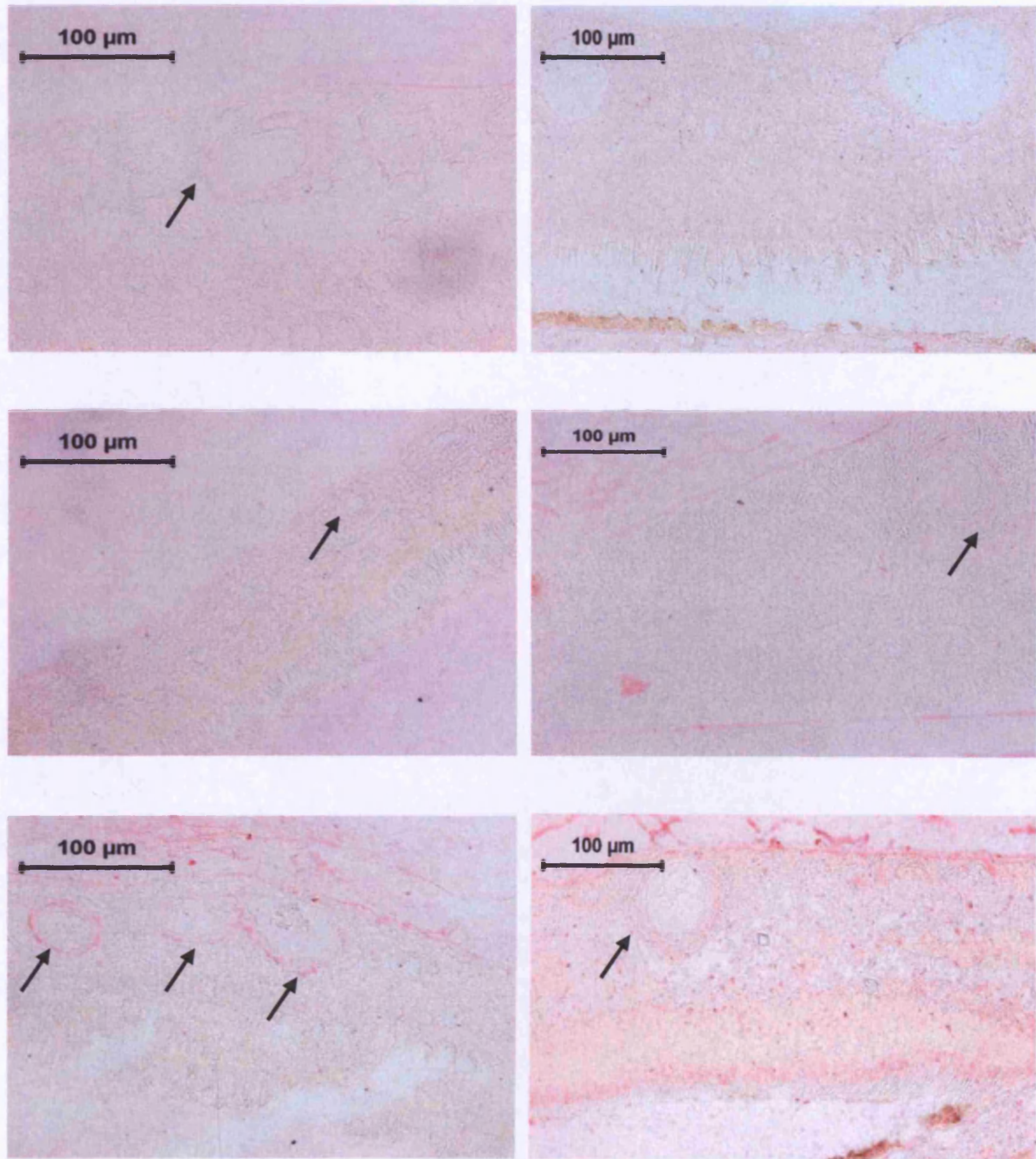


Figure 4.12 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Lasered Diabetic Retinas.

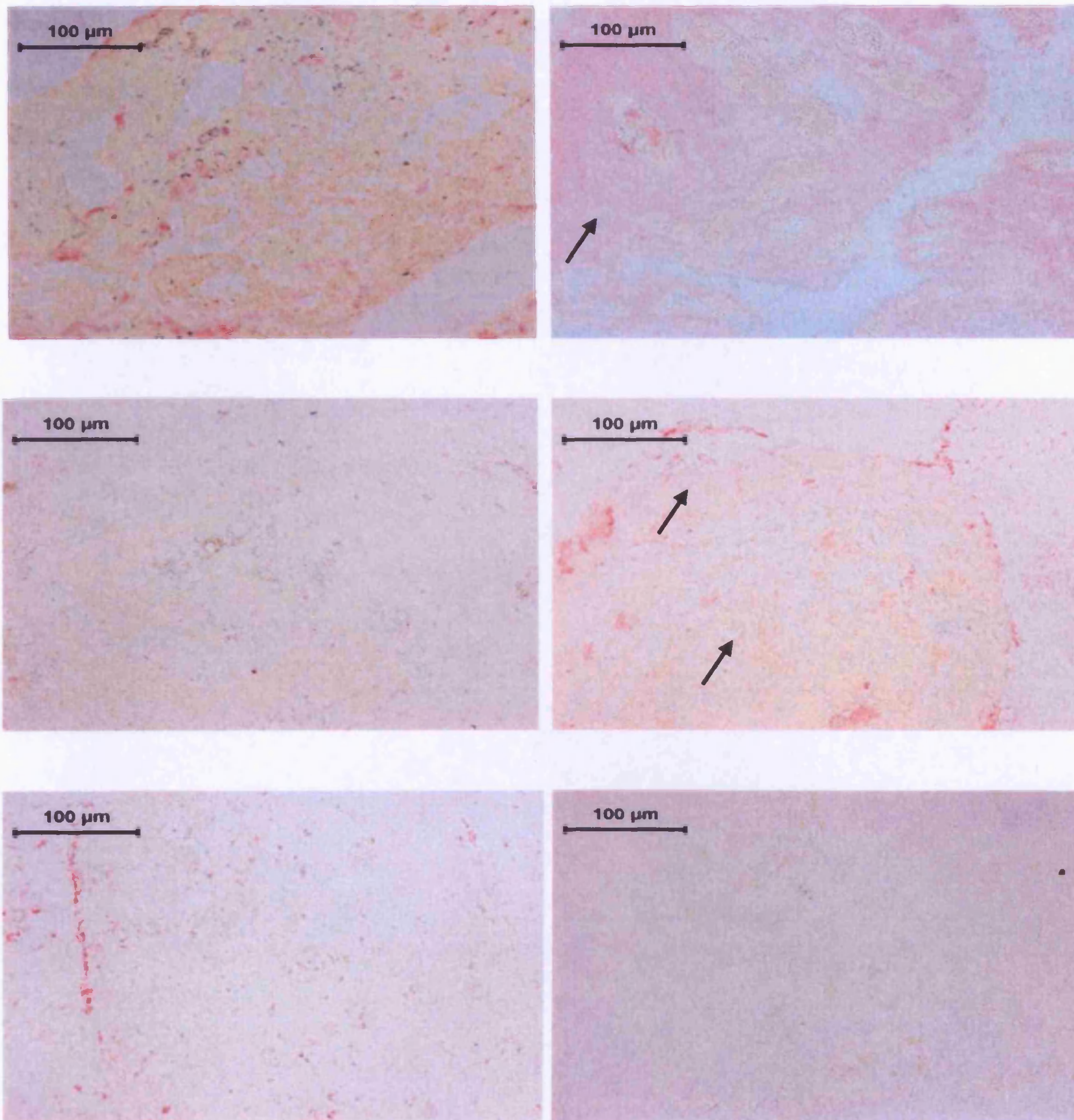


Figure 4.13 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Fibrovascular Membranes

4.3.2 VEGF receptor immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy immunostaining for all three receptors was observed in both non-diabetic and diabetic vascular and extravascular tissue. The staining pattern depended upon the specificity of the antibody being used and the category of tissue. Increased immunostaining was observed in preretinal and intra-retinal blood vessels of diabetic tissue as compared to non-diabetic tissue. For all the receptors variable staining of the vessels within each retina was observed with some staining positive and some staining negative. In some instances staining was associated with both endothelial cells and the perivascular region of the vessels. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in tables 4.3 to 4.5, but this did not correlate with either donor age, or time post mortem.

The average scores and standard deviations for VEGFR-1 immunostaining are represented in table 4.3. Statistical analysis demonstrated that significant differences were observed within the photoreceptors and the cell bodies of the outer retina across the tissue categories (P = <0.05%) but not within the other retinal layers or the retinal vessels.

In the non-diabetic retinas staining intensity for VEGFR-1 was generally minimal or weak within the photoreceptors, and the cell bodies of the outer retina. Weak to moderate staining was observed within the cell bodies of the inner retina (11/14), the GCL (11/14), and the retinal vessels (9/14) [Fig. 4.14].

In all the diabetic retinas with no overt retinopathy weak to moderate immunoreactivity for VEGFR-1 was associated with the cell bodies of the inner retina (12/12), the GCL (11/12), and the retinal vessels (8/12) [Fig. 4.15]. Immunoreactivity was slightly raised as compared to non-diabetic retinas. Staining within the other retinal layers was generally minimal or weak.

In the diabetic retinas showing vascular changes but no evidence of PDR staining for VEGFR-1 was raised, as compared with the non-diabetic and diabetic retinas with no overt retinopathy, within the cell bodies of the outer retina (3/5), the inner retina (5/5), the GCL (5/5), and the retinal vessels (4/5) [Fig. 4.16]. The most intense staining was observed within the GCL. Staining was again minimal or weak within the photoreceptors.

In the diabetic retinas with active neovascular PDR membranes on their surfaces intensity of staining for VEGFR-1 was similar within the cell bodies of the outer retina (4/6), the inner retina (5/6), the GCL (6/6), and within the retinal vessels (5/6) as compared to the retinas with vascular changes but no PDR (Fig. 4.17). The most intense immunoreactivity for VEGFR-1 was observed within the preretinal vessels of the membranes with all of them

demonstrating positive staining (6/6). In this tissue category staining of the intra-retinal vessels was associated both with the membranes and across the retina. Staining was weak within the non-vascular components of the membranes. Staining was absent from photoreceptors.

In those diabetic retinas which had undergone successful laser therapy staining was weak to moderate within the cell bodies of the inner retina (13/14), the outer retina (14/14), the GCL (14/14), and the retinal vessels (14/14). Staining was again generally minimal within the photoreceptors (Fig. 4.18).

VEGFR-1 immunostaining was weak to moderate within the preretinal vessels of 10/11 of the excised membranes but staining tended to be confined to a proportion of the vessels within each membrane with 4/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Weak staining was associated with the non-vascular components of the membranes (Fig. 4.19).

The average scores and standard deviations for VEGFR-2 immunostaining are represented in table 4.4. Statistical analysis demonstrated that significant differences were observed within the inner retina, the GCL, and the retinal vessels across the tissue categories ($P = <0.05\%$) but not within the other retinal layers.

In the non-diabetic retinas and the diabetic retinas with no obvious retinopathy VEGFR-2 immunoreactivity was absent or minimal within all the retinal layers and within the retinal vessels (Fig. 4.20; Fig. 4.21).

In the diabetic retinas showing vascular changes but no evidence of PDR increased staining was observed within the cell bodies of the inner retina (3/5) and the GCL (4/5) compared to the non-diabetic retinas and the diabetic retinas with no overt retinopathy. Staining was particularly apparent within the Müller cell endfeet in the GCL (Fig. 4.22). VEGFR-2 immunoreactivity was again absent or minimal in all the other retinal layers and within the retinal vessels.

In the diabetic retinas with active neovascular membranes on their surfaces VEGFR-2 immunoreactivity was reduced to minimal levels within the cell bodies of the inner retinal layer and the GCL as compared to the diabetic retinas with vascular changes. Staining was absent or minimal within the other retinal layers. In all retinas moderate to intense staining was observed within the retinal vessels (Fig. 4.23). Moderate staining was also observed within the preretinal vessels of most (5/6) of the membranes. In 4/6 diabetic retinas staining of the intra-retinal vessels was associated with the membranes but in 2/4 of these staining

was also observed in vessels across the retina. Minimal staining was associated with the non-vascular components of the membranes.

In those diabetic retinas which had undergone successful laser therapy staining was reduced to minimal levels within the retinal vessels in comparison to the diabetic retinas with PDR membranes on their surfaces (Fig. 4.24). Staining was again absent or minimal within the retinal layers.

VEGFR-2 staining was weak to moderate within the preretinal vessels of 7/11 of the excised membranes but staining tended to be confined to a proportion of the vessels within each membrane with 2/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Minimal staining was associated with the non-vascular components of the membranes (Fig. 4.25).

The average scores and standard deviations for VEGFR-3 immunostaining are represented in table 4.5. Statistical analysis demonstrated that significant differences were observed within the GCL and the retinal vessels across the tissue categories (P = <0.05%) but not within the other retinal layers.

In the non-diabetic retinas VEGFR-3 immunostaining was generally absent or minimal within all the layers and within the retinal vessels (Fig. 4.26).

In the diabetic retinas with no overt retinopathy all the retinas (12/12) demonstrated increased immunoreactivity for VEGFR-3 within the GCL as compared with the non-diabetic retinas, with most (11/12) also demonstrating increased immunoreactivity for VEGFR-3 within the cell bodies of the inner retina. Staining within the retinal vessels was generally weak with 9/12 retinas showing positive immunoreactivity to VEGFR-3. Staining within the other retinal layers was generally absent or minimal (Fig. 4.27).

In the diabetic retinas showing vascular changes but no evidence of PDR the intensity of staining for VEGFR-3 was similar to that observed for the eyes with no overt retinopathy. Again staining was weak to moderate within the cell bodies of the inner retina (4/5) and the GCL (4/5). Staining was particularly apparent within the Müller cell endfeet in the GCL (Fig. 4.28). Staining within the retinal vessels was weak (2/5). Staining within the other retinal layers was absent or minimal.

In the diabetic retinas with active neovascular PDR membranes on their surfaces the intensity of staining for VEGFR-3 was generally absent or minimal within the photoreceptors and the cell bodies of the outer retina. Staining intensity within the cell bodies of the inner retina was reduced to minimal levels as compared to the unlasered diabetic retinas. Staining intensity within the GCL was weak and so was also reduced compared to the unlasered

diabetic retinas. Weak to moderate staining intensity was observed within the retinal vessels with 5/6 showing positive immunoreactivity for VEGFR-3; staining was slightly raised as compared with that observed in the unlasered diabetic retinas with obvious vascular changes. Weak to moderate staining was also observed within the pre-retinal vessels of the membranes with 5/6 showing positive immunoreactivity for VEGFR-3 (Fig 4.29). In this tissue category staining of the intra-retinal vessels was associated with the membranes in 3/6 retinas but staining in 2/3 of these was also observed in vessels across the retina. Minimal staining was associated with the non-vascular components of the membranes.

In those diabetic retinas which had undergone successful laser therapy staining was minimal or weak and was generally reduced to the levels observed within the non-diabetic retinas (Fig. 4.30).

VEGFR-3 immunostaining was weak to moderate within the pre-retinal vessels of 10/11 of the excised membranes but staining tended to be confined to a proportion of vessels within each membrane with 2/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Minimal staining was associated with the non-vascular components of the membranes (Fig. 4.31).

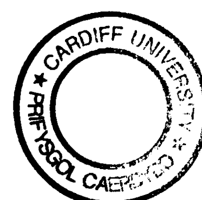

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TABLE 4.3 MEAN INTENSITY OF VEGFR-1 IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	0.6 (0.8)	0.2 (0.4)	1.2 (0.9)	1.6 (1.1)	1.1 (1.0)		
No Overt Retinopathy (n=12)	0.4 (0.5)	0.3 (0.5)	1.4 (0.5)	1.9 (0.9)	1.3 (0.9)		
Intraretinal Changes (n=10)	0.8 (1.2)	1.2 (1.2)	1.6 (0.8)	2.2 (0.8)	1.6 (1.0)		
PDR (n=9)	0 (0)	1.0 (0.8)	1.3 (0.7)	2.0 (0.8)	1.5 (1.1)	2.5 (0.5)	1.2 (0.4)
Laser-No Residual PDR (n=14)	0.2 (0.6)	1.5 (0.6)	1.5 (0.6)	1.9 (0.7)	1.7 (0.4)		
Excised Membranes (n=17)						1.6 (0.8)	1.1 (0.8)

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

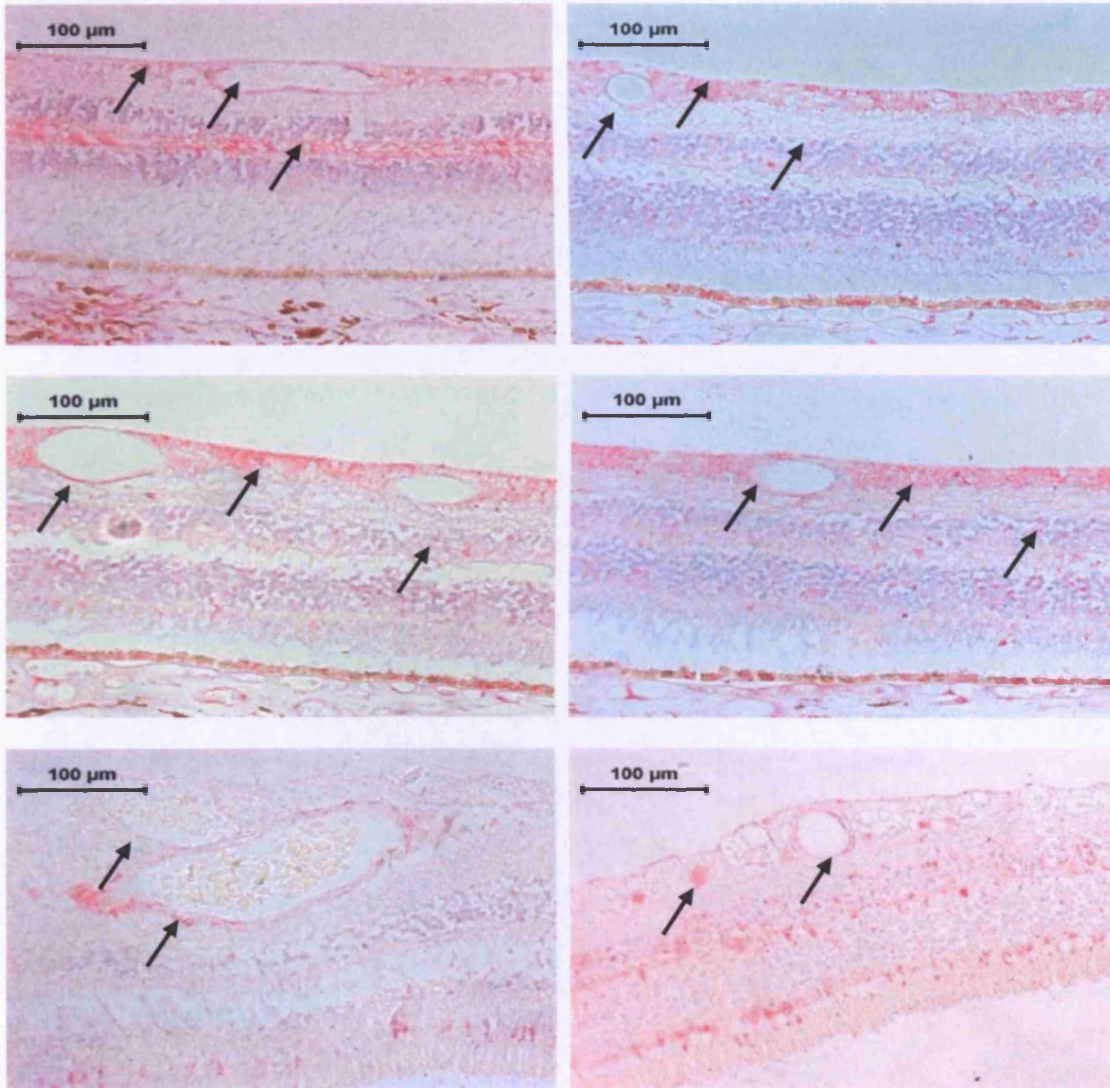

Figure 4.14 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Non-Diabetic Retinas.

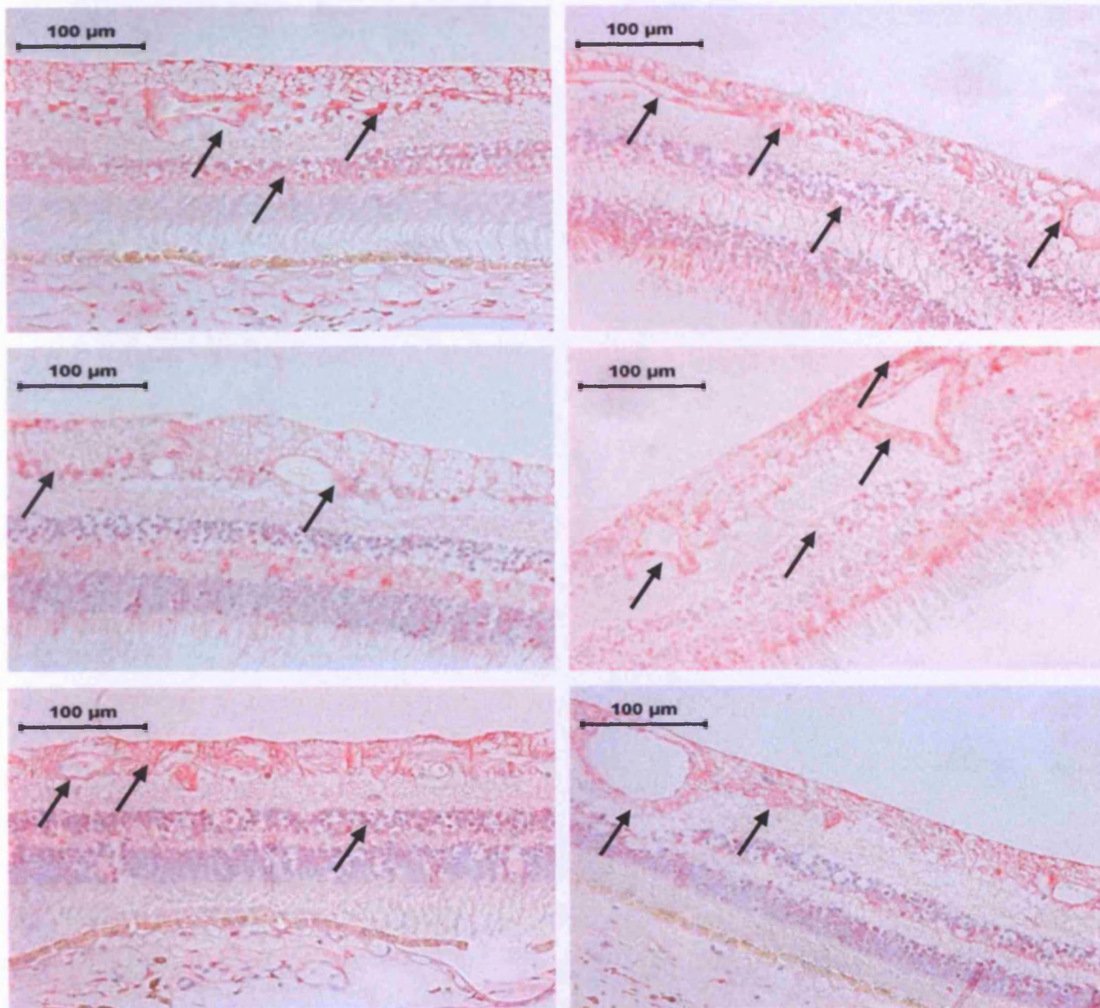

Figure 4.15 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.

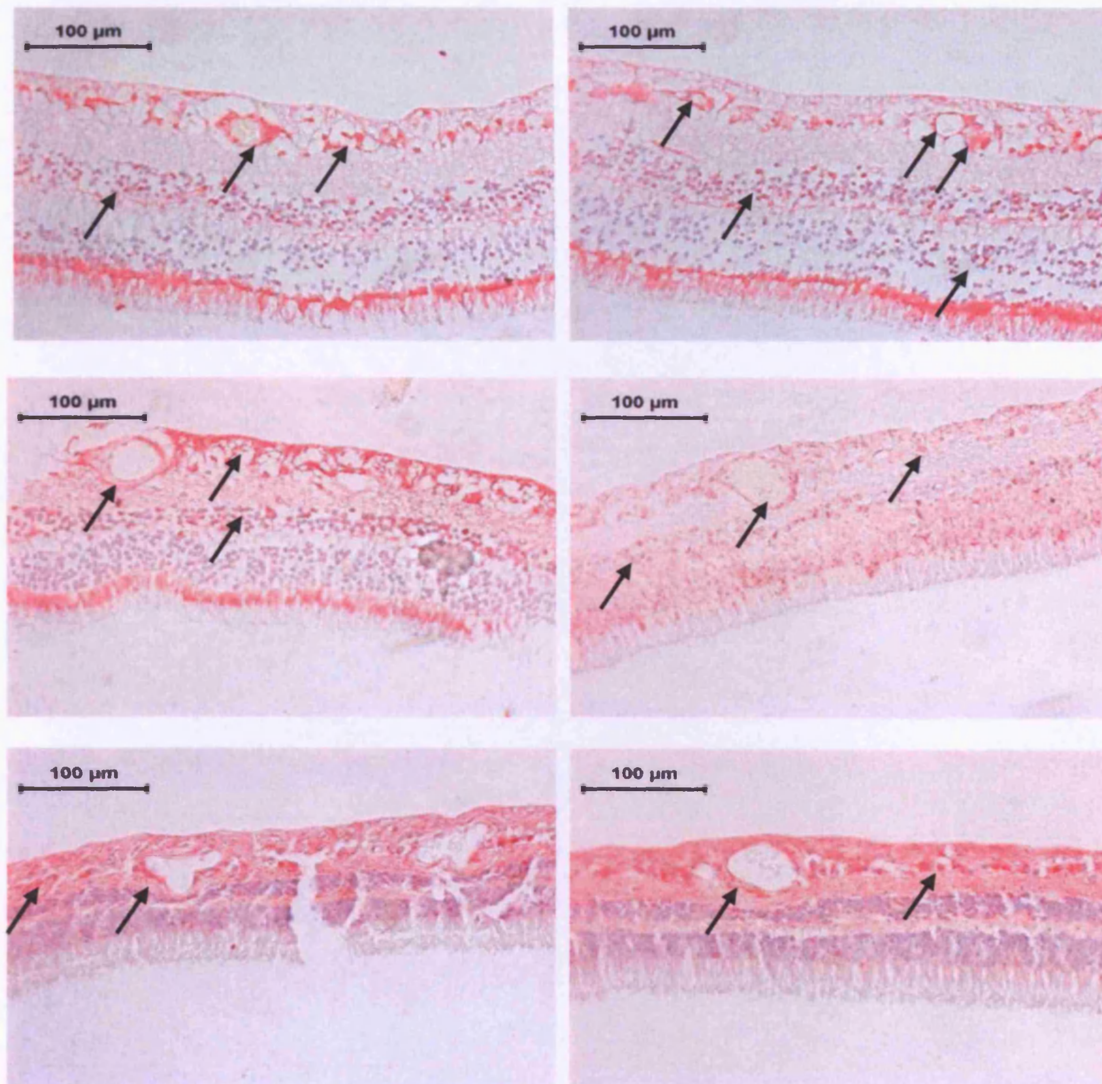

Figure 4.16 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Unlasered Diabetic Retinas with NPDR.

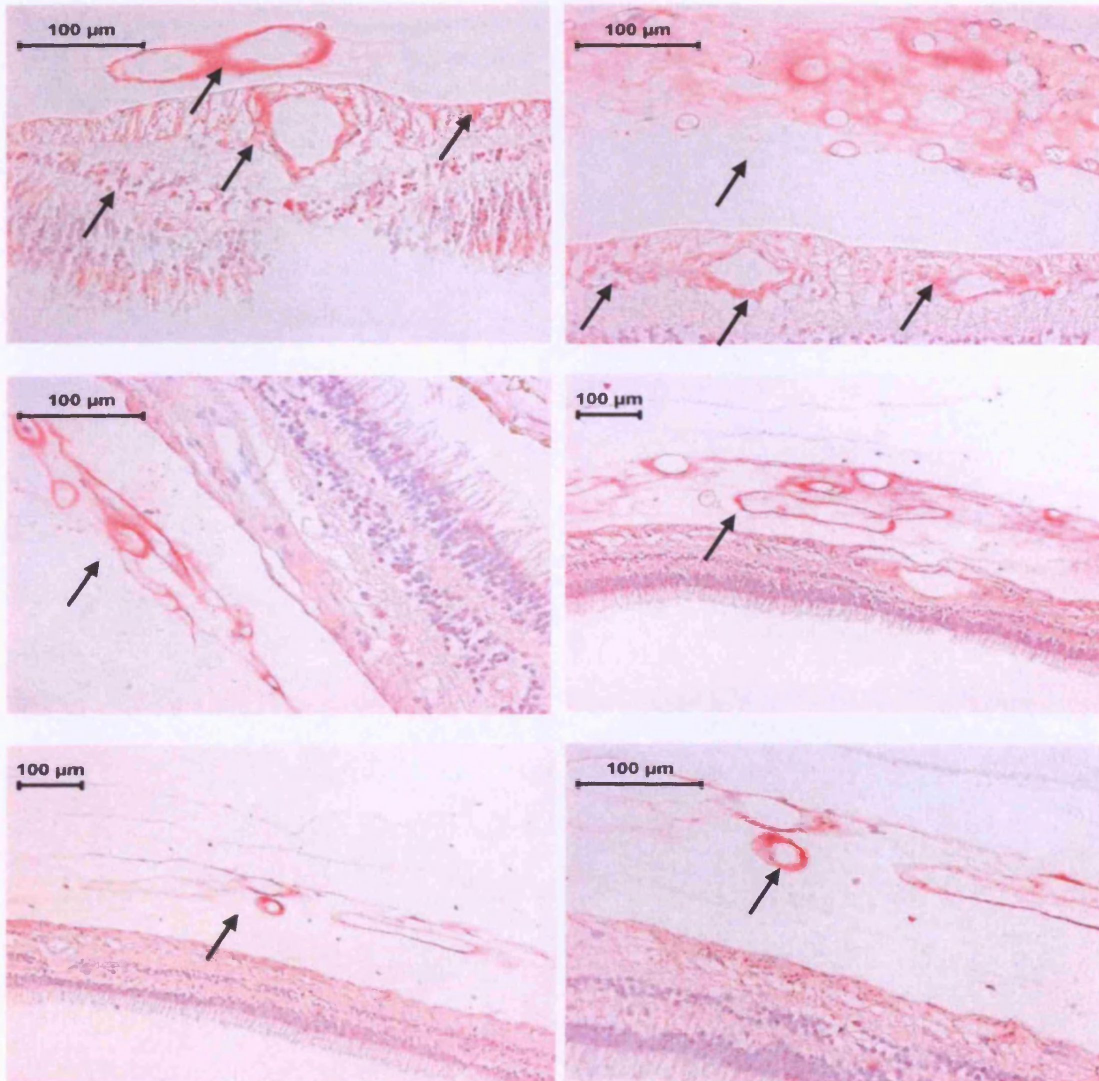

Figure 4.17 Transverse Sections Showing the Immunolocalisation of VEGFR-1 in Diabetic Retinas with PDR.

Arrows show location of staining in the retinal vessels and the preretinal membranes, and also in retinal layers where positive staining was observed

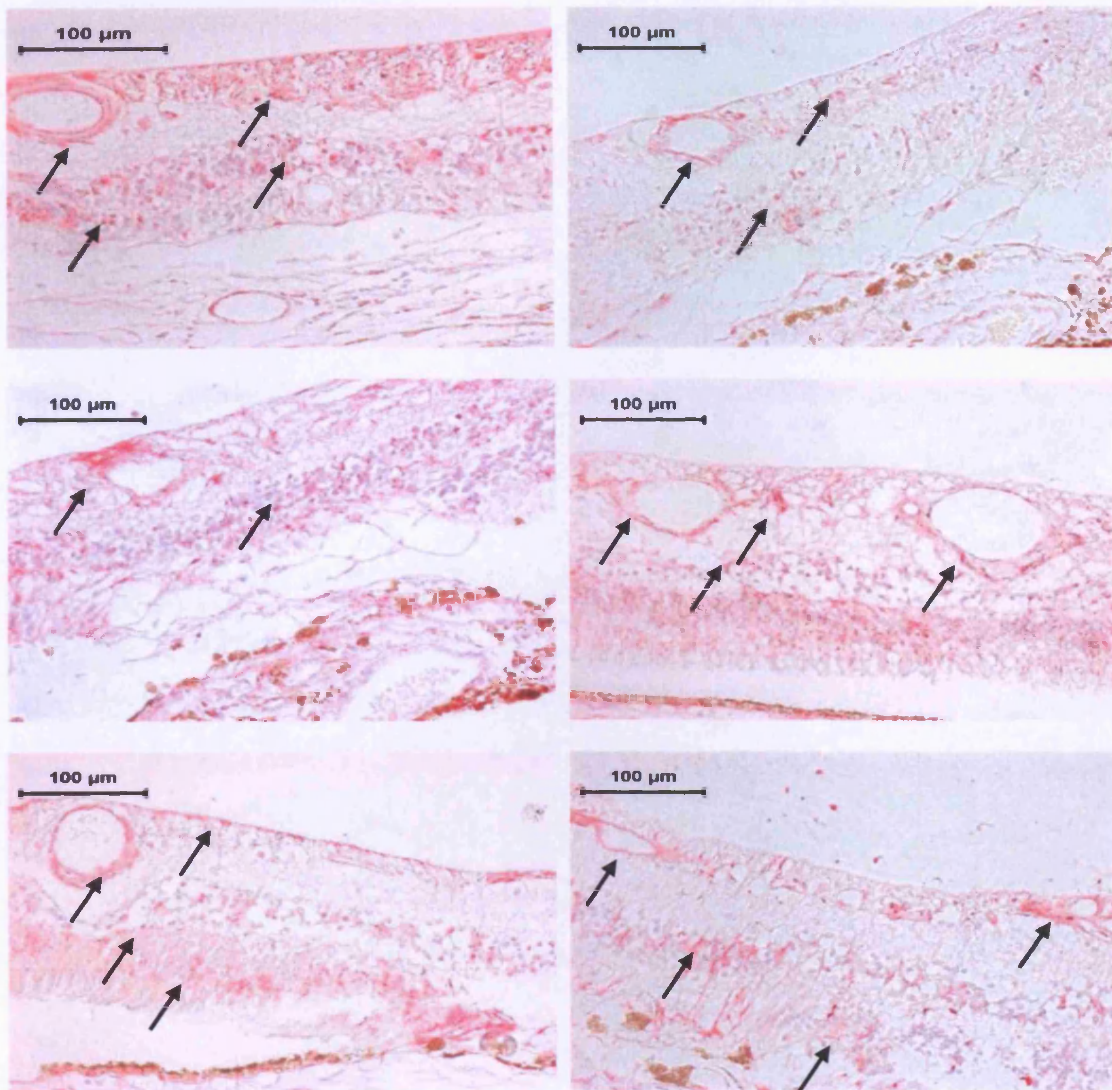

Figure 4.18 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Lasered Diabetic Retinas.

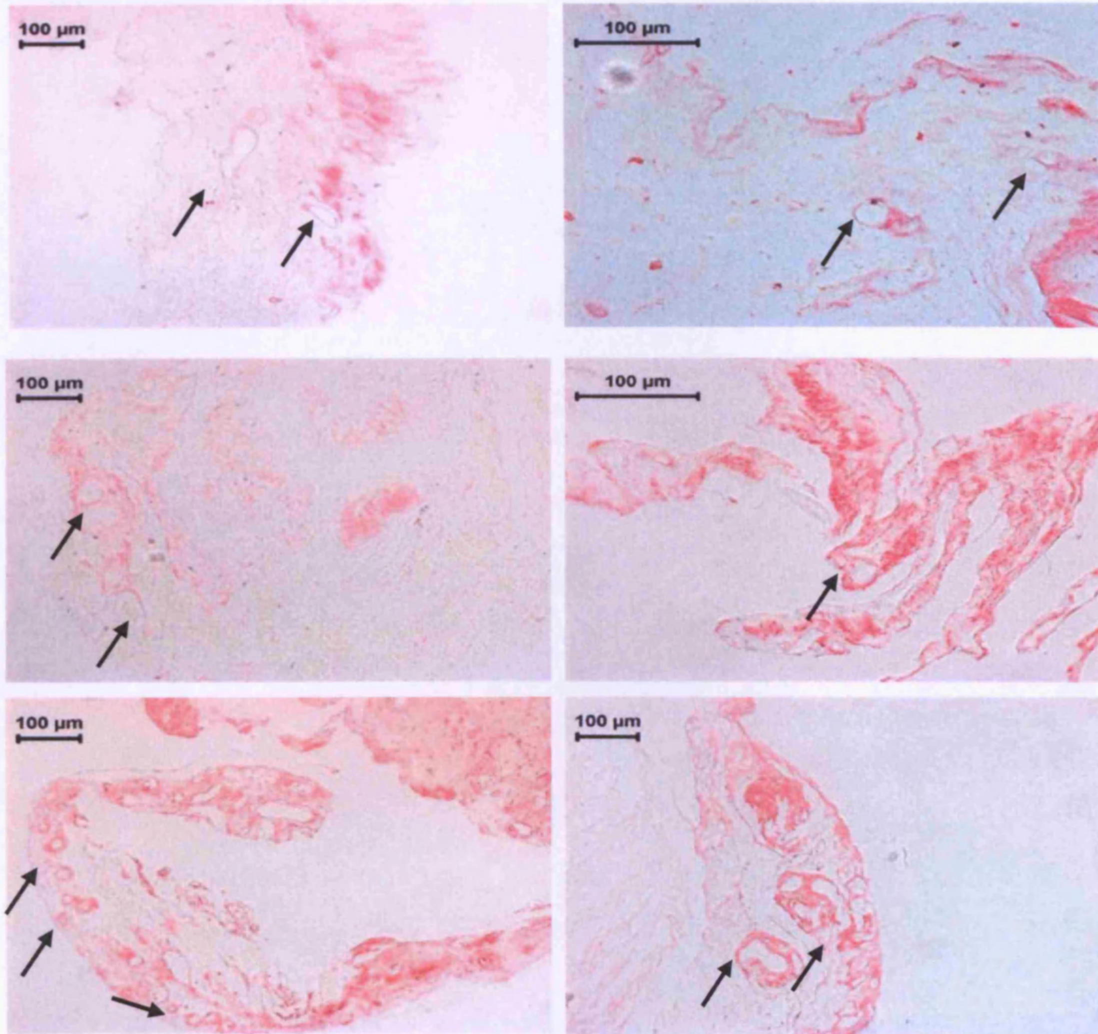

Figure 4.19 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Fibrovascular Membranes

TABLE 4.4 MEAN INTENSITY OF VEGFR-2 IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-Receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	0.1 (0.3)	0.1 (0.3)	0.5 (0.7)	0.3 (0.5)	0.4 (0.4)		
No Overt Retinopathy (n=12)	0.3 (0.4)	0 (0)	0.8 (0.6)	0.8 (0.4)	0.8 (1.0)		
Intraretinal Changes (n=10)	0.4 (0.9)	0 (0)	1.4 (1.3)	1.4 (0.9)	0.4 (0.6)		
PDR (n=6)	0 (0)	0 (0)	0.5 (1.1)	0.2 (0.4)	2.3 (0.8)	2.0 (1.2)	0.7 (1.1)
Laser-No Residual PDR (n=9)	0.1 (0.3)	0 (0)	0.7 (0.5)	0.3 (0.5)	0.5 (0.9)		
Excised Membranes (n=17)						1.6 (1.2)	0.6 (0.7)

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

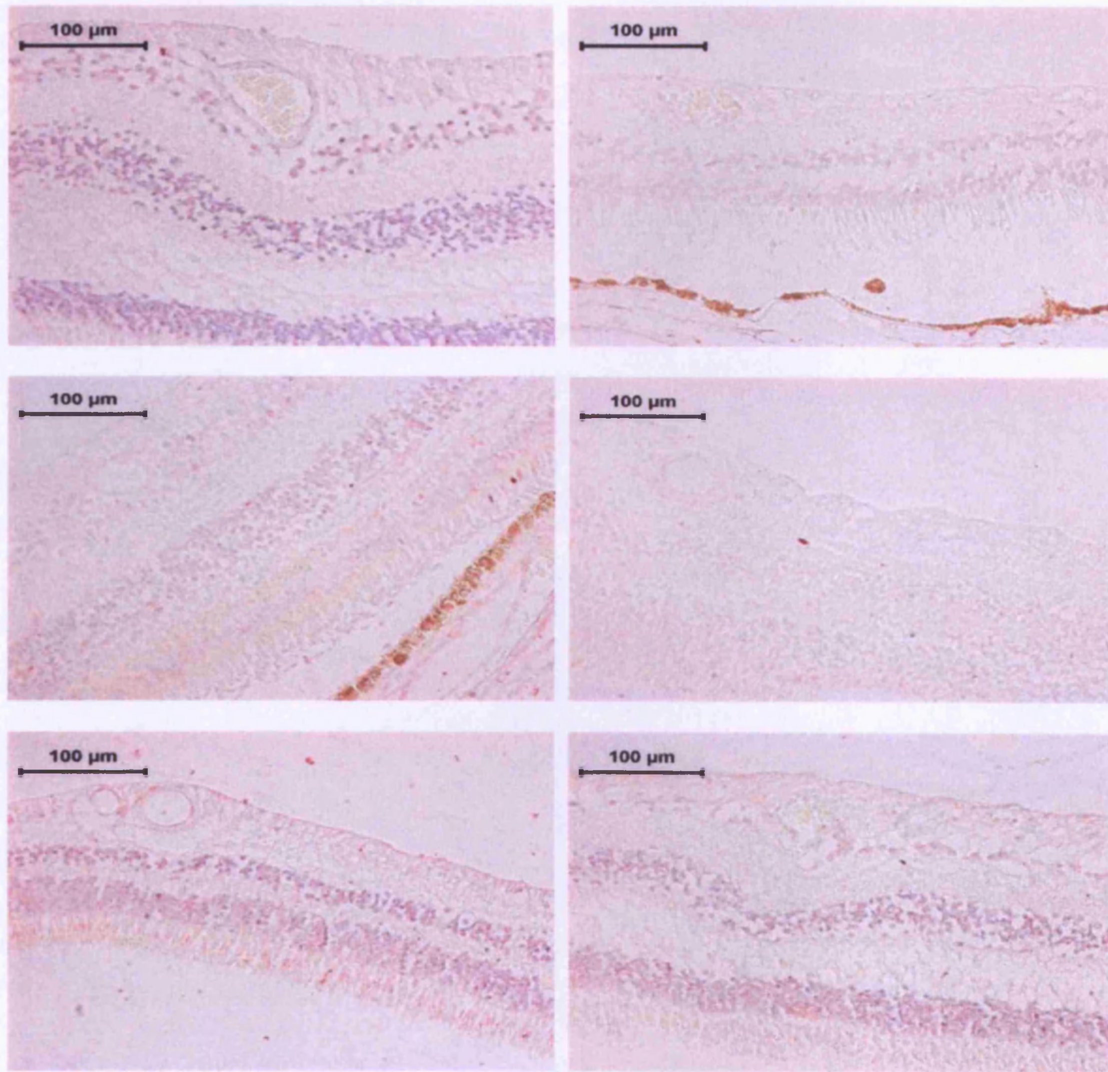

Figure 4.20 Transverse Sections Showing the Immunolocalisation of VEGFR-2 in Non-Diabetic Retinas.
Staining was absent or minimal.

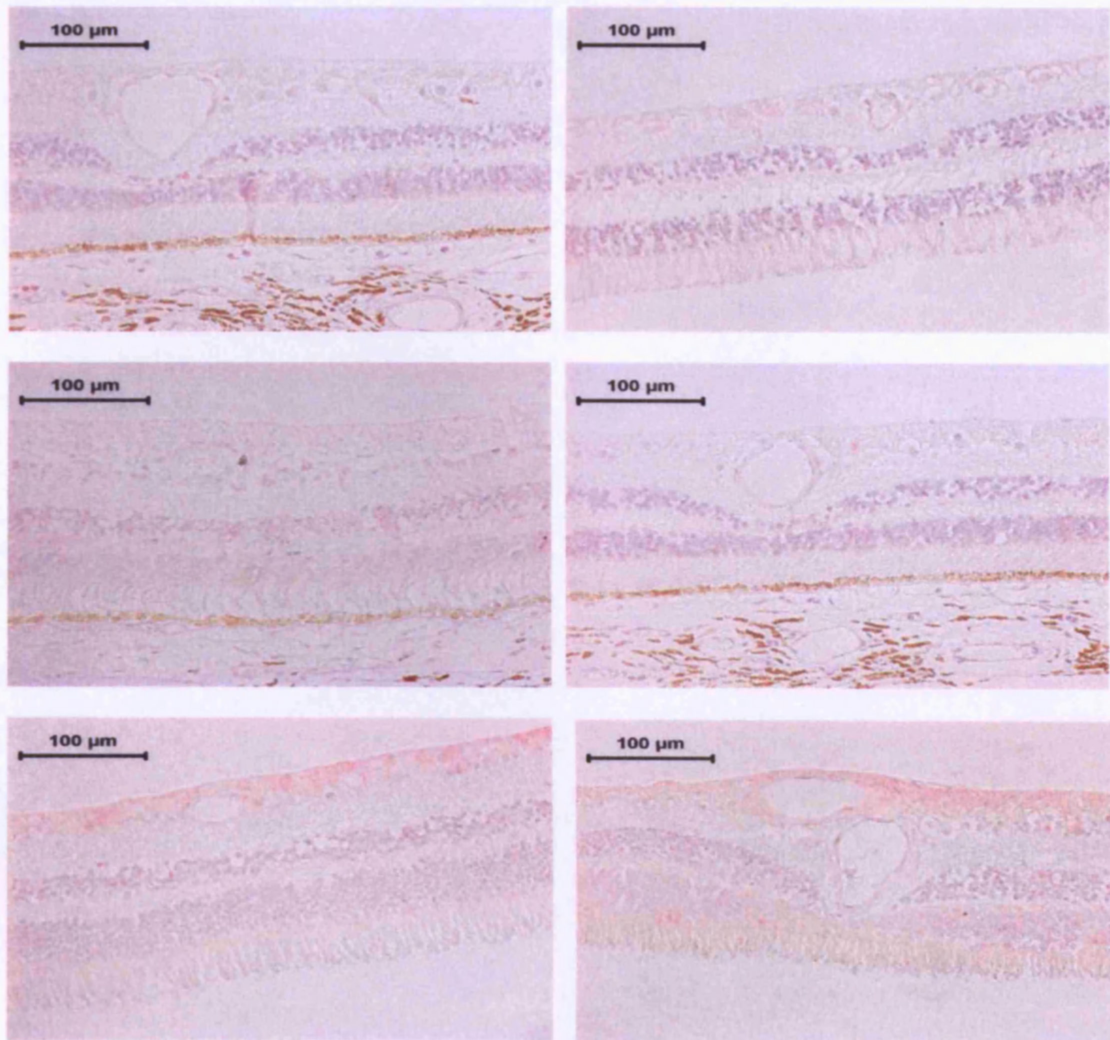

Figure 4.21 Transverse sections showing the immunolocalisation of VEGFR-2 in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities. Staining was absent or minimal.

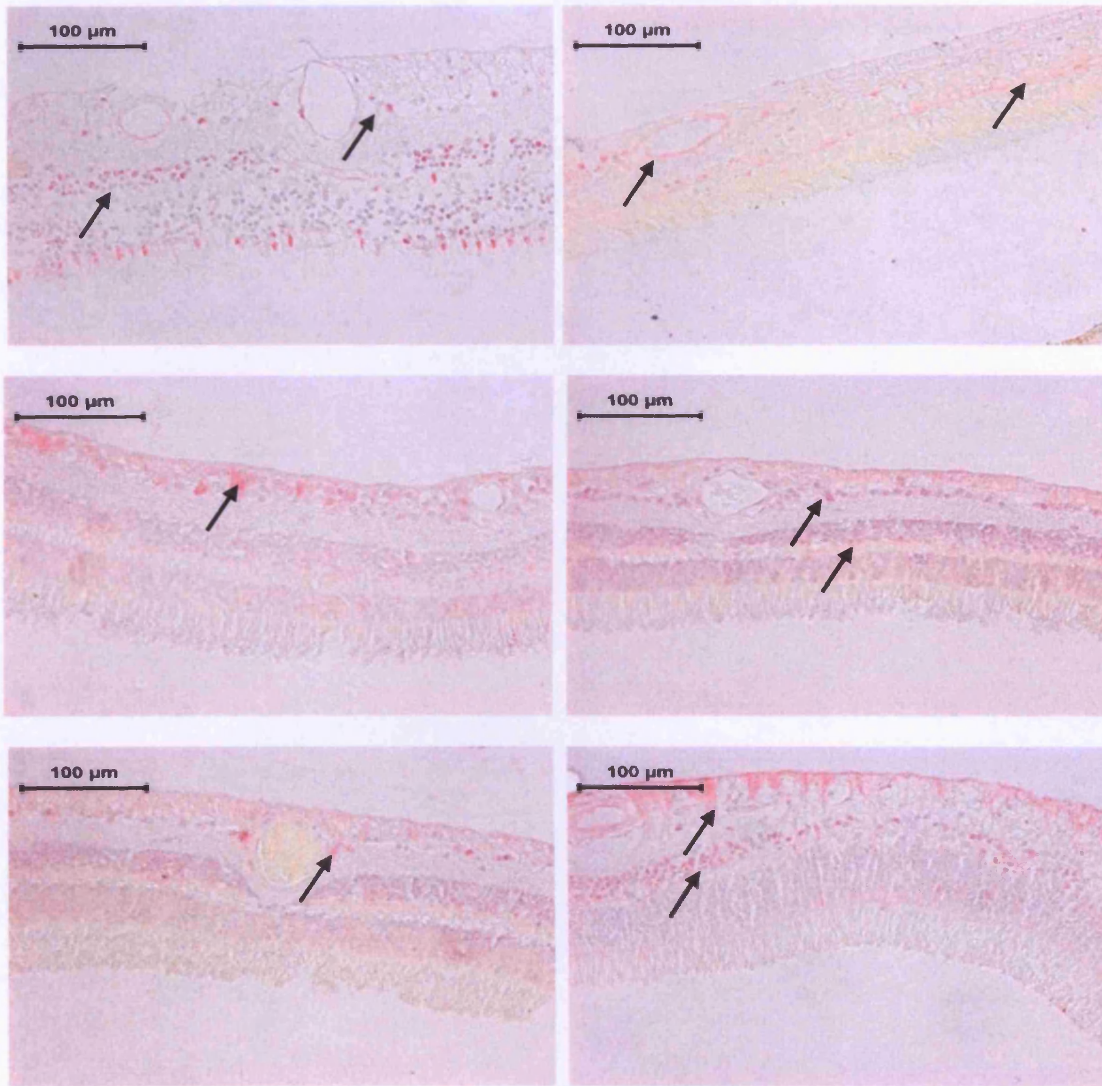

Figure 4.22 Transverse Sections Showing the Immunolocalisation of VEGFR-2 (Arrows) in Unlasered Diabetic Retinas with NPDR.

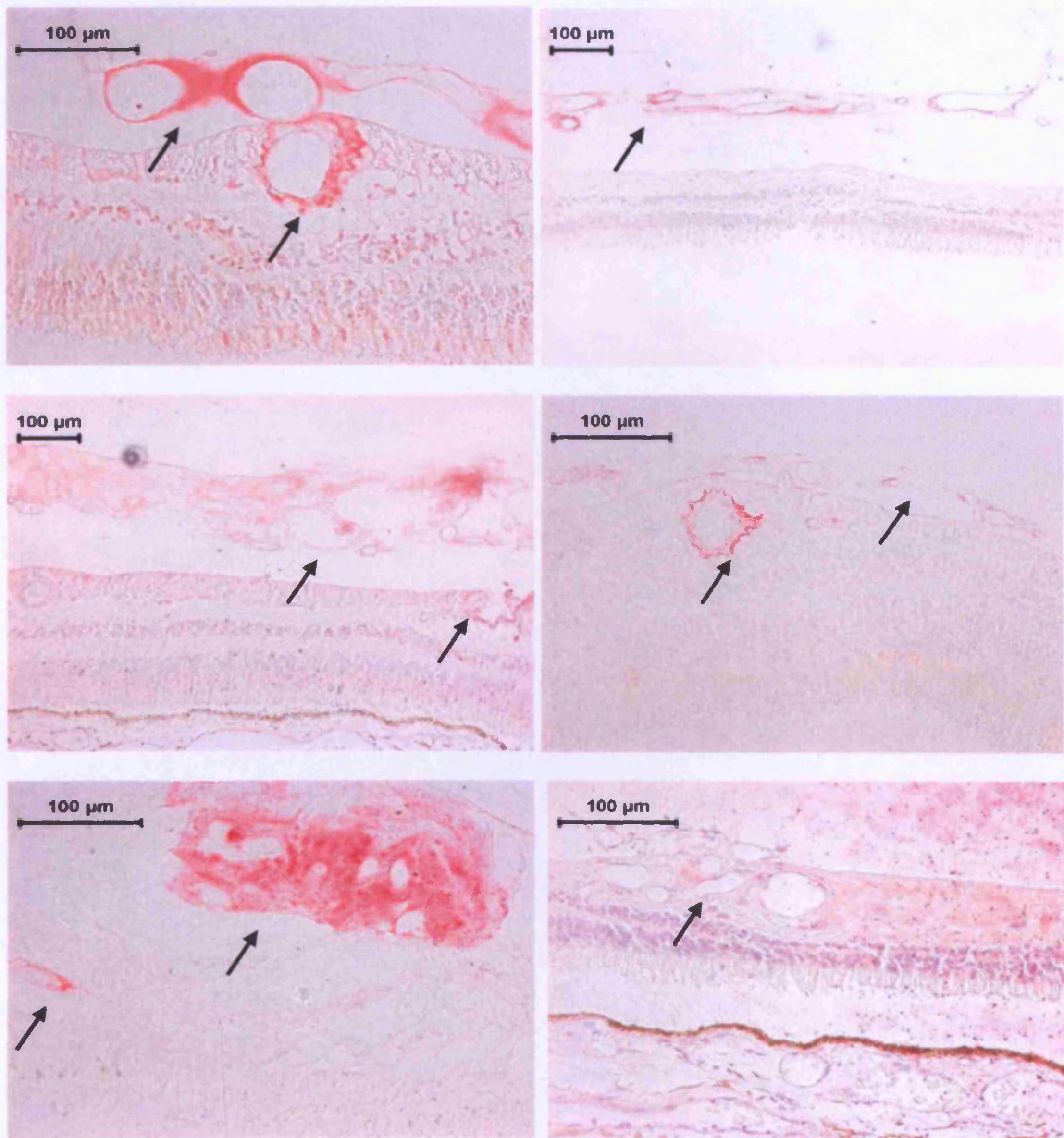

Figure 4.23 Transverse Sections Showing the Immunolocalisation of VEGFR-2 in Diabetic Retinas with PDR.
Arrows show staining around the retinal vessels and in the preretinal membranes.

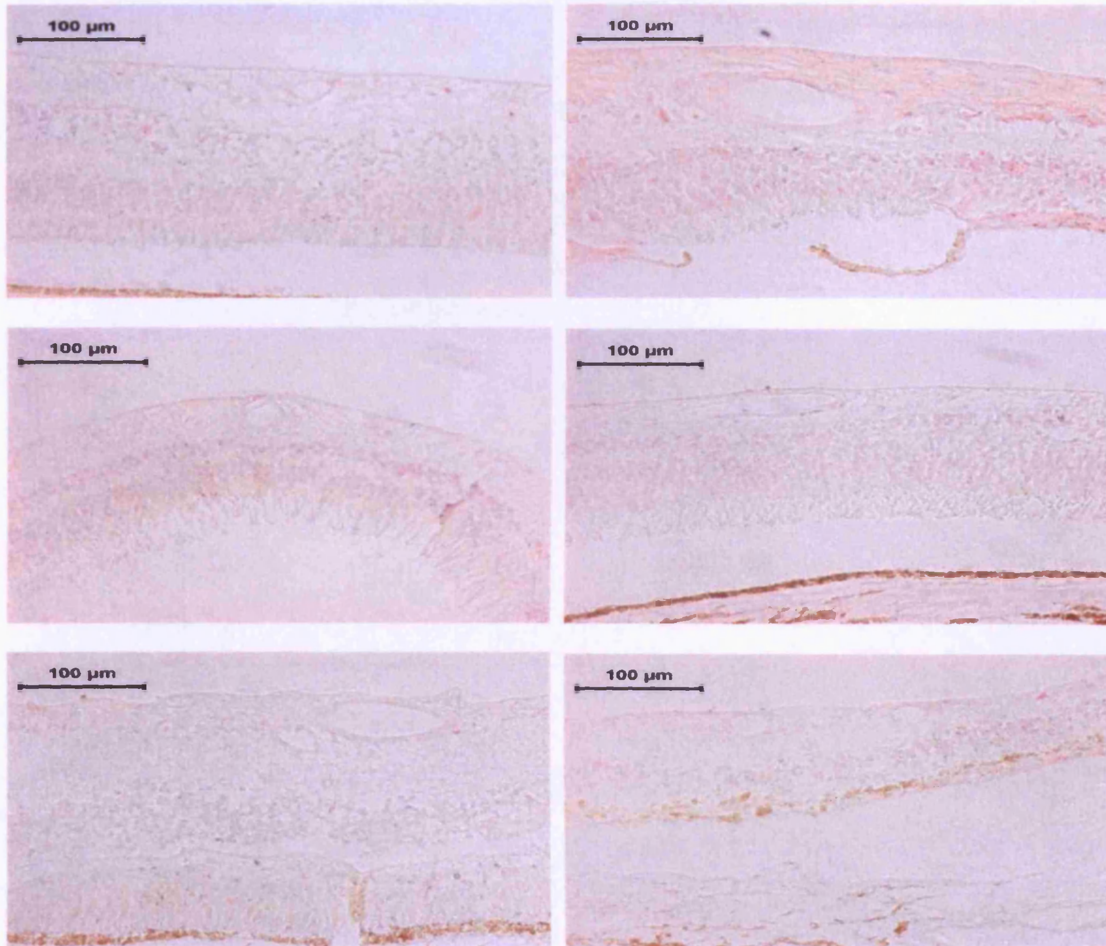

Figure 4.24 Transverse Sections Showing the Immunolocalisation of VEGFR-2 in Lasered Diabetic Retinas.
Staining was absent or minimal.

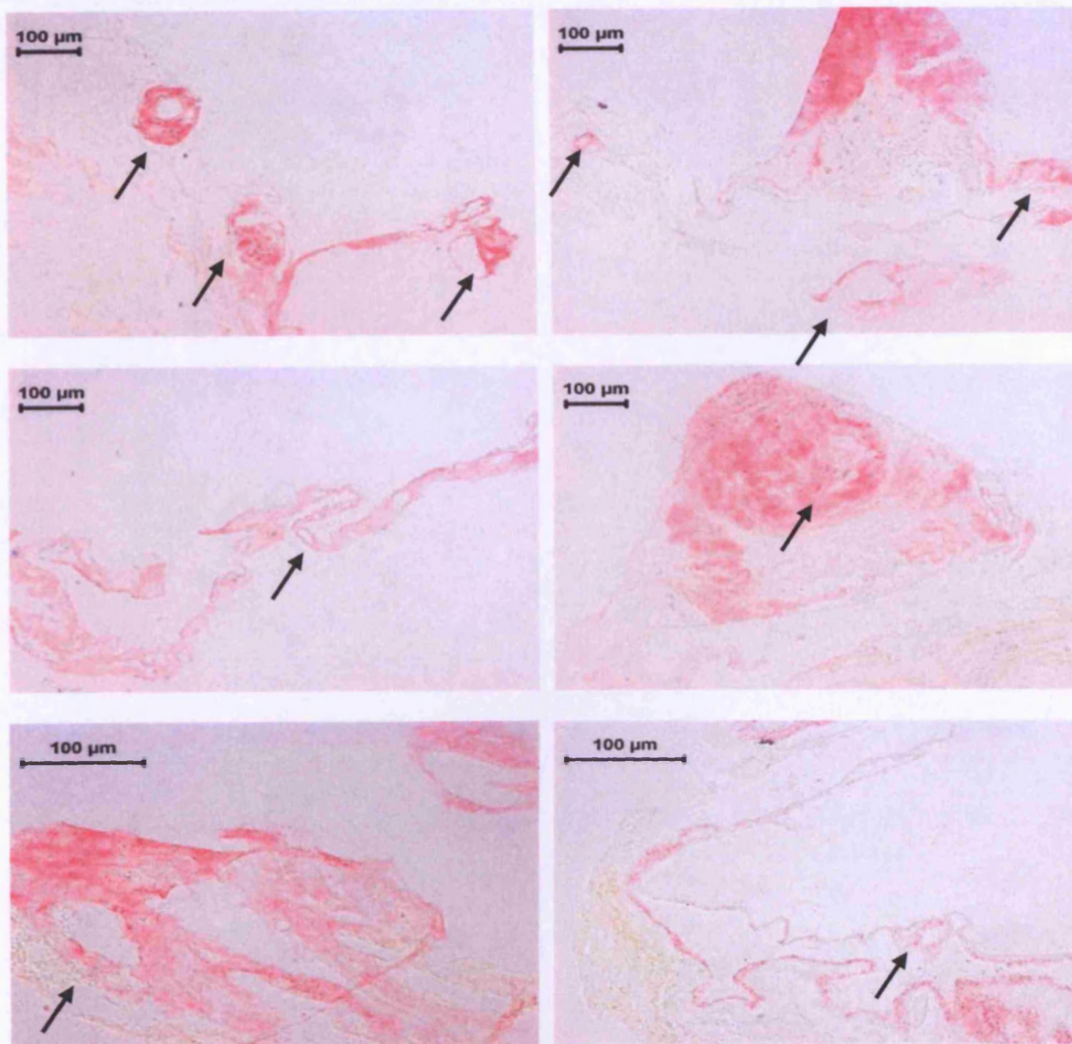

Figure 4.25 Transverse Sections Showing the Immunolocalisation of VEGFR-2 (Arrows) in Fibrovascular Membranes

TABLE 4.5 MEAN INTENSITY OF VEGFR-3 IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	0 (0)	0.1 (0.3)	0.9 (0.9)	0.6 (0.7)	0.8 (0.9)		
No Overt Retinopathy (n=12)	0.3 (0.8)	0 (0)	1.5 (0.8)	1.8 (0.8)	1.0 (0.7)		
Intraretinal Changes (n=10	0.6 (0.8)	0 (0)	1.8 (1.2)	2.0 (1.1)	1.2 (1.5)		
PDR (n=9	0 (0)	0 (0)	0.3 (0.5)	1.0 (0.8)	1.5 (1.1)	1.5 (1.3)	0.3 (0.5)
Laser-No Residual PDR (n=14)	0.2 (0.6)	0.2 (0.4)	0.9 (0.6)	0.8 (0.6)	0.9 (0.8)		
Excised Membranes (n=17)						1.7 (1.0)	0.2 (0.4)

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

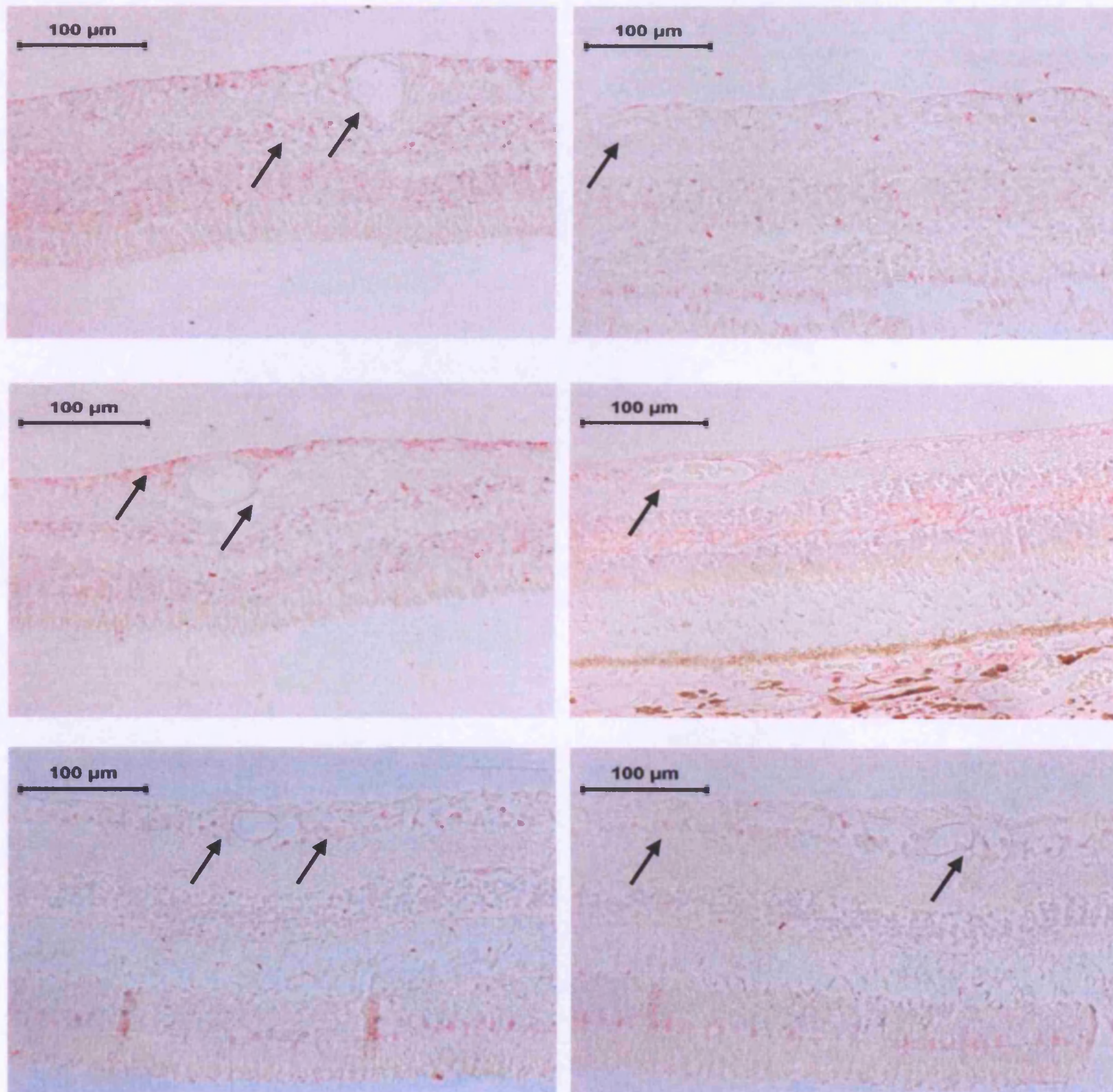

Figure 4.26 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Non Diabetic Retinas

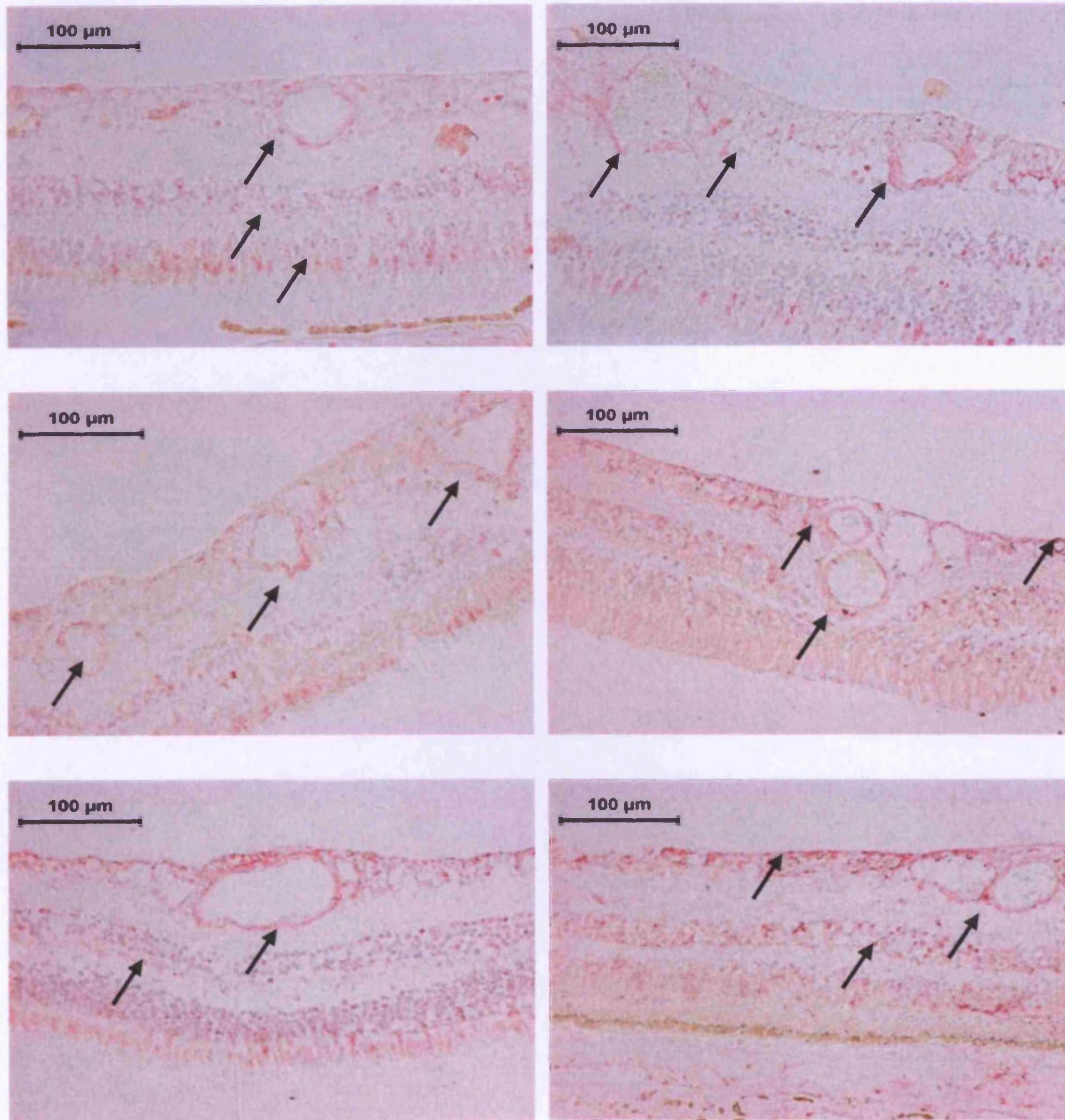

Figure 4.27 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities

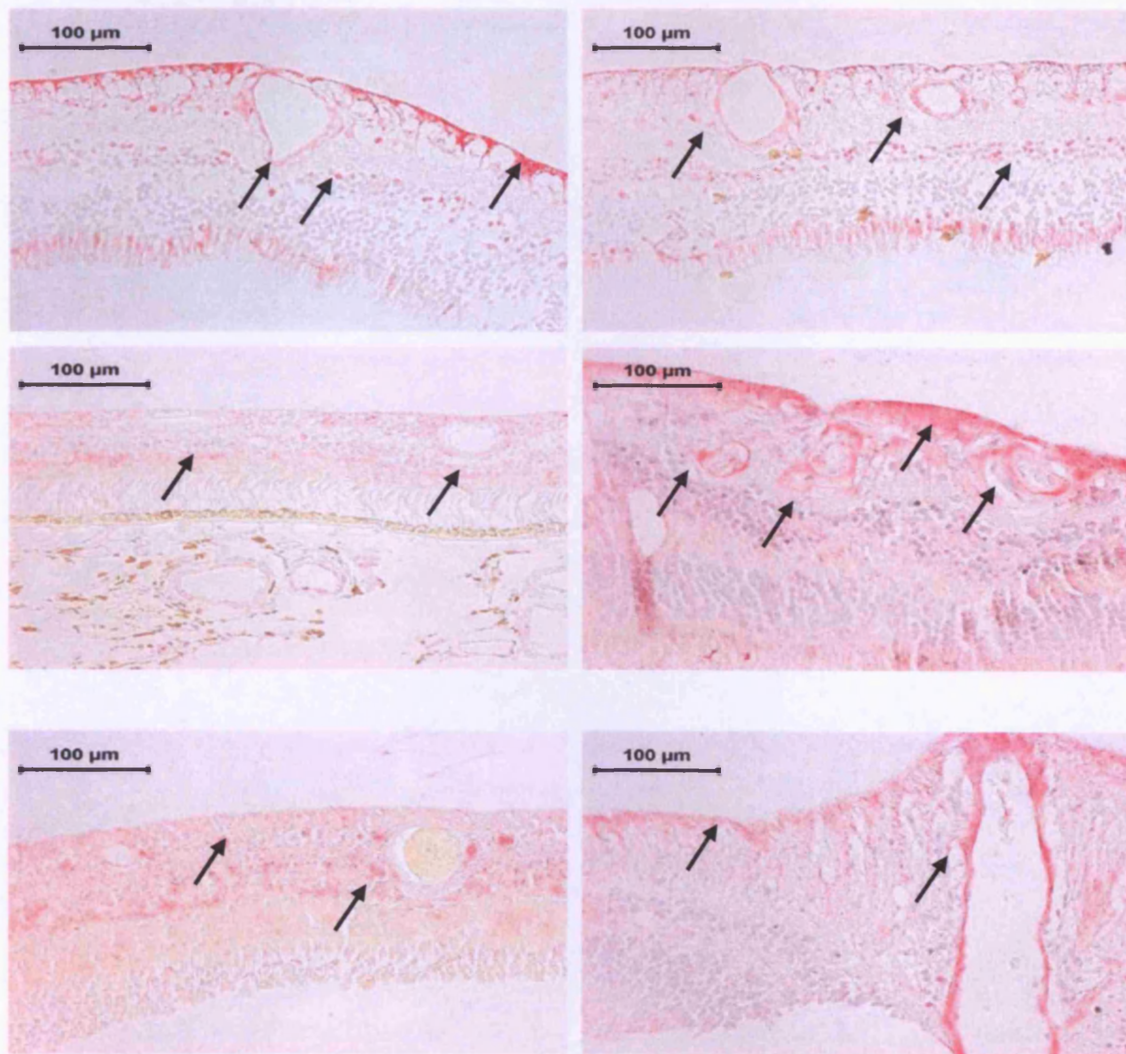

Figure 4.28 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Unlasered Diabetic Retinas with NPDR.

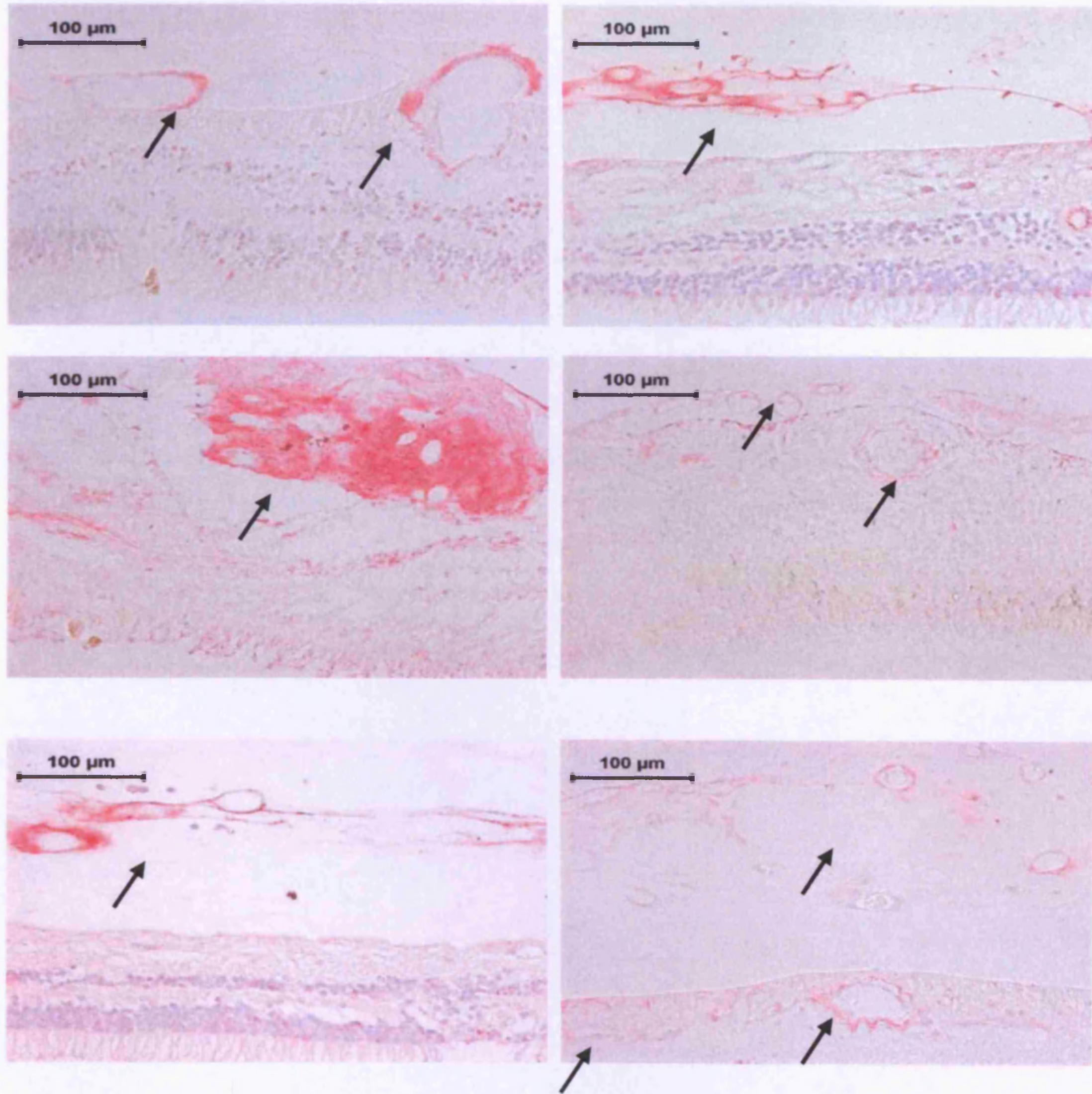

Figure 4.29 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Diabetic Retinas with PDR.

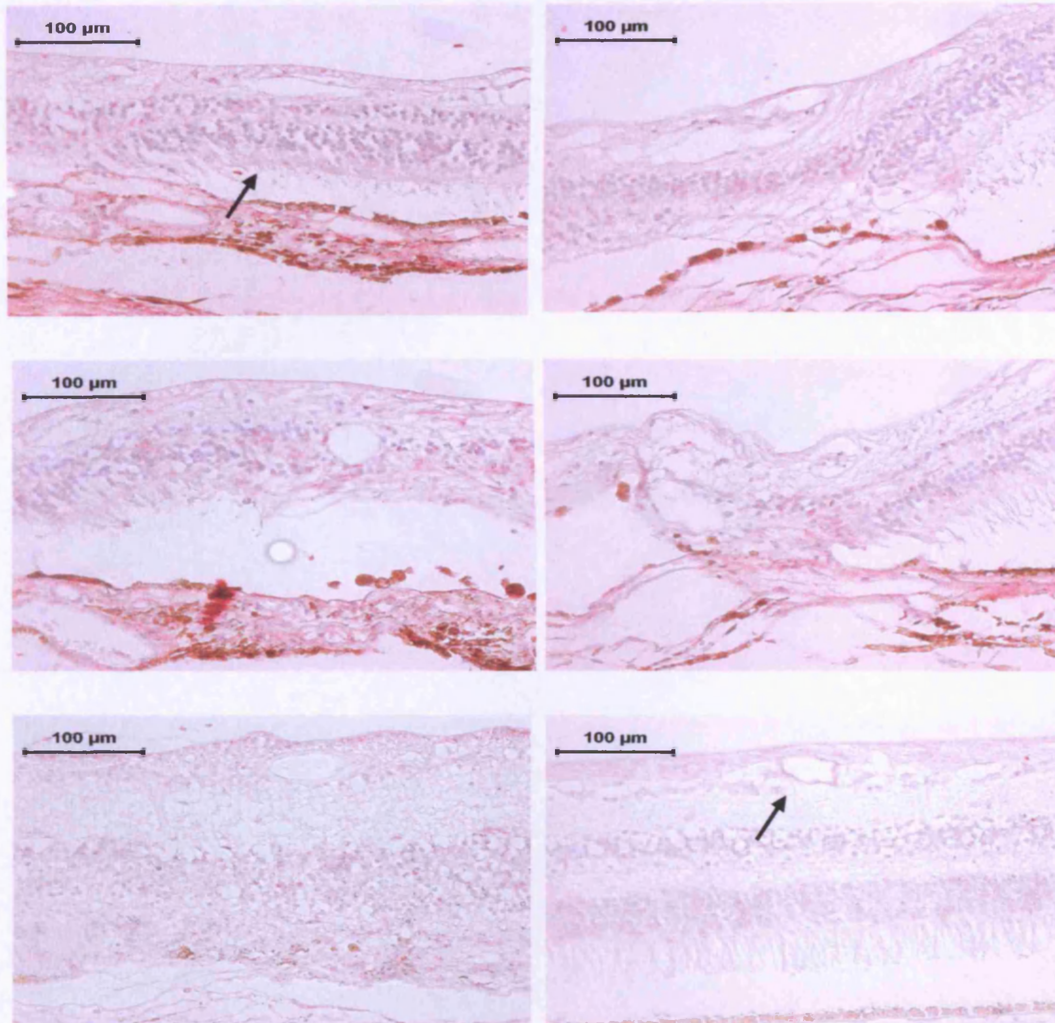

Figure 4.30 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Lasered Diabetic Retinas.

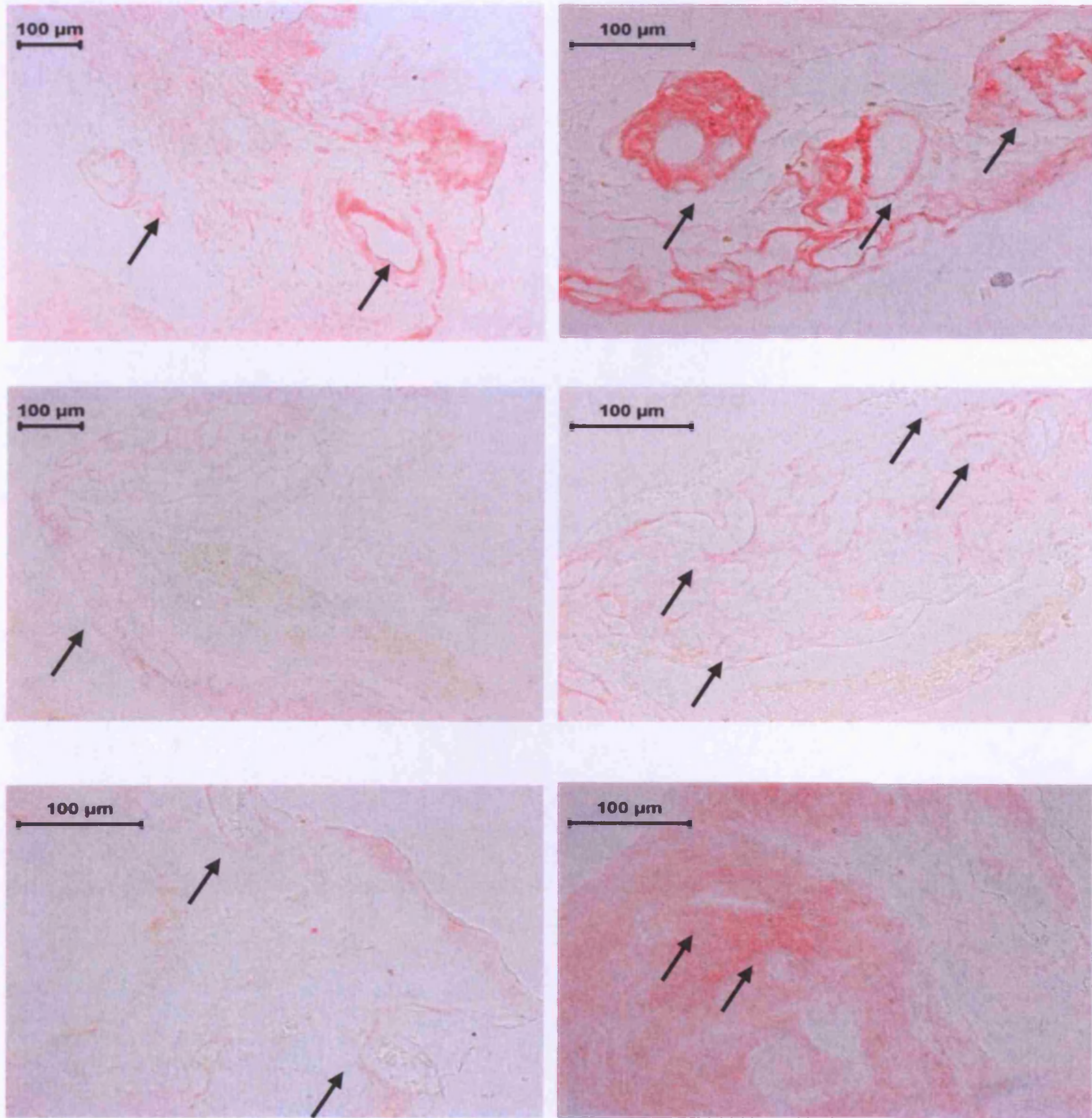

Figure 4.31 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Fibrovascular Membranes

4.3 Angiopoietin immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy, Ang-1 and Ang-2 staining was apparent in both non-diabetic and diabetic tissue. The staining pattern depended upon the specificity of the antibody being used and the category of tissue. Ang-1 immunoreactivity was generally confined to the vessels of the retina but not to the preretinal vessels of the membranes. Ang-2 immunoreactivity was confined to the preretinal vessels undergoing active neovascularization but only appeared to be associated with the retinal vessels of the non-diabetic retinas and diabetic retinas with no overt retinopathy. Variable staining of the vessels within each retina was observed with some staining positive and some staining negative. Ang-2 immunoreactivity was also observed within the GCL. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in tables 4.6 and 4.7, but this did not correlate with donor age, post-mortem time, or duration of diabetes.

The average scores and standard deviations for Ang-1 immunostaining are represented in table 4.6. Statistical analysis demonstrated that significant differences were observed within the retinal vessels across the tissue categories ($P = <0.05\%$) but not within the retinal layers.

In the non-diabetic retinas staining intensity for Ang-1 was generally absent or minimal within the photoreceptors, the cell bodies of the outer retina, the inner retina, and the GCL. Weak to moderate staining was observed within the retinal vessels of 10/14 of the non-diabetic retinas (Fig. 4.32).

In the diabetic retinas with no overt retinopathy Ang-1 staining intensity was reduced to weak levels within the retinal vessels as compared to the non-diabetic retinas. Staining was again absent or minimal within the photoreceptors, the cell bodies of the outer retina, the inner retina and the GCL (Fig. 4.33).

In the diabetic retinas showing vascular changes but no evidence of PDR, Ang-1 immunostaining was increased within the retinal vessels of 4/5 of the retinas, compared to that observed in the diabetic eyes with no overt retinopathy. Staining was again absent or minimal within the photoreceptors, and the cell bodies of the outer retina, inner retina, and GCL (4.34).

In the diabetic retinas with active neovascular PDR membranes on their surfaces staining was increased to moderate levels within the retinal vessels (5/6) compared to the retinas with intraretinal changes. Minimal staining was observed within the preretinal vessels of the membranes and no staining was apparent within the non-vasculr components of the

membranes. Staining was absent or minimal within the photoreceptors and the cell bodies of the outer retina, the inner retina, and the GCL.

In those diabetic retinas which had undergone successful laser therapy the staining intensity was reduced to weak to moderate levels within the retinal vessels. Staining was again absent or minimal within the photoreceptors, the cell bodies of the inner retina, the outer retina, and the GCL (Fig. 4.35).

Minimal staining was observed within the preretinal vessels of the excised membranes. Weak to moderate staining was associated with the non vascular components of most (9/11) of the membranes (Fig. 4.37).

The average scores and standard deviations for Ang-2 staining are represented in table 4.7. Statistical analysis demonstrated that no significant differences were observed within the retinal layers and the retinal vessels across the tissue categories.

In the non-diabetic retinas weak to moderate staining was observed within the GCL (11/14) and the retinal vessels (10/14). Minimal staining was demonstrated within the photoreceptors (6/14), and the cell bodies of the outer retina and the inner retina (Fig. 4.38).

In the diabetic retinas with no overt retinopathy, Ang-2 immunostaining was again weak to moderate within GCL (10/12), and the retinal vessels (8/12). Minimal staining was demonstrated within the photoreceptors and the cell bodies of the outer and inner retina (Fig. 4.39).

In the diabetic retinas showing vascular changes but no evidence of PDR, Ang-2 staining was reduced to minimal levels within the retinal vessels (2/5) as compared to the non-diabetic retinas and the retinas with no overt retinopathy. Ang-2 staining intensity was weak to moderate within the GCL (4/5). Minimal staining was demonstrated within the photoreceptors and the cell bodies of the outer retina and inner retina (Fig. 4.40).

In the diabetic retinas with active neovascular PDR membranes on their surfaces 4/6 retinas demonstrated increased immunoreactivity for Ang-2 within the cell bodies of the inner retina as compared with the other categories of tissue. Ang-2 staining intensity was again weak to moderate within the GCL (5/6). Minimal staining was demonstrated within the photoreceptors, the cell bodies of the outer and inner retina and the retinal vessels. The staining intensity was weak to moderate within the preretinal vessels of the membranes. In this tissue category staining of the intraretinal vessels was associated both with the membranes and across the retina. (Fig. 4.41).

In those diabetic retinas which had undergone successful laser therapy Ang-2 staining was again weak to moderate within the GCL (14/14). Minimal staining was

demonstrated within the photoreceptors, the cell bodies of the outer retina, and the inner retina, and the retinal vessels (Fig. 4.42).

Minimal staining was observed within the preretinal vessels and the non-vascular components of the membranes (Fig. 4.43).

TABLE 4.6 MEAN INTENSITY OF ANG-1 IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	0.5 (0.8)	0.1 (0.3)	0.1 (0.4)	0.4 (0.6)	1.6 (1.2)		
No Overt Retinopathy (n=12)	0.3 (0.4)	0 (0)	0 (0)	0.3 (0.6)	0.9 (1.0)		
Intraretinal Changes (n=10)	0.4 (0.9)	0 (0)	0.2 (0.5)	0.2 (0.5)	1.4 (1.1)		
PDR (n=9)	0 (0)	0 (0)	0.2 (0.4)	0.2 (0.4)	2.2 (1.3)	0.4 (0.9)	0 (0)
Laser-No Residual PDR (n=14)	0.1 (0.3)	0 (0)	0.1 (0.3)	0.1 (0.3)	1.2 (1.0)		
Excised Membranes (n=17)						0.5 (1.0)	1.6 (1.0)

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

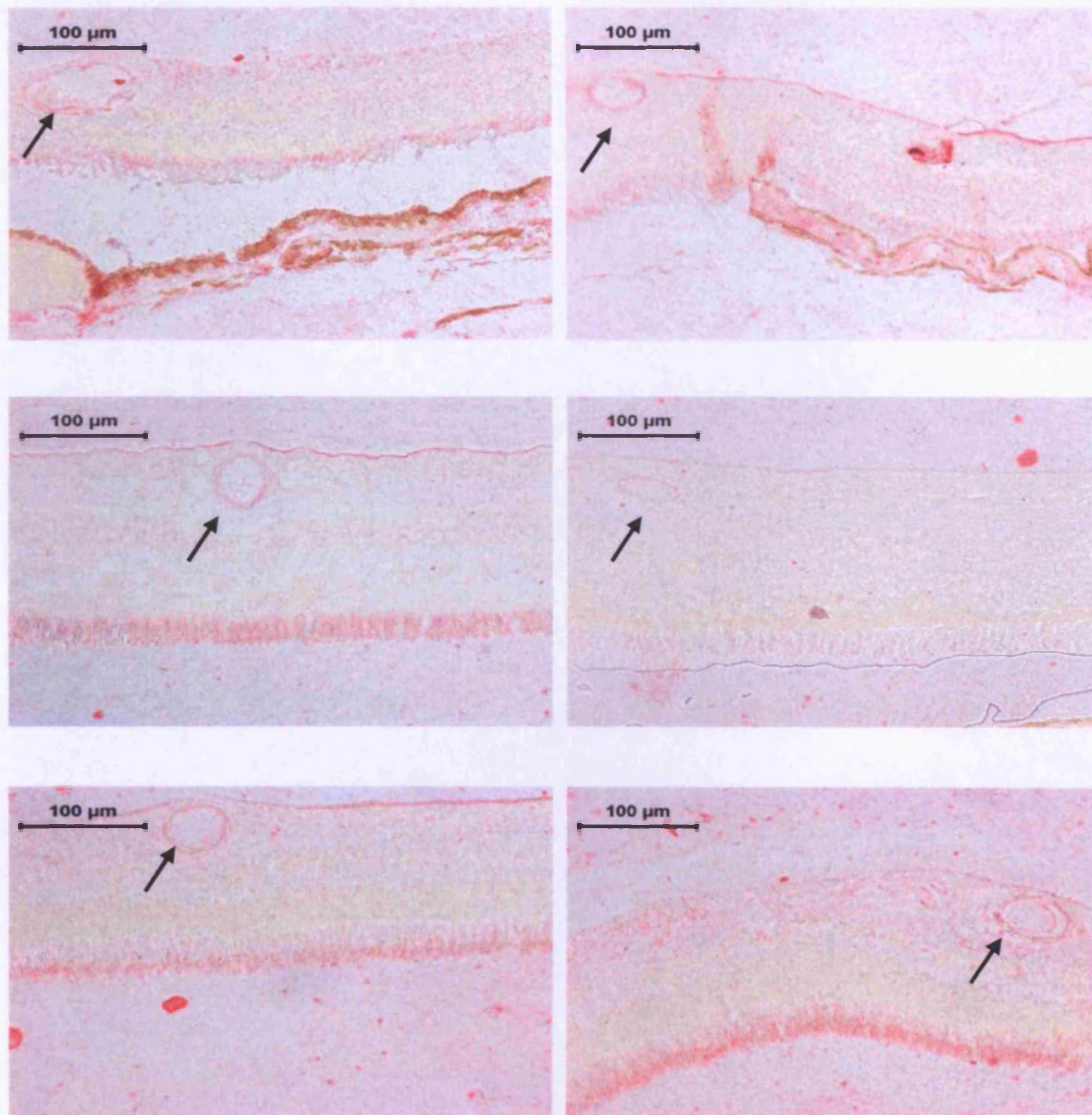

Figure 4.32 Transverse Sections Showing the Immunolocalisation of Ang-1 (Arrows) in Non-Diabetic Retinas.

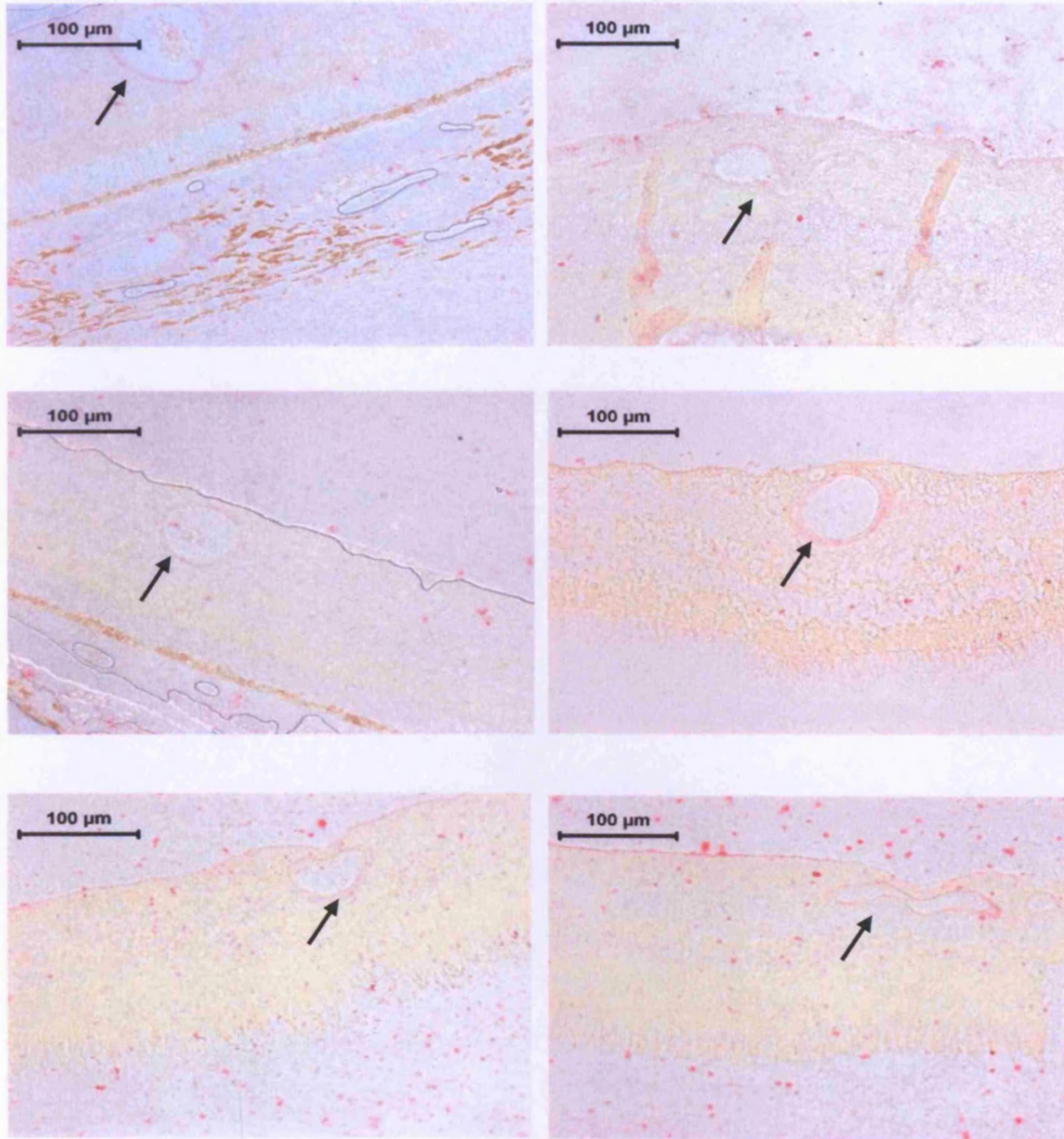

Figure 4.33 Transverse Sections Showing the Immunolocalisation of Ang-1 (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.

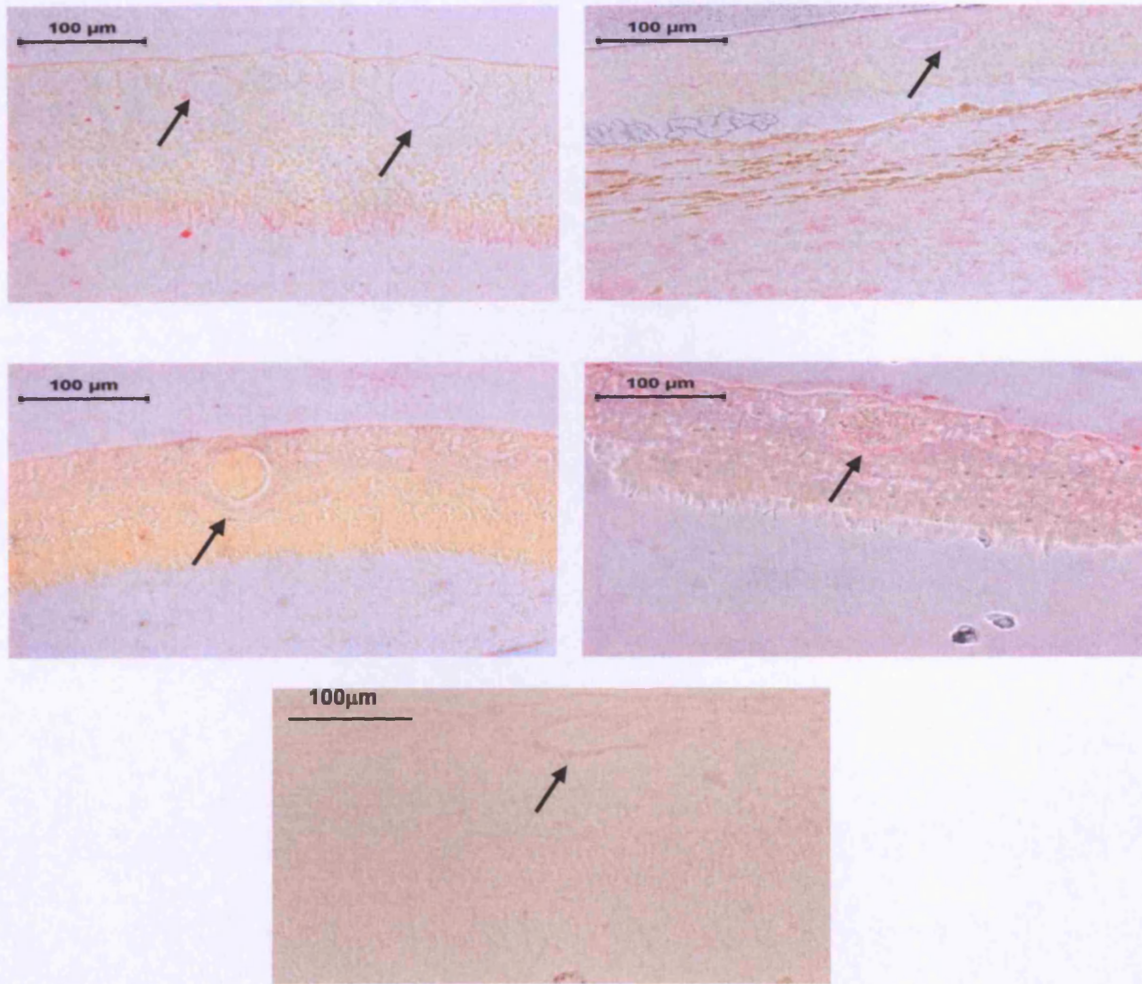

Figure 4.34 Transverse Sections Showing the Immunolocalisation of Ang-1 (where present) [Arrows] in Unlasered Diabetic Retinas with NPDR.

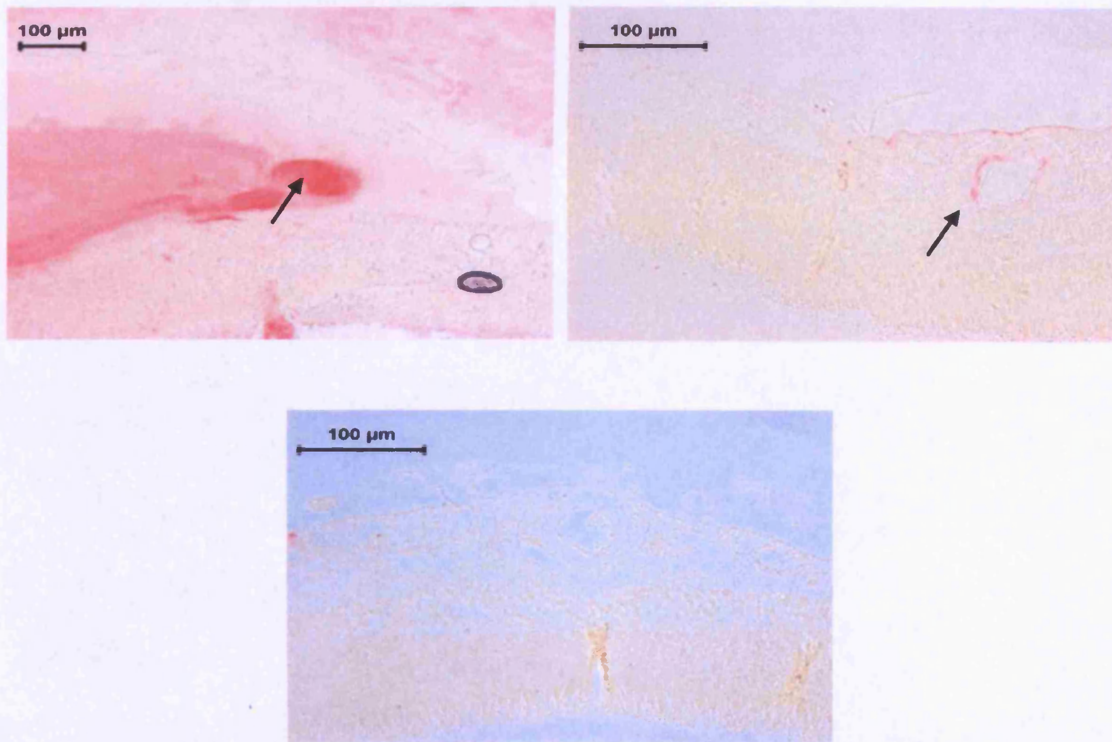

Figure 4.35 Transverse Sections Showing the Immunolocalisation of Ang-1 in Diabetic Retinas with PDR.

Photographs from sections with minimal antibody precipitates present are shown.

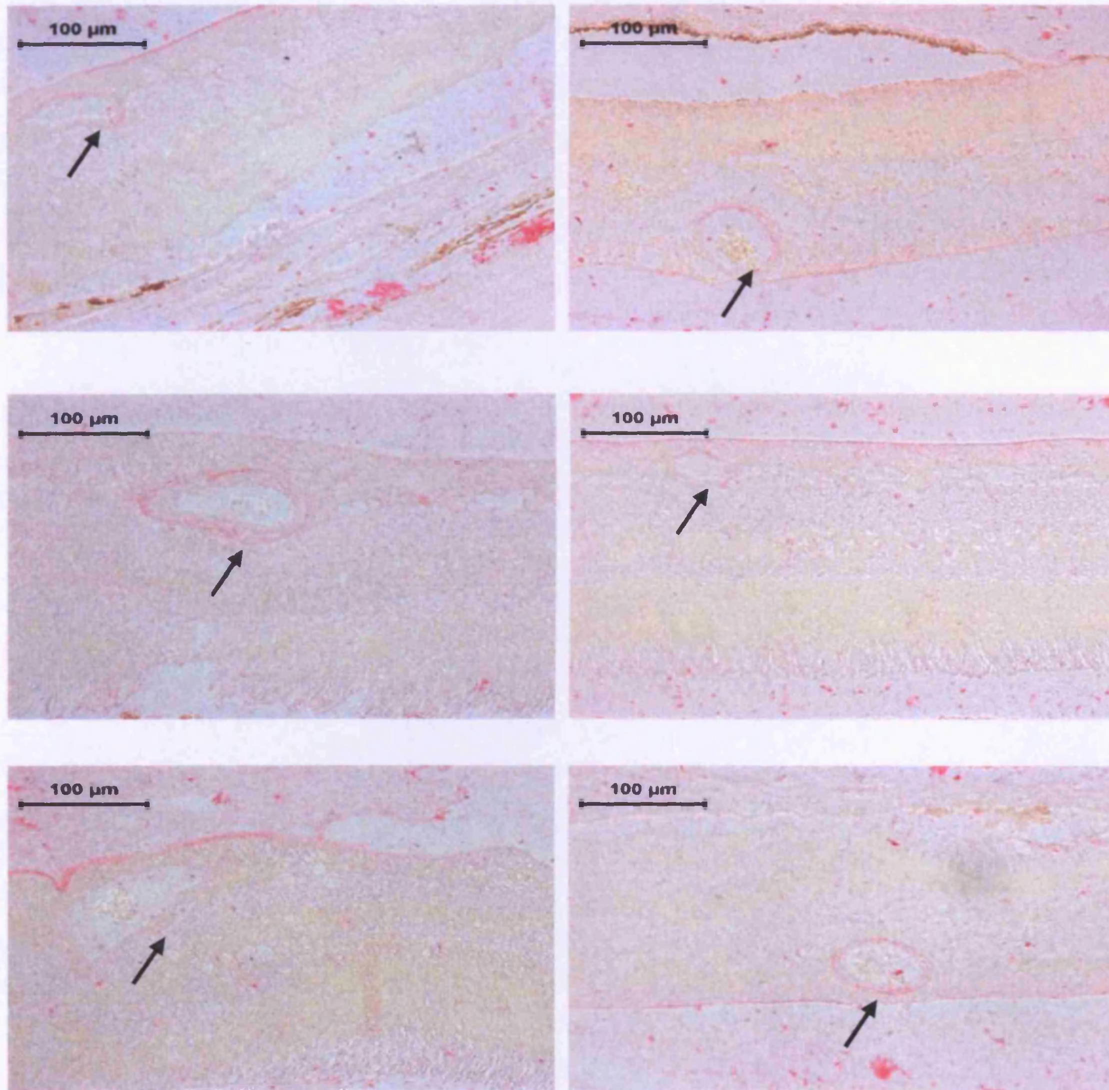

Figure 4.36 Transverse Sections Showing the Immunolocalisation of Ang-1 (Arrows) in Lasered Diabetic Retinas

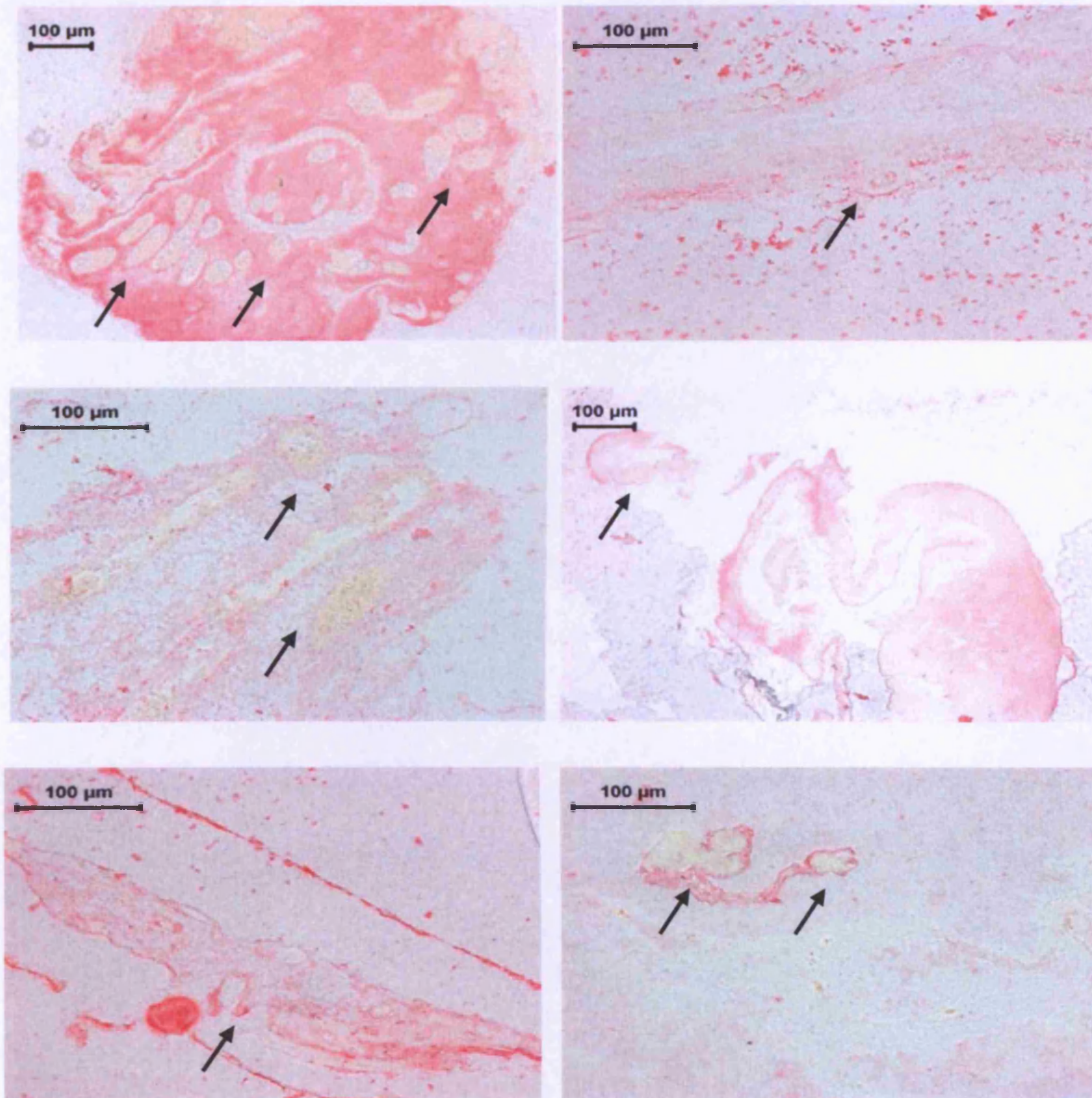

Figure 4.37 Transverse Sections Showing the Immunolocalisation of Ang-1 in Fibrovascular Membranes

TABLE 4.7 MEAN INTENSITY OF ANG-2 IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	0.6 (0.9)	0.2 (0.6)	0.1 (0.4)	1.5 (1.0)	1.6 (1.2)		
No Overt Retinopathy (n=12)	0.8 (0.6)	0.3 (0.5)	0.2 (0.4)	1.8 (1.1)	1.5 (1.2)		
Intraretinal Changes (n=10)	0.6 (0.9)	0.2 (0.5)	0.4 (0.6)	1.6 (1.1)	0.6 (0.9)		
PDR (n=6)	0.3 (0.5)	0.3 (0.8)	1.0 (1.1)	1.5 (1.1)	0.7 (1.2)	1.5 (1.6)	0.3 (0.5)
Laser-No Residual PDR (n=14)	0.6 (0.7)	0.4 (0.6)	0.6 (0.8)	1.6 (0.6)	0.9 (1.0)		
Excised Membranes (n=17)						0.7 (1.3)	0.9 (0.9)

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

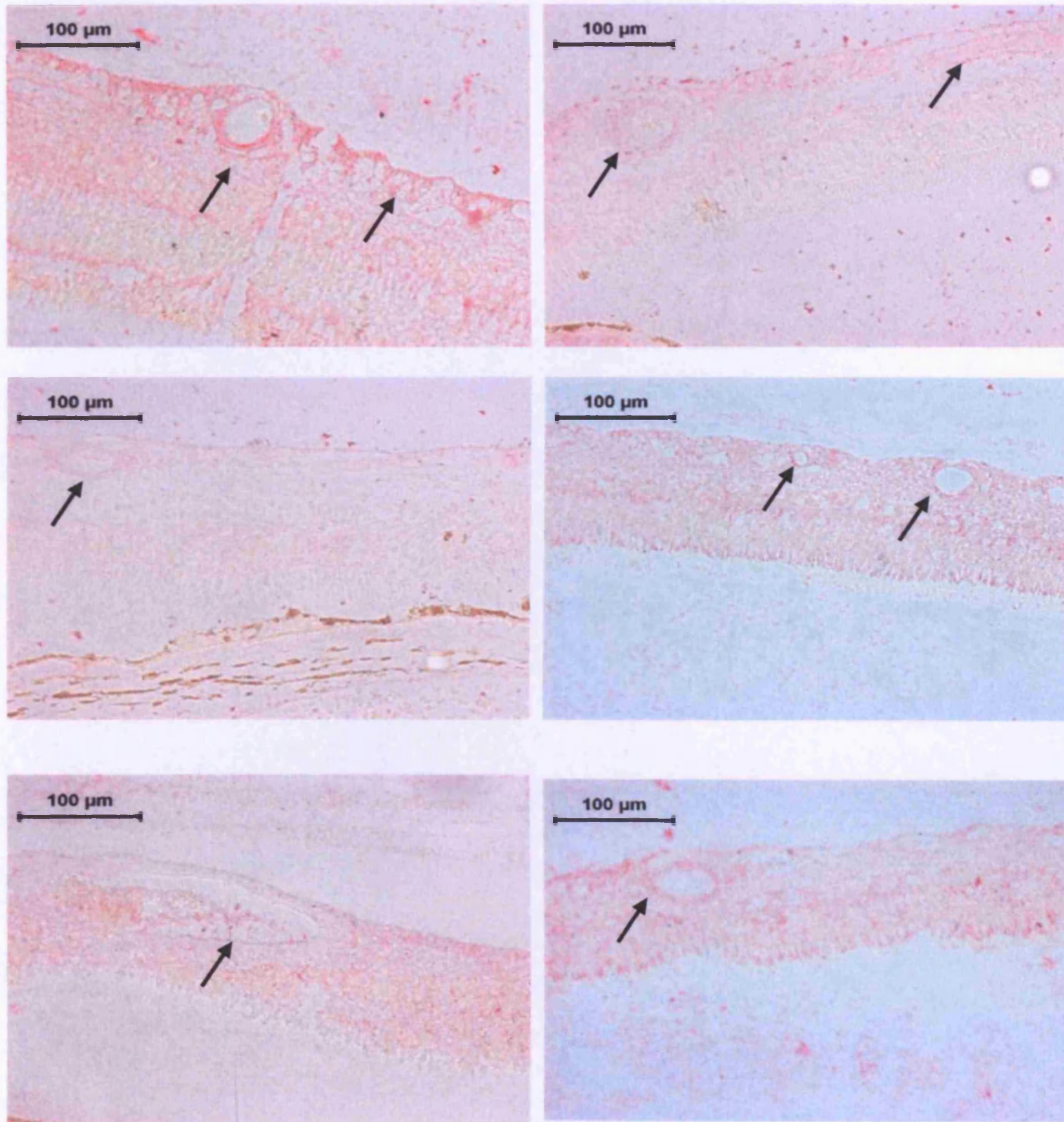

Figure 4.38 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Non-Diabetic Retinas

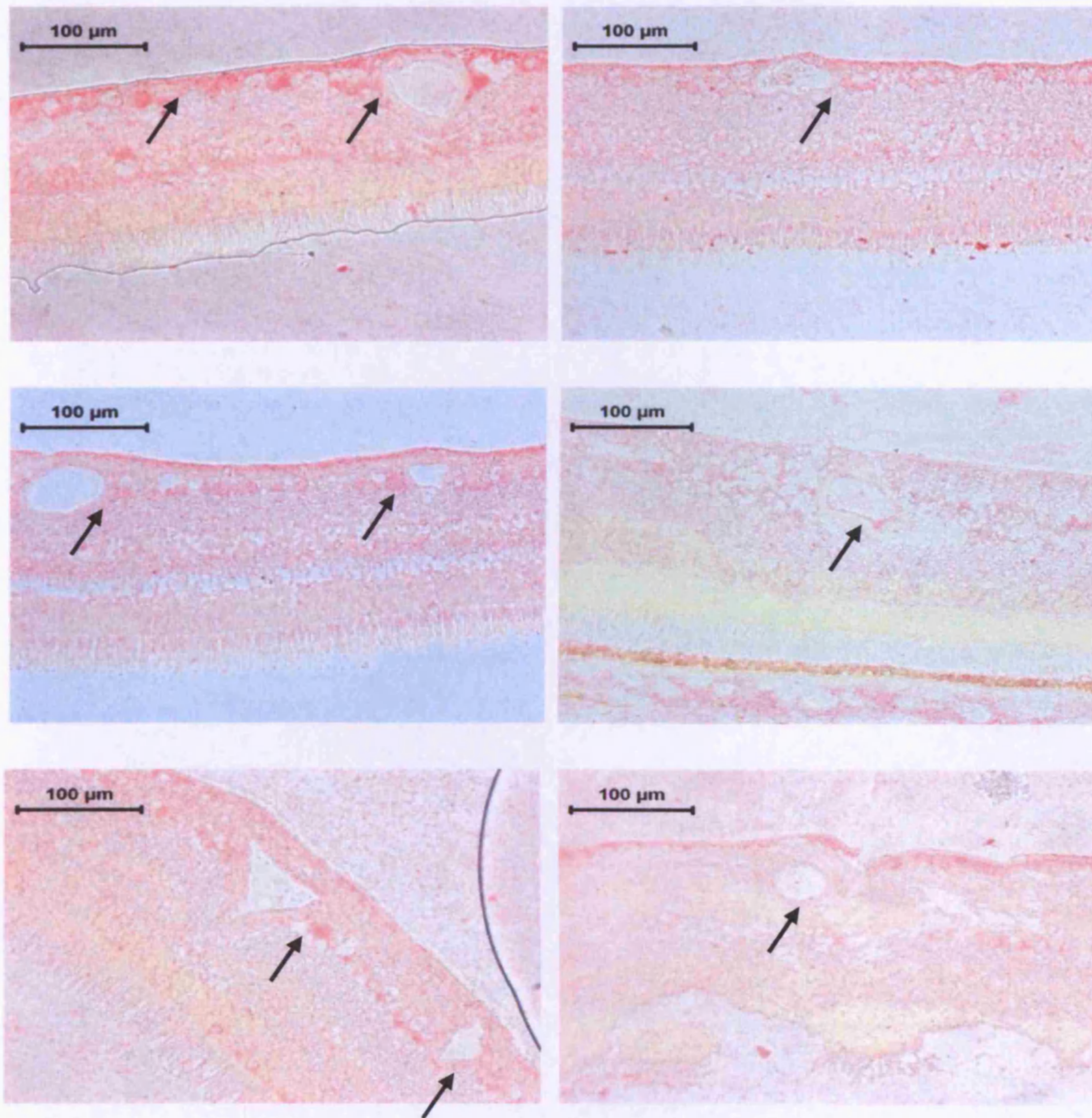

Figure 4.39 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities

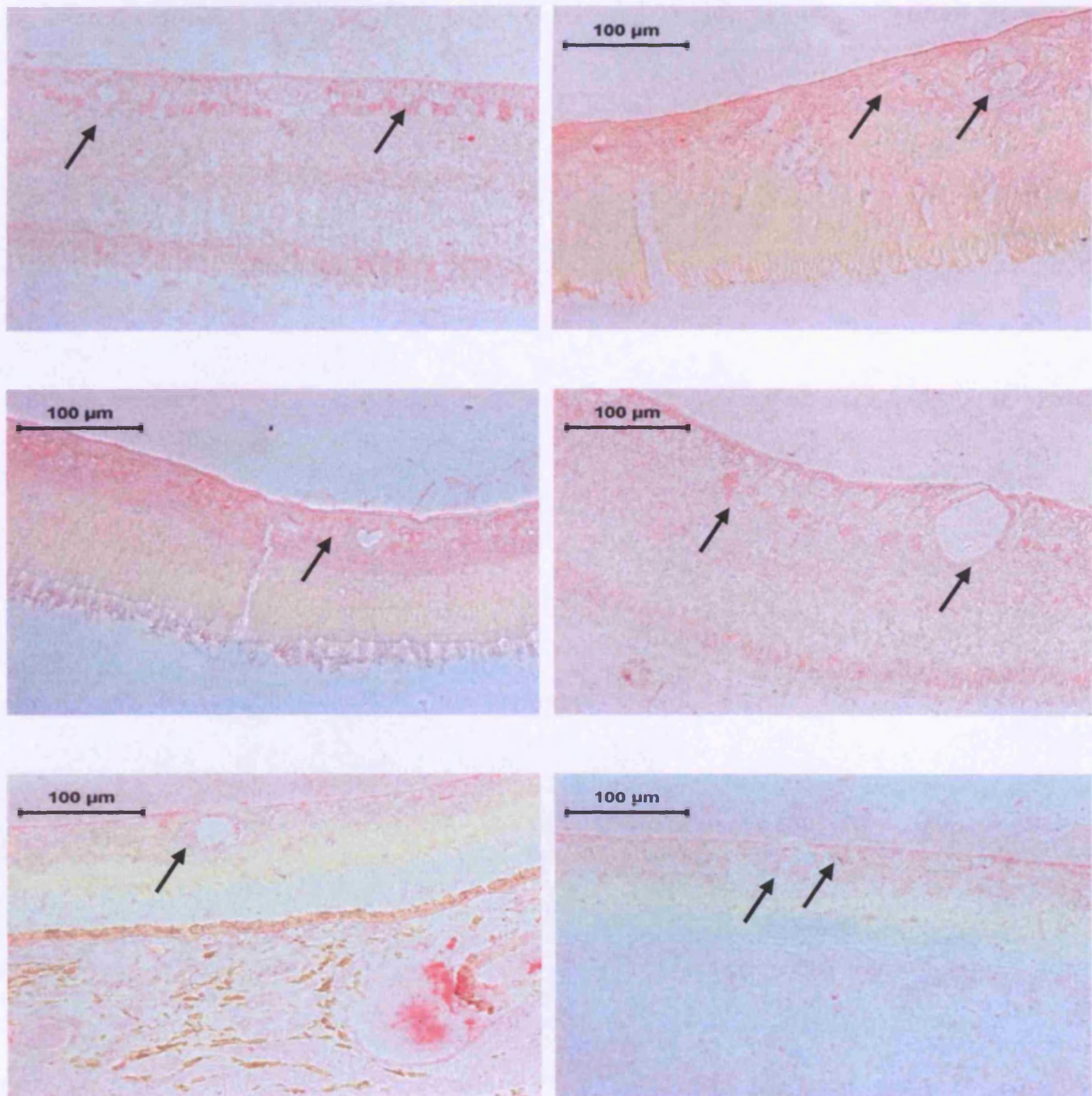

Figure 4.40 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Unlasered Diabetic Retinas with NPDR.

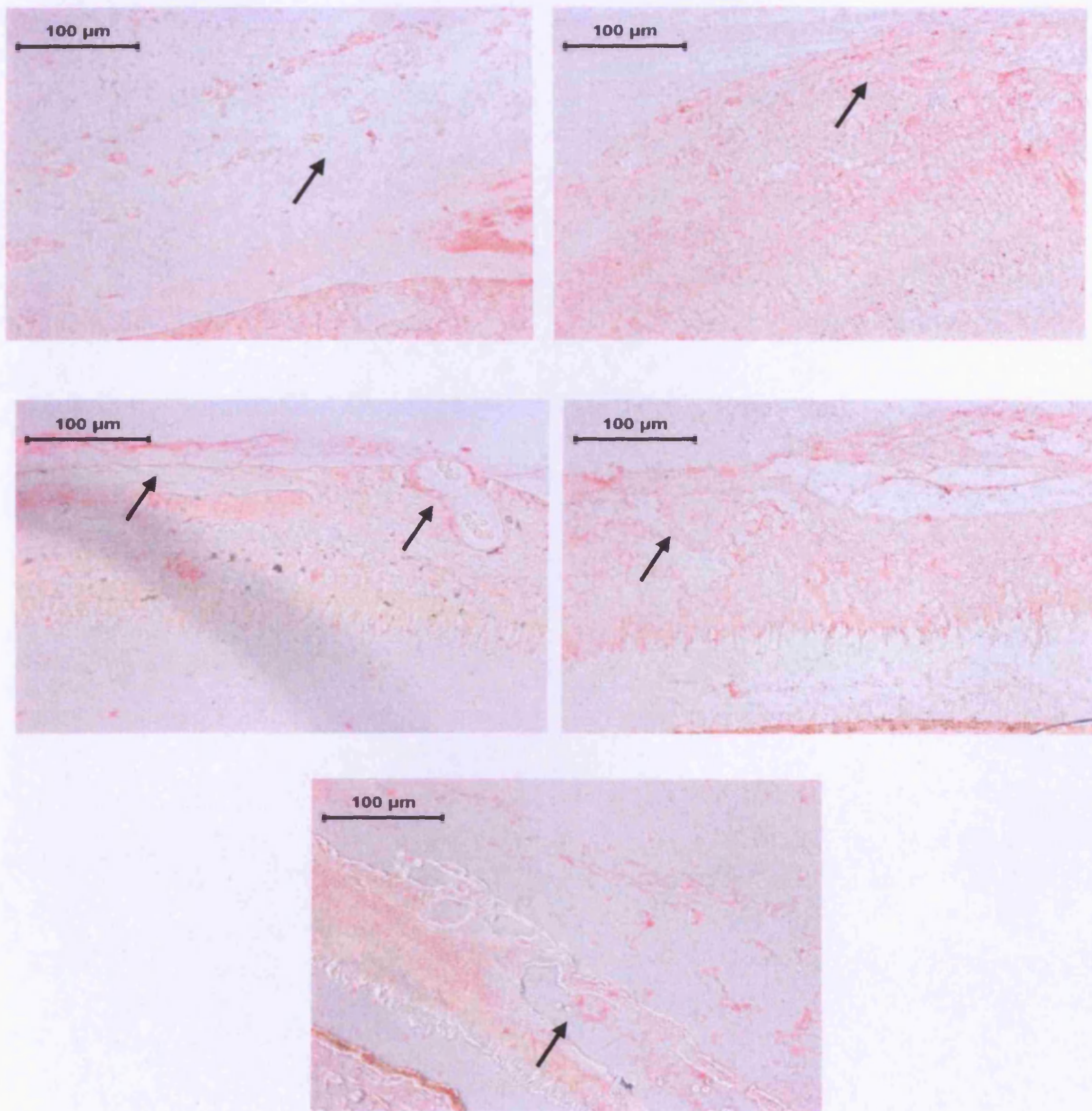

Figure 4.41 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Diabetic Retinas with PDR.

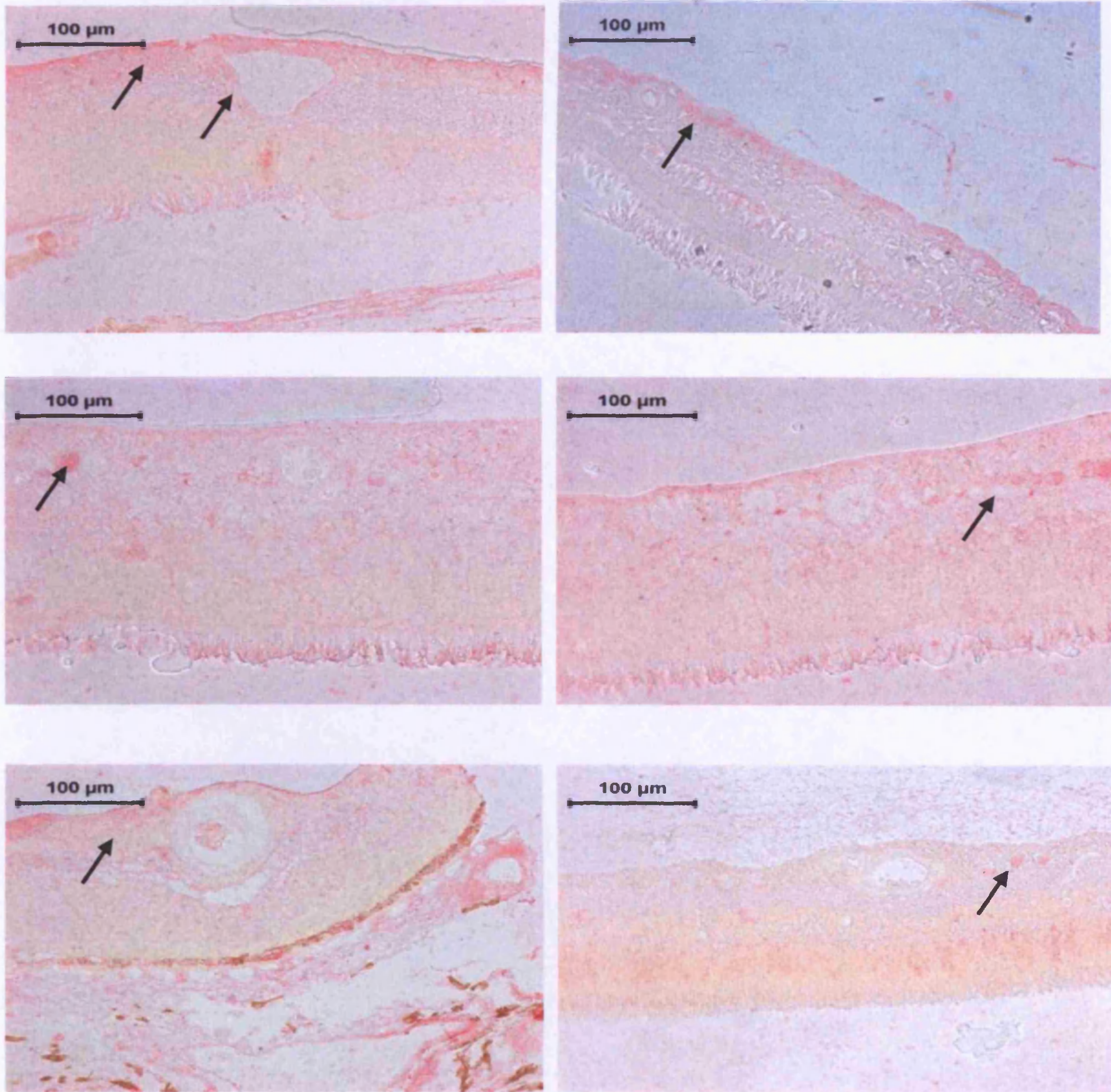

Figure 4.42 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Lasered Diabetic Retinas

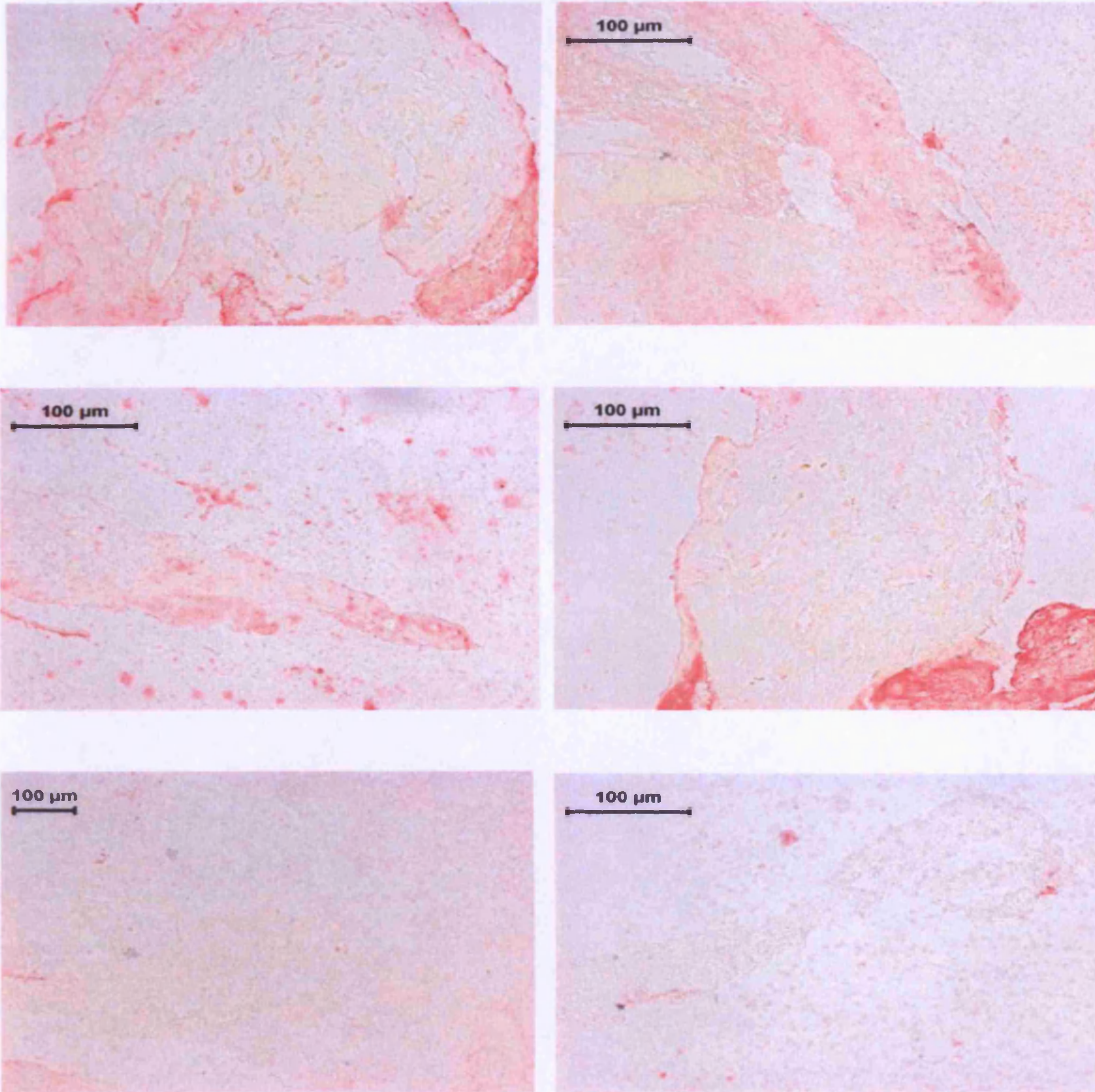

Figure 4.43 Transverse Sections Showing the Immunolocalisation of Ang-2 in Fibrovascular Membranes.
Staining was absent or minimal.

4.3.4 Tie-2 immunostaining of retinal sections and fibrovascular membranes

The average scores and standard deviations for Tie-2 immunostaining are represented in table 4.8. When examined by light microscopy, Tie-2 staining was apparent in most of the non-diabetic and diabetic vascular tissue which was confined to retinal endothelial cells and the perivascular region. Increased immunostaining was observed within the intraretinal vessels of diabetic tissue as compared to non-diabetic tissue. Variable staining of the vessels within each retina was observed with some staining positive and some staining negative. Staining was also associated with extravascular regions of the retina. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 4.8, but this did not correlate with either donor age, post-mortem time, or duration of diabetes. Statistical analysis demonstrated that significant differences were observed within the photoreceptors across all the tissue categories (P = <0.05%) but not within the retinal layers or the retinal vessels.

In the non-diabetic retinas weak staining for Tie-2 was observed within the retinal vessels (5/14). Weak to moderate staining was observed within the photoreceptors (11/14) and the cell bodies of the outer retina (13/14), the inner retina (12/14), and the GCL (13/14) [Fig. 4.44].

In the diabetic retinas with no overt retinopathy weak staining was demonstrated within the retinal vessels (6/12). Moderate staining was observed within the photoreceptors (10/12), and the cell bodies of the outer retina (11/12), and the GCL (11/12). However staining intensity was slightly reduced to weak to moderate levels within the cell bodies of the inner retina as compared to the non-diabetic retinas (11/12) [Fig. 4.45].

In the diabetic retinas showing vascular changes but no evidence of PDR the staining intensity was raised to weak to moderate levels within the retinal vessels (4/5) and to moderate levels within the cell bodies of the inner retina (5/5) as compared to the non-diabetic retinas and the retinas with no overt retinopathy. Staining intensity was reduced to weak to moderate levels within the photoreceptors (3/5) and the cell bodies of the outer retina (3/5) as compared to the non-diabetic retinas and the retinas with no overt retinopathy. Staining within the GCL was intense in all (5/5) of the retinas (Fig. 4.46).

In the diabetic retinas with active neovascular PDR membranes on their surfaces the staining intensity was increased to moderate to intense levels within the photoreceptors (6/6) and the cell bodies of the outer retina (6/6) as compared to the retinas with intraretinal changes. Staining intensity was again moderate within the cell bodies of the inner retina (4/6) and weak to moderate within the retinal vessels (3/6). In this tissue category staining of the

intra-retinal vessels was not specifically associated with the membranes. Weak to moderate staining was observed within the pre-retinal vessels of the membranes with 4/6 showing positive immunoreactivity for Tie-2 (Fig. 4.47). Weak to moderate staining was also associated with the non-vascular components of the membranes (3/6).

In those diabetic retinas which had undergone successful laser therapy the intensity of staining showed a similar pattern to that observed in the PDR retinas. However the level of staining was raised to moderate levels within the retinal vessels (11/14) [Fig. 4.48].

Staining for Tie-2 was minimal within the pre-retinal vessels of the excised fibrovascular membranes (2/11). Weak staining was demonstrated within the non-vascular component of the membranes (8/11) [Fig. 4.49].

TABLE 4.8. MEAN INTENSITY OF TIE-2 IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	1.7 (1.1)	1.7 (1.1)	1.7 (1.1)	1.7 (1.1)	1.1 (1.4)		
No Overt Retinopathy (n=12)	2.3 (1.2)	2.1 (0.1)	1.7 (1.0)	2.7 (0.9)	1.3 (1.5)		
Intraretinal Changes (n=10)	1.8 (1.6)	1.4 (1.5)	2.0 (1.0)	3.0 (0.0)	1.6 (1.3)		
PDR (n=9)	2.8 (0.5)	2.8 (0.5)	2.0 (1.6)	2.7 (0.5)	1.5 (1.6)	1.8 (1.5)	1.5 (1.6)
Laser-No Residual PDR (n=14)	2.5 (0.9)	2.5 (0.8)	2.3 (0.7)	2.7 (0.6)	2.1 (1.3)		
Excised membranes (n=17)						0.6 (1.2)	1.1 (0.9)

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

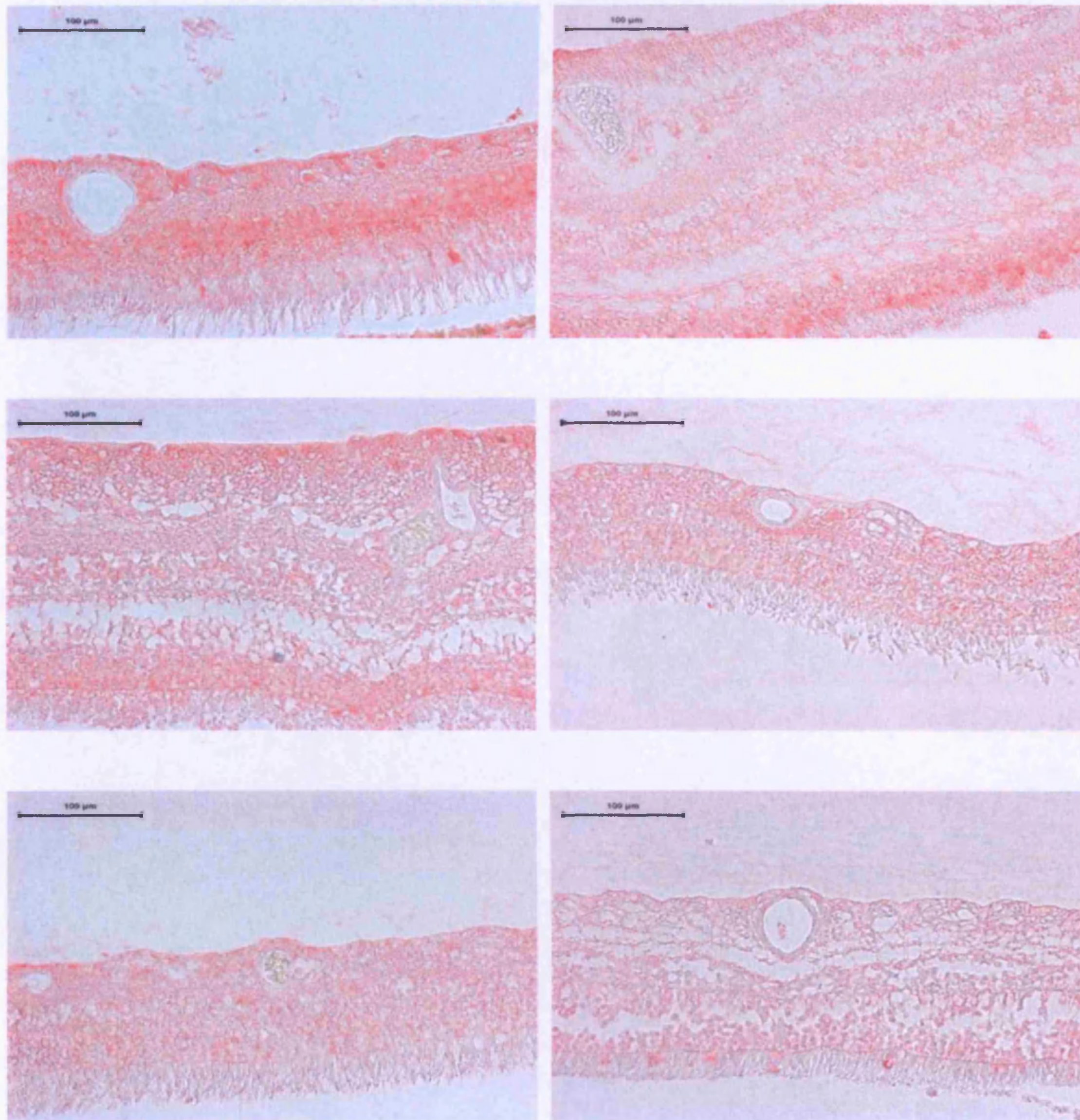

Figure 4.44 Transverse Sections Showing the Immunolocalisation of Tie-2 in Non-Diabetic Retinas.

Staining was observed in the retinal vessels and across all the retinal layers

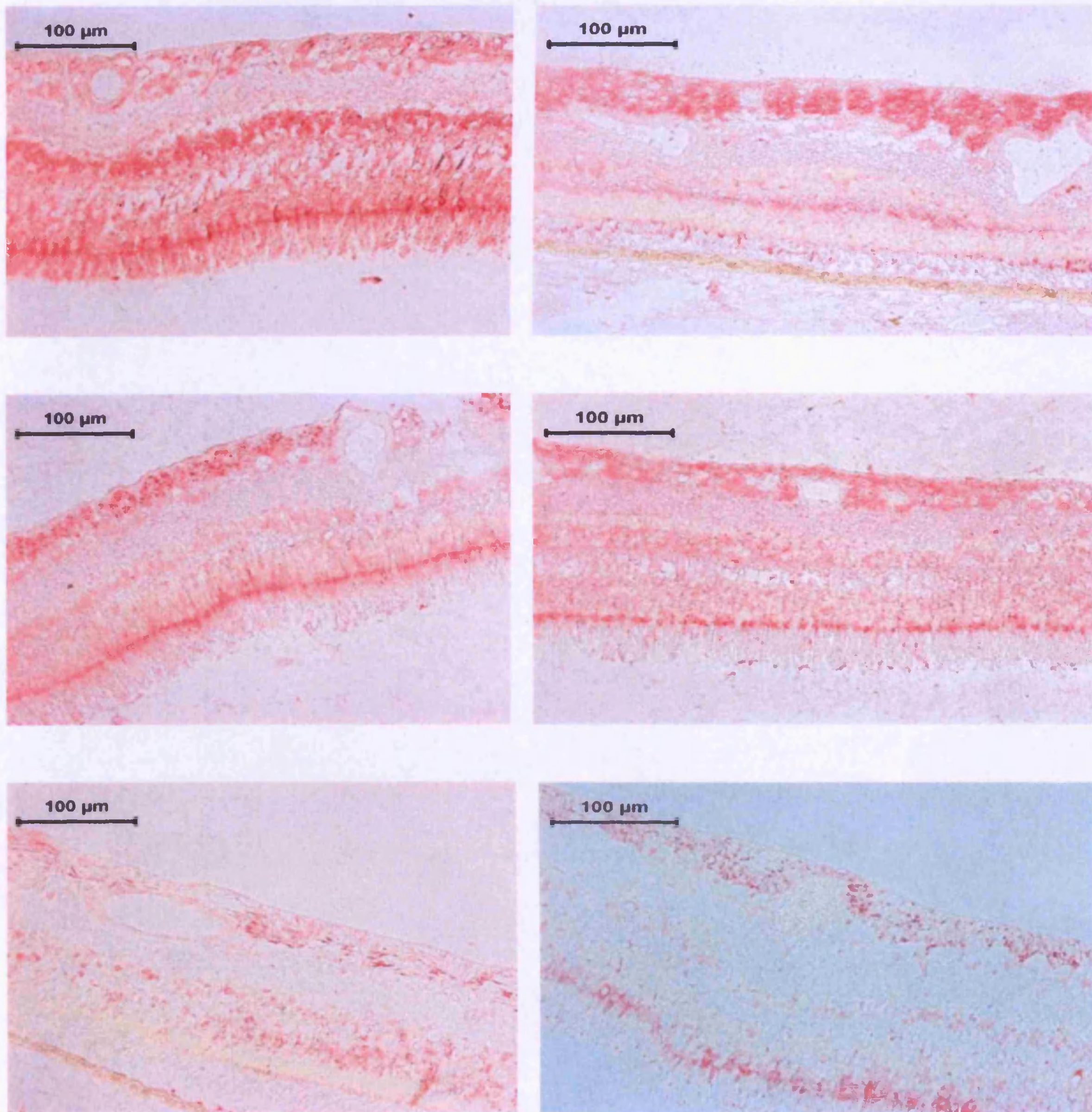

Figure 4.45 Transverse Tie-2 in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.

Staining was observed in the retinal vessels and across all the retinal layers.

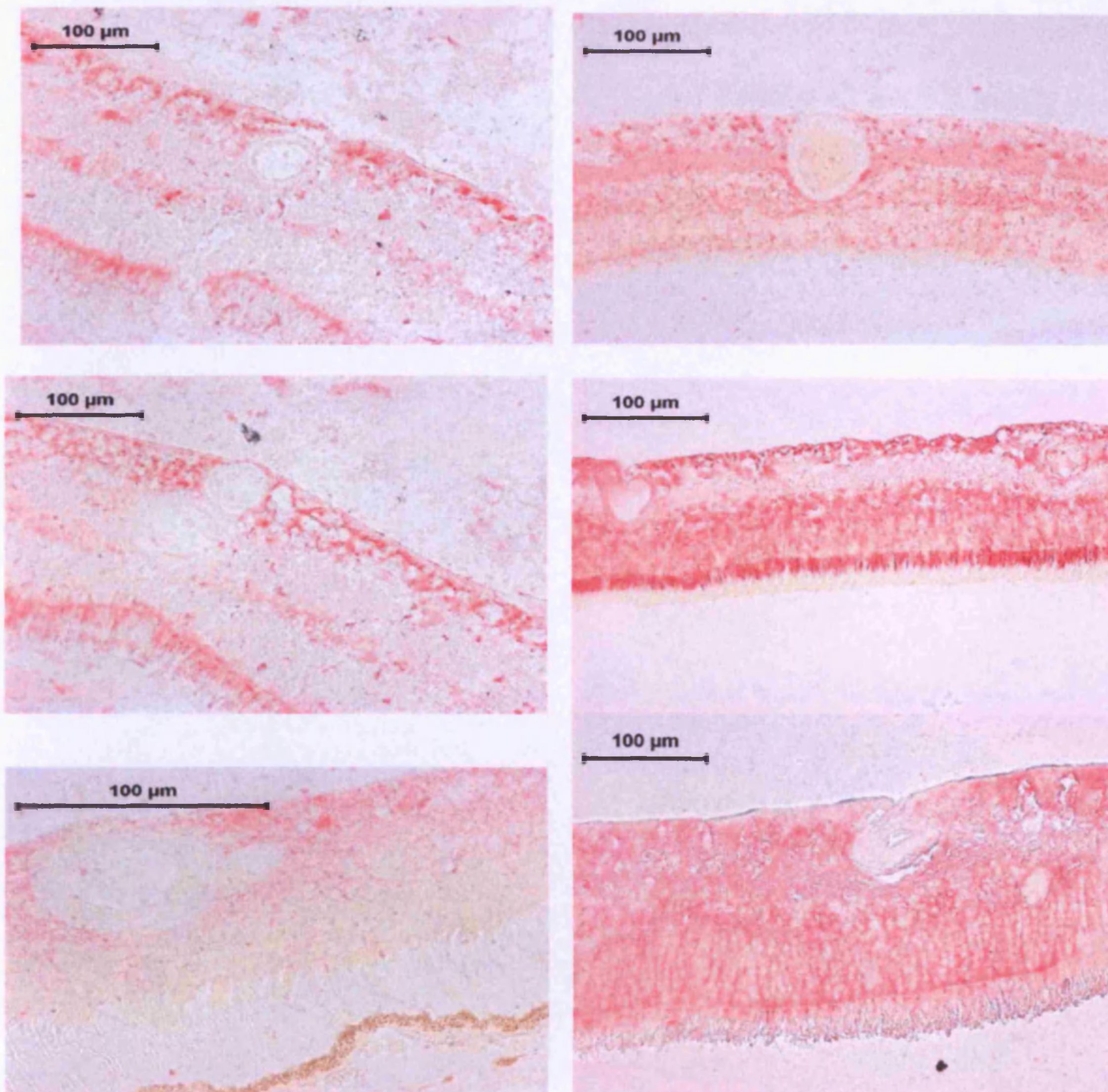

Figure 4.46 Transverse Sections Showing the Immunolocalisation of Tie-2 in Unlasered Diabetic Retinas with NPDR.

Staining was observed in the retinal vessels and across all the retinal layers.

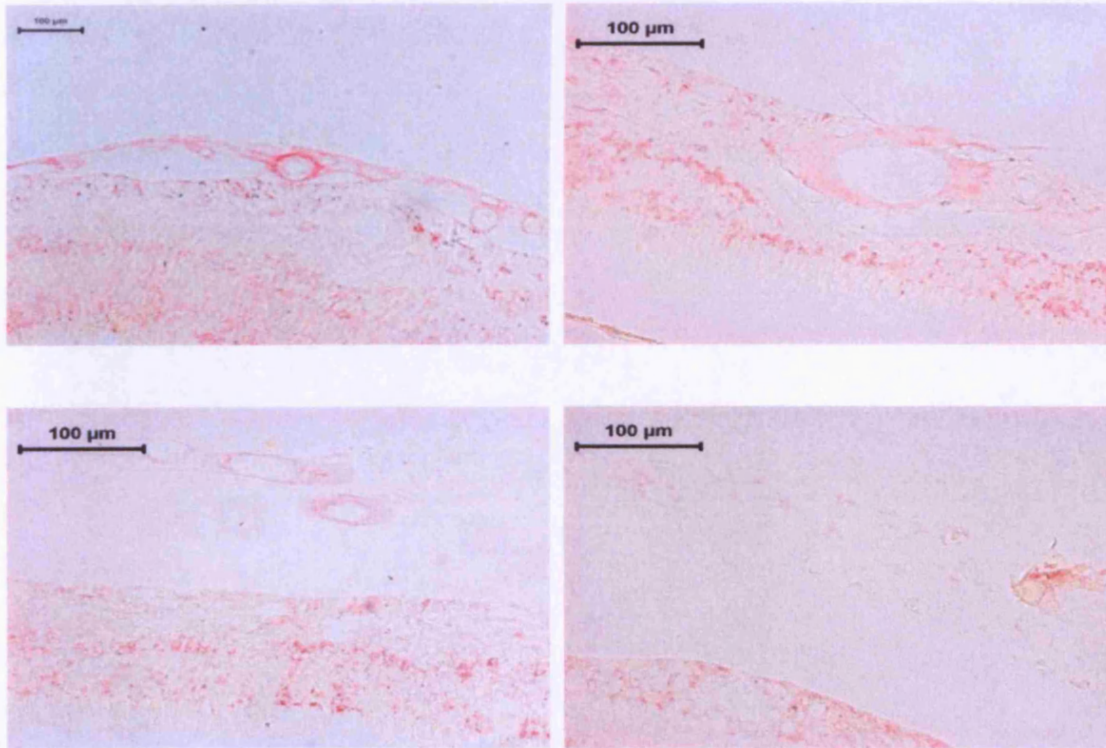

Figure 4.47 Transverse Sections Showing the Immunolocalisation of Tie-2 in Diabetic Retinas with PDR.

Sections are shown where few Tie-2 antibody precipitates are present. Staining was observed in the retinal vessels and across all the retinal layers.

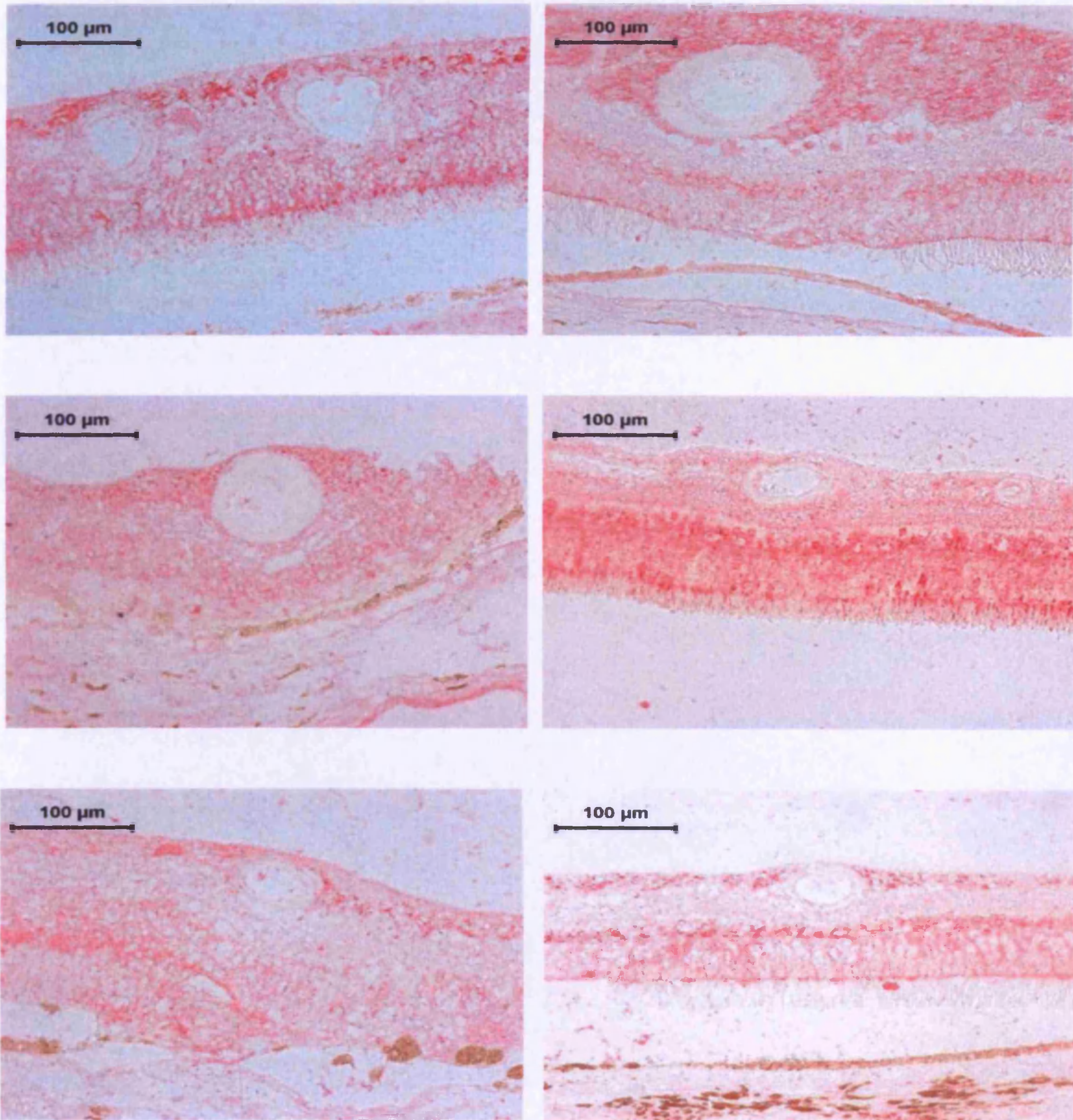

Figure 4.48 Transverse Sections Showing the Immunolocalisation of Tie-2 in Lasered Diabetic Retinas.

Staining was observed in the retinal vessels and across all the retinal layers.

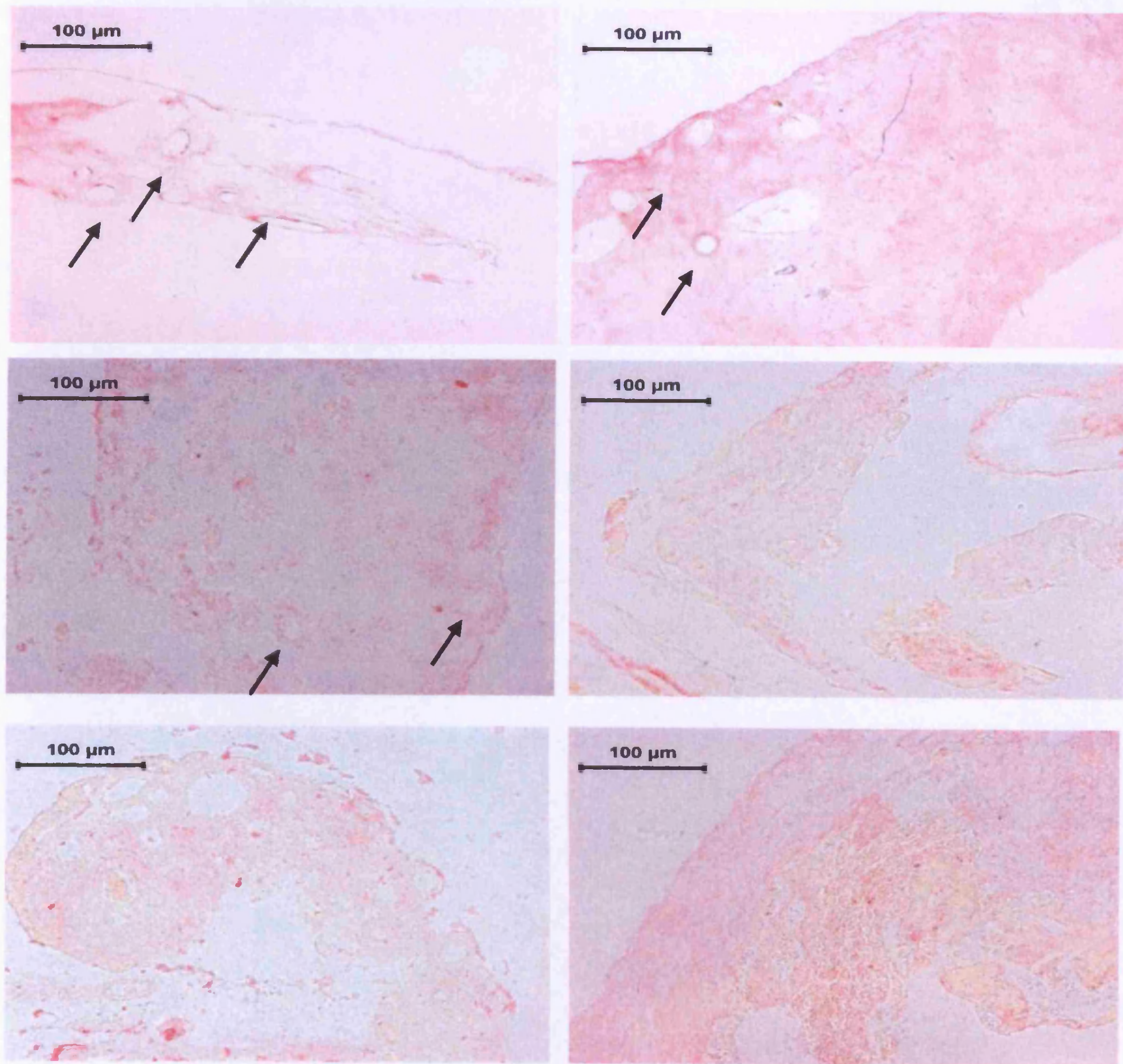

Figure 4.49 Transverse Sections Showing the Immunolocalisation of Tie-2 (Arrows) in Fibrovascular Membranes

4.3.5 TNF alpha immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy immunostaining for TNF-α was observed in both non-diabetic and diabetic vascular and extravascular tissue. Increased immunostaining was observed in preretinal and intraretinal blood vessels as compared to non-diabetic tissue. Variable staining of the vessels within each retina was observed with some staining positive and some staining negative. In some instances staining was associated with both ECs and the perivascular region of the vessel. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 4.9, but this did not correlate with either donor age, or time post mortem.

The average scores and standard deviations for TNF-α immunostaining are represented in table 4.9. Statistical analysis demonstrated that significant differences were observed within the outer retina across the tissue categories ($P = <0.05\%$) but not within the other retinal layers or the retinal vessels.

In the non-diabetic retinas staining intensity for TNF-α was generally minimal or absent within the photoreceptors, the cell bodies of the outer retina and the retinal vessels. Weak to moderate staining was observed within the cell bodies of the inner retina (9/11) and the GCL (9/11) [Fig. 4.50].

In the diabetic retinas with no overt retinopathy immunostaining was again generally minimal or absent within the photoreceptors and the cell bodies of the outer retina. Staining was reduced to minimal or absent in the cell bodies of the inner retina as compared to the non-diabetic retinas. Staining was again weak to moderate within the GCL (6/11). The most intense staining for TNF-α was observed within the retinal vessels with all retinas (11/11) showing positive staining [Fig. 4.51].

In the diabetic retinas showing vascular changes but no evidence of PDR staining for TNF-α was again minimal or absent within the photoreceptors and cell bodies of the outer retina. Staining also remained weak to moderate within the cell bodies of the inner retina (5/10) and the GCL (6/10). Staining was reduced to weak to moderate levels in the retinal vessels as compared with the diabetic retinas with no overt retinopathy (4/10) [Fig. 4.52].

In the diabetic retinas with active neovascular PDR membranes on their surfaces staining for TNF-α was absent within the photoreceptors and cell bodies of the outer retina. Staining was reduced within the cell bodies of the inner retina (3/5) and GCL (3/5) as compared to the retinas with vascular changes but no evidence of PDR. Staining was slightly raised in the retinal vessels (3/5) as compared to the retinas with vascular changes but no

evidence of PDR. Staining was weak to moderate within the preretinal vessels of the membranes (2/5) [Fig. 4.53].

In those retinas which had undergone successful laser therapy staining for TNF-α was absent or minimal within the photoreceptors. Staining was raised within the cell bodies of the outer retina as compared to all the other categories of tissue (7/13). Staining was raised to weak to moderate levels in the GCL as compared to the PDR retinas (10/13). Staining was raised within the retinal vessels (9/13) as compared to the PDR retinas (Fig. 4.54].

Weak staining was observed within the preretinal vessels of the excised membranes (5/13). Staining was absent within the non vascular components of the membranes [Fig. 4.55].

TABLE 4.9 MEAN INTENSITY OF TNF- α IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-Receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	0.1 (0.3)	0.5 (0.3)	1.1 (0.8)	1.5 (0.9)	0.8 (0.6)		
No Overt Retinopathy (n=12)	0.6 (0.4)	0.6 (0.4)	0.6 (0.4)	1.3 (1.1)	2.6 (0.3)		
Intraretinal Changes (n=10)	0.4 (0.3)	0.6 (0.5)	1.2 (1.0)	1.5 (1.0)	1.2 (0.8)		
PDR (n=9)	0 (0)	0 (0)	0.8 (0.4)	0.8 (0.4)	1.6 (1.2)	1.2 (0.6)	0.2 (0.1)
Laser-No Residual PDR (n=14)	0.5	1.5	1.5	1.8	2.0		
Excised Membranes (n=17)						1.0 (0.5)	0 (0)

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

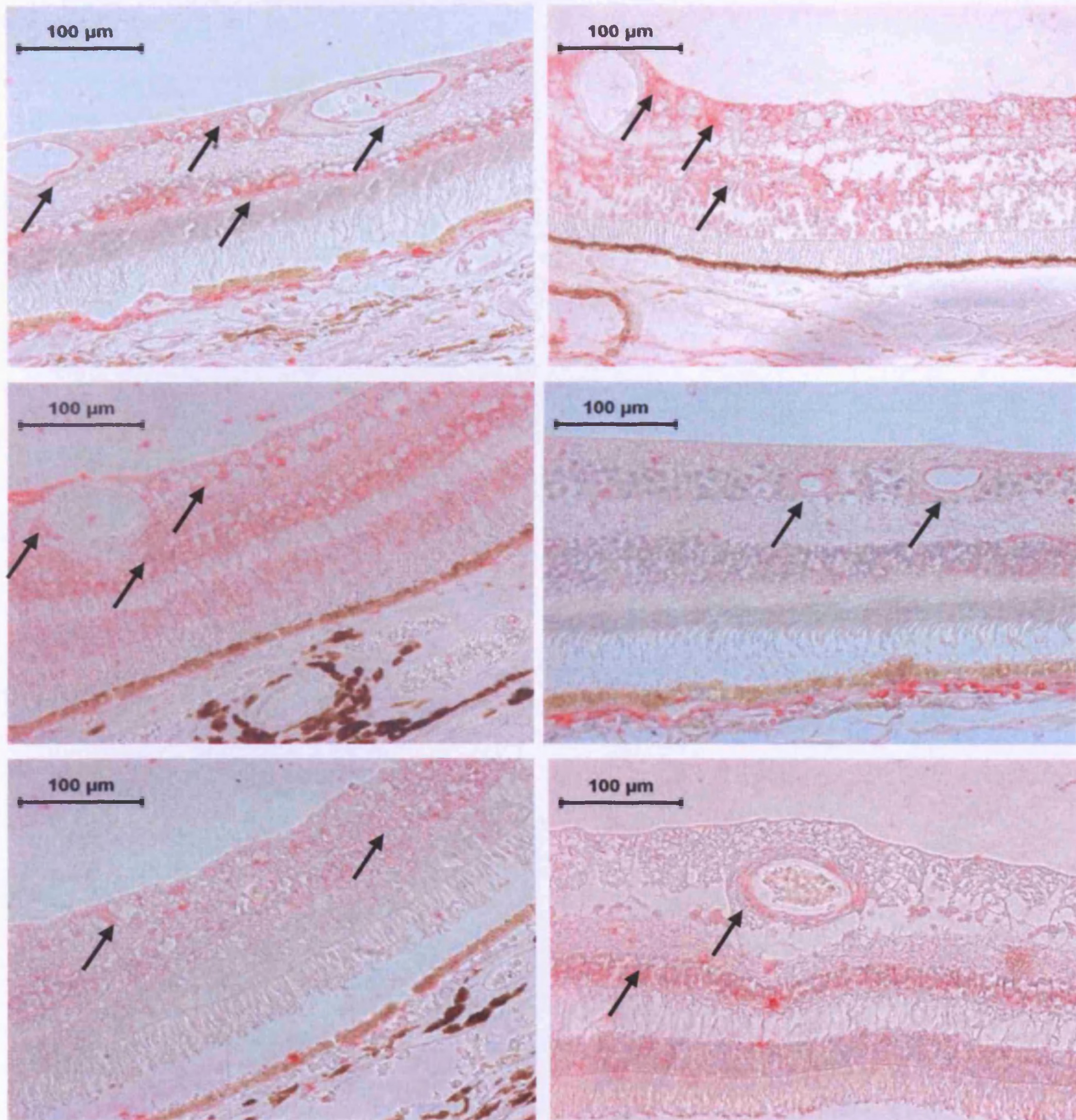

Figure 4.50 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Non-Diabetic Retinas.

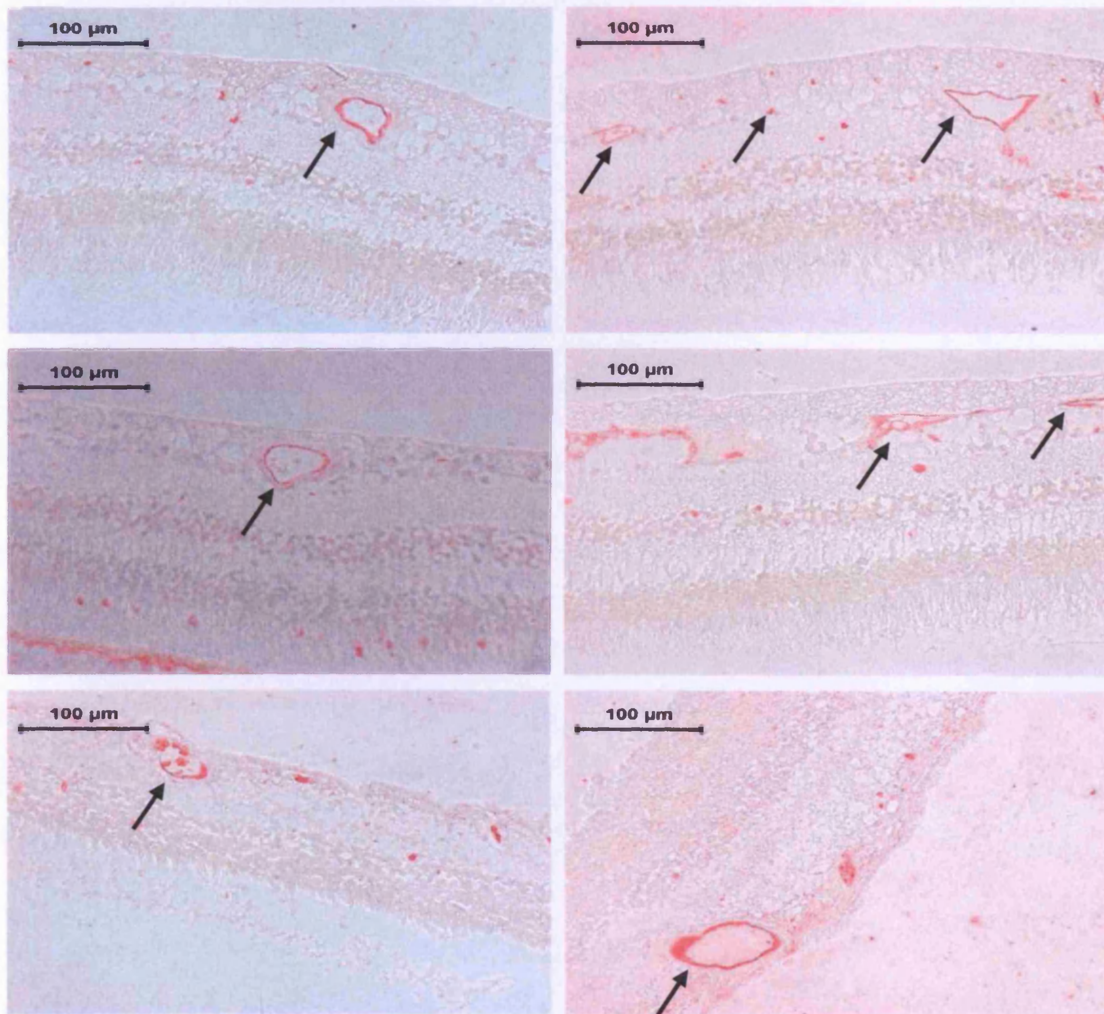

Figure 4.51 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.

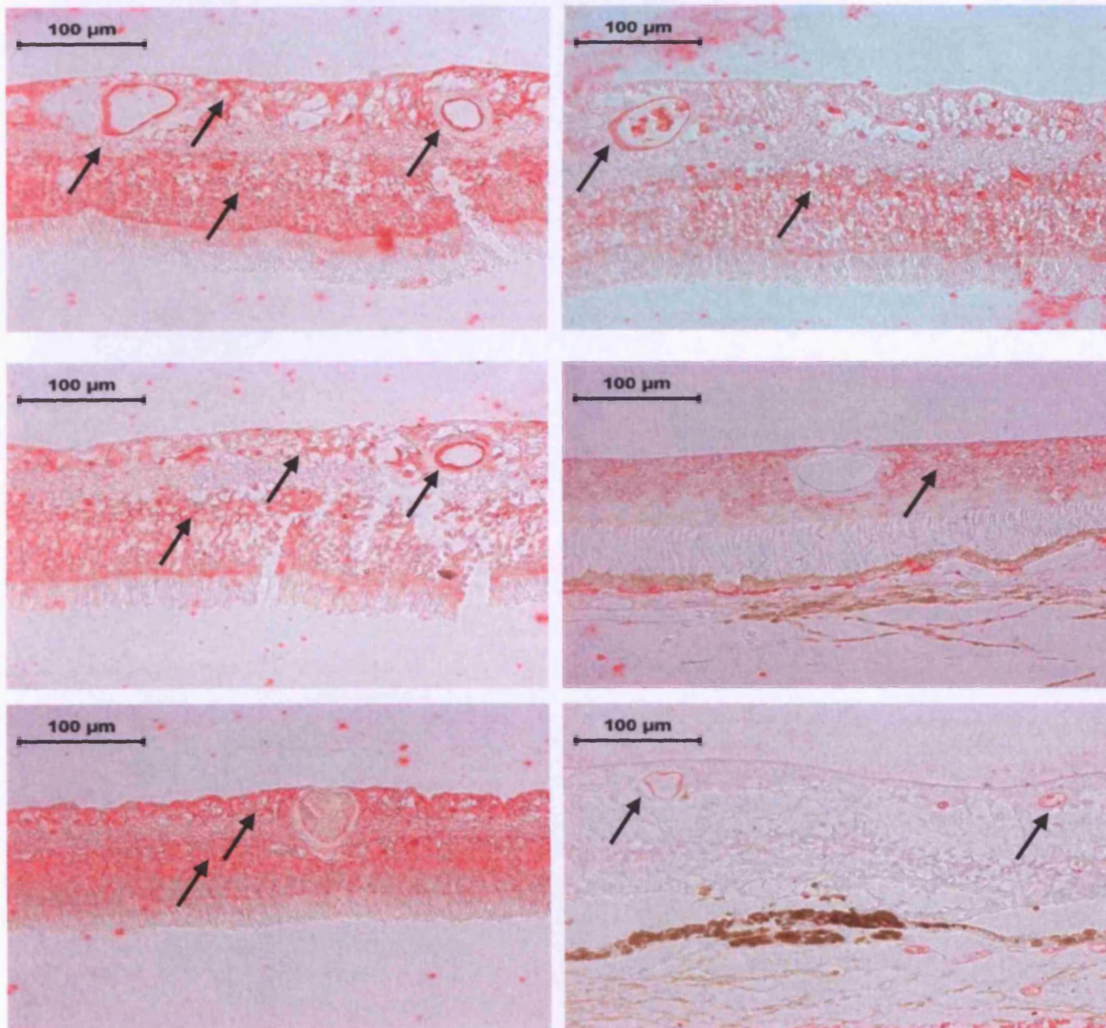

Figure 4.52 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Unlasered Diabetic Retinas with NPDR.

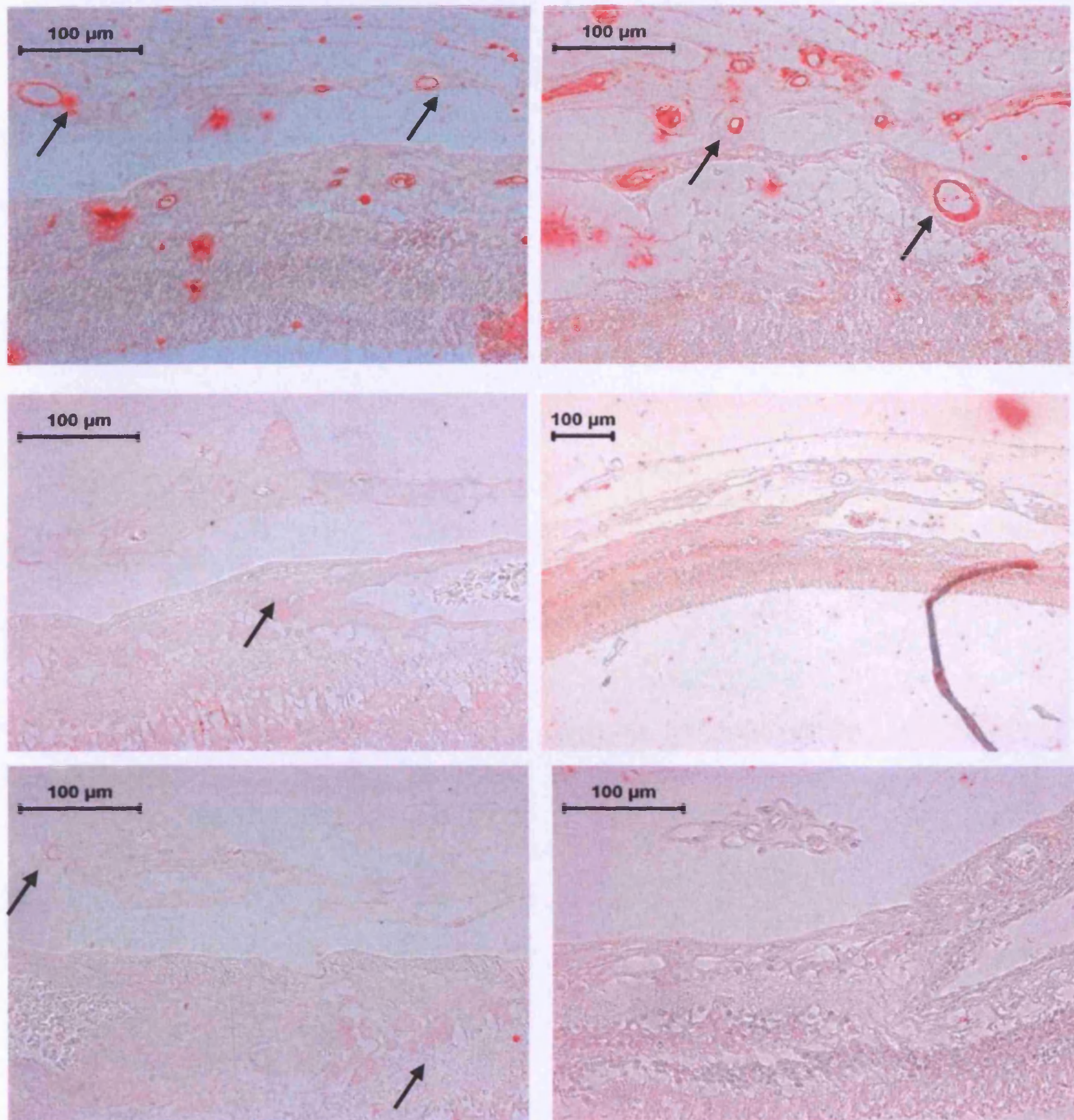

Figure 4.53 Transverse Sections Showing the Immunolocalisation of TNF-α in Diabetic Retinas with PDR.
Arrows showing the location of staining in most of the membranes.

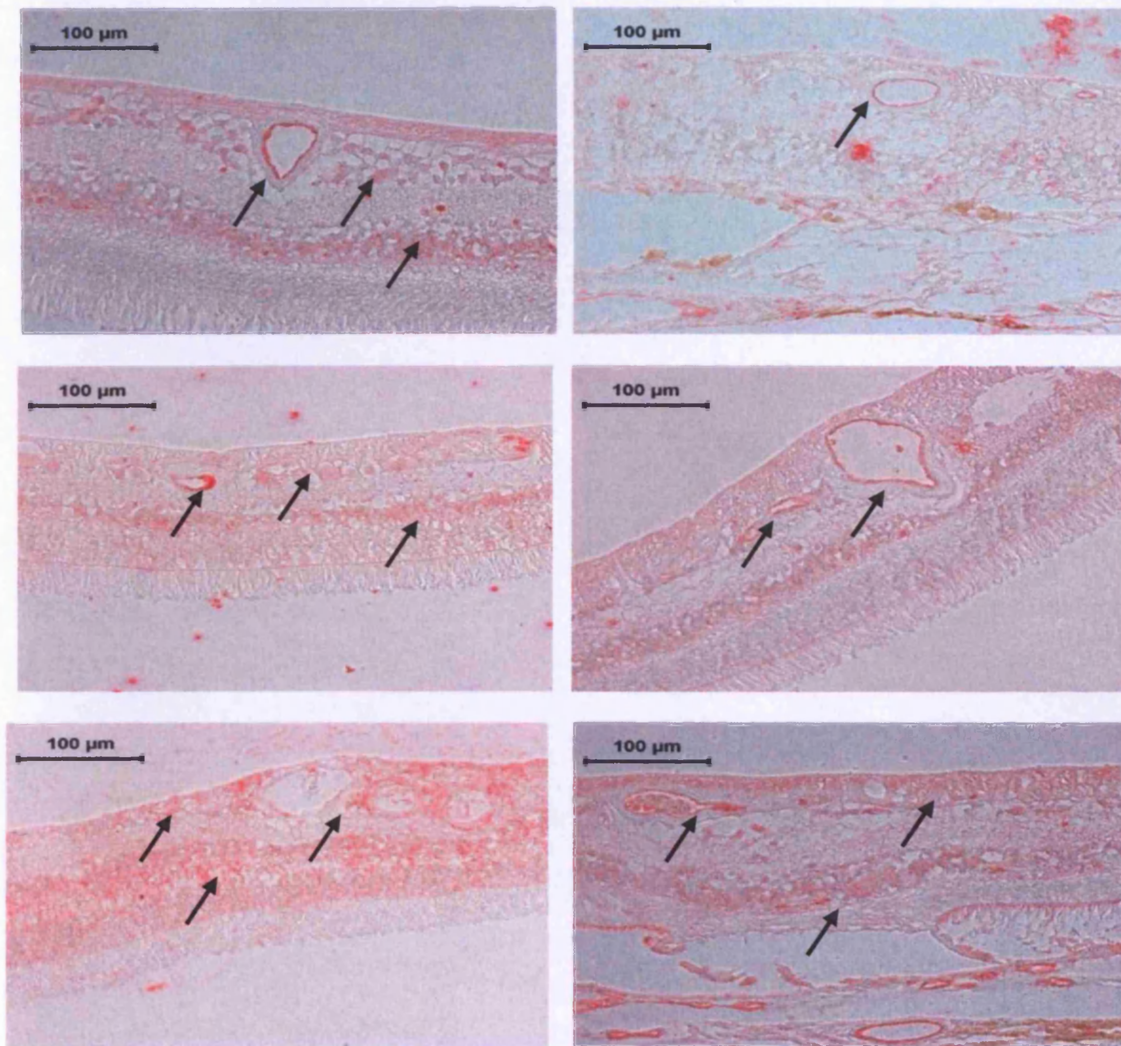

Figure 4.54 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Lasered Diabetic Retinas.

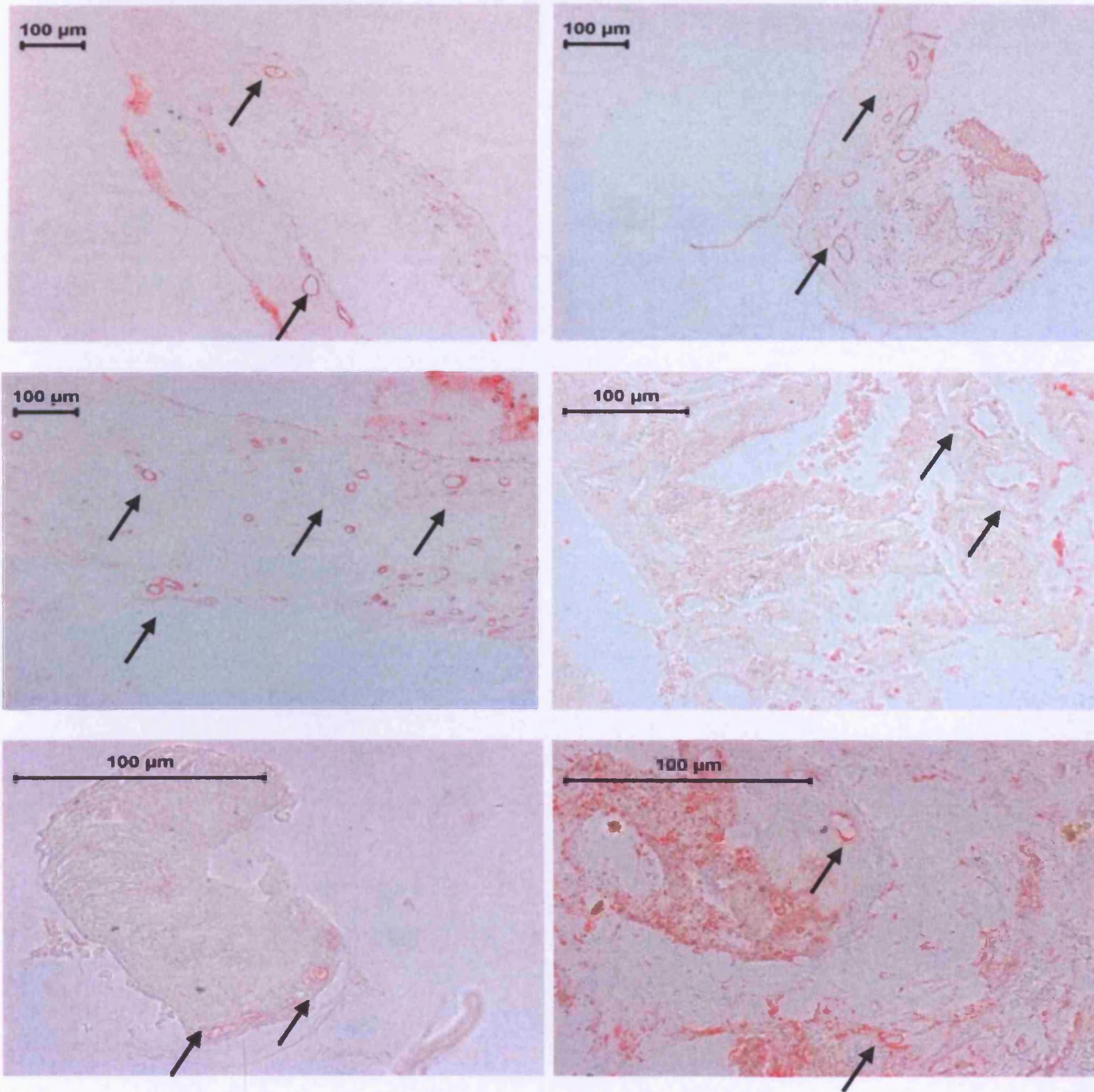

Figure 4.55 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Fibrovascular Membranes

4.4 DISCUSSION

The data presented in this study demonstrate that VEGF, the angiopoietins and TNF-α are localised in diabetic tissue and that they are upregulated in diabetic retinopathy. These observations add support for a role of VEGF and its receptors in diabetic retinopathy and indicate the importance of the angiopoietins, its receptor Tie-2 and TNF-α in pathological angiogenesis.

The VEGF/Receptor Family

Immunostaining for VEGF-A, VEGF-C and the VEGF receptors was performed using a dilution series of the primary antibodies, against a set number of sections, as recommended by the manufacturer. Initially only weak staining was observed. Successful staining for VEGF-A was eventually achieved using a highly concentrated dilution of the primary antibody and by exposing the angiogenic bindings sites with proteolytic digestion using 0.1% chymotrypsin. VEGF-C and the receptor antibodies were used at lower dilutions than VEGF-A and in addition proteolytic digestion was also needed to produce satisfactory staining for VEGF-C. Also some non-specific background staining was observed with all antibodies which was removed by blocking with a solution of 10% milk protein/10% rabbit serum prior to adition of the primary antibody. Deposits were observed when examining the sections stained with VEGF-C. After consultation with the manufacturer the VEGF-C antibody was spun at 25000 rpm at 4°C for 10 mins but this did not remove the deposits. Staining for VEGF-C was retried using both a new primary antibody, a new secondary antibody and new fast red substrate but again the deposits were observed. Photographs were taken of areas showing minimal deposits.

VEGF-A was present, albeit at low levels, within the vessels of non-diabetic retinas and diabetic retinas with no overt retinopathy. Several adult organs and tissues in guinea pig and man have been found to constitutively express VEGF-A mRNA (Ladoux *et al*., 1993b). Various other workers have shown that VEGF-A is expressed weakly within the retinal vessels of human and animal non-diabetic retinas (Murata *et al.*, 1995; Lutty *et al.*, 1996; Gerhardinger *et al.*, 1998; Spirin *et al.*, 1999; Kim *et al.*, 1999b; Witmer *et al.*, 2002). VEGF-A may be secreted in these adult organs in small amounts that are insufficient to induce angiogenesis but may be necessary for regulating baseline microvascular permeability which is essential for tissue nutrition and waste removal and/or maintaining the differentiated state of blood vessels. (Kevil *et al.,* 1998; Luo *et al.,* 1998; Zebrowski *et al.,* 1999; Bates and Harper., 2002; Fu and Shen, 2004; Shibuya, 2005). Kunz Mathews *et al.* (1997)

demonstrated that increased VEGF-A immunoreactivity was correlated with increased vascular permeability before morphologic changes occurred in the vasculature. It has been shown that the activity of specific Src family kinases is essential for the VEGF-induced enhancement of vascular permeability through the disruption of the VEGFR-2/cadherin/catenin complex (Eliceiri *et al.*, 1999; Weis *et al.*, 2004). Alternatively VEGF-A may be stored within the cells that synthesise it, perhaps awaiting an emergency situation that requires angiogenesis.

In this study the level of VEGF-A expression was raised considerably within the retinal vessels of the eyes with intraretinal vascular changes. This suggests that VEGF exerts its action(s) during the earlier stages of diabetic retinopathy before proliferation occurs. In animal models of background retinopathy and in studies of human diabetic retinas with pre-proliferative diabetic retinopathy VEGF-A expression within the retinal vessels was also shown to be significantly increased as compared to non-diabetic controls (Lutty *et al.*, 1996; Shima *et al.*, 1996; Amin *et al.*, 1997; Segawa *et al.*, 1997; Hammes *et al.*, 1998; Spirin *et al.*, 1999; Ellis *et al.*, 2000; van Eeden *et al.*, 2006; Kaur *et al.*, 2006) and VEGF was shown to induce diabetic induced early retinal abnormalities such as increased vascular permeability (El-Remessey *et al.*, 2003; Cukiernik *et al.*, 2004). VEGF-A was effective in inducing ICAM-1-mediated retinal leukostasis and BRB breakdown in vivo in diabetic rats, indicating that both are important in the pathogenesis of early diabetic retinopathy. (Joussen *et al.*, 2002b; Ishida *et al.*, 2003).

More direct evidence for VEGF-A as a primary candidate in pre-proliferative retinopathy (and PDR) comes from studies in which VEGF-A administration to animals was shown to be sufficient to produce many of the vascular abnormalities common to background diabetic retinopathy (Tolentino *et al.*, 1996; Tolentino *et al.*, 2002; Witmer *et al.*, 2004; Kinnunen *et al.*, 2006). Studies with VEGF inhibitors have confirmed that VEGF plays a central role in ischaemia-induced vascular permeability and intraocular neovascularization (Campochiaro and Hackett., 2003; Patel *et al.*, 2003).

Factors such as oxidative stress, cyclooxygenase-2, prostaglandin E_2, AGES, and IGF-1, which are correlated with diabetic retinopathy may also serve as the primary stimuli to increase retinal VEGF-A expression in background diabetic retinopathy (Segawa *et al.*, 1997; Ellis *et al.*, 2000; Mamputu and Renier, 2002; El-Remessy *et al.*, 2003; Ayalasomayajula *et al.*, 2004; Roybal *et al.*, 2005; Yokoi, 2005; Sreekumar *et al.*, 2006). AGES and Interleukin-6 have been shown to increase retinal VEGF-A expression from

Müller cells, RPE cells, ECS, and SMCs and pericytes (Hirata *et al.*, 1997; Lu *et al.*, 1998; Endo *et al.*, 2001; Yamagishi *et al.*, 2002; Li *et al.*, 2006; Yao *et al.*, 2006).

The highest level of VEGF-A expression was observed in the intraretinal vessels and preretinal vessels of subjects with active PDR which further supports a role of VEGF-A in PDR. This is consistent with the findings of other studies where VEGF-A was demonstrated in neovascular membranes (Malecaze *et al.*, 1994; Chen *et al.*, 1997; Schneeberger *et al.*, 1997; Armstrong *et al.*, 1998a; Funatsu *et al.*, 2003; Tsanou *et al.*, 2005). Levels of VEGF-A were also significantly higher in the vitreous and aqueous and plasma from PDR subjects than in non-diabetic subjects, subjects with non-proliferative retinopathy, quiescent retinopathy and those which had received laser therapy (Adamis *et al.*, 1994; Aiello *et al.*, 1994; Hernández *et al.*, 1998; Lin Lip *et al.*, 2000; Endo *et al.*, 2001; Umeda *et al.*, 2001; Hogeboom van Buggenum *et al.*, 2002; Mitamura *et al.*, 2002; Ogata *et al.*, 2002abc; Simó *et al.*, 2002; Funatsa *et al.*, 2003; Funatsu *et al.*, 2004; Lip *et al.*, 2004; Yokoi *et al.*, 2005; Ishizaki *et al.*, 2006).

In situ hybridization and immunohistochemical studies on human retinas have demonstrated that proliferation of vascular elements in PDR and neovascularization of the retina and/or iris secondary to central retinal vein occlusion, retinal detachment, and intraocular tumours were always accompanied by upregulation of VEGF-A mRNA (Murata *et al.*, 1995; Pe'er *et al.*, 1995; Lutty *et al.*, 1996; Pe'er *et al.*, 1996; Sueshi *et al.*, 1996; Amin *et al.*, 1997; Kunz Mathews *et al.*, 1997; Witmer *et al.*, 2002). Similarly in animal models of proliferative retinopathy, including ROP, VEGF-A expression was increased in retinal vessels and pre-retinal growths during the period of retinal hypoxia and remained elevated during the development of neovascularization (Pierce *et al.*, 1995; Dorey *et al.*, 1996; Stone *et al.*, 1996; Robbins *et al.*, 1997; Ozaki *et al.*, 1999; Witmer *et al.*, 2002; Bullard *et al.*, 2003). Ozaki *et al.* (1999) also demonstrated that increased levels VEGF in ischaemic retina showed a temporal and spatial correlation with increased expression of HIF-1α.

All these findings demonstrate that VEGF-A is expressed in the retina prior to the development of neovascularization, remains elevated until neovascularization develops and then declines as the neovascularization regresses. This demonstrates an especially strong correlation between VEGF-A expression and retinal neovascularization.

In this study the finding that immunostaining for VEGF-A is reduced in diabetic retinas that have no overt preretinal neovascularization following laser therapy is consistent with the findings that vitreous and plasma concentrations of VEGF-A decline after successful

laser therapy (Aiello *et al.*, 1994; Lin Lip *et al.*, 2000). It is also consistent with the finding that the levels of VEGF, VEGFR-2 and VEGFR-1 are reduced in neovascular membranes receiving cryotherapy as compared to membranes containing active proliferating vessels (Armstrong *et al.*, 1998a). Both therapies result in the destruction of a large area of ischaemic retinal tissue presumably resulting in a reduction of VEGF-A, the suppression of neovascularization leading to vessel regression and quiescence.

VEGF-C has been shown to stimulate EC migration, proliferation and chemotaxis and has a strong chemotactic effect on VEGFR-3 producing cells in vitro (Joukov *et al.*, 1996; Witzenbichler *et al.*, 1998b; Kroon *et al.*, 1999; Lohela *et al.*, 2003; Saharinen *et al.*, 2004). VEGF-C also stimulated the release of NO, a potential mediator of VEGF-induced angiogenesis, from ECs and increased vascular permeability in the Miles assay (Witzenbichler *et al.*, 1998b). In a rabbit ischaemic hindlimb model VEGF-C promoted angiogenesis (Witzenbichler *et al.*, 1998b). It was also detected in haematopoietic cells and platelets and in bone marrow samples of acute leukaemia patients (Wartivaara *et al.*, 1998). The investigators suggested that VEGF-C release from activated platelets may have a role in angiogenesis during wound healing, and possibly other pathological conditions, such as atherosclerosis, tumour growth, and metastasis. VEGF-C has also been shown to be associated with the regulation of angiogenesis in the lymphatic vasculature (Kukk *et al.*, 1996; Jussila and Alitalo, 2002; Karkkainen *et al.*, 2004). Although no other investigators have looked at VEGF-C expression in diabetic retinopathy, it may not be unreasonable to say that, from the findings of my study, VEGF-C does appear to play some part in the pathogenesis of diabetic retinopathy. It is expressed in the early stages of diabetic retinopathy which suggests its action could be to induce vascular permeability. It is strongly expressed in PDR retinas which suggests that it could be involved in proliferation and migration of endothelial cells. Its expression in the non-diabetics suggests it may also have a role in the quiescent vasculature.

The observation in this study that VEGFR-2 is greatly elevated in both intra- and preretinal vessels in PDR tissue and minimal in the retinal vessels of non-diabetic retina and the quiescent vessels of lasered diabetic retina with no evidence of PDR is in agreement with the view that VEGFR-2 is involved in PDR. VEGF-A and VEGF-C have both been shown to induce invasion, and tube formation when bound to VEGFR-2 (Tille *et al.*, 2003). It is consistent with the finding that VEGFR-2 is present in neovascular membranes and diabetic retinas and in animal models of ischaemia-induced retinal neovascularization (Malecaze *et al.*, 1994; Chen *et al.*, 1997; Armstrong *et al.*, 1998a; Suzuma *et al.*, 1998; Ishida *et al.*, 2000;

Ishimama *et al.*, 2001; Witmer *et al.,* 2002; Gerber and Ferrara., 2003; Cerdan *et al.,* 2004; Wilkinson-Berka *et al.,* 2006). Blockade of VEGFR-2 receptor signalling was sufficient to completely prevent retinal neovascularization (Ozaki *et al.,* 2000). VEGFR-2 may be associated with integrin-dependent migration of ECs, as it forms a complex with integrin áVβ3 upon binding VEGF (Soldi *et al.,* 1999; Hutchings *et al.,* 2003). An interaction between VEGFR-2 and VE-cadherin, a cell-cell adhesion molecule has also been described.

These findings also correlate with studies where VEGFR-2 levels were elevated in non-ocular pathologies that are characterised by neovascularization (Shweiki *et al.*, 1992; Brown *et al.*, 1993a; Brown *et al.*, 1993b; Fava *et al.*, 1994; Abu-Jawdeh *et al.*, 1996; Guidi *et al.*, 1996; Leung *et al.*, 1997; Samaniego *et al.*, 1998; Hiratsuka *et al.,* 2002; Inoue *et al.,* 2002; Stewart *et al.*, 2003; Takekosh *et al.,* 2004; Pallares *et al.,* 2006), and in embryogenesis where VEGFR-2 expression has been shown to be imperative for EC mitogenesis, the formation of blood vessels and for haematopoiesis. (Ferrara *et al.*, 1996).

Although VEGFR-2 gene expression appears to be upregulated in various pathologies characterised by hypoxia, *in vitro* studies have yielded conflicting results. The level of VEGFR-2 expression appears either to decline or not change by exposure to acute hypoxia (Brogi *et al.*, 1996; Takagi *et al.*, 1996a) whereas exposure to prolonged periods of hypoxia results in an increase in VEGF binding sites (Thieme *et al.*, 1995). Although the *in vivo* significance of increased VEGF-A expression, combined with initial decreased VEGFR-2 expression observed *in vitro*, is not certain, Takagi *et al.* (1996a) suggested that the physiological significance of the biphasic VEGF receptor response may be to regulate hypoxia induced neovascularization more tightly. Initial, possibly transient, decreases in oxygen concentrations, where VEGF-A levels can be dramatically elevated but where angiogenesis may not be urgently required, lead to a reduction of VEGFR-2 and thus an amelioration of VEGF-A's angiogenic stimuli. However, under conditions of chronic oxygen deficits, in which angiogenesis is a more appropriate response, VEGF receptors are increased and so potentially facilitate VEGF action. This is supported by the finding that hypoxia increases VEGF receptor number by 50% in cultured BRECs (Thieme *et al.*, 1995). *In vitro* studies have demonstrated that adenosine is a mediator of the angiogenic effects of VEGF-A through the regulation of VEGFR-2 expression during acute hypoxia (Takagi *et al.*, 1996a). Adenosine plays a major role in neuronal and vascular responses of the retina to alterations in oxygen delivery (Ghiardi *et al.*, 1999; Adair 2005).

The presence of VEGFR-1 in non-diabetic tissue adds support to the suggestion that VEGF-A has a function in endothelial maintenance and vascular permeability, for example, and that these effects are mediated through VEGF-A binding to the VEGFR-1 receptor.

The presence of VEGFR-1 in diabetic vessels, particularly in those undergoing active neovascularization, indicates that VEGFR-1 plays a role in both pre-proliferative retinopathy and PDR. As VEGFR-1 has previously been shown to promote vascular permeability (Kolch *et al.*, 1995), in diabetic retinopathy it may be involved in transducing signals within ECs which induce vascular leakage. It is upregulated during hypoxia (Gerber *et al.*, 1997; Partanen *et al.*, 1999. It may also be co-expressed with VEGFR-2 and it has been suggested that it may participate in VEGF-A induced mitogenesis by forming a heterodimer with VEGFR-2 (Waltenberger *et al.*, 1994). VEGFR-1-mediated signalling appears to modulate the reorganization of actin via p38 MAPK, whereas VEGFR-2 contributes to the reorganization of the cytoskeleton by phosphorylating FAK (focal adhesion kinase) and paxillin, suggesting a different contribution of the two receptors to the chemotactic response (Kanno *et al.*, 2000). VEGFR-1 has also been shown to act as a negative regulator of VEGF-A induced angiogenesis; a soluble form of VEGFR-1 can form a VEGF-A-stabilised ternary complex with the extracellular region of VEGFR-2 *in vitro* (Kendall *et al.*, 1996). In support of this, endogenous trophoblast DNA synthesis was shown to be increased 3-fold in the presence of anti-VEGFR-1 antibody but not in the presence of anti-VEGFR-2 antibody (Ahmed *et al.*, 1997).

VEGFR-1 has previously been detected in samples of neovascular membranes and diabetic retinas (Malecaze *et al.*, 1994; Chen *et al.*, 1997; Armstrong *et al.*, 1998a; Witmer *et al.*, 2002). In addition, these findings also correlate with studies where VEGFR-1 levels were elevated in other pathologies that are characterised by neovascularization (Peters *et al.*, 1993; Fava *et al.*, 1994; Leung *et al.*, 1997; Pallares *et al.*, 2006) and in developing embryos (Fong *et al.*, 1995; Ferrara *et al.*, 1996) indicating that VEGFR-1 is essential for endothelial differentiation, EC and vessel organisation, blood vessel growth, and vascular repair.

The findings from this study that VEGFR-3 is elevated in diabetic vessels, particularly in the intra- and preretinal vessels of the PDR retinas suggest that VEGFR-3 may a have a role in the pathogenesis of diabetic retinopathy. As mentioned previously, VEGF-C is a ligand for this receptor which is known to have angiogenic effects on endothelial cells (Joukov *et al.*, 1996; Witzenbichler *et al.*, 1998b). In developing mouse embryos, VEGFR-3 is specifically expressed in endothelial precursors although its expression does become confined to higher venules and the lymphatic system in adults (Kaipainen *et al.*, 1993;

Kaipainen *et al.*, 1994a; Lymboussaki *et al.*, 1998; Partanen *et al.*, 2000; Makinen *et al.*, 2001; Veikkola *et al.*, 2001). Targeted inactivation of the gene encoding VEGFR-3 resulted in defective blood vessel development in early mouse embryos indicating that VEGFR-3 is important for the remodelling and maturation of primary vascular networks into larger blood vessels (Dumont *et al.*, 1998). VEGFR-3 has also been detected in samples from patients with myeloid leukaemia, and in various tumour cell lines including a retinoblastoma cell line, indicating it plays a role in pathological neovascularization (Pajusola *et al.*, 1992; Fielder *et al.*, 1997; Partanen *et al.*, 1999; Valtola *et al.*, 1999; Skobe *et al.*, 2001ab; Witmer *et al.*, 2001; Clarijs *et al.*, 2002). A role for VEGFR-3 in adult angiogenesis was shown by Witmer *et al.*, 2004 who demonstrated that VEGFR-3 was expressed in pre-existing blood vessels in human tissues undergoing angiogenesis and in a VEGF-A induced model of iris neovascularization. VEGFR-3 has also been demonstrated in retinal vessels during early diabetic retinopathy (Witmer *et al.*, 2002). VEGFR-3 has recently been shown to heterodimerize with VEGFR-2 in ECs and stimulates VEGFR-2 signalling in response to VEGF-C (Alam *et al.*, 2004; Suzuki *et al.*, 2005). Together VEGFR-3 and VEGFR-2 induced the formation of capillary-like structures and the proliferation of human ECs. Use of an anti-human VEGFR-3 monoclonal antibody that antagonized the receptor activation by VEGF-C resulted in the reduction of tubule formation (Persaud *et al.*, 2004).

Various workers have demonstrated that production of VEGF and its receptors is not specifically confined to retinal vascular ECs in the diabetic retina. VEGF has been shown to be highly expressed in ganglion cells and glial cells (Müller cells and astrocytes) in non-diabetic retina (Famigietti *et al.*, 2003) and diabetic retina (Pe'er *et al.*, 1996; Sueshi *et al.*, 1996; Amin *et al.*, 1997; Hammes *et al.*, 1998). In addition VEGF expression is also significantly raised in these cells in animal models of ischaemic retinopathy (Shima *et al.*, 1996; Stone *et al.*, 1996; Robbins *et al.*, 1997; Kaur *et al.*, 2006) and during the development of the retinal vasculature (Stone *et al.*, 1995). RGCs have been shown to synthesise and release VEGF which is enhanced by hypoxia (Sueshi *et al.*, 1996; Jingjing *et al.*, 1999) and conditioned media from hypoxic RGCs stimulates *in vitro* angiogenesis in collagen gels (Jingjing *et al.*, 1999). Therefore VEGF appears to be released by Müller cells and astrocytes under hypoxic conditions. Increased VEGFR-2 and VEGFR-1 expression have also been demonstrated in the GCL, INL, and ONL in animal models of background retinopathy and ischaemia-induced retinal neovascularization (Hammes *et al.*, 1998; Suzuma *et al.*, 1998; van Eeden *et al.*, 2006) with VEGFR-1 mRNA also being present in cultured retinal glial cells (RGCs), and glial cells of epiretinal membranes. (Chen *et al.*, 1997).

Retinal capillaries are largely ensheathed by perivascular glial cells, which participate in the formation of barrier properties in capillaries (Janzer *et al.*, 1987; Tout *et al.*, 1993). In my study VEGF-A levels were raised in the GCL of the unlasered diabetic retinas with microvascular abnormalities. Therefore it may be reasonable to say that VEGF localised in glial cells plays some role as a vascular permeability factor both in normal retinal vessels and in the early stages of diabetic retinopathy. This VEGF expression may make the retinal vessels permeable so that the retinal cells can get a supply of oxygen and nutrition. This is supported by the finding of Sueshi *et al.* (1996) who demonstrated that VEGF expression in diabetic retina was associated with vascular hyperpermeability and that astrocytes intimately surrounded these blood vessels.

As glial cells and ganglion cells appear to be a major source of VEGF in ischaemic retina, the above studies suggest that glial cells and possibly ganglion cells are able to detect hypoxia and in response they secrete VEGF and increase their expression of the VEGF receptors. During retinal development and the associated network of blood vessels, astrocytes were shown to be sensitive to hypoxia and astrocytes only enter retinas in which the retinal vasculature will form (Stone *et al.*, 1987; Schnitzer, 1988ab In response to hypoxia the astrocytes are able to migrate ahead of the developing vessels and secret VEGF, inducing the formation of developing vessels toward the VEGF-producing astrocytes (Stone *et al.*, 1995). In support of this rat astrocytes in avascular retina promote fibronectin production, and fibronectin can provide guidance for migrating spindle cells (EC precursors and glial cells) and extending vessels (Jiang *et al.*, 1994). Astrocytes can also induce ECs to form capillary-like structures in culture (Laterra *et al.*, 1990). As the hypoxic pressures ease after the arrival of the blood vessels, the production of VEGF by astrocytes decreases. In the INL of the developing retina VEGF is also expressed transiently by cells which are presumed to be Müller cells. As Müller cells extend from the ILM to the OLM of the retina, they are thought to be affected by abnormal biologic activities in the vitreous. Penn *et al.* (1988) showed that the b-wave of electroretinograms, which is produced by Müller cell activity alone, is specifically affected in oxygen-induced rats. This finding points to a significant effect of variable oxygen levels on Müller cell function and suggest that Müller cells are important in building the vasculature, maintaining vascular homeostasis, and promoting neovascularization.

However, VEGF is present in glial cells of retinas from patients without proliferative retinopathy, indicating that hypoxia may not be the sole stimulus for VEGF expression from these cells (Sueshi *et al.*, 1996; Amin *et al.*, 1997; Hammes *et al.*, 1998). The production of

VEGF protein by hypoxic retinal glial cells *in vivo* may be influenced by glucose concentrations (Brooks *et al.*, 1998) and elevation of AGEs in the vitreous may also increase the expression and release of VEGF from Müller cells. Adenosine is also present in the GCL, INL, and the plexiform layers of the retina (Ghiardi *et al.*, 1999), and as it has been shown to regulate VEGFR-2 expression (Takagi *et al.*, 1996a), its release from glial cells and possibly other cell types under hypoxic conditions may be of relevance in the pathogenesis of diabetic retinopathy. Adenosine was also shown to increase VEGF-induced proliferation of canine retinal microvascular cells (Lutty *et al.*, 1998).

Müller cells and astrocytes also share the ability to form the glia limitans of the retina and of vessels (Holländer *et al.*, 1991). In ROP, preretinal vessels form when intensive hypoxia causes the degeneration of astrocytes and the strong expression of VEGF by other cells, particularly neurones (Chan-Ling *et al.*, 1992; Chan-Ling *et al.*, 1995ab; Stone *et al.*, 1996). Therefore the glia limitans becomes damaged, and with high expression of VEGF and the glia limitans breached, VEGF may diffuse in to the vitreous and induce vessel growth away from the retina. PDR may therefore be caused by exaggeration of the mechanisms that cause normal vascularization of the retina; preretinal vessels may from, as do normal vessels, by the hypoxia-induced secretion of VEGF and may be proliferative because of the high level of VEGF expression.

Takagi *et al.* (1996b) demonstrated that bovine retinal PCs (BRPCs) predominantly express VEGFR-1 in contrast to retinal ECs which predominantly express VEGFR-2. Witmer *et al.*, 2004 also demonstrated that activated pericytes express VEGFR-1 in a monkey model of iris neovascularization. Retinal PCs possess large numbers of high affinity VEGF binding sites, which result in tyrosine phosphorylation of intracellular substrates and weak growth promoting effects after long-term VEGF stimulation. This suggests that VEGF may mediate the response of retinal PCs as well as retinal ECs during the pathological angiogenesis characteristic of PDR and other ischaemic retinal disorders.

Hypoxia also increased VEGF expression in BRPCs. Although the role of PCs during angiogenesis is poorly understood, it is likely that hypoxia would turn them predominantly mitogenic, resulting in their loss of contact with endothelial cells, thereby promoting endothelial cell growth. AGES have been shown to upregulate the secretory forms of VEGF mRNA in retinal pericytes (Yamagishi *et al.*, 2002). This suggests that AGEs disturb retinal microvascular homeostasis by inducing pericyte apoptosis and VEGF overproduction, therefore playing a role in the pathogenesis of early diabetic retinopathy.

The Angiopoietin/Tie-2 Family

Immunostaining for Ang-1, Ang-2, and Tie-2 was performed using a dilution series of the primary antibodies, against a set number of sections, as recommended by the manufacturer. Initially only weak staining was observed for all antibodies. Successful staining was eventually achieved using a highly concentrated dilution of each primary antibody and by exposing the angiogenic binding sites with proteolytic digestion using 0.1% chymotrypsin. Deposits were observed when examining the sections stained with all three antibodies. After consultation with the manufacturer the antibodies were spun at 25000 rpm at 4°C for 10 mins but this did not remove the deposits. Staining was retried using new primary antibodies, a new secondary antibody and new fast red substrate but again the deposits were observed. Photographs were taken of areas showing minimal deposits. The sections were imaged several months after staining and some drying out of the mountant was observed which is evident from the photomicrographs.

In this study Ang-1 and Tie-2 were localised to the endothelial and perivascular cells of both the non-diabetic and diabetic retinas. In the vessels of the non-diabetic retinas and the diabetic retinas with no overt retinopathy this is consistent with the observation that Ang-1 is expressed in normal arterial and venous specimens (Witzenbichler *et al.*, 1998a) and in BRECs and BAECs (Oh *et al.*, 1999). Tie-2 is also expressed and phosphorylated in the entire spectrum of the quiescent vasculature (arteries, veins and capillaries) which also suggests a role for Tie-2 signalling in the maintenance of the quiescent adult vasculature (Wong *et al.*, 1997). Maisonpierre *et al.*, 1997 also demonstrated that during ovulation Ang-1 is expressed in early follicles where the vasculature is in a quiescent state.

The initiation of blood vessel growth involves focal reduction of intercellular interactions and interactions between the cells of the blood vessel and the surrounding ECM (Lauren *et al.*, 1998). This is associated with a loss of PCs and possibly of SMCs from the existing vessels (Risau, 1997). The maturation of newly formed vessels involves the accumulation of a basal lamina and tightly associated PCs or SMCs on the abluminal side.

In normal adult quiescent vessels, perivascular cells have previously been shown to constitutively secrete Ang-1, enhancing contact between neighbouring ECs and between ECs/perivascular cells, therefore maintaining endothelial integrity and orientation of ECs on the basal lamina leading to vessel stabilisation and maturation of the vasculature (Mandriota *et al.*, 1998; Gamble *et al.*, 2000; Hori *et al.*, 2004).

The presence of Ang-1 and Tie-2 in the diabetic vessels is also consistent with a proposed role for the Ang-1/Tie-2 system at all stages of diabetic retinopathy. Ang-1 is chemotactic for ECs and induces migration, tube formation, sprouting and survival, but not proliferation of ECs in vitro (Davis *et al.*, 1996; Witzenbichler *et al.*, 1998a; Koblizek *et al.*, 1998; Hayes *et al.*, 1999; Kwak *et al.*, 1999; Papapetropoulos *et al.*, 1999; Kim *et al.*, 2000a,b; Kwak *et al.*, 2000). Under conditions of postnatal angiogenesis, Ang-1 expression may be important for initiation of new capillary sprouting, the movement of ECs toward each other and the recruitment of perivascular cells required for fusion into capillary structures (Koblizek *et al.*, 1998; Fujikawa *et al.*, 1999; Kim *et al.*, 2000a, b; Papapetropoulos *et al.*, 2000; Harfouche *et al.*, 2002; Babaei *et al.*, 2003; Harfouche *et al.*, 2003; DeBusk *et al.*, 2004; Metheny-Barlow *et al.*, 2004; Saito *et al.*, 2004). In addition, Ang-1 also has anti-permeability and anti-inflammatory functions (Thurston *et al.*, 1999; Gamble *et al.*, 2000; Thurston *et al.*, 2000; Wang *et al.*, 2000; Kim *et al.*, 2001; Joussen *et al.*, 2002a; Pizurki *et al.*, 2003; Hori *et al.*, 2004; Li *et al.*, 2004; Wang et al., 2004; Jho *et al.*, 2005; Baffert *et al.*, 2006). The observations that Ang-1 and Tie-2 are expressed in endothelial cells of glioblastomas, in leukaemia cell lines, metastatic melanomas and gastric carcinoma (Kaipainen *et al.*, 1994b; Stratmann *et al.*, 1998; Witzenbichler *et al.*, 1998a; Yoshizaki *et al.*, 2004) further supports their role in angiogenesis. Ang-1 has also been shown to promote wound healing through enhanced angiogenesis in a diabetic mouse model (Cho *et al.*, 2006). Tie-2 is also expressed during active vasculogenesis in mice embryos and during vasculogenesis in the developing human placenta (Sato *et al.*, 1993; Schnurch and Risau, 1993; Kayisli *et al.*, 2006). Also, following on from the findings that Ang-1 is expressed in quiescent vasculature during ovulation, Maisonpierre *et al.* (1997) demonstrated that Ang-1 is also present, alongside that of Ang-2, in late pre-ovulatory follicles and the corpus luteum where angiogenesis is ongoing. Ang-1 when given intravitreally to newly diabetic rats, normalized retinal VEGF and intercellular adhesion molecule-1 mRNA and protein levels, leading to reductions in leukocyte adhesion, endothelial cell injury, and blood-retina barrier breakdown, early pathological changes observed in diabetic retinopathy showing that Ang-1 directly protects the retinal vasculature in diabetes.

Wong *et al.* (1997) also demonstrated that Tie-2 expression was upregulated in the endothelium of neovessels in rat tissues undergoing angiogenesis during hormonally stimulated follicular maturation and uterine development and in healing skin wounds. However, downregulation of Tie-2 was demonstrated during the later stages coinciding with regression of vessels. This appeared to be apparent in my study where Tie-2 was high in the

preretinal vessels undergoing active angiogenesis but downregulated in the preretinal vessels undergoing regression in the excised membranes. It was also consistent with my finding that Ang-1 was downregulated in the pre-retinal vessels of the excised membranes which supports the observation that the Ang-1 signal is blocked by Ang-2 during vascular regression in ovarian follicles (Maisonpierre *et al.*, 1997).

Ang-2 appears to initiate angiogenesis by binding to the Tie-2 receptor on ECs which results in weakening of the phosphorylation of, and blocking of, the chemotactic effects of Ang-1 (Maisonpierre *et al.*, 1997; Sato *et al.*, 1998; Witzenbichler *et al.*, 1998a). Because Ang-1 is thought to be important for stabilising the vessel wall, local Ang-2 expression might promote SMC/PC drop-off, which is thought to be a requirement for rendering and maintaining ECs accessible to angiogenic inducers. This is supported by the finding that Ang-2 destabilizes quiescent endothelial cells (Scharpfenecker *et al.*, 2004). This loss of PCs was associated with the upregulation of Ang-2 in the ECs of glioblastomas (Stratmann *et al.*, 1998). This role of Ang-2 is also supported by the demonstration that the addition of Ang-2 to VEGF induced neovascularization by promoting vascular destabilisation and sprouting in the corneal micropocket assay (Asahara *et al.*, 1998). Few perivascular cells were present in the vessels supporting the concept that loosening of contacts between ECs and perivascular cells initiates angiogenesis by recruiting VEGF (and possibly other growth factors). Similar findings were observed in the corpus luteum where in the presence of abundant VEGF, Ang-2 may promote vessel sprouting by blocking a constitutive (stabilising) Ang-1 signal, whereas in the absence of VEGF, Ang-2 inhibition of a constitutive Ang-1 signal can contribute to vessel regression (Maisonpierre *et al.*, 1997). Furthermore, Oh *et al.*, 1999 demonstrated that in BRECs VEGF and hypoxia induced an increase in Ang-2. They suggested that the angiogenic stimuli of hypoxia might deteriorate the integrity of the vasculature by suppressing Ang-1 activation of Tie-2. Ang-2 expression has been demonstrated in SMCs, PCs, and microvascular ECs (Mandriota *et al.*, 1998). BMEC Ang-2 mRNA levels were increased by VEGF, VEGF and bFGF in combination, by hypoxia and were decreased by Ang-1 and Ang-2 itself (Maisonpierre *et al.*, 1997; Mandriota *et al.*, 1998; Yamakawa *et al.*, 2003; Pichiule *et al.*, 2004). This suggests that the angiogenic effect of a number of regulators may be achieved in part through the regulation of an autocrine loop of Ang-2 activity in microvascular ECs. That Ang-2 was stimulated by hypoxia further supports it role in pathological diseases characterised by hypoxia. This points to Ang-2 as a potential important component of the angiogenic switch that characterises the passage of diabetic retinopathy from the avascular to the vascular phase, and provides strong evidence for a

collaboration between VEGF and Ang-2 in the regulation of neovascularization in ischaemic tissues. Ang-2 mRNA is also strongly expressed in highly vascularized tumours (Bunone *et al.*, Stratmann *et al.*, 1999; 1999; Brown *et al.*, 2000; Etoh *et al.*, 2001).

My finding that the level of Ang-2 staining was raised in the non-diabetic retinas and the diabetic retinas with no overt retinopathy as compared to the diabetic retinas with vascular changes was unexpected. Ang-2 would be expected to be downregulated in quiescent retina in order to allow Ang-1 to exert its stabilising effects on the vasculature. However, Maisonpierre *et al.* (1997) also reported the expression of Ang-2 in the normal quiescent wall which suggests a balance of vessel maintenance by positive and negative regulators and the distinct but overlapping expression pattern of Ang-1 and Ang-2 is consistent with the possibility that Ang-2 may regulate Ang-1 function at particular sites and stages of vascular development. Patel *et al.*, 2005 showed that Ang-2 concentrations were higher in the vitreous of patients with NPDR compared to Ang-1 but was found at low concentrations in patients with PDR. The suggested that the levels were lower in the PDR patients due to the established nature of the vitrectomy. That is in long standing and treated PDR, there is little active vessel replication. In established PDR, new vessels are mature and limited active angiogenesis is taking place. They showed that in patients with NPDR the Ang-1 concentrations were half that of Ang-2. Early neovascularization is initiated at this stage of retinopathy as intravascular changes are taking place where changes in Ang-2 levels relative to Ang-1 occur and this allows Ang-2 to predominate at the Tie-2 receptor. Ang-2 is able to competitively inhibit Ang-1 binding to Tie-2. The predominance of the Ang-2 at the Tie-2 receptor would promote increased vascular permeability leading to breakdown of the blood-retinal barrier and neovascularization.

I also demonstrated low levels of Ang-2 in the intra-retinal vessels of the diabetic retinas with vascular changes and in PDR retinas. This finding also contradicts the hypothesised role of Ang-2 as this factor may be expected to be upregulated during these stages of angiogenesis in order to antagonise the vessel stabilising effects of Ang-1. Also Ang-2 is known to promote inflammation and vascular leakage, both features of diabetic retinopathy (Roviezzo *et al.*, 2005). However, it has been shown that Ang-2 may lead to angiogenesis or vessel regression and apoptosis depending on the presence of VEGF (Hanahan, 1997; Lobov *et al.*, 2002). I also encountered some problems with staining using the Ang-1 and Ang-2 antibodies. Stain deposits were apparent on some of the retinal sections which were not removed by centrifuging the antibodies. These observations were less obvious with the Tie-2 antibody. Therefore it must be taken into account that in this study

some non-specific staining may possibly have been observed. Any future studies on immunohistochemistry using the angiopoietin and Tie-2 antibodies should involve some prior treatment of the antibodies (e.g. immunoprecipitation) in order to remove these deposits. Other types of studies such as *in situ* hybridisation may also produce more reliable results.

In this study Ang-2 was present in the pre-retinal vessels of diabetic retinas undergoing active proliferation which is consistent with its role in angiogenesis. Lower levels in the preretinal vessels of the excised membranes may reflect the fact that they are no longer undergoing active proliferation. This is consistent with the finding that Ang-2 is upregulated during angiogenesis and the progression of hepatocellular carcinoma (Zhang *et al.,* 2006). Also Ang-2 has been shown to stimulate endothelial progenitor cell (EPC) migration to areas of neovascularization (Gill and Brindle, 2005). EPCs have been shown to localize at sites of active angiogenesis and vessel remodelling such as healing wounds, tumours, and ischaemic retina, where they contribute to neovasculization (Asahara *et al.,* 1997; Lyden *et al.,* 2001; Grant *et al.,* 2002).

Ang-2 mRNA was shown to be upregulated in mice models of ischaemia-induced retinal neovascularization, in the INL, GCL, and in the neovascular vessels (Oh *et al*., 1999; Hackett *et al.,* 2000). When retinal neovascularization started to regress, strong staining was still observed in the GCL and the INL of the hypoxic retinas. Also Ang-2 deficient mice were shown to lack ischaemia-induced retinal neovascularization (Hackett *et al.,* 2002). These data suggest that both hypoxia- and VEGF-induced neovascularization might be facilitated by selective induction of Ang-2 which deteriorates the integrity of the pre-existing vasculature. In my study I also found Ang-2 protein in the GCL and the INL which suggests that the upregulation of Ang-2 precedes the development of neovascularization and parallels the temporal and spatial changes of neovascularization development, which suggests that Ang-2 plays a critical role in retinal neovascularization. Tie-2 was also located in glial cells and ganglion cells. Therefore the angiopoietins appear to have both paracrine and autocrine actions on ECs. Takagi *et al.,* 2002 demonstrated upregulation of Ang-2 and Tie-2 in highly vascularized regions of human epiretinal membranes. Ang-2 were shown to promote tube-forming activity and enhanced the effects of VEGF in cultured BRECs suggesting that in microvascular ECs, Ang-2 can probably induce at least some level of Tie-2 signalling, which contributes to endothelial angiogenic functions.

TNF-α

Immunostaining for TNF-α was performed using a dilution series of a primary antibody from Abcam, against a set number of sections, as recommended by the manufacturer. Initially I found that staining was inconsistent and weak, staining very few sections. I tried changing the dilutions of the antibody, followed by chymotrypsin pre-digestion but again minimal staining was observed. I repeated the same procedure with a different antibody from Autogen Bioclear but again had the same problems. I then purchased a different antibody from Abcam. This time I tried a dilution series followed by proteolytic predigestion with chymotrypsin but again successful staining wasn't achieved. Following consultation with the manufacturer I tried 2 different methods of proteolytic predigestion. The first method involved placing the sections in a solution of sodium citrate in a pressure cooker for either 1, 2, or 3 minutes. The second method involved placing the sections in a pressure cooker for 1, 2, or 3 minutes followed by chymotrypsin pre-treatment. I found that placing the sections in the pressure cooker for 3 minutes produced satisfactory results. However following this with chymotrypsin solution damaged the retinas. Therefore for the purposes of this study sections were placed in a pressure cooker for 3 minutes followed by incubation with the primary antibody.

Initially only weak staining was observed for all antibodies. Successful staining was eventually achieved using a highly concentrated dilution of each primary antibody and by exposing the angiogenic binding sites with proteolytic digestion using 0.1% chymotrypsin. In this study low levels of TNF-α were localised to the endothelial and perivascular regions of the non-diabetic retinas and higher levels were demonstrated in diabetic retinas indicating that it may play a role in the pathogenesis of both diabetes and diabetic retinopathy. This is in agreement with Tezel *et al.,* 2001 who demonstrated that TNF-α is constitutively and weakly expressed in normal human retinas.

The highest level of TNF-α immunostaining was observed in the vessels of the diabetic eyes without obvious microvascular changes and was then reduced in retinas with PDR. This supports the finding that high doses of TNF-α have been shown to inhibit angiogenesis whereas low doses were shown to induce angiogenesis (Fajardo *et al.,* 1992). TNF-α is increased during periods of hypoxia (Lahat *et al.,* 2003; Ben-Yosuf *et al.,* 2005) and was more specifically shown to be increased during hypoxia in mouse models of oxygen-induced retinopathy in mice, perhaps before microvascular changes become obvious (Yossuck *et al.,* 2001; Majka *et al.,* 2002). Kerkar *et al.,* 2006 also showed that TNF-α was

able to relax pericyte contractility, which may lead to pericyte dropout, an event which occurs early in diabetes before retinal microvascular changes become obvious.

TNF-α has been shown to play a role in diabetic retinopathy, because it alters the cytoskeleton of ECs, resulting in leaky barrier function and EC activation and an inflammatory response. Studies using diabetic animals have shown that increased leukocyte adhesion to retinal capillaries is an early event in diabetic retinopathy associated with areas of capillary nonperfusion and capillary obstruction and the development of EC damage (Camussi *et al.*, 1991; Kim *et al.*, 1992; Claudia *et al.*, 1994; Deli *et al.*, 1995; Bamforth *et al.*, 1996; de-Vries *et al.*, 1996; Luna *et al.*, 1997; Lutty *et al.*, 1997; Mark *et al.*, 1999; Miyamoto *et al.*, 1999; Joussen *et al.*, 2001; Mark *et al.*, 2001; Mayhan *et al.*, 2002; Trickler *et al.*, 2005; Kerkar *et al.*, 2006; Koss *et al.*, 2006). Joussen *et al.*, 2001 showed that TNF-α in diabetic plasma increases adherence of human leukocytes to retinal ECs, which was increased with the severity of diabetic retinopathy. Menon *et al.*, 2006 showed that TNF-α was able to disrupt VE-cadherin complexes at vascular EC junctions leading to gapping between ECs, causing increased vascular leakage in tumours.

These changes in retinal ECs are central in the progression of diabetic retinopathy. This may explain why it was slightly raised in the diabetic retinas with microvascular abnormalities in my study. The production of TNF-α has been shown to be significantly increased during long-term hyperglycaemia in spontaneously diabetic rats and mice, as well as in streptozotocin-induced diabetic rats. Ages, produced as a result of hyperglycaemia have been shown to promote mRNA expression and secretion of TNF-α in HUVEC (Rashid *et al.*, 2004).

In this study the level of TNF-α expression in the retinal vessels of the PDR retinas was below that observed in the diabetic retinas without microvascular abnormalities but was raised above that seen in the non-diabetic retinas. The level of TNF-α expression was also raised in the neovessels of the PDR retinas and the fibrovascular membranes. This suggests that TNF-α production plays some role in PDR. This is consistent with the findings of Spranger *et al.*, 1995 who demonstrated that there was an increase in TNF-α production in neovascular eye disease and PDR. Levels of TNF-α were shown to be elevated in animal models of ischaemia-induced retinal neovascularization compared with the retinas of non diabetic animals (Yossuck *et al.*, 2001; Joussen *et al.*, 2002c; Majka *et al.*, 2002). TNF-α has previously been detected in the neovessels and extracellular matrix of PDR membranes (Limb *et al.*, 1994; Limb *et al.* 1996; Armstrong *et al.*, 1998a). Armstrong *et al.*, 1998b showed that oxidative damage associated with tissue hypoxia stimulated retinal

neovascularization in rabbit retina through expression of TNF-α along with VEGF and PDGF. Inhibition of TNF-α or TNF-α knockout was shown to reduce pathological neovascularization in mouse models of oxygen-induced retinopathy by increasing physiological angiogenesis (Gardiner *et al.*, 2005; Kociok *et al.*, 2006). TNF-α has also been found in the vitreous of eyes with PDR (Limb *et al.*, 1991; Franks *et al.*, 1992; Limb *et al.*, 2001) and an association between the serum level of TNF-α and the development of PDR has also been demonstrated (Limb *et al.*, 1996).

TNF-α is a potent inducer of angiogenesis in vivo (Frater-Shroder *et al.*, 1987; Leibovich *et al.*, 1987; Montrucchio *et al.*, 1994). However, in vitro, TNF-α seems to inhibit in vitro angiogenic activities such as endothelial proliferation and tube formation (Frater-Shroder *et al.*, 1987; Sato *et al.*, 1987), suggesting that TNF-α may induce angiogenesis indirectly by activating other regulators of angiogenesis, e.g. inflammatory cell secretion of VEGF, IL-8 etc. TNF-α can induce Tie-2 expression (Willam *et al.*, 2000). Ang-1 is up-regulated by TNF-α (Scott *et al* 2002; Scott *et al.*, 2005). TNF-α upregulated Ang-2 in HUVECs (Kim *et al.*, 2000d). Therefore TNF-α-induced inflammation angiogenesis might be facilitated by the induction of Ang-2. TNF-α was found in human choroidal neovascular membranes and it colocalized with VEGF, Ang-1, and Ang-2 in cultured choroidal ECs (Hangai *et al.*, 2006). It increased Ang-2 mRNA and protein levels prior to those of Ang-1 and VEGF. These results raise the possibility that during neovascularization, TNF-α may modulate endothelial plasticity and survival by sequential inactivation of Tie-2 followed by activation of Tie-2 and VEGF receptors. Chen *et al.*, 2004 also showed that TNF-α induced a weak angiogenic response in a mouse cornea assay and systemic overexpression of Ang-1 or Ang-2 dramatically increased corneal angiogenesis induced by TNF-α.

In this study the level of TNF-α was raised in the lasered retinas compared to the PDR retinas and was raised significantly compared to the non-diabetic retinas. This is in contrast to other studies in diabetic eyes with intensive coagulation, TNF-α could not be detected (Kutty *et al.*, 1995; Platts *et al.*, 1995).

In this study production of TNF-α was not specifically confined to the retinal vessels. Staining was also observed within the inner retinal layer and the GCL. Staining was also observed within the outer retina in the lasered retinas. This is consistent with the findings of other workers (Fontane *et al.*, 2002; Majka *et al.*, 2002) who showed that in mouse models of retinal ischaemia, TNF-α was found mainly in the inner retina with a stronger expression in the GCL compared with cells of the INL particularly in neurons such as amacrine cells. TNF-α was also detected within structures of the ONL resembling Müller cell processes.

Significant increase of TNF-α expression was observed after reperfusion, in particular the GCL, and to a lesser extent, in INL cells (Fontane *et al.*, 2002) which is consistent with the observation that TNF-α was raised in the lasered retinas in my study. Other workers have shown that retinal Müller cells express TNF-α under hypoxic conditions (Fuchs *et al.*, 2005). TNF-α has been shown to increase the expression of MT1-MMP, MMP-3 and MMP-9 in cultured retinal Müller cells (Migita *et al.*, 1996; Zhang *et al.*, 1998; Majika *et al.*, 2002). MMP-9 has been implicated in cell migration and matrix degradation. The investigators suggested that TNF-α plays a role in the regulation of extracellular proteinase expression during retinal neovascularization. TNF-α binds to ECM proteins, including collagen, fibronectin, and laminin which constitute the main matrix components of epiretinal membranes (Scheiffarth *et al.*, 1988; Alon *et al.*, 1994; Hershkoviz *et al.*, 1995; Franitza *et al.*, 2000). Fontaine *et al.*, 2002 suggested that TNF-α could have neuroprotective or neurotoxic effects in ischaemic retina dependent upon whether it bound to TNF-R1 or TNF-R2.

TNF-α is secreted by glial cells in the CNS and has been shown to be released in activated cultured rat retinal microglia (Morigiwa *et al.*, 2000). Yoshida *et al.*, 2004 showed that TNF-α is produce by activated macrophages/microglia during post-ischaemic inflammation in a mouse model of ischemic retinal neovascularization. Tezel *et al.*, 2001 showed that retinal glial cells weakly stained for TNF-α in normal human retinas. In vitro studies using cocultures of retinal ganglion cells and glial cells showed that TNF-α is upregulated in retinal glial cells after exposure to simulated hypoxia (Tezel *et al.*, 2000).

CHAPTER 5 EXPRESSION OF THE ANTI-ANGIOGENIC GROWTH FACTOR PEDF IN NORMAL AND DIABETIC HUMAN RETINAS.

5.1 INTRODUCTION

To investigate spatial and temporal changes of PEDF expression in the progression of diabetic retinopathy, 25 human retinas were stained with anti-PEDF antibody. 5 non-diabetic retinas and 5 retinas from each of the diabetic groups, summarized in chapter 3 were stained. The intensity of staining was recorded for each retina and an average score calculated for each category of tissue.

5.2 Control Staining

To confirm specificity negative controls were incubated without primary antibody or with primary antibody after pre-absorption with recombinant PEDF. Staining was negative on all the control sections (see figure 5.1).

5.3 PEDF Immunostaining of Retinal Sections

When examined by light microscopy PEDF staining was observed in the non-diabetic retinas (mean 2.2), in retinas without ocular abnormalities (1.6), and in retinas with non-proliferative diabetic retinopathy (1.2) [see figure 5.1). Intraretinal staining was nearly abolished in patients with PDR (mean 0.4) compared with non-diabetic retinas, retinas with diabetes but without ocular disease and patients with NPDR. Retinas with previous scatter photocoagulation resulting in quiescent PDR had weak intraretinal immunochemical staining that was, on average, slightly more intense than that of the patients with active PDR (mean 1.0).

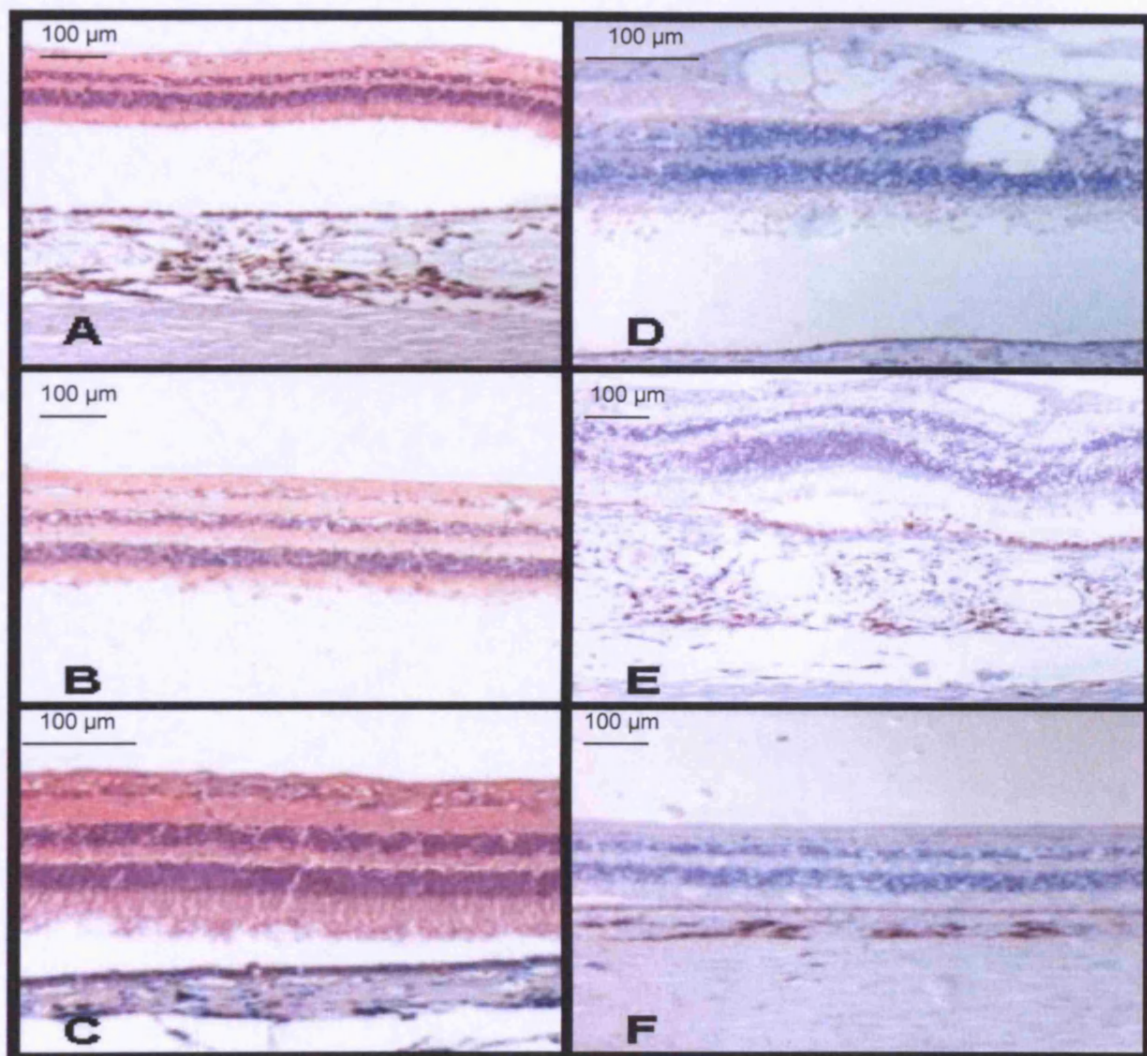

Figure 5.1 Transverse Sections Showing the Immunolocalisation of PEDF
PEDF immunostaining was localised to non-diabetic retina (A), unlasered diabetic retina with no obvious intraretinal vascular changes (B), unlasered retina with intraretinal vascular changes (C), diabetic retina with PDR (D), and lasered diabetic retina (E). Immunoreactivity was abolished in control retinas processed with normal pig serum (F).

5.4 Discussion

The data presented in this study demonstrate that PEDF was strongly expressed in non-diabetic retinas but that there was almost no staining in retinas with active proliferation. These observations support the view that induction of angiogenesis in diabetic human retinas requires not only elevation of growth factors such as VEGF (Aiello *et al.*, 1994) but also a decrease in angiogenesis inhibitors such as PEDF (Dawson *et al.*, 1999; King et *al.*, 2000).

Several other workers have shown that in normal foetal and adult human and animal retinas, PEDF is expressed in the RPE, photoreceptors, interphotoreceptor matrix (IPM) , retinal ganglion cells, cells of the inner retinal layer, nerve fibre layer, inner and outer plexiform layers. (Tombran-Tink *et al.*, 1995; Wu *et al.*, 1995; Ortego, 1996; Wu *et al.*, 1996; Alberdi *et al.*, 1998; Karakousis, 2001; Behling and Bennett, 2002; Ogata *et al.*, 2002bc Eichler *et al.*, 2004; Hattenbach *et al.,* 2005; Becerra, 2006). It has been suggested that secretion of PEDF in the retina, both during vascular development and in adults, accumulates in avascular spaces of the eye such as the aqueous and vitreous humor, and the interphotoreceptor matrix where it is responsible for excluding vessels from invading the retina, vitreous, and cornea (Dawson *et al.*, 1999; Karakousis, 2001; Behling and Bennett, 2002). PEDF has been shown to inhibit VEGF-induced proliferation and migration of microvessel ECs (Duh *et al.*, 2002) and has been shown to inhibit VEGF-induced retinal endothelial cell growth and migration in several animal models of ischaemia induced retinal neovascularization (Mori 2001; Stellmach *et al.*, 2001; Auricchio *et al.*, 2002; Duh *et al.*, 2002; Mori *et al.*, 2002c; Raisler *et al.*, 2002). The mechanism by which PEDF exerts its antiangiogenic action is not fully understood. However, several reports showed that it causes apoptosis of stimulated or proliferating ECs (Stellmach *et al.*, 2001; Guo, 2002; Volpert *et al.*, 2002; Chen *et al.*, 2006) and this may be the mechanism of inhibition of angiogenesis. Cai *et al.*, 2006 demonstrated that PEDF was able to inhibit VEGF-induced angiogenesis via γ-secretase cleavage of VEGFR-1. They also showed that PEDF was also able to inhibit VEGF-induced phosphorylation of VEGFR-1. Phosphorylation of VEGFR-1 was also shown to regulate VEGFR-2 signalling.

The presence of PEDF in the neuronal layers of the retina is also consistent with a role for PEDF as a neuronal survival and neuronal differentiating factor in the retina (Becerra, 1997; Cayouette *et al.*, 1999; Houenou *et al.*, 1999; Jablonski *et al.*, 2000; Cao *et al.*, 2001; Crawford *et al.*, 2001; Ogata *et al.*, 2001c; Becerra *et al.*, 2006; Li *et al.*, 2006). PEDF has been shown to promote photoreceptor outer segment formation and maturation,

and maintains steady state levels of opsin and glutamine synthetase expression in photoreceptor and Müller glial cells (Jablonski *et al.*, 2000; Jablonski *et al.*, 2001).

In this study there was a small reduction in staining intensity in diabetic retinas without microvascular abnormalities and in diabetic patients with NPDR compared with non-diabetic retinas. The finding that PEDF is reduced in patients with NPDR is consistent with the finding that under hypoxic conditions in culture, secretion of PEDF is decreased (Dawson, 1999). It has been shown that PEDF acts as an anti-inflammatory and anti-permeability in the eye (Mori *et al.*, 2001; Stellmach *et al.*, 2001; Liu *et al.*, 2004; Zhang *et al.*, 2005). Yamagishi *et al.*, 2006 showed that PEDF could inhibit AGE-induced retinal vascular hyperpermeability. Down-regulation of PEDF expression has been shown to increase the secretion of VEGF and TNF-α in retinal Müller cells suggesting that a decrease in ocular PEDF levels may play contribute to inflammation and vascular leakage in the eye (Zhang *et al.*, 2005). PEDF has also been shown to protect cultured retinal pericytes from advanced glycation endproduct-induced injury. As pericyte loss from retinal blood vessels is an early feature NPDR a reduction in PEDF, as observed in this study, could lead to pericyte dropout (Yamagishi *et al.*, 1995; Yamagishi *et al.*, 2002).

The finding that there was almost no staining for PEDF in the retinas undergoing active proliferation is consistent with the view that PEDF loss creates a permissive environment for angiogenesis that my contribute to the progression of ocular neovascular disease. Ischaemia- induced retinal neovascularization has been shown to cause a significant reduction in retinal PEDF and a substantial increase in VEGF mRNA and protein in rat models (Gao *et al.*, 2001; Crossen, 2002). A decrease in levels of PEDF has also been described in the vitreous of patients with PDR (Holekamp *et al.*, 2002; Ogata *et al.*, 2001a; 2002a). Vitreous levels of PEDF were shown to be low in eyes with proliferative vitreoretinopathy whereas VEGF levels were raised supporting the view that lower levels of PEDF and higher levels of VEGF may be related to ocular proliferation. However, recently Apte *et al.*, 2004 showed that PEDF stimulated choroidal neovasculariation at high concentrations but at low concentrations it decreased neovascularization. Therefore the concentration of PEDF appears to be important, whether this applies to diabetic retinopathy remains to be determined.

It therefore appears that there is a direct correlation between PEDF and the extent of neovascularization and that there is an equilibrium shift between PEDF and VEGF in the uncontrolled growth of blood vessels in the eye (Gao *et al.*, 2001; Ohno-Matsui *et al.*, 2001; Gao and Ma, 2002; Ogata *et al.*, 2001a; 2002a, b, c). Eichler *et al.*, 2004 showed that

exposure of Müller cells to VEGF suppressed PEDF release in a dose-dependent manner. These findings suggest that in ischaemic retina Müller cells generate a permissive condition for angiogenesis by secreting more VEGF and less PEDF.

More direct evidence for PEDF as an anti-angiogenic factor in PDR comes from studies in which delivery of PEDF protein through virus-mediated gene transfer and direct protein introduction was used successfully to inhibit angiogenesis in a number of animal models of ischaemia-induced retinopathy and choroidal neovascularization (Mori, 2001; Rasmussen, 2001; Stellmach, 2001; Auricchio, 2002; Duh, 2002; Mori *et al.,* 2002ab; Raisler, 2002).

Retinas with quiescent retinal neovascularization who had had retinal photocoagulation had higher levels of PEDF compared with retinas with active PDR. Photocoagulation induces regression of retinal neovascularization and has been shown to be associated with a reduction in the incidence of severe visual loss and retinal neovascularization (Early Treatment Diabetic Retinopathy Study Research Group, 1991). The findings suggests therefore that the positive effects of retinal photocoagulation are mediated at least in part by the re-establishment of near-normal PEDF levels. Hattenbach *et al.*, 2005 observed a considerable upregulation of PEDF mRNA and protein and an increased secretion into the culture medium of RPE cells. PEDF has also been shown to be secreted by RPE cells into the interphotoreceptor matrix of the retina (Siegel *et al.*, 1994; Wu *et al.*, 1995; Wu *et al.*, 1996). Ogata *et al.*, 2001b demonstrated an increased expression of various angiogenic and antiangiogenic cytokines including PEDF mRNA in photocoagulated human RPE cells. The findings from these studies and my study supports the hypothesis that antiangiogenic factors play a critical role in the regression of intraocular neovascularization observed after photocoagulation (Diabetic Retinopathy Study Research Group, 1978; Doft *et al.*, 1984).

CHAPTER 6 EXPRESSION OF CAVEOLIN-1, -2, AND -3 IN THE NORMAL AND DIABETIC RETINA

6.1 INTRODUCTION

Sections were stained to localise and assess the extent of caveolin-1 presence in the retina and preretinal membranes. Sections were also stained to localise and assess the extent of caveolin-2 and -3 in normal retinas. Immunostained sections of retina were examined by light microscopy to determine if there was a temporal and spatial relationship between staining intensity and the various pathological changes associated with diabetic retinopathy.

6.2 CONTROL STAINING

To confirm specificity of the immunostaining control sections were processed with omission of the primary antibodies. Staining was negative on all the control sections (see figure 6.1).

6.3 Immunolocalisation of Caveolin-1, -2, and -3

Sections were stained to localise and assess the extent of Caveolin-1, -2, and -3 presence in the retina and preretinal membranes. Immunostained sections of retina were examined by light microscopy to determine if there was a temporal and spatial relationship between staining intensity and the various pathological changes associated with diabetic retinopathy.

6.3.1 Caveolin-1 immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy staining for caveolin-1 was observed in both non-diabetic and diabetic extravascular tissue. However, caveolin-1 immunoreactivity was generally absent or weak around the intraretinal vessels. Where staining was observed within the vessels, in some instances staining was associated with both endothelial cells and the perivascular region of the vessels. The staining pattern depended upon the category of tissue. Variability of staining within retinal layers and intra-retinal vessels across all categories was observed, with some cells staining negative and some staining positive. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 6.1 to 6.6 but this did not show a correlation with either donor age, the type of glycaemic control in the case of diabetic groups or time post-mortem.

In the non-diabetic retinas staining intensity for caveolin-1 was generally minimal or weak within the photoreceptors and the retinal vessels. Only 4/12 retinas demonstrated

staining within the vessels. Weak to moderate staining was observed within the cell bodies of the outer retina (12/12), and the cell bodies of the inner retina (12/12). Moderate staining was observed within the GCL (12/12) [figure 6.1].

In the diabetic retinas with no overt retinopathy minimal to weak staining was again observed within the photoreceptors and the retinal vessels. In comparison to the non-diabetic retinas the number of retinas showing staining for caveolin-1 within the retinas was raised (9/11). As in the non-diabetic retinas, weak to moderate immunoreactivity was associated with the cell bodies of the outer retina (10/11), and the cell bodies of the inner retina (11/11). Staining in the GCL was slightly raised to moderate to intense levels as compared to the non-diabetic retinas (11/11) [figure 6.2].

In the diabetic retinas showing vascular changes but no evidence of PDR staining was raised in the photoreceptors (8/9) and the cell bodies of the inner retina (9/9), as compared with the non-diabetic and diabetic retinas with no overt retinopathy. Staining within the cell bodies of the outer retina was slightly reduced, as compared with the non-diabetic and diabetic retinas with no overt retinopathy (9/9). Staining was again moderate to intense within the GCL (9/9). Staining was still weak within the retinal vessels (5/9) [figure 6.3].

In the diabetic retinas with active neovascular PDR membranes on their surfaces intensity of staining for caveolin-1 was raised in all the retinal layers as compared to all the other previous categories of tissue. Staining was moderate to intense within the photoreceptors (5/5), the cell bodies of the outer retina (5/5), the cell bodies of the inner retina (5/5) and the GCL (5/5). Staining was reduced slightly within the retinal vessels as compared to the other categories of tissue (3/5).Within the pre-retinal membranes staining was weak to moderate within the pre-retinal vessels (5/5) and weak within the non-vascular components of the membranes (4/5) [figure 6.4].

In those diabetic retinas which had undergone successful laser therapy staining was reduced within all the retinal layers as compared to the PDR retinas. Staining was minimal to weak within the photoreceptors (5/11) and the retinal vessels (6/11).Staining was weak to moderate within the cell bodies of the outer retina (10/11), the cell bodies of the inner retina (10/11) and the GCL (10/11) [figure 6.5].

Caveolin-1 staining was weak to moderate within the pre-retinal vessels of 15/17 of the excised membranes. Weak to moderate staining was also associated with the non-vascular components of the membranes [figure 6.6].

TABLE 6.1. MEAN INTENSITY OF CAVEOLIN-1 IMMUNOSTAINING

Tissue Category	Retinal Layer				Retinal Vessels	Membrane	
	Photo-Receptors	Outer Retina	Inner Retina	GCL		Vessels	Matrix
Non-diabetic (n=14)	0.9	1.6	1.9	2.0	0.9		
No Overt Retinopathy (n=12)	0.7	1.4	1.8	2.3	0.9		
Intraretinal Changes (n=10)	1.3	1.1	2.2	2.3	1.0		
PDR (n=9)	2.2	2.4	2.4	2.4	0.6	1.4	1.0
Laser-No Residual PDR (n=14)	0.9	1.1	1.9	1.9	0.6		
Excised Membranes (n=17)						1.4	1.3

GCL = ganglion cell layer

ILM = internal limiting membrane

0 = background staining

1 = weak staining

2 = moderate staining

3 = intense staining

Values in parenthesis = +/- standard deviation

6.3.2 Caveolin-2 Immunostaining of retinal sections

Of the 8 non-diabetic retinas tested, staining was absent in the RPE, however staining was observed in the outer segments of the photoreceptors in 7/8 of the retinas. Staining was also observed in the outer region of 2/8 of the retinas, in the inner region of 3/8 of the retinas and in the ganglion cell layer of 4/8 of the retinas. Perivascular staining was observed in 3/8 of the retinas and in one of the retinas one vessel showed endothelial cell staining [figure 6.7].

6.3.3 Caveolin-3 immunostaining of retinal sections

Of the 8 non-diabetic retinas tested only one stained positively for caveolin-3 where immunostaining was localised to the outer and inner regions of the retina and the ganglion cell layer [figure 6.7].

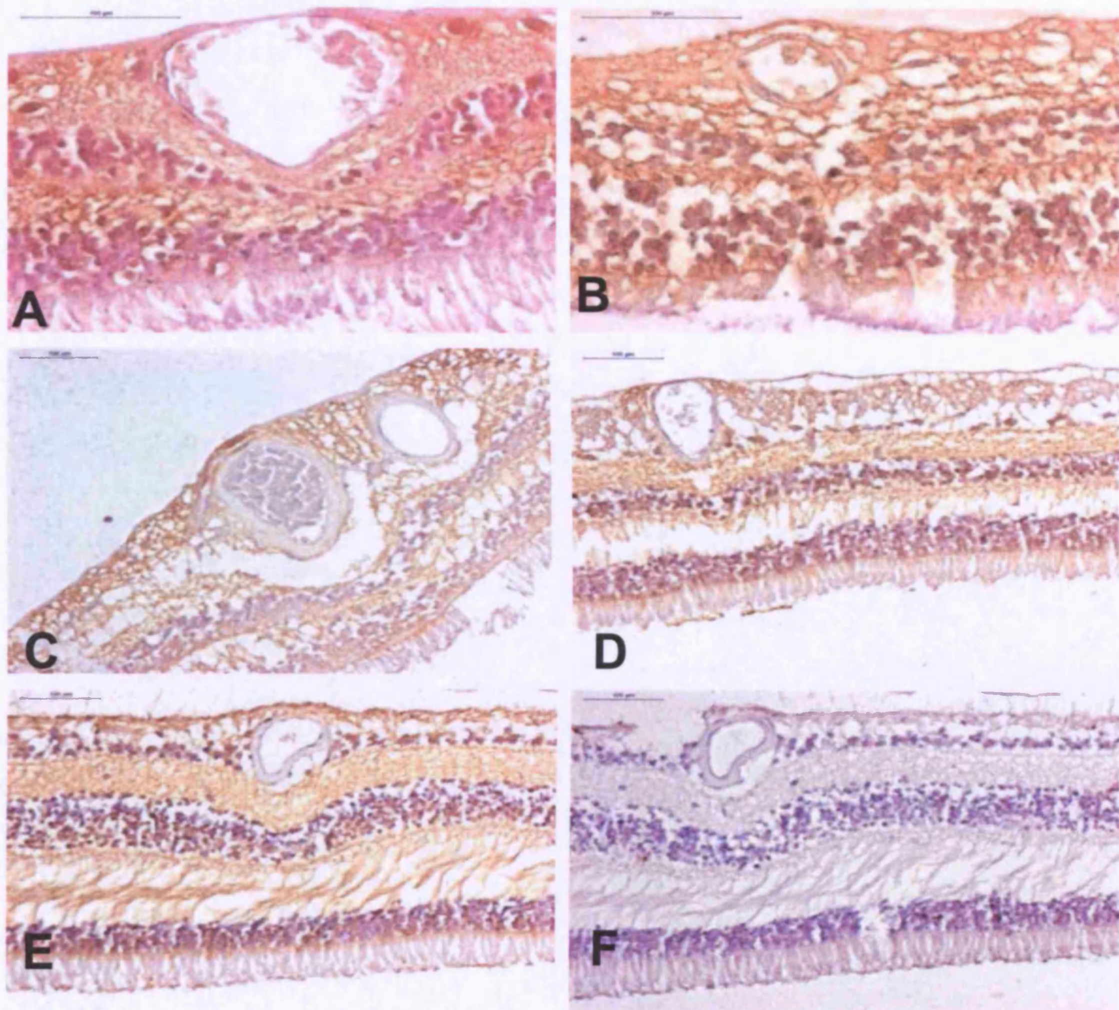

Figure 6.1 Transverse Sections Showing the Immunolocalisation of Caveolin-1 in Non-Diabetic Retinas (A-E) and Negative Control Section (F).
Staining was observed in the retinal vessels and across all the retinal layers.

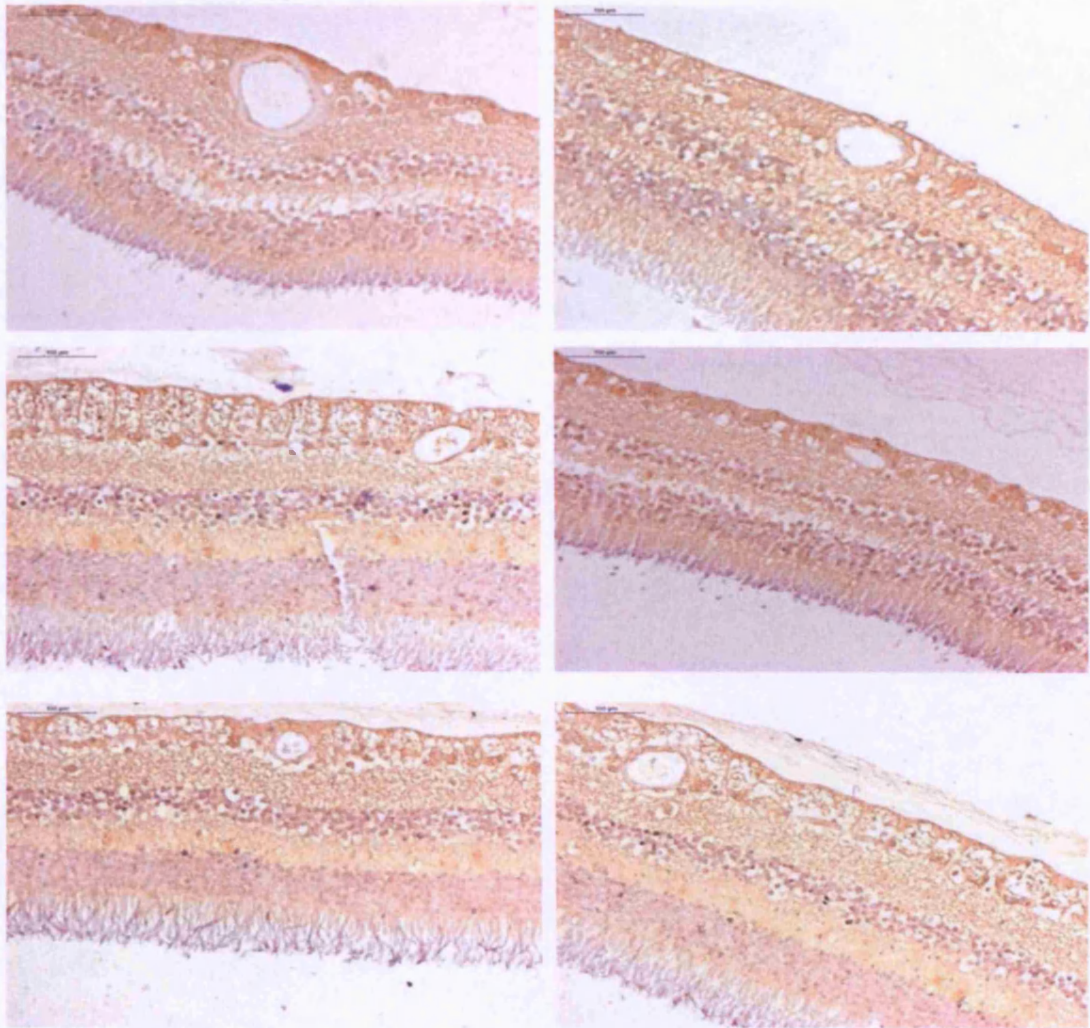

Figure 6.2 Transverse Sections Showing the Immunolocalisation of Caveolin-1 in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities. Staining was observed in the retinal vessels and across all the retinal layers.

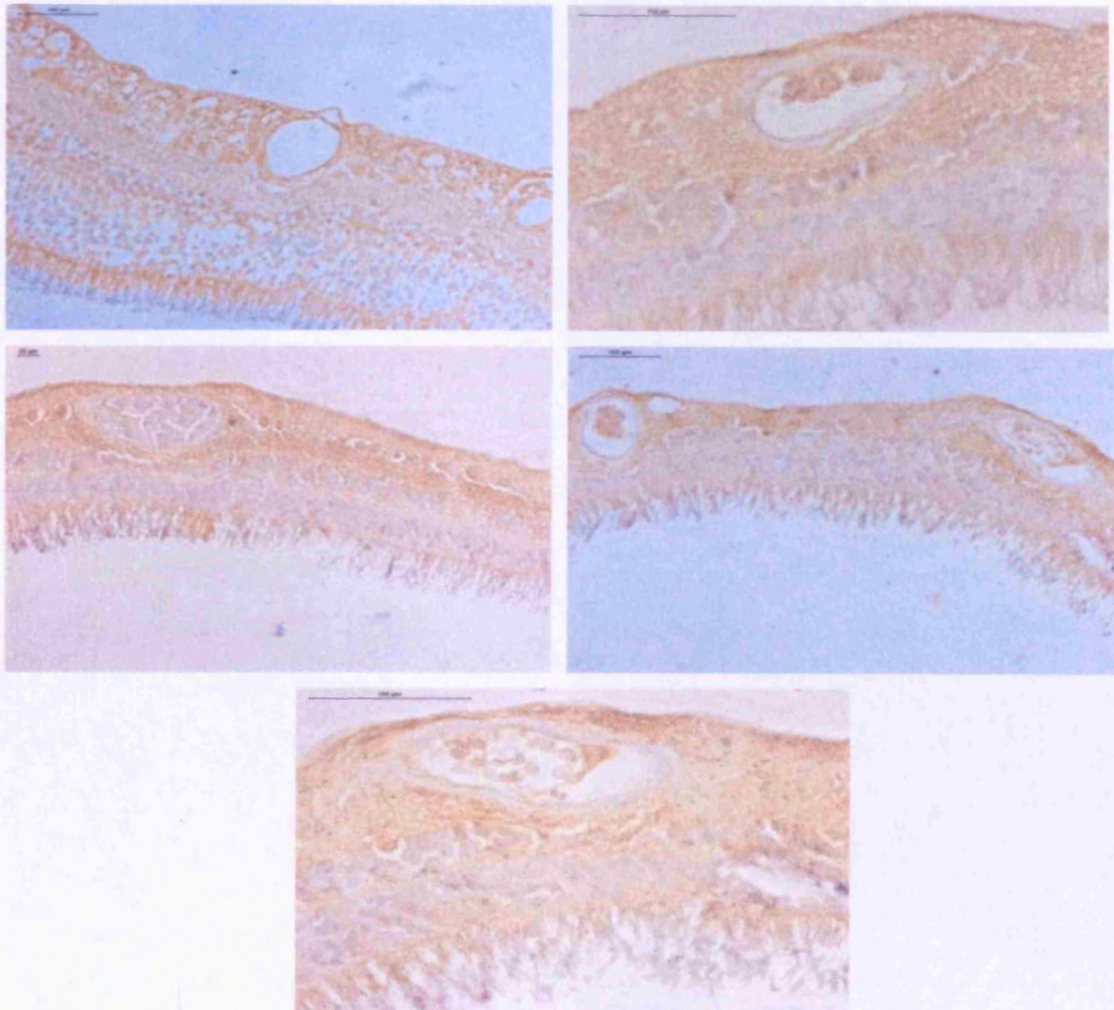

Figure 6.3 Transverse section showing the immunolocalisation of Caveolin-1 in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.
Staining was observed in the retinal vessels and across all the retinal layers.

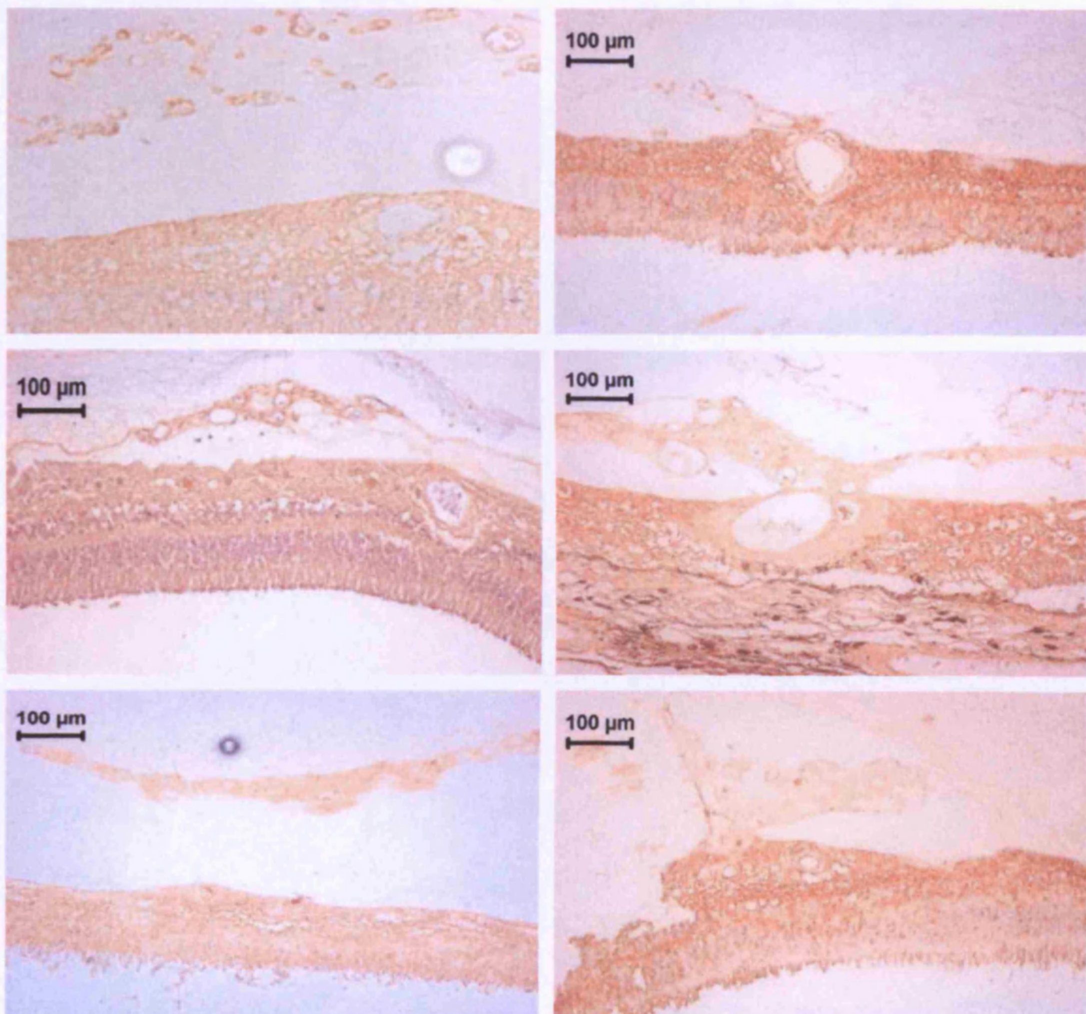

Figure 6.4 Transverse Sections Showing the Immunolocalisation of Caveolin-1 in Diabetic Retinas with PDR.
Staining was observed in the retinal vessels and across all the retinal layers.

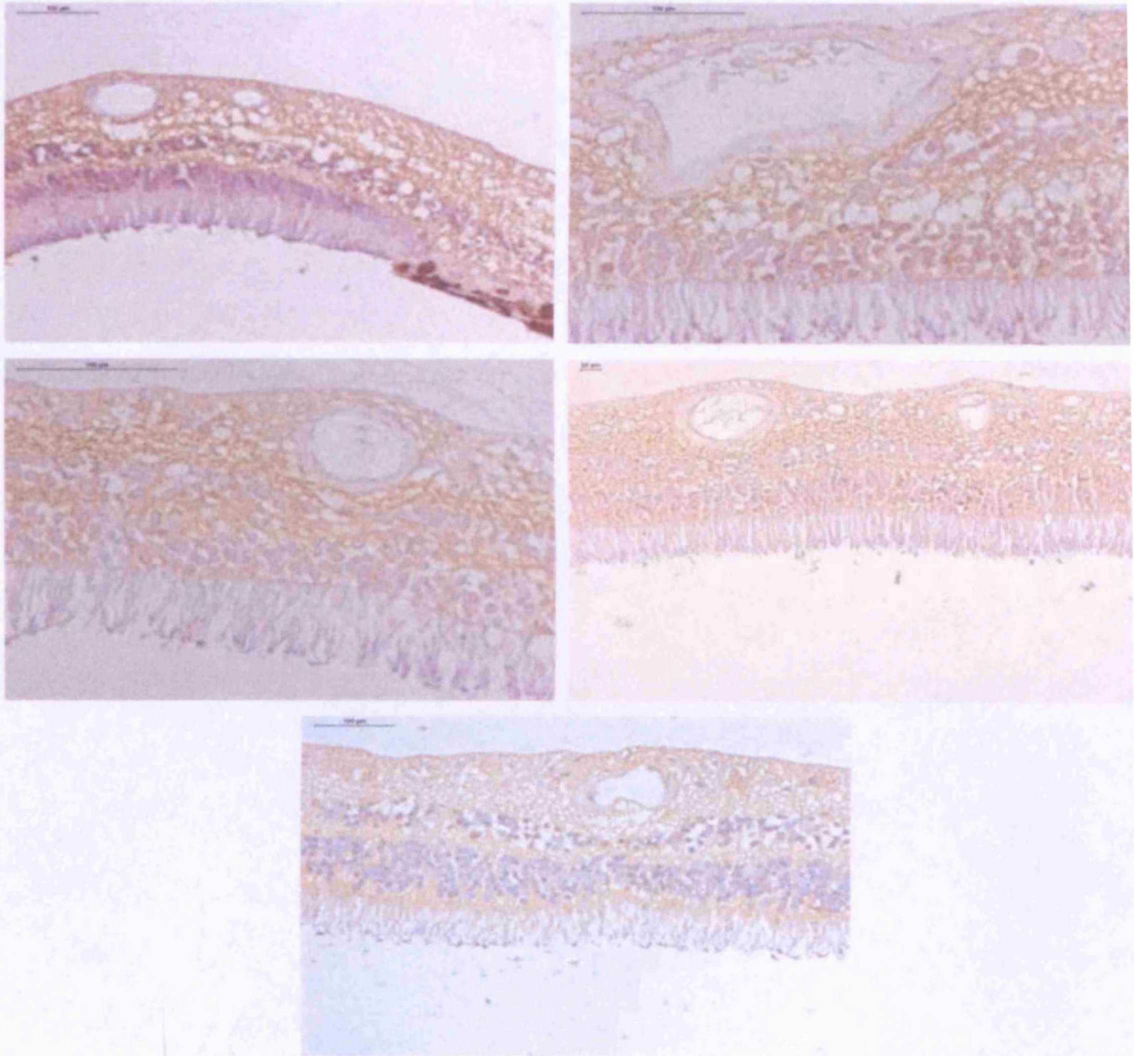

Figure 6.5 Transverse Sections Showing the Immunolocalisation of Caveolin-1 in Lasered Diabetic Retinas.

Staining was observed in the retinal vessels and across all the retinal layers.

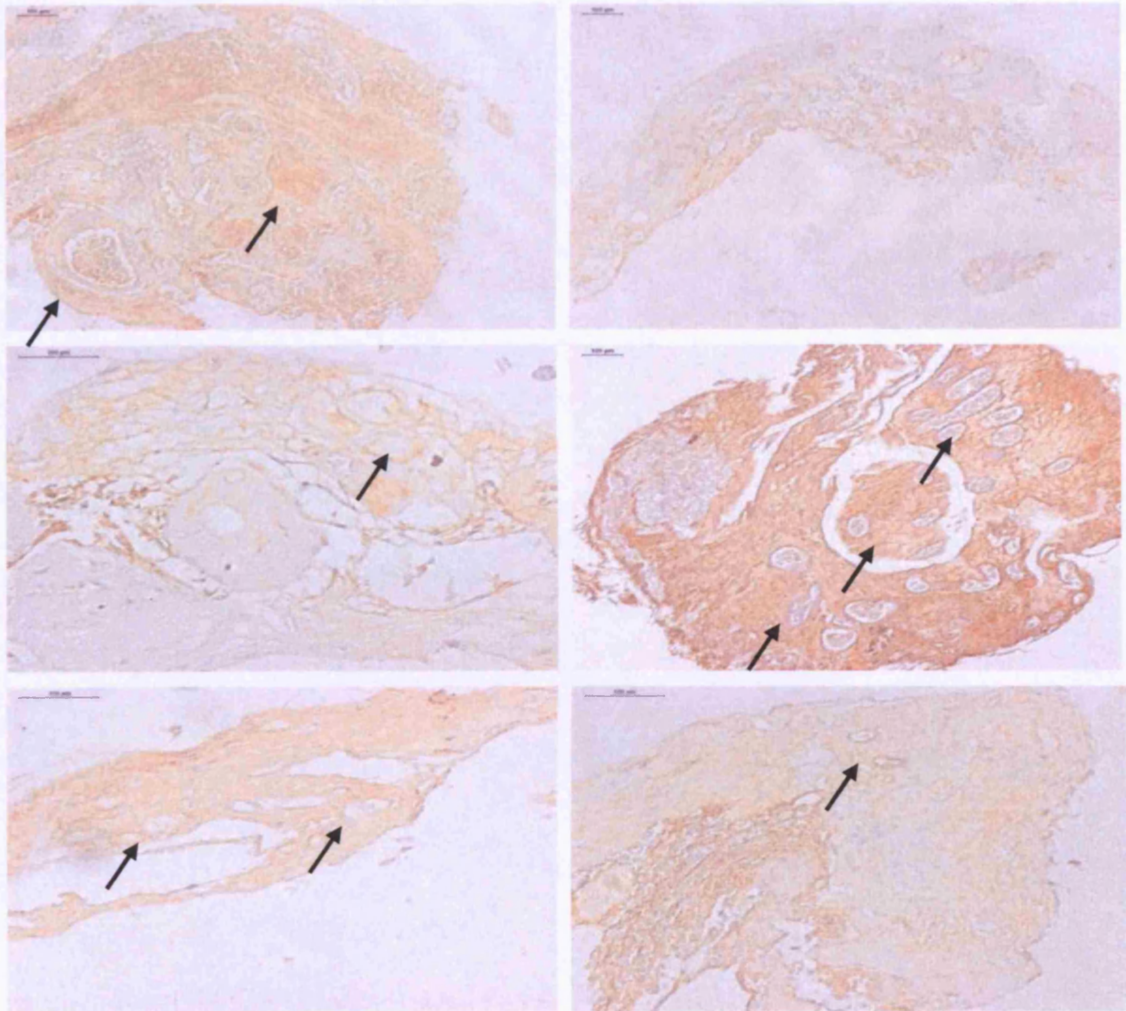

Figure 6.6 Transverse Sections Showing the Immunolocalisation of Caveolin-1 (Arrows) in Fibrovascular Membranes

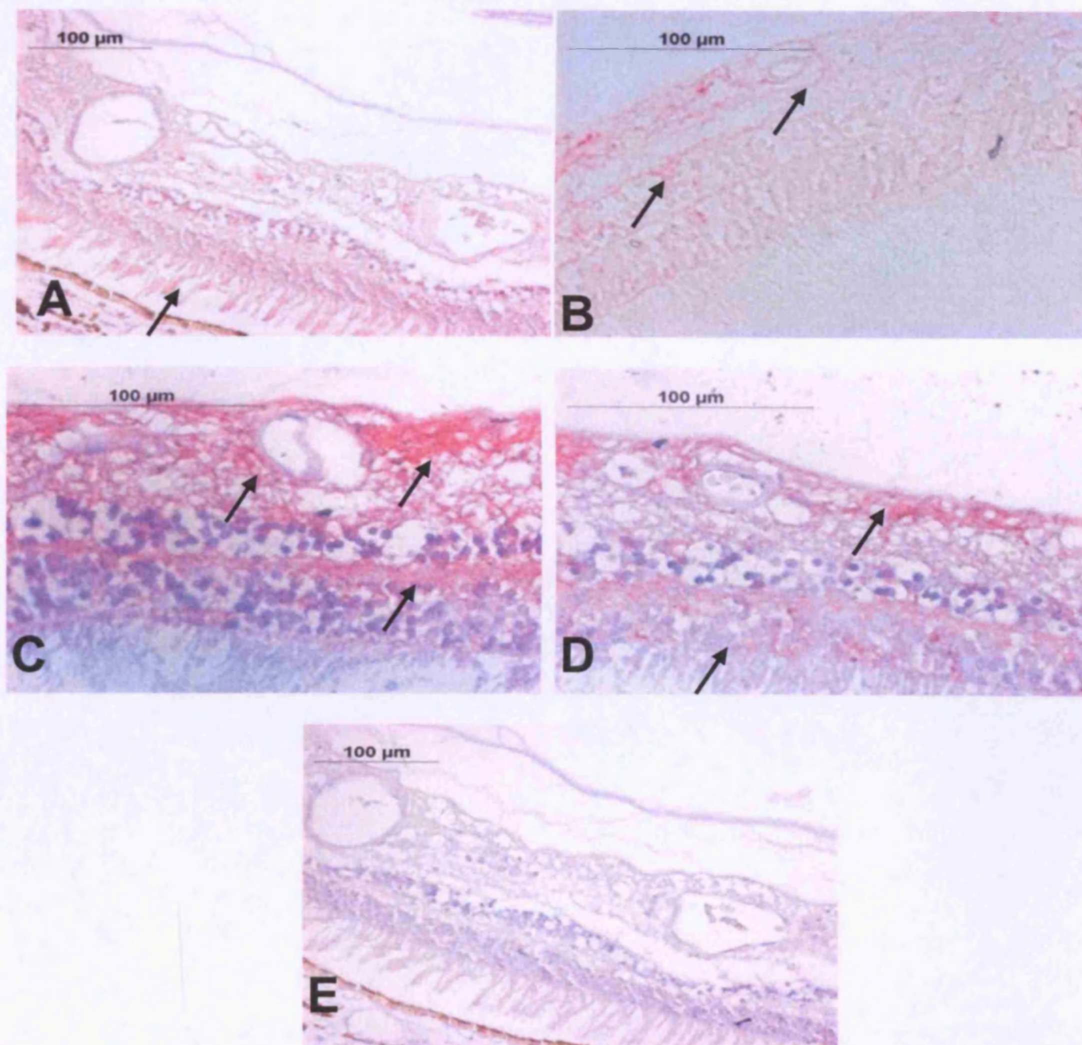

Figure 6.7 Transverse Sections Showing the Immunolocalisation of Caveolin-2 and Caveolin-3

Caveolin-2 immunsotaining was localised to non-diabetic retina (A, B). Caveolin-3 immunostaining was localised to non-diabetic retina (C,D). Immunoreactivity was abolished in control retina processed with normal goat serum (E).

6.4 DISCUSSION

Immunostaining for caveolin-1, caveolin-2 and caveolin-3 was performed using a dilution series of the primary antibodies, against a set number of sections, as recommended by the manufacturer. Initially only weak staining was observed for all antibodies. Proteolytic digestion using 0.1% chymotrypsin or proteinase K also only produced weak staining. Successful staining was eventually achieved using a highly concentrated dilution of each primary antibody and by exposing the angiogenic binding sites with proteolytic digestion using 0.2% triton X-100. I also found that there was a lot of background staining for caveolin-1 using the alkaline phosphatase/fast red system. I therefore substituted that enzyme/substrate complex for horseradish peroxidase/diaminobenzidine which produced more specific staining.

The data presented in this study demonstrate that caveolin is present in human retina. In addition caveolin-1 was shown to be present in diabetic retinas and fibrovascular membranes. These observations support a role for caveolin in the normal functioning of the retina and in diabetic retinopathy.

Caveolin-1 was present at low levels in the retinal vessels of the non-diabetic retinas and at all stages of diabetic retinopathy. Staining was slightly raised in the neovessels of the microvascular membranes of the PDR retinas. Several workers have demonstrated caveolin-1 expression in retinal endothelial cells (Gardiner and Archer, 1986a, b; Feng *et al.,* 1999b; Bridges *et al.,* 2001; Kim *et al* 2006) indicating that it is involved in the normal physiological functioning of the retina.

Cav-1 and caveolae have been shown to serve as flow-activated mechanosensors or transducers of physiological responses in intact blood cells (Yu *et al.,* 2006). Caveolae are also believed to be responsible for transcytosis, the process by which plasma proteins (e.g. albumin) are transported across capillary endothelium (Palade *et al.,* 1979; Vasile *et al.,* 1983; Ghitescu *et al.,* 1986; Milici *et al.,* 1987; Schnitzer *et al.,* 1995; Predescu *et al.,* 1998).

Cav-1 is the major protein coat of endothelial caveolae and is necessary for caveolae assembly. Several studies have shown a complete loss of caveolae organelles in blood vessels, adipocytes and fibroblasts obtained from cav-1-deficient mice confirming the necessity of this protein in caveolae biogenesis (Drab *et al.,* 2001; Razani *et al.,* 2001; Rizani and Lisanti, 2001; Zhao *et al.,* 2002; Cao *et al.,* 2003). Cav-1 appears to play an important role in cholesterol homeostasis and caveolae has been shown to play a role in cholesterol trafficking as has been observed within lens epithelial cells and fibre cells (Lo *et al.*, 2001; Sexton *et al.,* 2004; Cenedealla *et al.,* 2006).

Caveolae are able to form mobile signalling platforms which concentrate and organize signal transduction cascades that regulate tissue structure, vascular tone, and basal permeability (Anderson, 1993; Lisanti *et al.,* 1994; Parton, 1996). In caveolae, cav-1 can interact with itself to form homo-oligomers as well as with several other signalling molecules.

Caveolae have been implicated in signalling through the p42/44 MAP kinase pathway which is involved in the proliferation, survival, and migration of ECs. Other components of the p42/MAP kinase cascade are localized within caveolae membranes. These include receptor tyrosine kinases (VEGFR-2, Tie-2, EGFR, PDGFR, InsR) (Smart *et al.,* 1995; Liu *et al.*, 1996; Mineo *et al.,* 1996; Liu *et al.,* 1997; Liu *et al.,* 1999; Cho *et al.,* 2004b; Sonveaux *et al* 2004), H-Ras (Mineo *et al.,* 1996; Song *et al.,* 1996; Yoon *et al.,* 2003), Raf kinase (Mineo *et al.,* 1996), ERK (Lisanti *et al.,* 1994; Liu *et al.,* 1996), Shc (Liu *et al.,* 1996), Grb-2 (Liu *et al.,* 1996) and Nck (Liu *et al.,* 1996) as well as PI-3 kinase, PLCγ and protein kinase C and phosphatidylinositol (Lisanti *et al.,* 1994; Liu *et al.,* 1995; Pike *et al.,* 1996; Liu *et al.,* 1997; Gingras *et al.,* 1998; Okamoto *et al.,* 1998; Ahn *et al.,* 1999; Murthy and Makhlouf, 2000; Predescu *et al.,* 2001; Kawamura *et al.,* 2003; Labrecque *et al.,* 2003;). Both in vitro and cell culture experiments indicate that cav-1 can directly interact with and maintain some of these signalling molecules in an inactive conformation (Anderson, 1998; Okamoto *et al.,* 1998) acting as a scaffolding protein, able to negatively regulate the activity of other molecules by binding to and releasing them in a timely fashion holding them in a quiescent or inhibited state. Down regulation of cav-1 expression and caveolae organelles may be a prerequisite for EC proliferation and subsequent angiogenesis. This is supported by the finding that exposure of Ecs to VEGF, has been shown to result in a reduced EC expression of cav-1 and caveolae which was mediated by a negative feedback mechanism through the VEGFR-2 and subsequent downstream p42/44 MAP kinase pathway (Liu *et al.,*1999; Labrecque *et al.,* 2003). Cav-1 overexpression has been shown to reduce VEGF-mediated angiogenesis with defects in vasodilatation, permeability, and angiogenesis (Brouet *et al.,* 2004; Bauer *et al.,* 2005). Sonveaux *et al.,* 2004 showed that cav-/- Ecs formed tubes on Matrigel on VEGF stimulation which was dramatically repressed when compared with cav+/+ Ecs. With the exception of insulin receptor which appears to be stimulated by cav-1 (Yamamoto et al., 1998), studies have demonstrated that cav-1 bound to and inhibited growth factor receptor kinase activity (Couet *et al.,* 1997; Bilderback *et al.,* 1999; Yamamoto *et al.,*

1999) as well as other important secondary messengers and substrates (Shaul and Anderson, 1998).

Liu *et al.,* 2002 showed that cav-1 is down-regulated by endothelial growth factors that stimulate the initial proliferative phase but upregulated during the differentiation phase into tubular networks. Under conditions of cell confluence, they showed that cav-1 expression stimulates EC differentiation and tubule formation. They hypothesize that caveolin acts a both a pro- and anti-angiogenic factor by serving as a "differentiation sensor" that monitors and responds to changes in the relative balance of positive and negative factors to "tell" a target cell whether to remain quiescent or to become proliferative. This may explain why I observed the same levels of cav-1 staining across all tissue categories. In the PDR membranes the neovessels may have already undergone proliferation and so cav-1 may now be acting as an anti-angiogenic factor.

I also observed cav-1 staining in the photoreceptors, the ganglion cell layer and the inner and outer regions of both non-diabetic and diabetic retinas. Interestingly levels were raised in the diabetic retinas as compared to the non-diabetic retinas. Kim *et al.,* 2006 also observed cav-1 immunostaining in the photoreceptors, ganglion cell layer, the inner plexiform layer and outer plexiform layer of normal rat retinas which is in agreement with my findings. This may indicate that cav-1 plays a role in signal transduction in glial and neuronal cells. As far as I am aware, no other studies have looked at the expression of cav-1 in the diabetic retina and so its role in diabetic retinopathy is still to be determined. Photoreceptor detergent resistant membranes (DRMs) are enriched in cav-1 (Elliott *et al.,* 2003) and cav-1 has been shown to be localized to photoreceptor synaptic ribbons. DRMs from rod outer segments contain several proteins involved in phototransduction (Seno *et al.,* 2001). It was suggested that cav-1 may therefore be involved with regulating transmitter release and visual transduction and the formation of synaptic ribbons (Kachi *et al.,* 2001).

In my study I observed cav-2 staining in the photoreceptors, the neuronal layers of the retinas and the ganglion cell layer of non-diabetic retinas, some staining was also observed around the vessels. As well as cav-1 expression endothelial cells also express cav-2 but not cav-3 (Garcia-Cardena *et al.,* 1997). Cav-2 has to heteroligomerize with cav-1 to form the caveolar coat but cannot form the caveolar coat by itself (Monier *et al.,* 1995; Sargiacomo *et al.,* 1995; Scherer *et al.,* 1997; Scheiffele *et al.,* 1998; Mora *et al.,* 1999; Parolini *et al.,* 1999). Cav-2 has been shown to be expressed in adult rat retina where it showed similar but weaker staining than cav-1 (Kim *et al.,* 2006) but staining was more intensely detected in vessels as compared to cav-1. As in my study cav-2 was present in the inner plexiform layer,

inner nuclear layer, outer plexiform layer in the processes of glial cells and Müller cells. Also in the retinal neuronal cells including the ganglion cells, amacrine cells, bipolar cells, horizontal cells, and photoreceptor cells. Based on the functional role of caveolins, these molecules may be assumed to play an important role in the cholesterol homeostasis of the retina. Cav-2 may also be involved in synaptic signal transduction in the retina and cav-2 has also been shown to be expressed in brain astrocytes (Cameron *et al.*, 1997; Ikezu *et al.*, 1998). Cav-2 has also been shown to be present in the RPE collected from rat eyes and in human RPE cell lines (Mora *et al.*, 2006) which was in contrast to my study where immunostaining was not observed. Cav-2 has a non-polar distribution in RPE cells, consistent with the observation that cav-2 expression may be necessary in certain cells for the efficient assembly of plasmalemmal caveolae (Lahtinen *et al.*, 2003; Sowa *et al.*, 2003). The determination of the precise role of cav-2 in the retina will require further study.

Cav-3 is primarily expressed on skeletal muscle and smooth muscle cells (Song *et al.*, 1996; Tang *et al.*, 1996; Kogo *et al.*, 2006). In my study I showed that in one retina, cav-3 was detected in the outer and inner regions of the retina and the ganglion cell layer indicating that it is expressed by cells other than muscle cells. Cav-3 was also shown to be expressed in brain astroglial cells (Ikezu *et al.*, 1998) and has been shown to be upregulated in reactive astrocytes surrounding senile plaques in the brains of Alzheimer's patients (Nishiyama *et al.*, 1999). Cav-3 shows high homology with cav-1 (85% similarity) (Tang *et al.*, 1996) and either is sufficient for the formation of caveolae invaginations (Li *et al.*, 1998; Hagiwara *et al.*, 2000; Galbiati *et al.*, 2001; Park *et al.*, 2002). Cav-3 can interact with adenyl cyclase, eNOS and insulin receptor signalling (Yamamoto *et al.*, 1998).

There is therefore some indication that cav-2 and cav-3 are involved both in the normal physiological functioning of the retina, as shown by my study, and in various disease processes but further studies need to be carried out to determine their precise roles.

7. GENERAL DISCUSSION

7.1 MICROVASCULAR COMPLICATIONS AND DIABETIC RETINOPATHY

Retinopathy is a frequent microvascular complication of diabetes mellitus (DM) that remains a major therapeutic challenge (Lobo *et al.,* 2004). To prevent and improve the treatment of DR, it is fundamental that we know the evolution of the earliest changes that occur in the retina affected by DM and how these changes relate to the progression of retinopathy. In this study retinas were selected for immunostaining based on clinical assessment and by microscopical examination of sections for microvascular abnormalities associated with diabetic retinopathy.

The overall purpose of this project was to try to demonstrate that the spatial and temporal expression of angiogenic growth factors was altered during the progression of diabetic retinopathy. In recent years it has become increasingly obvious that inhibitors of angiogenesis are just as important as pro-angiogenic factors in the pathogenesis of diabetic retinopathy. I therefore also examined the expression of the well known anti-angiogenic factor PEDF during different stages of diabetic retinopathy. Finally, this study was also undertaken to determine if the levels of caveolin changed during the progression of diabetic retinopathy as caveolae have been shown to play an important role in the compartmentalization of second messengers involved in growth factor receptor signal transduction pathways.

7.2 GROWTH FACTOR EXPRESSION DURING DIABETIC RETINOPATHY

The findings from my study clearly show that VEGF, the angiopoietins and TNF-α play different roles in the pathogenesis of diabetic retinopathy. It therefore appears that growth factors may act alone or in concert to bring about the microvascular abnormalities observed in diabetic retinopathy.

The specific localization of VEGFR-2 in PDR retina supports the previous suggestion that VEGF binding to the VEGFR-2 receptor sends a classic proliferative signal (Malecaze *et al.*, 1994; Chen *et al.*, 1997; Armstrong *et al.*, 1998a; Suzuma *et al.*, 1998; Ishida *et al.*, 2000; Ozaki *et al.,* 2000; Ishimama *et al.*, 2001; Witmer *et al.,* 2002; Gerber and Ferrara., 2003; Cerdan *et al.,* 2004; Wilkinson-Berka *et al.,* 2006). The demonstration that VEGFR-1 is expressed in both non-diabetic retina and diabetic retina supports the concept that VEGF is involved in vascular maintenance as well as in other endothelial cell functions such as maintenance of vascular permeability and endothelial cell proliferation (Kolch *et al.*, 1995).

This study adds to the limited knowledge about the role of VEGFR-3 in pathological angiogenesis (Pajusola *et al.*, 1992; Fielder *et al.*, 1997; Partanen *et al.*, 1999; Valtola *et al.*, 1999; Skobe *et al.*, 2001ab; Witmer *et al.*, 2001; Clarijs *et al.*, 2002). VEGF-C can induce angiogenesis under certain circumstances which could be due in part to its capacity to bind to an activate VEGFR-2, in addition to VEGFR-3 (Tille *et al.*, 2003). Clearly VEGF-C binding to VEGFR-3 does play a role in diabetic retinopathy.

VEGF increases permeability and is mitogenic for ECs, acting early and at most points of the angiogenic cascade (Lim *et al.*, 2003). In contrast, Ang-1 has vessel-maturing activates and acts at a later stage which involves recruitment of vessel-supporting cells, strengthening of intercellular junctions, and establishment of leakage-resistant vessels (Suri *et al.*, 1996; Davis and Yancopoulos, 1999; Fujikawa *et al.*, 1996b; Hanahan, 1997; Papapetropoulas *et al.*, 1999; Thurston *et al.*, 1999; Gamble *et al.*, 2000). It has been shown that a combination of Ang-1 and VEGF recruits smooth muscle actin-α-positive cells to vascular walls (Asahara *et al.*, 1998). Co-induction of endothelial Ang-1 and VEGF may exert such vessel-maturing effects on the sprouting vessels in an autocrine manner in the microenvironment of active sprouting. Ang-1 exerts a vascular endothelial barrier protective effect by blocking the action of VEGF (Gamble *et al.*, 2000; Jho *et al.*, 2005). I did find low levels of Ang-1 in PDR membranes which supports this role of Ang-1 however, a high level of Ang-1 immmunostaining was observed in the PDR retinal vessels. Perhaps it was localized to a subset of retinal vessels that were no longer undergoing active angiogenesis. However in the presence of VEGF, Ang-1 has been shown to significantly potentiate VEGF-induced neovessel sprouting (Zhu *et al.*, 2002).

Ang-2 signals cause vessel structures to become loosened, reducing endothelial cell contacts with the matrix and disassociation of perivascular support cells. This loosening appears to render the endothelial cells more accessible and responsive toward angiogenic inducers such as VEGF (and likely other angiogenic inducers) leading to capillary sprouting and the formation of new vessels. I only showed low levels of Ang-2 expression in the retinal vessels which was an unexpected finding. Again, perhaps it was localized to a subset of vessels that were not undergoing active angiogenesis. The action of Ang-2 has become controversial as in some instances Ang-2 can either promote angiogenesis or induce regression of vessels (Maisonpierre *et al.*, 1997; Holash *et al.*, 1999; Yu and Stamenkoviv, 2001). This appears to be dependent upon the presence of VEGF. When VEGF is present, Ang-2 appears to induce angiogenesis, but in the absence of VEGF it promotes vessel regression (Asahara et al., 1998; Lobov *et al.*, 2002; Oshima *et al.*, 2004). Hangai *et al.*, 2006

speculate that sequential induction of Ang-2 and then Ang-1 and VEGF in ECs may provide precise and stage-appropriate autocrine and/or paracrine angiogenic signals to ECs.

The findings from this study that TNF-α was upregulated in the vessels of PDR membranes and diabetic retinas supports the suggestion that it may also play some role in diabetic retinopathy (Spranger *et al.*, 1995; Limb *et al.*, 1996; Armstrong *et al.*, 1998a; Limb *et al.*, 2001; Yossuck *et al.*, 2001; Majka *et al.*, 2002). Studies of the angiogenic properties of TNF-α yield conflicting results. In vivo it appears to be a potent inducer of angiogenesis (Frater-Shroder *et al.*, 1987; Leibovich *et al.*, 1987; Montrucchio *et al.*, 1994). However, in vitro, TNF-α seems to inhibit angiogenic activities such a endothelial proliferation and tube formation (Frater-Shroder *et al.*, 1987; Sato *et al.*, 1987; Yang *et al.*, 2004), apart from in one study in HUVECs where TNF-α was shown to upregulate Ang-2 expression (Kim *et al.*, 2000d) . It has been suggested that TNF-α may induce angiogenesis indirectly by activating other regulators of angiogenesis. TNF-α has been shown to induce Ang-1, Ang-2, Tie-2 VEGF, and VEGF-C expression (Kim *et al.*, 2000d; Willam *et al.*, 2000; Scott *et al.*, 2002; Chen *et al.*, 2004; Scott *et al.*, 2005; Hangai *et al.*, 2006; Zhao *et al;* 2006; Zhu *et al.*, 2006). Results from our laboratory also showed that TNF-α was also able to upregulate KDR (Zhao *et al.*, 2006). TNF-α was also shown to co-localize with VEGF and the angiopoietins in choroidal neovascular membranes and it increased Ang-2 levels prior to those of Ang-1 and VEGF. This suggests that during neovascularization TNF-α may modulate endothelial plasticity and survival by sequential inactivation of Ang-2 followed by activation of Tie-2 and the VEGF receptors.

VEGF, Ang-2 and TNF-α have been shown to be hypoxia inducible (Oh *et al.*, 1999). As VEGF, the angiopoietins and TNF-α were localized to vascular endothelial cells and non-vascular cells this supports the hypothesis that the retinal response to the hypoxic environment would be to upregulate the receptors and to stimulate the synthesis and secretion of VEGF, Ang-2 and TNF-α in retinal PCs, ECs, glial cells, ganglion cells, and possibly other cell types. The sustained production of VEGF, Ang-1, Ang-2 and TNF-α would eventually lead to an angiogenic response. Sustained production of these growth factors may be maintained by a positive feedback mechanism to the receptors on the retinal cells. The presence of Ang-1 in retinal vessels not involved in retinal angiogenesis may allow a shift in the local balance of Ang-1/Ang-2 back in favour of Ang-1, to effect maturation and stabilization of the newly formed vessels. Therefore there appears to be a collaboration between VEGF, Ang-1, Ang-2, and TNF-α to elicit angiogenesis, all of which appear to exert their effects on retinal cells via both autocrine and paracrine mechanisms.

7.3 THE EXPRESSION OF PEDF IN DIABETIC RETINOPATHY

The findings from this study support the findings of other workers that PEDF is highly expressed in non-diabetic retinas and diabetic retinas with NPDR. (Tombran-Tink *et al.*, 1995; Wu *et al.*, 1995; Ortego, 1996; Wu *et al.*, 1996; Alberdi *et al.*, 1998; Karakousis, 2001; Behling and Bennett, 2002; Ogata *et al.*, 2002bc; Eichler *et al.*, 2004; Hattenbach *et al.*, 2005; Becerra, 2006). This study also showed that PEDF is downregulated in PDR which confirms findings obtained from previous studies (Holekamp *et al.*, 2002; Ogata *et al.*, 2001a; 2002b, c).

It appears therefore that under normal physiological conditions, secretion of PEDF into avascular spaces in the eye such as the aqueous and vitreous, excludes vessels from invading the retina, vitreous and cornea (Dawson *et al.*, 1999; Karakousis, 2001; Behling and Bennett, 2002). It may do this by inhibiting VEGF-induced proliferation and migration of ECS (Mori 2001; Stellmach *et al.*, 2001; Auricchio *et al.*, 2002; Duh *et al.*, 2002; Mori *et al.*, 2002c; Raisler *et al.*, 2002; Cai *et al.*, 2006).

The general consensus is that loss of PEDF creates a permissive environment for angiogenesis that may contribute the progression of ocular neovascular disease. This is supported by the findings that ischaemia- induced retinal neovascularization caused a significant reduction in retinal PEDF and a substantial increase in VEGF mRNA and protein in rat models (Gao *et al.*, 2001; Crossen, 2002). Down-regulation of PEDF expression has also been shown to increase the secretion of both VEGF and TNF-α from retinal Müller cells (Zhang *et al.*, 2005). It therefore appears that there is a direct correlation between PEDF and the extent of neovascularization and that there is an equilibrium shift between PEDF and VEGF in the uncontrolled growth of blood vessels in the eye (Gao *et al.*, 2001; Ohno-Matsui *et al.*, 2001; Gao and Ma, 2002; Ogata et al., 2002a, b, c; Eichler *et al.*, 2004). However, recently Apte *et al.*, 2004 showed that PEDF stimulated choroidal neovasculariation at high concentrtations but at low concentrations it decreased neovascularization. Therefore the concentration of PEDF appears to be important, whether this applies to diabetic retinopathy remains to be determined and therefore caution should be taken when considering using PEDF for the treatment of PDR.

7.4 THE EXPRESSION OF CAVEOLINS IN DIABETIC RETINOPATHY

The observation that only cav-1 and cav-2 were observed in the retinal vessels is consistent with the findings from other studies where cav-1 and cav-2 have both been shown to be expressed by retinal endothelial cells but not cav-3 (Garcia-Cardena *et al.*, 1997; Feng

et al., 1999b; Bridges *et al.,* 2001; Kim *et al.,* 2006). The presence of cav-1 and cav-2 in non-diabetic retina supports previous observations that under normal physiological conditions caveolae act as flow-activated sensors in ECs (Yu *et al.,* 2006). Shear stress is recognized by the endothelial cells and regulates vascular tone, vessel wall remodelling, cell adhesion, coagulation, and fibronolysis (Dewey *et al.,* 1981; Davies *et al.,* 1995). Caveolae are also believed to be involved in transcytosis, the process by which proteins (e.g. albumin) are transported across capillary endothelium (Palade *et al.,* 1979; Vasile *et al.,* 1983;1989; Predescu *et al.,* 1998). In addition cav-1 and cav-2 hetrodimerize to form the protein coat of caveolae and both are necessary for caveolae assembly (Drab *et al.*, 2001; Razani *et al.,* 2002).

As well as their physiological roles in vascular endothelium, caveolae are able to form mobile signalling platforms which concentrate and organize signal transduction cascades. (Anderson, 1993; Lisanti *et al.,* 1994; Parton, 1996). Several receptor tyrosine kinases are located in caveolae including VEGFR-2 and Tie-2 as well as the TNF receptors I and II (Feng *et al.,* 1999a; Liu *et al.,* 1999; Yoon *et al.,* 2003; Cho *et al.,* 2004b; D'Allessio *et al.,* 2005). Cav-1 has been shown to interact with and maintain some of these signalling molecules in an inactive conformation acting as a scaffolding protein, able to negatively regulate the activity of these molecules by binding to and releasing them in a timely fashion holding them in a quiescent or inhibited state. Down regulation of cav-1 may be a prerequisite for EC proliferation and subsequent angiogenesis. Down-regulation of cav-1 was demonstrated in the retinal vessels of the PDR retinas in this study and exposure of ECs to VEGF has been shown to result in a reduced EC expression of cav-1 which was mediated by a negative feedback mechanism through the VEGFR-2 receptor and subsequent downstream p42/44MAP kinase pathway (Liu *et al.,* 1999; Labrecque *et al.,* 2003;). This does not support the findings that cav-1 expression was increased in the neovessels of the PDR retinas. These conflicting findings for cav-1 expression may be explained by the fact that cav-1 expression appears to be down-regulated during the proliferative phase of angiogenesis, and then markedly upregulated during the differentiation phase (Liu *et al.,* 2002). Liu *et al.,* 2002 showed that cav-1 is downregulated by endothelial growth factors that stimulate the initial proliferative phase but upregulated during the differentiation stage into tubular networks. They hypothesized that caveolin acts a both a pro- and anti-angiogenic factor by serving as a "differentiation sensor" that monitors and responds to changes in the relative balance of positive and negative factors to "tell" a target cell whether to remain quiescent or to become

proliferative. In my study in the PDR membranes the neovessels may have already undergone proliferation and so cav-1 may now be acting as an anti-angiogenic factor.

The presence of the caveolins in the ganglion cells and glial cells agrees with the findings of other workers and may indicate that the caveolins play some role in signal transduction in these cells.(Kachi *et al.,* 2001).

7.5 FUTURE WORK

Examination of immmunostained retinal sections by light microscopy provided only limited information on the exact cellular localization of these growth factors and their receptors in diabetic retinopathy. Further studies at the ultrastructural level using either electron microscopy or confocal microscopy should help. It would also have been helpful to undertake staining of all the diabetic tissue with cav-2 and cav-3 antibodies as this will further clarify their roles in diabetic retinopathy and also with antibodies to TNF receptors I and II. This should be supplemented with *in situ* hybridization studies which would provide information on the exact cellular localization of cytokine production.

The current methods of treatment for PDR, including laser photocoagulation, vitrectomy and cryotherapy are expensive and cause destruction of viable retinal tissue. Non-invasive therapies are currently being sought to replace these surgical interventions. Over the last decade much research has concentrated on using inhibitors of the VEGF signalling pathway for the treatment of retinal neovascular diseases such as PDR and age related macula degeneration (AMD) and some success has been achieved using aptamers and antibodies which bind VEGF (Afzal *et al.,* 2007). Magugen®, an oligonucleitide aptamer and Lucentis®, a humanized antibody fragment have both been approved by the FDA for use in reversing the neovascularization associated with AMD and have been used successfully to treat macula oedema in clinical trials (Brown *et al.*, 2006; Rosenfield *et al.*, 2006). However these molecules are large and complex, are difficult to administer intravitreally and the regression of neovascularization is rarely permanent, which requires multiple applications of drug which is expensive. Bevacizumab (Avastin®), a humanized full-length antibody against VEGF, is a less expensive alternative which has been used in clinical trials for diabetic retinopathy and diabetic macula oedema where it was shown to exert its effects by blocking increased vascular permeability and angiogenesis (Averbukh *et al.,* 1998; Cunningham *et al.,* 2005). Several reports describe the use of off-label Bevacizumab in various proliferative retinopathies with subsequent good visual and structural outcomes (Abraham-Marin *et al.*, 2006; Rich *et al.*, 2006). Various molecules which are smaller, can be administered less

invasively and are less expensive are currently under investigation. Theses include Vatalanib (VEGFR tyrosine kinase inhibitor) (Steeghs *et al.,* 2007), Sirna-027 (SiRNA which down regulates VEGFR-1), VEGFR-SiRNA (SiRNA which down-regulates VEGF) [Reich *et al.*, 2003] and T2-TrpRS (a proteolytic fragment of tryptophan tRNA synthetase) [Banin *et al.,* 2006].

Precise understanding of the biologic roles of the VEGF, the angiopoietin family members, TNF, PEDF and caveolins and of how they may collaborate with each other and other growth factors may lead to novel and therapeutically significant strategies for promoting or inhibiting neovascularization. However, development of long-term therapeutic interventions based on the blocking of Tie-2, VEGFR-1, and possibly VEGFR-3, signalling should proceed with caution due to the possible disruption of crucial roles of these receptors in vascular maintenance. Further investigation into the specific signalling pathways used for vascular growth and maintenance may also lead to more specific and effective strategies for therapy.

ABBREVIATIONS

α	Alpha
Ab	Antibody
ABComplex	Avidin-Biotinylated Alkaline Phosphatase Complex
AGES	Advanced Glycation End Products
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
Ang-II	Angiotensin-II
APES	3-Aminopropyltriethoxysilane
β	Beta
BAEC	Bovine Aortic Endothelial Cell
bFGF	Basic Fibroblast Growth Factor
BM	Basement Membrane
BMEC	Bovine Microvascular Endothelial Cell
BREC	Bovine Retinal Endothelial Cell
BRPC	Bovine Retinal Pericyte
BSMC	Bovine Smooth Muscle Cell
Cav-1	Caveolin-1
Cav-2	Caveolin-2
Cav-3	Caveolin-3
°C	Degrees Centrigrade
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
DCCT	Diabetes Control and Complications Trial
ddH_2O	Double Distilled Water
EC	Endothelial Cell
ECGF	Endothelial Cell Growth Factor
EGF	Epidermal Growth factor
ELM	External Limiting Membrane
EPO	Erythropoietin
ERM	Epiretinal Membrane

Etc.	Et cetera [and other things]
Et al.	Et alia [and others]
FGF	Fibroblast Growth Factor
Fig	Figure
5'	Five Prime
γ	Gamma
GABA	Gabaaminobutyric acid
GCL	Ganglion Cell Layer
GH	Growth Hormone
HBGF	Heparin Binding Growth Factor
HIF	Hypoxia Inducible Factor
H_2O	Water
H and E	Haematoxylin and Eosin
Hr	Hour
HSMC	Human Smooth Muscle Cell
HSC	Haematopoietic Stem Cells
hUVECs	Human Umbilical Vein Endothelial Cells
Ig	Immunoglobulin
IgL	Immunoglobulin-like
IGF	Insulin-Like Growth Factor
IL-1	Interleukin-1
IL-4	Interleukin-4
IL-6	Interleukin-6
ILM	Internal Limiting Membrane
IMS	Industrial Methylated Spirit
INL	Inner Nuclear Layer
kb	Kilobase
kDa	Kilodalton
KDR	Kinase Insert Domain Containing Receptor
MAPK	Map Kinase
mg	Milligram
ml	Millilitre
mm	Millimetre

MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
min	Minute
NBF	Neutral Buffered Formalin
NDRI	National Disease Research Interchange
NO	Nitric Oxide
NPDR	Non-proliferative Diabetic retinopathy
NOS	Nitric Oxide Synthase
ONL	Outer Nuclear layer
%	Percentage
PA	Plasminogen Activator
PAEC	Porcine Aortic Endothelial Cell
PAI-1	Plasminogen Activator Inhibitor-1
PC	Pericyte
PCR	Polymerase Chain Reaction
PDECGF	Platelet Derived Endothelial Cell Growth Factor
PDGF	Platelet Derived Growth Factor
PDR	Proliferative Diabetic Retinopathy
PlGF	Placental Growth Factor
pH	$-\text{Log}_{10}$ Hydrogen Ion Concentration
PKC	Protein Kinase C
PLC-γ	Phospholipase C-gamma
pO_2	Oxygen Tension
PSMC	Porcine Smooth Muscle Cell
RGC	Retinal Glial Cell
ROP	Retinopathy of Prematurity
ROS	Reactive Oxygen Species
RPE	Retinal Pigmented Epithelium
RTK	Receptor Tyrosine Kinase
sFlt-1	Soluble Fms-like Tyrosine Kinase-1
SMC	Smooth Muscle Cell
TEK	Tunica Interna Endothelial Cell Kinase

Tie-2	Tyrosine Kinase With Ig-like loops and epidermal growth factor homology domains-2
TBS	Tris Buffered Saline
TGF-α	Transforming Growth Factor-Alpha
TGF-β	Transforming Growth Factor-Beta
TNF-α	Tumour Necrosis Factor-Alpha
μm	Micrometre
uPA	Urokinase-Type Plasminogen Activator
UV	Ultraviolet
VAS	Vasculotropin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VPF	Vascular Permeability Factor
v/v	Volume by Volume

APPENDIX I: MATERIALS AND SUPPLIERS

Material	Supplier
ABComplex/Alkaline Phosphatase	DAKO
Aminopropyltriethoxysilane	Sigma
Anti-Angiopoietin-1 Antibody	Santa Cruz
Anti-Angiopoietin-2 Antibody	Santa Cruz
Anti-Caveolin-1 Antibody	BD Transduction Laboratories
Anti-Caveolin-2 Antibody	BD Transduction Laboratories
Anti-Caveolin-3 Antibody	BD Transduction Laboratories
Anti-TNF-α Antibody	Abcam
Anti-VEGFR-1 Antibody	Santa Cruz
Anti-VEGFR-2 Antibody	Santa Cruz
Anti-VEGFR-3	Santa Cruz
Anti-Tie-2 Antibody	Santa Cruz
Anti-$VEGF_{165}$ Antibody	Santa Cruz
Anti-VEGF-C Antibody	Santa Cruz
Biotinylated Goat Anti-Rabbit Immunoglobulin (IgG)	Sigma
Biotinylated Rabbit Anti-Goat Immunoglobulin (IgG)	Sigma
Chloroform	BDH
α-Chymotrypsin	Sigma
Cover Slips	Raymond Lamb
Disodium Hydrogen Orthophosphate	BDH
Eosin	BDH
Fast Red/TR Naphol AS-MX Tablets	Sigma
Filters (0.2μm)	BDH
Formaldehyde	BDH
Glass Microscope Slides	Raymond Lamb
Haematoxylin(Harris and Mayers)	BDH
Hydrochoric Acid	Sigma
Hydromount	National Diagnostics

Industrial Methylated Spirit	Genta Medical
Loctite Adhesive	Till and Whitehead
Milk Protein	Premier Beverages
Normal Goat Serum	Sigma
Normal Pig Serum	Sigma
Normal Rabbit Serum	Sigma
Paraffin Wax	Lamb
Potassium Dihydrogen Orthophosphate	Sigma
Practamount	ASCO Laboratories
Sodium Chloride	BDH
Sodium Citrate	Sigma
Tris (Hydroxymethyl) Methylamine	BDH
Tris-HCl	BDH
TritonX-100	Sigma
Xylene	BDH

APPENDIX II- Constituents and Preparation of Buffers and Solutions

Neutral Buffered Formalin

1. Dissolve 510mg disodium hydrogen orthophosphate, 325mg potassium dihydrogen orthophosphate and 9g sodium chloride in 900ml ddH_2O.
2. Add 100ml formaldehyde.
3. Adjust to pH 6.8 using hydrochloric acid.

3-Aminopropyltriethoxysilane Coated Slides

1. Wash slides in 1% extran (v/v in ddH_20) for 1 hour.
2. Rinse for 2 hours in hot running water.
3. Air dry.
4. Place in 1% 3-aminopropyltriethoxysilane (v/v in acetone) for 5 minutes.
5. Rinse slides in acetone for 5 minutes.
6. Wash in running tap water for 5 minutes.
7. Dry at room temperature overnight or $60^{o}C$ for 1 hour

10x Tris Buffered Saline (pH 7.6 at $25^{o}C$)

1. Dissolve 87.6g sodium chloride, 60.6g Tris-HCl, and 13.9 g Tris-base in 1 litre ddH_2O.
2. Adjust pH to 7.6

0.1% α-Chymotrypsin Solution

1. Place sections in pre-warmed Tris buffered saline at $37^{o}C$ for 5 minutes.
2. Place in solution containing 300ml Tris buffered saline, 30mg α-chymotrypsin, and 300mg calcium chloride at $37^{o}C$ for 20 minutes.
3. Rinse slides with Tris buffered saline.

Sodium Citrate Solution (Pressure Cooker)

1. Dissolve 5.88g of tri-sodium citrate solution in 1965ml of distilled water
2. Add 44ml of 0.2M hydrochloric acid solution
3. Adjust pH to 6.0
4. Bring to the boil in a pressure cooker
5. Add slides in a metal rack and place lid on pressure cooker and bring to pressure

6. Time for 3 minutes

7. Run running tap water over the pressure cooker

8. Release pressure, remove lid, and run water into the pressure cooker for 10 minutes.

9. Remove slides and place in Tris buffered saline for 5 minutes

0.2% Triton-X 100

1. Place sections in pre-warmed Tris buffered saline at 37°C for 5 minutes.

2. Place in solution containing 300ml Tris buffered saline and 600μl Triton-X-100 at 37°C for 30 minutes.

3. Rinse slides with Tris buffered saline.

ABComplex/Alkaline Phosphatase Kit

1. Mix 1 drop avidin and 1 drop biotin with 5ml ddH_2O.

2. Vortex and leave for at least 30 minutes before use.

Substrate Solution For Phosphatase Staining (Imunohistochemistry)

1. Dissolve 1 Tris buffered saline tablet and 1 Fast red tablet in 10ml ddH_2O.

2. Filter through a 0.2μm filter.

REFERENCES

Abe R, Shimizu T, Yamagishi S, Shibaki A, Amano S *et al.* (2004). Overexpression of pigment epithelium-derived factor decreases angiogenesis and inhibits the growth of human malignant melanoma cells in vivo. *Am J Pathol.* **164:**1225-1232

Abedi H, Zachary I. (1997). Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem.* **272**:15442-15451

Abraham JA, Whang JL, Tumolo A. (1986). Human basic fibroblast growth factor: nucleotide sequence and genomic organization. *EMBO J.* **5**:2523-2528

Abraham-Marin ML, Cortes-Luna CF, Alvarez-Rivera G, Hernandez-Rojas M, Quiroz-Mercado H *et al.* (2007). Intravitreal bevacizumab therapy for neovascular age-related macular degeneration: a pilot study. *Graefes Arch. Clin. Exp. Ophthalmol.* **245**:651-655

Abu-Jawdeh GM, Faix JD, Niloff J, Tognazzi K, Manseau E *et al.* (1996). Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasms. *Lab Invest.* **74**:1105-1115

Achen M, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. (1998). Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt-4). *Proc Natl Acad Sci.* **95**:548-553

Acker T, Beck H, Plate KH. (2001). Cell type specific expression of vascular endothelial growth factor and angiopoietin-1 and -2 suggests an important role in astrocytes in cerebellar vascularization. *Mech Dev.* **108:**45-57

Adair TH. (2005). Growth regulation of the vascular system: an emerging role for adenosine. *Am J Physiol Regul Integr Comp Physiol.* **289:**R283-R296

Adamis AP, Miller JW, Bernal M, D'Amico DJ, Folkman J *et al.* (1994) Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol.* **118**:445-450

Adamis AP, Shima DT, Tolentino M, Gragoudas ES, Ferrara N *et al.* (1996). Inhibition of VEGF prevents retinal ischaemia-associated iris neovascularization in a primate. *Arch Ophthalmol.* **114**:66-71

Adamis A, Shima DT, Yeo K, Yeo T, Brown LF *et al.* (1993). Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. *Biochem Biophys Res Comm.* **193**: 631-638

Afzal A, Shaw LC, Ljubimov AV, Boulton ME, Segal MS, Grant MB. (2007). Retinal and choroidal microangiopathies: Therapeutic opportunities. *Microvasc Res.* In Press

Ahmad SA, Liu W, Jung YD, Fan F, Wilson M *et al.* (2001). The effects of angiopoietin-1 and -2 on tumor growth and angiogenesis in human colon cancer. *Cancer Res.* **61**:1255-1259

Ahmed A, Dunk C, Kniss D, Wilkes M. (1997). Role of VEGF receptor-1 (Flt-1) in mediating calcium-dependent nitric oxide release and limiting DNA synthesis in human trophoblast cells. *Lab Invest.* **76**:779-791

Ahn S, Maudsley S, Luttrell LM, Lefkowitz RJ, Daaka Y. (1999). Src-mediated tyrosine phosphorylation of dynamin is required for beta2-adrenergic receptor internalization and mitogen-activated protein kinase signaling. *J Biol Chem.* **274**:1185-8.

Aiello LP. (1997). vascular endothelial growth factor and the eye: Biochemical mechanisms of action and implications for novel therapies. *Ophthalmic Res.* **29**:354-362

Aiello LP, Cavallerano J, Bursell S. (1996). Diabetic eye disease. *Endocrinol Metab clinics N Am.* **25**:271-291

Aiello LP, Gardner TW, King GL, Blankenship G, Cavallerano JD *et al.* (1998). Diabetic retinopathy. *Diabetes Care.* **21**:143-156

Aiello LP, Northrup JM, Keyt BA, Takagi H, Iwamoto MA. (1995). Hypoxic regulation of vascular endothelial growth factor in retinal cells. *Arch Ophthalmol.* **113**:1538-1544

Alam A, Herault J, Barron P, Favier B, Fons P *et al.* (2004). Heterodimerization with vascular endothelial growth factor receptor-2 (VEGFR-2) is necessary for VEGFR-3 activity. *Biochem Biophys Res Commun.* **324**:909-915

Alberdi E, Ayemerich MS, Becerra SP. (1999). Binding of pigment epithelium-derived factor (PEDF) to retinoblastoma cells and cerebellar granule neurons. Evidence for a PEDF receptor. *J Biol Chem.* **24**:31605-31612

Alberdi E, Hyde CC, Becerra SP. (1998). Pigment epithelium-derived factor (PEDF) binds to glycosaminoglycans: analysis of the binding site. *Biochem.* **28**:10643-10652.

Alikacem N, Yoshizawa T, Nelson KD, Wilson CA. (2000). Quantitative MR imaging study of intravitreal sustained release of VEGF in rabbits. *Invest Ophthalmol Vis Sci.* **41**:1561-1569

Aloisi F, Care A, Borsellino G, Gallo P, Rosa S. (1992). Production of hemolymphopoietic cytokines (IL-6, IL-8, colony-stimulating factors) by normal human astrocytes in response to IL-1β and tumor necrosis factor-α. *J Immunol.* **149**:2358-2366

Alon R, Cahalon L, Hershkoviz R, Elbaz D, Reizis D *et al.* (1994). TNF-α binds to the N-terminal domain of fibronectin and augments the β_1-integrin-mediated adhesion of $CD4^+$ T lymphocytes to the glycoprotein. *J Immunol.* **152**:1304-1313

Altshuler G, Ornoy A. (1986). Thickness of renal glomerular capillary basement membrane in the offspring of diabetic rats fed a regular or high-sucrose diet. *Acta Anat (Basel).* **126**:237-239.

Amin RH, Frank RN, Kennedy A, Eliott D, Puklin JE *et al.* (1997). Vascular endothelial growth factor is present in glial cells of the retina and optic nerve of human subjects with non proliferative diabetic retinopathy. *Invest Ophthalmol Vis Sci.* **38**:36-47

Anagnostou Z, Liu Z, Steiner M, Chin L, Lee ES *et al.* (1994). Erythropoietin receptor mRNA expresssion in human endothelial cells. *Proc Natl Acad Sci USA.* **91**:3974-3978

Anderson RG. (1993) Caveolae: where incoming and outgoing messengers meet. *Proc Natl Acad Sci U S A.* **90**:10909-10913

Anderson RG. (1998). The caveolae membrane system. *Annu Rev Biochem* **67:**199-225

Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM *et al.* (1998). Vascular permeability in experimental diabetes is associated with reduced endothelial occlucin content. Vascular endothelial growth factor decreases ocludin in retinal endothelial cells. *Diabetes.* **47**:1953-1959

Antonetti DA, Barber AJ, Hollinger LA, Wolpert EB, Gardner TW, (1999). Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumours. *J Biol Chem.* **274:**23463-23467

Aprelikova O, Pajusola K, Partanen J, Armstrong E, Alitalo R *et al.* (1992). FLT4, a novel class III receptor tyrosine kinase in chromosome 5q33-qter. *Cancer Res.* **52**:746-748

Apte RS, Barreiro RA, Duh E, Volpert O, Ferguson TA. (2004). Stimulation of neovascularization by the anti-angiogenic factor PEDF. *Invest Ophthalmol vis Sci.* **45:**4491-4497

Araki T, Taniwaki T, Becerra SP, Chader GJ, Schwartz JP. (1998). Pigment epithelium-derived factor (PEDF) differentially protects immature but not mature cerebelar granule cells against apoptopic cell death. *J Neursci Res.* **53:**7-15

Armstrong D, Augustin AJ, Spengler R, Al-Jada A, Nickola T *et al.* (1998a). Detection of vascular enothelial growth factor and tumour necrosis factor alpha in epiretinal membranes of proliferative diabetic retinopathy, proliferative vitreoretinopathy and macular pucker. *Ophthalmologica.* **212**:410-414

Armstrong D, Ueda T, Ueda T, Aljada A, Browne R *et al.* (1998b). Lipid hydroperoxide stimulates retinal neovascularization in rabbit retina through expression of tumor necrosis factor-α, vascular endothelial growth factor and platelet-derived growth factor. *Angiogenesis.* **2:**93-104

Asahara T, Chen D, Takahashi T, Fujikawa K *et al.* (1998). Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. *Circ Res.* **83**:233-240

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R *et al.* (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* **275:**964-967

Ashton N. (1957). Retinal vascularization in health and disease. *Am J Ophthalmol.* **44**:7-24

Athanassiades A, Lala PK. (1998). Role of placenta growth factor (PIGF) in human extravillous trophoblast proliferation, migration and invasiveness. *Placenta.* **7:**465-473

Audero T, Cacone I, Zanon I, Previtali SC, Piva R, *et al.* (2001). Expression of angiopoietin-1 in human glioblastomas regulates tumor-induced angiogenesis: in vivo and in vitro studies. *Arterioscler Thromb Vasc Biol.* **21:**536-541

Auricchio A, Behling K, Maguire A, O'Connor E, Bennett J. (2002). Inhibition of retinal neovascularization by intraocular viral-mediated delivery of anti-angiogenic agents. *Mol Ther.* **6:**490-494

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L *et al.* (2003). Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *Nat Med.* **9:**936-943

Averbukh E, Weiss O, Halpert M, Yanko R, Moshe R *et al.* (1998). Gene expression of insulin-like growth factor-1, its receptor and binding proteins in retina under hypoxic conditions. *Metabolism.* **47**: 1331-1336

Ayalasomayajula SP, Amrite AC, Kompella UB. (2004). Inhibition of cyclooxygenase-2, but not cyclooxygenase-1, reduces prostaglandin E_2 secretion from diabetic rat retinas. *Eur J Pharmacol.* **498:**275-278

Aymerich MS. Alberdi EM, Martinez A, Becerra SP (2001). Evidence for pigment epithelium-derived factor receptors in the neural retina. *Invest Ophthalmol Vis Sci.* **42:**3287-3293

Babei S, Teichert-Kuliszewska K, Zhang Q, Jones N, Dumont DJ *et al.* (2003). Angiogenic actions of angiopoietin-1 requuire endothelium-derived nitric oxide. *Am J Pathol.* **162:**1927-1936

Baffert F, Le T, Thurston G, McDonald DM. (2006). Angiopoietin-1 decreases plasma leakage by reducing number and size of endothelial gaps in venules. *AJP-Heart.* **290:**107-118

Balkwill F. (2002). Tumor necrosis factor or tumor promoting factor? *Cytokine Growth Factor Rev.* **13:**135-141

Bamforth SD, Lightman S, Greenwood J. (1996). The effect of TNF-α and IL-6 on the permeability of the rat blood-retinal barrier in vivo. *Acta Neuropathol.* **91:**624-632

Bangstad HJ, Osterby R, Hartmann A, Berg TJ, Hanssen KF. (1999). Severity of glomerulopathy predicts long-term urinary albumin excretion rate in patients with type 1 diabetes and microalbuminuria. *Diabetes Care.* **22:**314-319.

Banin E, Dorrell MI, Aguilar E, Ritter MR, Aderman CM *et al.* (2006). T2-TrpRS inhibits preretinal neovascularization and enhances physiological vascular regrowth in OIR as assessed by a new method of quantification. *Invest Ophthalmol Vis Sci.* **47**:2125-2134

Barna BP, Estes ML, Jacobs BS, Hudson S, Ransohoff RM. (1990). Human astrocytes proliferate in response to tumor necrosis factor α. *J Neuroimmunol.* **30:**239-243

Barreiro R, Schadlu R, Herndon J, Kaplan HJ, Ferguson TA. (2003). The role of Fas-FasL in the development and treatment of ischemic retinopathy. *Invest Ophthalmol vis Sci.* **44:**1282-286

Battegay EJ. (1995). Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospects. *J Mol Med.* **73**:333-346

Bates DO, Harper SJ. (2002). Regulation of vascular permeability by vascular endothelial growth factors. *Vascul Pharmacol.* **39:**225-237

Becerra AP. (2006). Focus on molecules: Pigment epithelium-derived factor (PEDF). *Exp Eye Res.* **82:**739-740

Becerra SP. (1997). Structure-function studies on PEDF. Anoninhibitory serpin with neurotrophic activity. *Adv Exp Med Biol.* **425:**223-237

Becerra SP, Sagasti A, Spinelli P, Notario V. (1995). Pigment epithelium-derived factor behaves like a noninhibitory serpin: neurotrophic activity does not require the serpin reactive loop. *J Biol Chem.* **270:**25992-25999

Behling KC, Surace EM, Bennett SJ. (2002). Pigment epithelium-derived factor expression in the developing mouse eye. *Mol Vis.* **8:**449-454

Ben-Yosef Y, Miller A, Shapiro S, Lahat N. (2005). Hypoxia of endothelial cells leads to MMP-2-dependent survival and death. *Am J Physiol Cell Physiol.* **289:**1321-1331

Bergstrom JD, Bostedor RG, Masarachia PJ, Reska AA, Rodan G. (2000). Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. *Arch Biochem.* **373:**231-241

Bilak MM, Becerra SP, Vincent AM, Moss BH, Aymerich MS, Kuncl RW. (2002). Identification of the neuroprotective molecular region of pigment epithelium-derived factor and its binding sites on neurons. *J Neurosci.* **22:**9378-9386

Bilak MM, Corse AM, Bilak SR, Lehar M, Tombran-Tink J *et al.* (1999). Pigment epithelium-derived factor (PEDF) protects motor neurons from chronic glutamate-mediated neurodegeneration. *J Neuropathol Exp Neurol.* **58:**719-728

Bilderback TR, Gazula VR, Lisanti MP, Dobrowsky RT. (1999). Caveolin interacts with Trk A and p75(NTR) and regulates neurotrophin signaling pathways. *J Biol Chem.* **274:**257-263.

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL. (1997a). A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature* **385:**729-733

Black R, Rauch CT, Kozlosky K *et al.* (1997b). A metalloproteinase-disintegrin releases TNF from cells. *Mol Biol Cell.* **8:**35-36

Blanks JC. (1994). Morphology of the retina. In *Retina.* TE Ogden [ed]. Mosby, St Louis. pp 37-51

Blobel CP. (1997). Metalloproteinase-disintegrins: links to cell adhesion and cleavage of TNF alpha and Notch. *Cell.* **90:**589-592

Bolton SJ, Anthony DC, Perry VH. (1998). Loss of tight junction proteins occluding and zonula occludins-1 from cerebral vascular ndothelium during neutrophil-induced blood-brain barrier breakdown in vivo. *Neuroscience.* **86:**1245-1257

Boocock CA, Charnock-Jones DS, Sharkey AM, Mclaren J, Barker PJ *et al.* (1995). Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Natl Cancer Inst.* **87**:506-516

Borg J, deLapeyriere O, Tetsuro N, Rottapel R, Dubreuil P *et al.* (1995). Biochemical characterization of two isoforms of FLT4, a VEGF receptor-related tyrosine kinase. *Oncogene.* **10**:973-984

Bost LM, Aotaki-Keen A, Hjelmeland LM. (1992). Co-expression of FGF-5 and bFGF by the retinal pigment epithelium *in vitro*. *Exp Eye Res.* **55**:727-734

Bouck N. (2002). PEDF: anti-angiogenic guardian of ocular function. *Trends Mol Med.* **8:**330-334

Breier G. (2000). Endothelial receptor tyrosine kinases involved in blood vessel development and tumor angiogenesis. *Adv Exp Med Biol.* **476:**57-66

Breier G, Albrecht U, Sterre S, Risau W. (1992). Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Devel.* **114**:521-532

Brennan FM, Feldman M. (1996). Cytokines in autoimmunity. *Curr Opin Immunol.* **8:**872-877

Bridges CC, El-Sherbeny A, Roon P, Ola MS, Kekuda R *et al.* (2001). A comparison of caveolae and caveolin-1 to folate receptor alpha in retina and retinal pigment epithelium. *Histochem J.***33:**149-158

Brock TA, Dvorak HF, Senger DR. (1991). Tumor-secreted vascular permeability factor increases cytosolic Ca2+ and von Willebrand factor release in human endothelial cells. *Am J Pathol.* **138**:213-221

Brogi E, Schatteman G, Wu T, Kim EA, Varticovski L *et al.* (1996). Hypoxia induced paracrine regulation of vascular endothelial growth factor receptor expression. *J Cell Invest.* **97**:469-476

Brooks SE, Gu A, Kaufmann PM, Marcus DM, Caldwell RB. (1998). Modulation of VEGF production by pH and glucose in retinal Müller cells. *Curr Eye Res.* **17**:875-882

Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL *et al.* (2001). Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statins. *Circ Res.* **89:**866-873.

Brouet A, DeWever J, Martinive P, Havaux X, Bouzin C *et al.* (2005). Antitumor effects of in vivo caveolin gene delivery are associated with the inhibition of the proangiogenic and vasodilatory effects of nitric oxide. *FASEB J.* **19:**602-4.

Brown DL, Robbins R. (1999). Developments in the therapeutic applications of bisphosphonates. *J Clin Pharmacol.* **39:**651-660

Brown DM, Kaiser PK, Michels M, Soubrane G, Heier JS *et al.* (2006). Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med.* **355**: 1432-1444

Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ *et al.* (1993a). Increased expression of vascular permability factor (Vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. *Am J Pathol.* **143**:1255-1262

Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ *et al.* (1993b). Expression of vascular permeability factor (Vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res.* **53**:4727-4735

Brown LF, Dezube BJ, Tognazzi K, Dvorak HF, Yancopoulos GD. (2000). Expression of Tie1, Tie-2, and angiopoietins 1, 2, and 4 in Kaposi's sarcoma and cutaneous angiosarcoma. *Am J Pathol.* **156:**2179-2183

Brown LF, Yeo B, Berse TK, Yeo DR, Senger HF *et al.* (1992). Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med.* **176**:1375-1379

Browning AC, Gray T, Amoaku WM. (2005). Isolation, culture, and characterisation of human macular inner choroidal microvascular endothelial cells. *Br J Ophthalmol.* **89:**1343-1347

Brownlee M, Cerami A, Vlassara H. (1988). Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med.* **318**:1315-1321

Bucci M, Gratton JP, Rudic RD, Acevedo L, Roviezzo F *et al.* (2000). In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat Med.* **6:**1362-1367

Bullard LE, Qi X, Penn JS. (2003). Role for extracellular signal-responsive kinase-1 and -2 in retinal angiogenesis. *Invest Ophthalmol Vis Sci.* **44:**1722-1731

Bunone G, Vigneri P, Mariani L, Buto S, Collini P *et al.* (1999). Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. *Am J Pathol.* **155:**1967-1976

Burgering BM, Bos JL. (1995). Regulation of ras-mediated signalling: more than one way to skin a cat. *Trends Biochem Sci.* **20**:18-22

Cai J, Jiang WG, Grant MB, Boulton M. (2006). Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. *J Biol Chem.* **281:**3604-3613

Caldwell RB, Slapnick SM, McLaughlin BJ. (1985). Lanthanum and freeze-fracture studies of retinal pigment epithelial cell junctions in the streptozotocin diabetic rat. *Curr Eye Res.* **4**:215-227

Calera MR, Venkatakrishnan A, Kazlauskas A. (2004). VE-cadherin increases the half-life of VEGF receptor 2. *Exp Cell Res.* **300:**248-256

Cameron PL, Ruffin JW, Bollag R, Rasmussen H, Cameron RS. (1997). Identification of caveolin and caveolin-related proteins in the brain. *J Neurosci.* **17:**9520-9535

Campochiaro P, Hackett SF. (2003) Ocular neovascularization: a valuable model system. *Oncogene.* **22:**6537-6548

Camussi G, Turello E, Bussolino F, Baglioni C. (1991). Tumor necrosis factor alters cytoskeletal organization and barrier function of endothelial cells. *Int Arch Allergy Appl Immunol.* **96:**84-91

Cantley LC, Auger KR, Carpeneter C, Duckworth B, Graziani A *et al.* (1991). Oncogenes and signal transduction. *Cell.* **64**:281-302

Cao Y, Linden P, Farnebo J, Cao R, Eriksson A *et al.* (1998). Vascular endothelial growth factor C induces angiogenesis in vivo. *Proc Natl Acad Sci USA.* **95:**14389-14394

Cao W, Tombran-Tink J, Elias R *et al.* (2001). In vivo protection of photoreceptors from light damage by pigment epithelium-derived factor. *Invest Ophthalmol Vis Sci.* **42:**1646-1652

Cao G, Yang G, Timme TL, Saika T, Truong LD *et al.* (2003). Disruption of the caveolin-1 gene impairs renal calcium reabsorption and leads to hypercalciuria and urolithiasis. *Am J Pathol.* **162:**1241-1248.

Carlson EC, Audette JL, Klevay LM, Nguyen H, Epstein PN. (1997). Ultrastructural and functional analyses of nephropathy in calmodulin-induced diabetic transgenic mice. *Anat Rec.* **247:**9-19.

Carlson EC, Audette JL, Veitenheimer NJ, Risan JA, Laturnus DI *et al.* (2003). Ultrastructural morphometry of capillary basement membrane thickness in normal and transgenic diabetic mice. *Anat Rec A Discov Mol Cell Evol Biol.* **271:**332-341.

Carmeliet P. (2003). Angiogenesis in health and disease. *Nat Med.* **9:**653-660

Carmeliet P, Ferraira V, Breier G, Pollefeyt S, Kieckens L, Gertenstein M *et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* **380**:435-439

Carmeliet P, Storkebaum E. (2002). Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. *Semin Cell Dev Biol.* **13:**39-53

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N *et al.* (1975). An endotoxin-inducced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA.* **72:**3666-3670

Cayouette M, Smith SB, Becerra SP *et al.* (1999). Pigment epithelium-derived factor delays the death of photoreceptors in mouse models of inherited retinal degeneration. *Neurobiol Dis.* **6:**523-532

Cenedella RJ, Neely AR, Sexton P. (2006). Multiple forms of 22 kDA caveolins-1 alpha present in bovine lens cells could reflect variable palmitoylation. *Exp Eye Res.* **82:**229-235

Cerdan C, Rouleau A, Bhatia M. (2004). VEGF-A165 augments erythopoietic development from humanembryonic stemmcells. *Blood.* **103:**2504-2512

Chan-Ling T, Gock B, Stone J. (1995a). The effect of oxygen on vasoformative cell division: Evidence that 'physiological hypoxia' is the stimulus for normal retinal vasculogenesis. *Invest Ophthalmol Vis Sci.* **36**:1204-1215

Chan-Ling T, Gock B, Stone J. (1995b). Supplemented oxygen therapy: Basis for noninvasive treatment of retinopathy of prematurity. *Invest Ophthalmol Vis Sci.* **36**: 1215-1230

Chan-Ling T, Stone J. (1992). Degeneration of astrocytes in feline retinopathy of prematurity causes failure of the blood-retinal barrier. *Invest Ophthalmol Vis Sci.* **33**:2148-2159

Charnock Jones DS, Sharkey AM, Rajput Williams J, Burch D, Schofield JP *et al.* (1993). Identification of alternatively spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. *Biol Reprod.* **478**:1120-1128

Chen JX, Chen Y, DeBusk L, Lin W, Lin PC. (2004). Dual functional roles of Tie-2/angiopoietin in TNF-alpha-mediated angiogenesis. *Am J Physiol Heart Circ Physiol.* **287:**H187-95

Chen L, Zhang S, Barnstable CJ, Tombran-Tink J. (2006). PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases. *Biochem Biophys Res Commun.* **348:**1288-1295

Chen Y, Hackett SF, Schoenfeld C, Vinores MA, Vinores SA, Campochiaro PA. (1997). Localisation of vascular endothelial growth factor and its receptors to cells of vascular and avascular epiretinal membranes. *Br J Ophthalmol.* **81**:919-926

Chew EY, Klein ML, Ferris FL 3rd, Remaley NA, Murphy RP *et al.* (1996). Association of elevated serum lipid levels with retinal hard exudate in diabetic retinopathy. Early Treatment Diabetic Retinopathy Study (ETDRS) Report 22. *Arch Ophthalmol.* **114:**1079-1084.

Cho CH, Kammerer HJ, Lee MO, Steinmetz YS, Ryu SH et al. (2004a). Ang-1: a designed angiopoietin-1 variant with nonleaky angiogenic activity. *Proc Natl Acad Sci USA.* **101:**5547-5552

Cho CH, Lee CS, Chang M, Jang IH, Kim SJ *et al.* (2004b). Localization of VEGFR-2 and PLD2 in endothelial caveolae is involved in VEGF-induced phosphorylation of MEK and ERK. *Am J Physiol Heart Circ Physiol.***286:**H1881-1888.

Cho C, Sung H, Kim K, Cheon HG, Oh GT *et al.* (2006). COMP-angiopoietin-1 promotes wound healing through enhanced angiogenesis, lymphangiogenesis and blood flow in a diabetic mouse model. *PNAS.* **103:**4946-4951

Christofori G, Naik P, Hanahan D. (1995). Vascular endohelial growth factor and its receptors, *flt-1 and flk-1*, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. *Mol Endo*. **9**:1760-1770

Claudio L, Martiney J, Brosnan C. (1994). Ultrastructural studies of the blood-retina barrier after exposure to interleukin-1 or tumor necrosis factor-alpha. *Lab Invest.* **70:** 850-861

Claffey KP, Wilkinson WO, Spiegelman BM. (1992). Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways. *J Biol Chem.* **267**:16317-16322

Clarijs R, Schalkwijk L, Hofman UB, Ruiter DJ, de Waal R. (2002). Induction of vascular endothelial growth factor receptor-3 expression on tumor microvasculature as a new progression marker in human cutaneous melanoma. *Cancer Res.* **62:**7059-7065

Clauss M, Weich H, Brier G, Knies U, Rockl W *et al.* (1996) The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem.* **271:**17629-17634

Cohen T, Nahari D, Ceremi L, Neufield G, Levi B. (1996). Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem.* **271**:736-741

Colombo ES *et al.* (2001). Pigment epithelium-derived factor (PEDF), and endogenous angiogenesis inhibitor is down regulated in ocular tissues in proliferative retinopathy. *Invest Ophthalml Vis Sci.* **42:**S809

Conn G, Soderman DD, Schaffer MT, Wile M, Hatcher VB, Thomas KA. (1990). Purification of a glycoprotein vascular endothelial growth factor from a rat glioma-derived cell line. *Proc Natl Acad Sci USA.* **87**:1323-1327

Connolly DT, Olander JV, Heuvelman D, Nelson R, Monsell R, Siegel N *et al.* (1989b). Human vascular permeability factor. Isolation from U937 cells. *J Biol Chem.* **264**:20017-20024

Couet J, Sargiacomo M, Lisanti MP. (1997). Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. *J Biol Chem.* **272:**30429-30438.

Crawford SE, Stellmach V, Ranalli M, Huang L, Volpert O *et al.* (2001). Pigment epithelium-derived factor (PEDF) in neuroblastoma: a multifunctional mediator of Schwann cell antitumor activity. *J Cell Sci.* **114:**4421-4428

Croll SD, Ransohoff RM, Cai N, Zhang Q, Martin FJ *et al.* (2004). VEGF-mediated inflammation prcedes angiogenesis in adult brain. *Exp Neurol.* **187:**388-402

Cruss HJ. (1996). Molecular, structural, and biological characteristics of the tumor necrosis factor ligand superfamily. *Int J Clin Lab Res.* **26:**143-159

Cukiernik M, Hileeto D, Evans T, Mukherjee S, Downey D *et al.* (2004). Vascular endothelial growth factor in diabetes induced early retinal abnormalities. *Diabetes Res Clin Prac.* **65:**197-208

Cunningham AJ, Murray CA, O'Neill LA, Lynch MA, O'Connor JJ. (1996). Interleukin-1β (IL-1β) and tumour necrosis factor (TNF) inhibits long-term potentiation in the rat denate gyrus in vitro. *Neurosc Lett.* **203:**17-20

Cunningham Jr., ET, Adamis AP, Altaweel M, Aiello LP, Bressler NM *et al.* (2005). A phase II randomized double-masked trial of pegaptanib, an anti-vascular endothelial growth factor aptamer, for diabetic macular oedema. *Ophthalmol.* **112:**1747-1757

Cunningham SA, Waxham MN, Arrate PM, Brock TA. (1995). Interaction of the Flt-1 tyrosine kinase receptor with the p85 subunit of phosphatidylinositol 3-kinase. Mapping of a novel site involved in binding. *J Biol Chem.* **270**:20254-20257

Cuthbertson RA, Mandel TE. (1987). The effect of murine fetal islet transplants on renal and retinal capillary basement membrane thickness. *Transplant Proc.* **19:**2919-21.

D'Alessio, Al-Lamki RS, Bradley JR, Pober JS. (2005). Caveolae participate in tumor necrosis factor 1 signaling and internalization in a human endothelial cll line. Am J Pathol. **166:**1273-1282

De La Cruz JP, Gonzalez-Correa JA, Guerrero A, de la Cuesta FS. (2004). Pharmacological approach to diabetic retinopathy. *Diabetes Metab Res Rev.* **20:**91-113

De Vries HE, Blom-Roosemalen MCM, Oosten MV, de-Boer AG, Berkel TJCV *et al.* (1996). The influence of cytokines on the integrity of the blood-brain barrier in vitro. *J Neuroimmunol.* **64:**37-43

De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N *et al.* (1992). The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science.* **255**: 989-991

Daly C, Wong V, Burova Y, Wei S, Zabski S *et al.* (2004). Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). *Genes Dev.* **18:**1060-1071

Danowski TS, Fisher ER, Khurana RC, Nolan S, Stephan T. (1972). Muscle capillary basement membrane in juvenile diabetes mellitus. *Metabolism.* **21:**1125-1132.

Darland DC, Massingham LJ, Smith SR, Piek E, Saint-Geniez M. (2003). Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. *Devel Biol.* **264:**275-288

Das A, Fanslow W, Cerretti D, Warren E, Talarico N, McGuire P. (2003). Angiopoietin/Tek interactions regulate mmp-9 expression and retinal neovascularization. *Lab Invest.* **83:**1637-1645

Das K, Lewis RY, Scherer PE, Lisanti MP. (1999). The membrane spanning domains of caveolins 1 and 2 mediate the formation of caveolin hetero-oligomers. Implications for the assembly of caveolae membranes in vivo. *J Biol Chem.***274:**18721-18728

Davies S, Aldrich TH, Jones PF, Acheson A, Compton DL *et al.* (1996). Isolation of Angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell.* **86**:1161-1169

Davies PF, Mundel T, Barbee KA. (1995). A mechanism for heterogeneous endothelial responses to flow in vivo and in vitro. *J Biomech.* **28:**1553-1560.

Davies S, Papadopoulos TH, Aldrich PC, Maisonpierre T, Huang L *et al.* (2003). Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. *Nat Struct Biol.* **10:**38-44

Davis MD. (1992). Diabetic retinopathy. A clinical overview. *Diabetes Care.* **15:**1844-1874

Davis S, Yancopoulos GD. (1999). The angiopoietins: Yin and yang in angiogenesis. *Curr Top Microbiol Immunol.* 273:173-184

Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H *et al.* (1999). Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science.* **285:**245-248

DeBusk LM, Hallahan DE, Lin PC. (2004). Akt is a major angiiogenic mediator downstream of the Ang1/Tie2 signaling pathway. *Exp Cell Res.* **298:**167-177

Dejana E. (1996). Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. *J Clin Invest.* **98:**1949-1953

Deli M, Descamps L, Dehouck M, Cecchlli R, Joo F *et al.* (1995). Exposure of tumor necrosis factor-α to luminal membrane of bovine brain capillary endothelial cells co-cultures with astrocytes induces a delayed increase of permeability and cytoplasmic stress fiber formation of actin. *J Neurosci Res.* **41:**717-726

Del Maschio A, Zanetti A, Coroda M, Rival Y, Ruco L *et al.* (1996). Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-cell adherens junctions. *J Cell Biol.* **135:**497-510

Denk A, Goebeler M, Schmid S, Berberich I, Ritz O *et al.* (2001). Activation of NF-κB via the IκB kinase complex is both essential and sufficient for proinflammatory gene expression in primary endothelial cells. *J Biol Chem.* **276:**28451-28458

DeRubertis FR, Craven PA. (1994). Activation of protein kinase C in glomerular cells in diabetes. *Diabetes.* **43**:1-8

Desai TR, Leeper NJ, Hynes KL, Gewertz BL. (2002). Interleukin-6 causes endothelial barrier dysfunction via the protein kinase C pathway. *J Surg Res.* **104:**118-23

Detmar M, Brown LF, Claffey KP, Yeo K, Kocher O *et al.* (1994). Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J Exp Med.* **180**:1141-1146

Dewey CF Jr, Bussolari SR, Gimbrone MA Jr, Davies PF. (1981). The dynamic response of vascular endothelial cells to fluid shear stress. *J Biomech Eng.* **103:**177-185.

Doolittle RF. (1984). Fibrinogen and fibrin. *Annu Rev Biochem.* **53**:195-229

Diabetic Retinopathy Study Research Group. (1978). Photocoagulation treatment of proliferatiive diabetic retinopathy: the second report of diabetic retinopathy study findings. *Ophthalmol.* **85:**82-106

Doft BH, Blankenship G. (1984). Retinopathy risk factor regression after laser panretinal photocoagulation for proliferative diabetic retinopathy. *Ophthalmol.* **85:**82-106

Dopp JM, Mackenzie-Graham A, Otero GC, Merrill JE. (1997). Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. *J Neuroimmunol.* **75:**104-112

Dorey CK, Aouididi S, Reynaud X, Dvorak HF, Brown LF. (1996). Correlation of vascular permeability factor/Vascular endothelial growth factor with extraretinal neovascularization in the rat. *Arch Ophthalmol.* **114**:1210-1217

Drab M, Verkade P, Elger M, Kasper M, Lohn M *et al.* (2001). Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice.*Science.* **293:**2449-52.

Duh EJ, Yang HS, Suzuma K, West K, Davarya S *et al.* (2002). Pigment epithelium-derived factor suppresses ischaemia-induced retinal neovascularization and VEGF-induced migration and growth. *Invest Ophthalmol Vis Sci.* **43:**821-829

Dumont DJ, Gradwohl GJ, Fong GH, Auerbach R, Breitman ML. (1993). The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. *Oncogene.* **8**:1293-1299

Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M *et al.* (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**:1897-

Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K *et al.* (1998). Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science.* **282**:946-949

Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM *et al.* (2001). *J Pharmacol Exp Ther.* **296:**235-242

Dvorak HF, Brown LF, Detmar M, Dvorak AM (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol.* **146**:1029-1039

Early Treatment Diabetic Retinopathy Study Research Group (1991): ETDRS No 10. Grading diabetic retinopathy from stereoscopic color fundus photographs: an extension of the modified Airlie House classification. *Ophthalmol.* **98:**786-806

Early Treatment Diabetic Retinopathy Study Research Group (1995): ETDRS No 19. Focal photocoagulation treatment of diabetic macular edema: relationship of treatment effect to fluorescein angiographic and other retinal characteristics at baseline. *Arch Ophthalmol.* **113:**1144-1155

Eichler W, Yafai Y, Keller T, Wiedemann P, Reichenbach A. (2004). PEDF derived from glial cells: a possible regulator of retinal angiogenesis. *Exp Cell Res.* **299:**68-78

Eichler W, Yafai Y, Kuhrt H, Grater R, Hoffmann S *et al.* (2001). Hypoxia- modulation of endothelial cell proliferation by soluble factors released by retinal cells. *Neuroreport.* **12:**4103-4108

Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J *et al.* (1999). Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell.* **4:**915-924

Elis EA, Guberski DL, Somogyi-Mann M, Grant MB. (2000). Increased H_2O_2, vascular endothelial growth factor and receptors in the retina of the BBZ/WOR diabetic rat. *Free Rad Biol Med.* **28:**91-101

Elliott MH, Fliesler SJ, Ghalayini AJ. (2003). Cholesterol-dependent association of caveolin-1 with the transducin alpha subunit in bovine photoreceptor rod outer segments: disruption by cyclodextrin and guanosine 5'-O-(3-thiotriphosphate). *Biochemistry.* **42:**7892-7903

El-Remessy AB, Behzadian MA, Abou-Mohamed G, Franklin T, Caldwell RW. (2003). Experimental diabetes causes breakdown of the blood-retina barrier by a mechanism involving tyrosine nitration and increases in expression of vascular endothelial growth factor and urokinase plasminogen activator receptor. *Am J Pathol.* **162:**1995-2004

Emslie-Smith D. Vision. (1988). In *Textbook of Physiology*. Emslie-Smith D, Paterson CR, Scratchers T, Read NW [Eds]. Longman Group, Singapore. pp 444-453

Enaida H, Ito T, Oshima Y, Sakamoto T, Yago K *et al.* (1998). Effect of growth factors on expression of integrin subtypes in microvascular endothelial cells isolated from bovine retinas. *Fukushima J Med Sci.* **44**:43-52

Endo M, Yanagisawa K, Tsuchida K, Okamoto T, Matsushita T. (2001). Increased levels of vascular endothelial growth factor and advanced glycation end products in aqueous humor of patients with diabetic retinopathy. *Horm Metab Res.* **33:**317-322

Engelman JA, Wykoff CC, Yasuhara S, Song KS, Okamoto T *et al.* (1997). Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *J Biol Chem.***272:**16374-16781

Enholm B, Paavonen K, Ristimäki A, Kumar V, Gunji Y *et al.* (1997). Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene.* **14**:2475-2483

Eriksson A, Cao R, Pawliuk R, Berg SM, Tsang M *et al.* (2002). Placenta growth factor-1 antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers. *Cancer Cell.* **1:**99-108

Esser S, Lampugnani M, Corada E, Dejana E. (1998). Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci.* **111:**1853-1865

Etoh T, Inoue H, Tanaka S, Barnard GF, Kitano S *et al.* (2001). Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: possible in vivo regulation via induction of proteases. *Cancer Res.* **61:**2143-2153

Fajardo LF, Kwan HH, Kowalski J, Prionas SD, Allison AC. (1992). Dual role of tumor necrosis factor-alpha in angiogenesis. *Am J Pathol.* **140:** 539-544

Famiglietti EV, Stopa EG, McGookin ED, Song P, LeBlanc V *et al.* (2003). Immunocytochemical localisation of vascular endothelial growth factor in neurons and glial cells of human retina. *Brain Res.* **969:**195-204

Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M *et al.* (1992). Distinct phosphotyrosinases on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell.* **69**:413-423

Fava R, Olsen NJ, Spencer-Green G, Yeo K, Yeo T *et al.* (1994). Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. *J Exp Med.* **180**:341-346

Feingold KR, Lee TH, Chung MY, Siperstein MD. (1986). Muscle capillary basement membrane width in patients with vacor-induced diabetes mellitus. *J Clin Invest.* **78:**102-107.

Feit-Leichman RA, Kinouchi R, Takeda M, Zhigang F. (2005). Vacsular damage in a mouse model of diabetic retinopathy: relation to neuronal and glial changes. *Invest Ophthalmol Vis Sci.* **46:**4281-4287

Feng Y, Venema VJ, Venema RC, Tsai N, Behzadian MA et al. (1999b).VEGF-induced permeability increase is mediated by caveolae. *Invest Ophthalmol Vis Sci.* **40:**157-167.

Feng Y, Venema VJ, Venema RC, Tsai N, Caldwell RB. (1999a). VEGF induces nuclear translocation of Flt-1/KDR, endothelial nitric oxide synthase, and caveolin-1 in vascular endothelial cells. *Biochem and Biophys Res Comm.* **256**:192-197

Ferrara N. (1995a). The role of vascular endothelial growth factor in pathological angiogenesis. *Breast Cancer Research and Treatment.* **36**:127-137

Ferrara N. (2001). Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am *J Physiol Cell Physiol.* **280:**C1358-1366.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L *et al.* (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature.* **380**:439-442

Ferrara N, Gerber HP, LeCouter J. (2003). The biology of VEGF and its receptors. *Nat Med.* **9:**669-676

Ferrara N, Henzel W. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Comm.* **161**:851-858

Ferrara N, Houck K, Jakeman L, Leung DW. (1992). Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrinol Rev.* **13**:18-32

Ferrara N, Heinsohn H, Walder CE, Bunting S, Thomas R. (1995b). The regulation of blood vessel growth by vascular endothelial growth factor. *Ann NY Acad Sci.* **752**:246-256

Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW. (1991). The vascular endothelial growth factor family of polypeptides. *J Cell Biochem.* **46**:211-218

Ferris FL 3rd, Davis MD, Aiello LM. (1999). Treatment of diabetic retinopathy. *N Engl J Med.* **341:**667-678

Ferris FL 3rd, Patz A. (1984). Macular edema. A complication of diabetic retinopathy. *Surv Ophthalmol.* **28 Suppl:**452-461.

Fielder W, Graeven U, Ergün S, Verago S, Kilic N *et al.* (1997). Expression of FLT4 and its ligand VEGF-C in acute myeloid leukemia. *Leukemia.* **11**:1234-1237

Filleur S, Volz K, Nelius T, Mirochnik Y, Huang H *et al.* (2005). *Cancer Res.* **65:**5144-5152

Fischer VW, Barner HB, Leskiw ML. (1979). Capillary basal laminar thichness in diabetic human myocardium. *Diabetes.***28:**713-719.

Fischer VW, Leskiw ML, Barner HB. (1981). Myocardial structure and capillary basal laminar thickness in experimentally diabetic rats. *Exp Mol Pathol.* **35:**244-256

Fisher JW, Nakashima J. (1992). Kidney regulation of erythropoietin production. In *Fisher JW* [ed] Springer, New York. pp33-48

Folkman J, Haudenschild C. (1980). Angiogenesis *in vitro. Nature.* **288**:551-556

Fong G, Rossant J, Gertsenstein M, Breltman ML. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature.* **376**:66-70

Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K *et al.* (2002). Neurodegenerative and neuroprotective effects of tumo necrosis factor (TNF) in retinal ischaemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J Neurosci.* **22:**1-7

Forrester JV, Shafiee A, Schroder S, Knott R, McIntosh L. (1993). The role of growth factors in diabetic retinopathy. *Eye.* **7**:276-287

Foster A. (1988). World distribution of blindess. *J Common Eye Health.* **1**:2-3

Fournier E, Dubreuil P, Birnbaum D, Borg JP. (1995). Mutation at tyrosine residue 1337 abrogates ligand-dependent transforming capacity of Flt-4 receptor. *Oncogene.* **11**:921-931

Fra AM, Mastroianni N, Mancini M, Pasqualetto E, Sitia R. (1999). Human caveolin-1 and caveolin-2 are closely linked genes colocalized with WI-5336 in a region of 7q31 frequently deleted in tumors. *Genomics.* **56:**355-6

Fra AM, Pasqualetto E, Mancini M, Sitia R. (2000). Genomic organization and transcriptional analysis of the human genes coding for caveolin-1 and caveolin-2. *Gene.* **243:**75-83

Fra AM, Williamson E, Simons K, Parton RG. (1995). De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc Natl Acad Sci USA.* **92:**8655-8659

Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB *et al.* (1998). *Circ.* **98:**699-710

Franitza S, Hershkoviz R, Kam N, Lichtenstein N, Vaday GG *et al.* (2000). TNF-alpha associated with extracellular matrix fibronectin provides a stoop signal for chemotacticlly migrating T cells. *J Immunol.* **165:**2738-2747

Frank RN. (1986). Diabetic retinopathy: Current concepts of evaluation and treatment. *Endocrinol Metab Clin North Am.* **15**:1512-1523

Frank RN. (2004). Diabetic retinopathy.*N Engl J Med.* **350:**48-58.

Franks WA, Limb GA, Stanford MR, Ogilvie J, Wolstencroft RA *et al.* (1992). Cytokines in human intra-ocular inflammation. *Curr Eye Res.* **11:**187-191

Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P. (1987). Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proc Natl Acad Sci USA.* **84:**5277-5281

Fu BM, Shen D. (2004). Acute VEGF effect on solute permeability of mammalian microvessels in vivo. *Microvasc Res.* **68:**51-62

Fuchs C, Forster V, Balse E, Sahel JA, Picaud S *et al.* (2005). Retinal-cell-conditioned medium prevents TNF-alpha-induced apoptosis of purified ganglion cells. *Invest Ophthalmol Vis Sci.* **46:**2983-91.

Fujikawa K, de Aos Scherpenseel I, Jain SK, Presman E, Christensen RA *et al.* (1999). Role of PI 3-kinase in angiopoietin-1-mediated migration and attachment-dependent survival of endothelial cells. *Exp Cell Res.* **253:**663-672

Fujikawa K, Presman E, Isner J, Varticovski L. (1996). Expression of Tie1 and Tie2 proteins during reendothelialization in balloon-injured rat carotid artery. *J Vascular Res.* **36:**272-281

Funatsu H, Yamashita H, Nama H, Mimura T, Sakata K *et al.* (2004). Risk evaluation of outcome of vitreous surgery for proliferative diabetic retinopathy based on vitreous level of vascular endothelial growth factor and angiotensin

Funatsu H, Yamashita H, Nama H, Mochizuki H, Mimura T *et al.* (2003). Outcomes of vitreous surgery and the balance between vascular endothelial growth factor and endostatin. *Invest Ophthalmol Vis Sci.* **44:**1042-1047

Galbiati F, Engelman JA, Volonte D, Zhang XL, Minetti C *et al.* (2001). Caveolin-3 null mice show a loss of caveolae, changes in the microdomain distribution of the dystrophin-glycoprotein complex, and t-tubule abnormalities. *J Biol Chem.* **276:**21425-33.

Galbiati F, Volonte D, Engelman JA, Watanabe G, Burk R *et al.* (1998). Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *EMBO J.* **17:**6633-6648

Galbiati F, Volonte D, Liu J, Capozza F, Frank PG *et al.* (2001). Caveolin-1 expression negatively regulates cell cycle progression by inducing G(0)/G(1) arrest via a p53/p21(WAF1/Cip1)-dependent mechanism. *Mol Biol Cell.* **12:**2229-2244.

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q *et al.* (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev Cell.* **3:**411-423

Galland F, Karamysheva A, Matteï MG, Rosnet O, Marchetto S *et al.* (1992). Chromosomal localisation of FLT4, a novel receptor-type tyrosine kinase gene. *Genomics.* **13**:475-478

Galis ZS, Sukhova GK, Lark MW, Libby P. (1994). Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest.* **94**:2493-2503

Gamble JR, Drew J, Trezise L, Underwood A, Parsons M *et al.* (2000). Angiopoietin-1 is an antipermeability and anti-inflammtory agent in vitro and targets cell junctions. *Circ Res.* **87**:603-607

Gao G, Li Y, Gee S, Dudley A, Fant J *et al.* (2002). Down-regulation of vascular endothelial growth factor and up-regulation of pigment epithelium-derived factor. A possible mechanism for the anti-angiogenic activity of plasminogen kringle 5*. *J Biol Chem.* **277**:9492-9497

Gao G, Li YL, Zhang D, Gee S, Crosson C. (2001). Unbalanced expression of VEGF and PEDF in ischaemia-induced retinal neovascularization. *FEBS Letts.* **489**:270-276

Gao G, Ma J. (2002). Tipping the balance for angiogenic disorders. *Drug Discov Today.* **7**:171-172

Garcia-Cardena G, Martasek P, Masters BS, Skidd PM, Couet J et al. (1997). Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *J Biol Chem.* **272**:25437-25440.

Gardiner TA, Anderson HR, Stitt AW. (2003). Inhibition of advanced glycation end-products protects against retinal capillary basement membrane expansion during long-term diabetes. *J Pathol.* **201**:328-333.

Gardiner TA, Archer DB. Endocytosis in the retinal and choroidal microcirculation.
Br J Ophthalmol. 1986 May;70(5):361-72.

Gardiner TA, Gibson DS, de Gooyer TE, de la Cruz VF, McDonald DM *et al.* (2005). Inhibition of tumor necrosis factor-alpha improves physiological angiogenesis and reduces pathological neovascularization in ischemic retinopathy. *Am J Pathol.* **166:** 637-44.

Garner A. (1994). Vascular diseases. In *Pathobiology of Ocular Disease A Dynamic Approach.* Garner A, Klintworth GK [eds]. Marcel Dekker, London. 1625-1710

Gerber HP, Coporelli F, Park J, Ferrara N. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes, Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem.* **272:**23659-23667

Gerber HP, Ferrara N. (2003). The role of VEGF in normal and neoplastic hematopoiesis. *J Mol Med.* **81:**20-31

Gerhardinger C, Brown LF, Roy S, Mizutani M, Zucker CL *et al.* (1998). Expression of vascular endothelial growth factor in the human retina and in nonproliferative diabetic retinopathy. *Am J Pathol.* **152**:1453-1462

Ghiardi GJ, Gidday JM, Roth S. (1999). The purine nucleoside adenosine in retinal ischaemia-reperfusion injury. *Vis Res.* **39**:2519-2535

Ghitescu L, Fixman A, Simionescu M, Simionescu N. (1986). Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: a receptor mediated transcytosis. *J Cell Biol.* **102:**1304-1311

Gill KA, Brindle NPJ. (2005). Angiopoietin-2 stimulates migration of endothelial progenitors and their interaction with endothelium. *Biochem Biophys Res Commun.* **336:**392-396

Gingras D, Gauthier F, Lamy S, Desrosiers RR, Beliveau R. Localization of RhoA GTPase to endothelial caveolae-enriched membrane domains. Biochem Biophys Res Commun. 1998 Jun 29;247(3):888-93.

Glaser BM, D'Amore PA, Michels RG, Patz A, Fenselau A. (1980). Demonstration of vasoproliferative activity from mammalian retina. *J Cell Biol.* **84**:298-304

Goldberg MA, Schneider TJ. (1994). Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J Biol Chem.* **269**:4355-4359

González-Clemente JM, Maurico D, Richart C, Broch M, Caixàs *et al.* (2005). Diabetic neuropathy is associated with activation of the TNF-α system in subjects with type 1 diabetes mellitus. *Clin Endocrinol.* **63:**525-529

Goetz FW, Planas JV, MacKenzie S. (2004). Tumor necrosis factors. *Devel Compar Immunol.* **28:**487-497

Grant MB, Mames RN, Fitgerald C, Ellis EA, Caballero S *et al.* (1993). Insulin-like growth factor I as an angiogenic agent: In vivo and in vitro studies. *Ann NY Acad Sci.* **692**:230-242

Grant MB, May WS, Caballero S, Brown GA, Guthrie SM *et al.* (2002). Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med.* **8:**607-612

Greene DA, Lattimer SA, Sima AA. (1987). Sorbitol, phosphoinositides, and sodium-potassium-ATPase in the pathogenesis of diabetic complications. *N Engl J Med.* **316**:599-606

Griffoni C, Spisni E, Santi S, Riccio M, Guarnieri T *et al.* (2000). Knockdown of caveolin-1 by antisense oligonucleotides impairs angiogenesis in vitro and in vivo. *Biochem Biophys Res Commun.* **276:**756-61.

Grossnklaus HE, Ling JX, Wallace TM, Dithmar S, Lawson DH *et al.* (2002). Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization. *Mol Vis.* **8:**119-126

Guan M, Pang CP, Yam HF, Cheung KF, Liu WW *et al.* Inhibition of glioma invasion by overexpression of pigment-epithelium-derived factor. *Cancer Gene Ther.* **11:**325-332

Guerrin M, Moukadiri H, Chollet P, Moro F, Dutt K *et al.* (1995). Vasculotropin/Vascular endothelial growth factor is an autocrine growth factor for human retinal pigment epithelial cells cultured in vivo. *J Cell Physiol.* **164**:385-394

Guidi AJ, Abu-Jawdeh G, Tognazzi K, Dvorak HF, Brown LF. (1996). Expression of vascular permeability factor (Vascular endothelial growth factor) and its receptors in endometrial carcinoma. *Cancer*. **78**:454-60

Gustin JA, Pincheira R, Mayo LD, Ozes ON, Kessler KM *et al.* (2004). Tumor necrosis factor activates CRE-binding protein through a p38 MAPK/MSK1 signaling pathway in endothelial cells. *Am J Physiol Cell Physiol.* **286:**C547-C555

Guto F, Guto K, Weindel K, Folkman J. (1993). Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. *Lab Invest*. **69**:508-517

Hackett SF, Wiegand S, Yancopoulos G, Campochiaro PA. (2002). Angiopoietin-2 plays an important role in retinal angiogenesis. *J Cell Physiol.* **192:**182-7.

Hagiwara Y, Sasaoka T, Araishi K, Imamura M, Yorifuji H *et al.* (2000). Caveolin-3 deficiency causes muscle degeneration in mice. *Hum Mol Genet.* **9:**3047-3054

Hainsworth DP, Katz ML, Sanders DA, Sanders DN, Wright EJ *et al.* (2002). Retinal capillary basement membrane thickening in a porcine model of diabetes mellitus. *Comp Med.* **52:**523-529.

Hammes H, Lin J, Bretzel RG, Brownlee M, Breier G. (1998). Upregulation of the vascular endothelial growth factor/vascular endothelial growth factor receptor sysyem in experimental background diabetic retinopathy of the rat. *Diabetes*. **47**:401-406

Hammes H, Lin J, Wagner P, Feng Y, vom Hagen Y *et al.* (2004). Angiopoietin-2 causes pericyte dropout in the normal retina. Evidence for involvement in diabetic retinopathy. *Diabetes.* **53:**1104-1110

Hangai M, He S, Hoffmann S, Lim JI, Ryan SJ *et al.* Hinton DR. (2006). Sequential induction of angiogenic growth factors by TNF-alpha in choroidal endothelial cells. *J Neuroimmunol.* **171:** 45-56.

Hangai M, Murata T, Miyawaki N, Spee C, Lim JI *et al.* (2001). Angiopoietin-1 upregulatiion by vascular endothelial growth factor in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* **42:**1617-1625

Hanahan D. (1997). Signaling vascular morphogenesis and maintenance. *Science.* **277**:48-50

Hannehan A, deJuan E, Lutti GA, Fox GM, Schiffer S *et al.* (1991). Altered distribution of basic fibroblast growth factor in diabetic retinopathy. *Arch Ophthalmol.* **109**:1005-1011

Harfouche R, Hassessian HM, Guo Y, Faivre V, Srikant CB *et al.* (2002). Mechanims which mediate the antiapoptopic effects of angiopoietin-1 on endothelial cells. *Microvasc Res.* **64:**135-147

Harfouche R, Gratton JP, Yancopoulos GD, Noseda M, Karsan A *et al.* (2003). Angiopoietin-1 activates both anti- and proapoptopic mitogen-activated protein kinases. *FASEB J.* **17:**1523-1525

Hashimoto T, Lam T, Boudreau NJ, Bollen AW, Lawton MT *et al.* (2001). Abnormal balance in the angiopoietin-tie2 system in human brain arteriovenous malformations. *Circ Res.* **89:**111-113

Hatva E, Kaipainen A, Mentula P, Jaaskelainen J *et al.* (1995). Expression of endothelial cell-specific receptor tyrosine kinases and growth factors in human brain tumours. *Am J Pathol.* **146**:368-378

Hattenbach L, Beck K, Pfeilschifter J, Koch F, Ohrloff C. (2005). Pigment-epithelium-derived factor is upregulated in photocoagulated human retinal pigment epithelial cells. *Ophthalmic Res.* **37:**341-346

Hauser S, Weich HA. (1993). A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors.* **9**:259-268

Hayes AJ, Huang WQ, Mallah J, Yang D, Lippman ME *et al.* (1999). Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells. *Microvasc Res.* **58:**224-237

Hayes AJ, Huang WQ, Yu J, Maisonpierre PC, Liu A *et al.* (2000). Expression and function of angiopoietin-1 in breast cancer. *Br J Cancer.* **83:**1154-1160

Hershkoviz R, Goldkorn I, Lider O. (1995). Tumour necrosis factor-alpha interacts with laminin and functions as a pro-adhesive cytokine. *Immunology.* **85:**125-130

Hewett PW, Murray JC. (1996). Coexpression of flt-1, flt-4 and KDR in freshly isolated and cultured human endothelial cells. *Biochem Biophys Res Comm.* **221**:697-702

Hirata C, Nakano K, Nakamura N, Kitagawa Y, Shigeta H *et al.* (1997). Advanced glycation end products induce expression of vascular endothelial growth factor by retinal Müller cells. *Biochem Biophys Res Comm.* **236**:712-715

Hiratsuka S, Nakamura K, Iwai S. (2002). MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell.* **2:**289-300

Hiscott P, Gray R, Grierson I, Gregor Z. (1994). Cytokeratin-containing cells in proliferative diabetic retinopathy membranes. *Br J Ophthalmol.* **78**:219-222

Hofman P, Blaauwgeers HG, Tolentino MJ, Adamis AP, Nunes Cardozo BJ *et al.* (2000). Vrensen GF, Schlingemann RO. VEGF-A induced hyperpermeability of blood-retinal barrier endothelium in vivo is predominantly associated with pinocytotic vesicular transport and not with formation of fenestrations. Vascular endothelial growth factor-A. *Curr Eye Res.* 21:637-645

Hogeboom van Buggenum IM, Polak BCP, Reichert-Theon JWM, de Vries-Knoppert WAEJ, van Hinsburgh VWM. (2002). Angiotensin converting enzyme inhibiting therapy is associated with lower vitreous vascular endothelial growth factor concentrations in patients with proiferative diabetic retinopathy. *Diabetologia.* **45:**203-209

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR *et al.* (1999a). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science.* **284:**1994-1998

Holash J, Wiegand SJ, Yancopoulos GD (1999b). New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene.* **18:**5356-5362

Holekamp NM, Bouck N, Volpert O. (2002). Pigment epithelium-derived factor is deficient in the vitreous of patients with choroidal neovascularization due to age-related macular dgeneration. *Am J Ophthalmol.* **134:**20-227

Holländer H, Makarov F, Dreher Z, van Driel D, Chan-Ling T *et al.* (1991). Functions of the macroglia of the retina: The sharing and division of labour between astrocytes and Müller cells. *J Comp Neurol.* **313**:587-603

Hoefen RJ, Berk BC. (2002). The role of MAP kinases in endothelial activation. *Vascul Pharmacol.* **38:**271-273

Hollenberg SM, Cunnion RE, Parrillo JE. (1991). The effect of tumor necrosis factor on vascular smooth muscle. *Chest.* **100:**1133-1137

Hori S, Ohtsuki S, Hosoya K, Nakashima E, Terasaki T. (2004). A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. *J Neurochem.* **89:**503-13.

Houck KA, Ferrara N, Winer J. (1991). The vascular endothelial growth factor family: four molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol.* **5**:1806-14

Houenou LJ, D'Costa AP, Li L, Turgeon VL, Enyadike C *et al.* (1999). Pigment epithelium-derived factor promotes the survival and differentiation of developing spinal motor neurons. *J Comp Neurol.* **412:**506-514

Huang I, Turck CW, Rao P, Peters KG. (1995). *Oncogene.* **11**:2097-2103

Hui YN, Goodnight R, Zhang XJ, Sorgente N, Ryan SJ. (1988). Glial epiretinal membranes and contraction. Immunohistochemical and morphological studies. *Arch Ophthalmol.* **106:**1280-1285.

Hulit J, Bash T, Fu M, Galbiati F, Albanese C *et al.* (2000). The cyclin D1 gene is transcriptionally repressed by caveolin-1. *J Biol Chem.* **275:**21203-9

Hunt JV, Smith CC, Wolff SP. (1990). Autooxidative glygosylation and possible involvement of peroxidases and free radicals in LDL modification by glucose. *Diabetes.***39**:1420-1424

Hutchings H, Ortega N, Plouet J. (2003). Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration and survival through integrin ligation. *FASEB J.* **17:**1520-1522

Idriss HT, Naismith JH. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech.* **50:**184-195

Igarashi K, Isohara T, Kato T, Shigeta K, Yamano T *et al.* (1998). Tyrosine 1213 of Flt-1 is a major binding site of Nck and SHP-2. *Biochem Biophys Res comm.* **246**:95-99

Iizasa H, Bse SH, Asashima T, Kitano T, Matsunaga N *et al.* (2002). Augmented expression of the tight junction protein occluding in brain endothelial cell line TR-BBB by rat angiopoietin-1 expressed in baculovirus-infected Sf plus insect cells. *Pharm Res.* **19:**1757-1760

Infeld D.A. and O'Shea J.G. (1998). Diabetic retinopathy. *Postgrad Med J.* **74**:129-133

Inoue M, Hager JH, Ferrara N, Gerber HP, Hanahan D. (2002). VEGF-Ahas a critical, non-redundant role in angiogenic switching and pancreatic beta cell carcinogenesis. *Cancer Cell.* **1:**193-202

Ishida S, Shinoda K, Kawashima S, Oguchi Y, Okada Y *et al.* (2000). Coexpression of VEGF receptors VEGF-R2 and neuropilin-1 in proliferative diabetic retinopathy. *Invest Ophthalmol Vis Sci.* **41:**1649-1656

Ishida S, Usui T, Yamashiro K, Kaji Y, Ahmed E. (2003). $VEGF_{164}$ is proinflammatory in the diabetic retina. *Invest Ophthalmol Vis Sci.***44:**2155-2162

Ishihama H, Ohbayashi M, Kurosawa N, Kitsukawa T, Matsuura O *et al.* (2001). Colocalisation of neuropilin-1 and Flk-1 in retinal neovascularization in a mouse model of retinopathy. *Invest Ophthalmol Vis Sci.* **42:**1172-1178

Ishizaki E, Takai S, Ueki M, Maeno T, Maruichi M *et al.* (2006). Correlatio between angiotensis-converting enzyme, vascular endothelial growth factor, and matrix metalloproteinase-9 in the vitreous of eyes with diabetic retinopathy. *Am J Ophthalmol.* **141:**129-134

Itabashi H, Ohneda A, Iimura Y. (1981). Thickening of basement membrane of muscle capillary in spontaneously diabetic KK mice.*Tohoku J Exp Med.* **133:**339-348.

Iwama A, Hamaguchi I, Hashiyama M, Murayama Y *et al.* (1993). Molecular cloning and characterization of mouse *tie* and *tek* receptor tyrosine kinase genes and their expression in haematopoietic stem cells. *Biochem Biophys Res Comm.* **195**:301-309

Ikezu T, Ueda H, Trapp BD, Nishiyama K, Sha JF *et al.* (1998). Affinity-purification and characterization of caveolins from the brain: differential expression of caveolin-1, -2, and -3 in brain endothelial and astroglial cell types. *Brain Res.* **804:**177-192

Jablonski MM, Tombran-Tink J, Mrazek DA, Iannaccone A. (2000). Pigment epithelium-derived factor supports normal development of photoreceptor neurons and opsin expression after retinal pigment epithelium removal. *J Neurosci.***20:**7149-7157

Jablonski MM, Tombran-Tink J, Mrazek DA, Iannaccone A . (2001). Pigment

Pigment epithelium-derived factor supports normal Müller cell development and glutamine synthetase expression after removal of the retinal pigment epithelium removal. *Glia.* **35:**14-25

Jackson RL, Esterly JA, Guthrie RA, Hewett JE, Waiches HB. (1982). Capillary basement membrane changes in adolescents with type 1 diabetes. *JAMA.* **248:**2143-2147

Janzer RC, Raff MC. (1987). Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature*. **325**:253-257

Jawa A, Kcomt J, Fonseca VA. (2004). Diabetic nephropathy and retinopathy. *Med Clin North Am.* **88:**1001-1036

Jaye M, Howk R, Burgess W. (1986). Human endothelial cell growth factor: cloning nucleotide sequence, chromosome localization. *Science.* **233**:541-545

Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M *et al.* (1997). Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science.* **276:**1423-1425

Jiang B, Liou GI, Behzadian MA, Caldwell RB. (1994). Astrocytes modulate retinal vasculogenesis: effects on fibronectin expression. *J Cell Sci.* **107**:2499-2508

Jingjing L, Xue Y, Agarwal N, Roque RS. (1999). Human Müller cells express VEGF183, a novel spliced variant of vascular endothelial growth factor. *Invest Opthalmol Vis Sci.* **40**:752-759

Joh D, Mehta D, Ahmmed G, Gao X, Tiruppathi C *et al.* (2005). Angiopoietin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca^2+ influx. *Circ Res.* **96:**1282-1290

Joh D, Mehta D, Ahmmed G, Gao X, Tiruppathi C *et al.* (2005). Angiopoietin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca^{2+} influx. *Circ Res.* **96:**1282-1290

Johnson PC. (1983). Thickening of the human dorsal root ganglion perineurial cell basement membrane in diabetes mellitus. *Muscle Nerve.* **6:**561-565.

Joo HJ, Oh DK, Kim YS, Lee KB, Kim SJ. (2004). Increased expression of caveolin-1 and microvessel density correlates with metastasis and poor prognosis in clear cell renal cell carcinoma. *BJU Int.* **93:**291-296

Joukov V, Pajusola K, Kaipainan A, Chilov D, Lahtinen I *et al.* (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J.* **15**:290-298

Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L *et al.* (1997). Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J.* 16:3898-3911.

Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE *et al.* (2001). Leukocyte-ediated endothelial cell injury and death in the diabetic retina. *Am J Pathol.* **158:** 147-152

Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C *et al.* (2004). A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J.* **18:**1450-1452.

Joussen AM, Poulaki V, Mitsiades N, Kirchhof B, Koizumi K *et al.* (2002c). Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. *FASEB J.* **16:**438-440

Joussen AM, Poulaki A, Tsujikawa W, Qin T, Quam Q *et al.* (2002a). Suppression of diabetic retinopathy with angiopoietin-1. *Am J Pathol.* **160:**1683-1693

Joussen AM, Poulaki V, Qin W, Kirchhof B, Mitsiades N *et al.* (2002b). Retinal vascular endothelial growth factor induces intercellular adhesion molecule-1 and endothelial nitric oxide synthase expression and initiates early diabetic retinal leukocyte adhesion in vivo. *Am J Pathol.* **160:**501-9.

Jussila L, Alitalo K. (2002). Vascular growth factors and lymphangiogenesis. *Physiol Rev.* **82:**673-700

Kachi S, Yamazaki A, Usukura J. (2001). Localization of caveolin-1 in photpreceptor synaptic ribbons. *Invest Ophthalmol Vis Sci.* **42:**850-852

Kaipainen A, Korhonen J, Mustonen T, van Hinsberg G, Fang D *et al.* (1994a). Expression of the FLT-4 receptor tyrosine kinase becomes restricted to endothelium of lymphatic vessels and some high endothelial venules during development. *Proc Natl Acad Sci USA*. **92**:3566-3570

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH *et al.* (1995). Expression of the fms-like tyrosine kinase FLT4 gene becomes restricted to endothelium of lymphatic vessels during development. *Proc Natl Acad Sci USA.* **92:**3566-3570.

Kaipainen A, Korhonen J, Pajusola K, Aprelikova O, Persico MG *et al.* (1993). The related FLT4, Flt1 and KDR receptor tyrosine kinases show distinct expression patterns in human foetal endothelial cells. *J Exp Med.* **178**:2077-2088

Kaipainen A, Vlaykova T, Hatva E, Bohling T *et al.* (1994b). Enhanced expression of the tie receptor tyrosine kinase messenger RNA in the vascular endothelium of metastatic melanomas. *Cancer Res*. **54**:6571-6577

Kakehashi A, Saito Y, Mori K, Sugi N, Ono R *et al.* (2006). Characteristics of diabetic retinopathy in SDT rats. *Diabetes Metab Res Rev.* **22:**455-461

Kanno S, Oda N, Abe M. (2000). Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. *Oncogene.* **19:**2138-2146

Karakousis PC, John SK, Behling KC, Surace EM, Smith JE. (2001). Localization of pigment epithelium derived factor (PEDF) in developing and adult human ocular tissues. *ol Vis.* **7:**154-163

Karayiannakis AJ, Syrigos KN, Polychronidis A, Pitiakoudis M, Bounovas A *et al.* (2001). Serum levels of tumor necrosis factor-alpha and nutritional status in pancreatic cancer patients. *Anticancer Res.* **21:**1355-1358

Karkainnen MJ, Ferrell RE, Lawrence EC, Kimak MA. Levinson KL (2000). Missence mutations interfere with VEGFR-3 signalling in primary lymphodema. *Nat Genet.* **25:**153-159

Karkainnen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC *et al.* (2001). A model for gene therapy of human hereditary lymphedema. *Proc natl Acad Sci USA.* **98:**12677-12682

Kaur C, Sivakumar V, Foulds WS. (2006). Early response of neurons and glial cells to hypoxia in the retina. *Invest Ophthalmol Vis Sci.* **47:**1126-1141

Kawamura S, Miyamoto S, Brown JH. (2003). Initiation and transduction of stretch-induced RhoA and Rac1 activation through caveolae: cytoskeletal regulation of ERK translocation. (2003). *Biol Chem.* **278:**31111-31117.

Kayisli UA, Cayli S, Seval Y, Tertemiz F, Huppertz B *et al.* (2006). Spatial and temporal distribution of Tie-1 and Tie-2 during very early development of the human placenta. *Placenta.* **27:**648-659

Kazlauskas A, Kashishian A, Cooper JA, Valius M. (1992). GTPase-activating protein and phosphatidylinositol-3-kinase bind to distinct regions of the platelet-derived growth factor receptor beta subunit. *Mol Cell Biol.* **12**:2534-44

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T *et al.* (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science.* **246**:1309-1312

Kendall RL, Wang G, Thomas KA. (1996). Identification of a natural soluble form of the vascular endothelial growth factor receptor, Flt-1, and its heterodimerization with KDR. *Biochem Biophys Res Comm.* **226**:324-328

Kerkar S, Williams M, Blocksom JM, Wilson RF, Tyburski G *et al.* (2006). TNF alpha and IL-1beta increase pericyte/endothelial cell co-culture permeability. *J Surg Res.* **132:**40-45

Kevil CG, Payne DK, Mire E, Alexander JS. (1998). Vascular permeability factor/ vascular endothelial cell growth factor-mediated permeability occurs through dosorganization of endothelial junctional proteins. *J Biol Chem.* **273:**15099-15103

Kilo C, Vogler N, Williamson JR. (1972). Muscle capillary basement membrane changes related to aging and to diabetes mellitus. *Diabetes.* **21:**881-905

Kim YN, Dam P, Bertics PJ. (2002b). Caveolin-1 phosphorylation in human squamous and epidermoid carcinoma cells: dependence on ErbB1 expression and Src activation. *Exp Cell Res.* **280:**134-147

Kim SY, Johnson MA, McLeod DS, Alexander T, Otsuji T *et al.* (2004). Retinopathy in monkeys with spontaneous type 2 diabetes. *Invest Ophthalmol Vis Sci.* 45:4543-4553

Kim I, Kim HG, Moon SO, Chae SW, So JN et al. (2000b). Angiopoietin-1 regulates endothelial survival through the phosphayidylinositol 3'-kinase/Akt signal transduction pathway. *Cir Res.* **86:**24-29

Kim I, Kim HG, So JN, Kim JH, Kwak HJ *et al.* (2000a). Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway. *Circ Res.* **86:**24-29

Kim I, Kim J, Ryu Y, Liu M, Koh G. (2000d). Tumor necrosis factor-α upregulates angiopietin-2 in human umbilical vein endothelial cells. *Biochem Biophys Res Commun.* **269:**361-365

Kim I, Kim JH, Ryu YS, Jung SH, Nah JJ *et al.* (2000c). Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway. *Oncogene.* **19:**4549-4552

Kim I, Kwak J, Ahn J, So J *et al.* (1999a). Molecular cloning and caracterization of a novel angiopoietin family protein, angiopoietin-3. *FEBS Letters.* **443**:353-356

Kim H, Lee T, Lee J, Ahn M, Moon C *et al.* (2006). Immunohistochemical study of caveolin-1 and -2 in the rat retina. *J Vet Sci.* **7:**101-104.

Kim I, Moon SO, Park SK, Chae SW, Koh GY. (2001). Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial clls by reducing ICAM-1, VCAM-1, and E-selectin expression. *Circ Res.* **89:**477-479

Kim I, Oh JL, Ryu YS, SO JN, Sessa WC *et al.* (2002a). Angiopoietin-1 negatively regulates expression and activity of tissue factor in endothelial cells. *FASEB J.* **16:**126-128

Kim I, Ryan R, Rohan S, Amano S, Agular S *et al.* (1999b). Constitutive expression of VEGF, VEGFR-1, and VEGFR-2 in normal eyes. *Invest Ophthalmol Vis Sci.* **40:**2115-2121

Kim K, Wass C, Cross A, Opal S. (1992). Modulation of blood-brain barrier permeability by tumor necrosis factor and antibody to tumor necrosis in the rat. *Lymphokine Cytokine Res.* **11:**293-298

King GL, Kiyoshi S. (2000). Pigment epithelium-derived factor: a key coordinator of retinal neuronal and vascular functions. *N Engl J Med.* **342:**349-351

Kinnunen K, Korpisalo P, Rissanen TT, Heikura T, Viita H *et al.* (2006). Overexpression of VEGF-A induces neovascularization and increased vascular leakage in rabbit eye after intravitreal adenoviral gene transfer. *Acta Physiol.* **187:**447-457

Klein HA, Moorehead HB (1970). Statistics on blindness in the model reporting area, 1969-1970. Betheseda. US Dept Health, Education and Welfare

Klein R, Klein BEK. (1985). Vision disorders in diabetes. In *National diabetes data group: diabetes in America;* Diabetes data compiled 1984. NIH Publ No 85-1468. Bethseda. US Dept Health and Human Services: 1-2

Klein R, Klein BE, Moss SE, Davis MD, DeMets DL. (1984a). The Wisconsin epidemiologic study of diabetic retinopathy. II. Prevalence and risk of diabetic retinopathy when age at diagnosis is less than 30 years. *Arch Ophthalmol.* **102:**520-526.

Klein R, Klein BE, Moss SE, Davis MD, DeMets DL. (1984b). The Wisconsin epidemiologic study of diabetic retinopathy. III. Prevalence and risk of diabetic retinopathy when age at diagnosis is 30 or more years. *Arch Ophthalmol.* **102:**527-532.

Klein R, Meuer SM, Moss SE, Klein BE. (1995). Retinal microaneurysm counts and 10-year progression of diabetic retinopathy. *Arch Ophthalmol.* **113:**1386-1391.

Koblizek TI, Weiss C, Yancopoulas GD, Deutsch U, Risau W. (1998). Angiopoietin-1 induces sprouting angiogenesis *in vitro.Curr Biol.* **8**:529-532

Koch E, Harlow I, Haines GK, Amento EP, Unemori EN *et al.* (1994). Vascular endothelial growth factor: a cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol.* **152**:4149-4155

Kociok N, Radetzky S, Krohne TU, Gavranic C, Joussen AM. (2006). Pathological but not physiological retinal neovascularization is altered in TNF-Rp55-receptor-deficient mice. *Invest Ophthalmol Vis Sci.* **7:**5057-5065

Kogo H, Ito S, Moritoki Y, Kurahashi H, Fujimoto T. (2006). Differential expression of caveolin-3 in mouse smooth muscle cells in vivo. *Cell Tiss Res.* **324:**291-300

Kohn S, Nagy JA, Dvorak HF, Dvorak AM. (1992). Pathways of macromolecular tracer transport across venules and small veins. Structural basis for the hyperpermeability of tumour blood vessels. *Lab Invest.* **67**:596-607

Kohner EM. (1993). Diabetic retinopathy. *BMJ.* **307**:1195-9

Kohner EM, Sleightholm M. (1986). Does microaneurysm count reflect severity of early diabetic retinopathy? *Ophthalmol.* **93:**586-589.

Kolch W, Martiny-Baron G, Keiser A, Marmé. (1995). Regulation of the expression of the VEGF/VPS and its receptors: role in tumour angiogenesis. *Breast Cancer Res and Treatment.* **36**:139-155

Korff T, Kimmina S, Martiny-Baron G, Augustin HG. (2001). Blood vessel maturation in a 3-dimensional spheroid coculture model; direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J.* **15:**447-457

Korhonen J, Partanen J, Armstrong E, Vaahtokari A, Elenius K *et al.* (1992). Enhanced expression of the *tie* receptor tyrosine kinase in endothelial cells during neovascularization. *Blood.* **80**:2548-2555

Korhonen J, Polvi A, Partanen J, Alitalo K. (1994). The mouse tie receptor tyrosine kinase gene: expression during embryonic angiogenesis. *Oncogene.* **9**:395-403

Korpelainen EI, Karkkainen M, Gunji Y, Vikkula M *et al.* 1999. Endothelial receptor tyrosine kinases activate the STAT signaling pathway: mutant Tie-2 causing venous malformations signals a distinct STAT activation response. *Oncogene.* **18**:1-8

Kosana H, Okano T, Katsura Y, Noritake M, Kado S *et al.* (1999). ProMMP-9 (92 kDa Gelatinase) in vitreous fluid of patients with proliferative diabetic retinopathy. *Life Sciences.* **64**:2307-2315

Krady JK, Basau A, Allen CM, Xu Y, LaNoue KF *et al.* (2005). Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy. *Diabetes.* **54:**1559-1565

Kroll J, Waltenberger J. (1997). The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. *J Biol Chem.* **272**:32521-325-27

Kroll J, Waltenberger J. (1998). VEGF-A induces expression of eNOS and iNOS in endothelial cells through VEGF receptor-2 (KDR). *Biochem Biophys Res Commun.* **252:**743-746

Kroon ME, Koolwijk P, van Goor H, Weidle UH, Collen A *et al.* (1999). Role and localization of urokinase receptor in the formation of new microvascular structures in fibrin matrices. *Am J Pathol.* **154:**1731-1742

Kubo A, Nishitani Y, Minamino N, Kikumoto K, Kurioka H *et al.* (2000). Adrenomedullin gene transcription is decreased in peripheral blood mononuclear cells of patients with IgA nephropathy. *Nephron.* **85:**201-206.

Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V, Alitalo K. (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Dev.* **122**:3829-3837

Kukk E, Waritvaara U, Gunji Y, Kaukonen J *et al.* (1997). Analysis of Tie receptor tyrosine kinase in haemopoietic progenitor and leukaemia cells. *Br J Haem.* **98**:195-203

Kuncl RW, Bilak MM, Bilak SR, Corse AM, Royal W *et al.* (2002). Pigment epithelium-derived factor is elevated in CSF of patients with amyotrophic lateral sclerosis. *J Neurochem.* **81:**178-184

Kunz Mathews M, Merges C, Mcleod DS, Lutty GA. (1997). Vascular endothelial growth factor and vascular permeability changes in human diabetic retinopathy. *Invest Ophthalmol Vis Sci.* **38**:2729-2741

Kutty RK, Kutty G, Hooks JJ, Wiggert B, Naginneni CN. (1995). Transforming growth factor beta inhibits the cytokine-mediated expression of the inducible nitric oxide synthase mRNA in human retinal pigment epithelial cells. *Biochem Biophys Res Commun.* **215:**386-393

Kwak HJ, Lee SJ, Lee YH, Ryu CH, Koh KN *et al.* (2000). Angiopoietin-1 inhibits irradiation- and mannitol-induced apoptosis in endothelial cells. *Circ.* **101:**2317-2324

Kwak HJ, So JN, LeeSJ, Kim I, Koh GY. (1999). Angiopoietin-1 is an apoptosis survival factor for endothelial cells. *FEBS Lett.* **448:**249-253

Labrecque L, Royal I, Surprenant DS, Patterson C, Gingras D *et al.* (2003). Beliveau R. Regulation of vascular endothelial growth factor receptor-2 activity by caveolin-1 and plasma membrane cholesterol. *Mol Biol Cell.* **14:**334-347.

Ladoux A, Frelin C. (1993a). Expression of vascular endothelial growth factor by cultured endothelial cells from brain microvessels. *Biochem Biophys Res Commun.* **194**:799-803

Ladoux A, Frelin C. (1993b). Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart. *Biochem Biophys Res Commun.* **195**:1005-1010

Lahat N, Rahat MA, Ballan M, Weiss-Cerem L, Engelmayer M *et al.* (2003). Hypoxia reduces CD80 expression on monocytes but enhances their LPS-stimulated TNF-α secretion. *J Leukoc Biol.* **74:**1360-1367

Lahtinen U, Honsho M, Parton RG, Simons K, Verkade P. (2003). Involvement of caveolin-2 in caveolar biogenesis in MDCK cells. *FEBS Lett.* **538:**85-88

Lai CM, Dunlop SA, May LA, Gorbatov M, Brankov M *et al.* (2005). Generation of transgenic mice with mild and severe retinal neovascularisation. *Br J Ophthalmol. 89:*911-916.

Larkins RG, Dunlop ME. (1992). The link between hyperglycaemia and diabetic nephropathy. *Diabetologica.* **35**:499-504

Laterra J, Guerrin C, Goldstein E. (1990). Astrocytes induce neural microvascular endothelial cells to form capillary-like structures *in vitro*. *J Cell Physiol.* **144**:204-215

Laurén J, Gunji Y, Alitalo K. (1998). Is Angiopoietin-2 necessary for the initiation of tumour angiogenesis. *Am J Path.* **153**:13331339

Lahat N, Rahat MA, Ballan M, Weiss-Cerem L, Engelmayer M *et al.* (2003). Hypoxia reduces CD80 expression on monocytes but enhances their LPS-stimulated TNF-α secretion. *J Leukoc Biol.* **74**: 197-205

Lee TS, Saltsman KA, Ohashi H, King GL. (1989). Activation of protein kinase C by elevation of glucose concentration: proposal for a mechanism in the development of diabetic vascular complications. *Proc Natl Acad Sci USA*. **86**:5141-5145

Leek RD, Landers R, Fox SB, Ng F, Harris AL *et al.* (1998). Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. *Br J Cancer.* **77**:2246-2251

Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V *et al.* (1987). Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. *Nature.* **329**:630-632

Lemieux C, Maliba R, Favier J, Theoret J, Merhi Y *et al.* (2005). Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflmmatory responses. *Blood.* **105**:1523-1530

Leong K, Karsan A. (2000). Signaling pathways mediated by tumor necrosis factor alpha. *Histol Histopathol.* **15**:1303-1325

LeRoith D, Roberts CT Jr. (1993). Insulin-like growth factors. *Ann NY Acad Sci.* **692**:1-9

Leung DW, Cachianes G, Kuang W, Goddel DV, Ferrara N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science.* **246**:1306-1312

Leung SY, Chan ASY, Wong MP, Yuen ST, Chung LP (1997). Expression of vascular endothelial growth factor and its receptors in pilocytic astrocytoma. *Am J Surg Path.* **21**:941-50

Levy AP, Levy NS, Wegner S, Goldberg MA. (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem.* 270:13333-3340

Levy AP, Tamargo R, Brem H, Nathans D. (1989). An endothelial growth factor from the mouse neuroblastoma cell line NB41. *Growth Factors.* **2**:9-19

Lewen RM. (1988). Subretinal neovascularization complicating laser photocoagulation of diabetic maculopathy. *Ophthalmic Surg.* **10**:734-737.

Li H, Tran VV, Hu Y, Saltzman WM, Barnstable CJ *et al.* (2006). A PEDF N-terminal peptide protects the retina from ischemic injury when delivered in PLGA nanospheres. *Exp Eye Res.* **83:**824-833

Li L, Renier G. (2006). Activation of nicotinamide adenine dinucleotide phosphate (reduced form) oxidase by advanced glycation end products links oxidative stress to altered vascular endothelial growth factor expression. *Metabolism.* **55:**1516-1523

Li S, Galbiati F, Volonte D, Sargiacomo M, Engelman JA *et al.* (1998). Mutational analayis of caveolin-induced vesicle formation. Expression of caveolin-1 recruits caveolin-2 to caveolae membranes. *FEBS Lett.* **434:**127-134

Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE *et al.* (1995). Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *J Biol Chem.* **270:** 15693-15701

Li X, Hahn CN, Parsons M, Drew J, Vadas MA *et al.* (2004). Role of protein kinase Czeta in thrombin-induced endothelial permeability changes: inhibition by angiopoietin-1. *Blood.* **104:**1716-1724

Lim H, Lip GYH, Blann AD. (2005). Angiopoietin-1 and angiopoietin-2 in diabetes mellitus: relationship to VEGF, glycaemic control, endothelial damage/dysfunction and atherosclerosis. *Atheroscl.* **180:**113-118

Limb GA, Alam E, Earley O, Chignell AH, Dumonde DC. (1994). Distribution of cytokine proteins within epiretinal membranes in proliferative vitreoretinopathy. *Curr Eye Res.* **13:**791-798

Limb GA, Chignell AH, Green W, LeRoy F, Dumonde DC. (1996). Distribution of TNFα and vascular adhesion molecules in fibrocellular membranes of proliferative diabetic retinopathy. *Br J Ophthalmol.* **80:**168-173

Limb GA, Hollifield RD, Webster L, Charteris DG, Chignell AH. (2001).Soluble TNF receptors in vitreoretinal proliferative disease. *Invest Ophthalmol Vis Sci.* **42:**1586-1591

Limb GA, Little BC, Meager A, Ogilvie JA, Wolstencroft RA *et al.* (1991). Cytokines in proliferative vitreoretinopathy. *Eye.* **5**:686-693

Lin D, Zhou J, Zelenka PS, Takemoto DJ. (2003). Protein kinase Cgamma regulation of gap junction activity through caveolin-1-containing lipid rafts. *Invest Ophthalmol Vis Sci.* **44**:5259-5268.

Lip P, Belgore F, Blann AD, Hope-Ross M, Gibson JM *et al.* (2000). Plasma VEGF and soluble VEGF receptor FLT-1 in proliferative retinopathy: relationship to endothelial dysfunction and laser treatment. *Invest Ophthalmol Vis Sci.* **41**:2115-2119

Lip PL, Chatterjee S, Caine GJ, Hope-Ross M, Gibson J *et al.* (2004). Plasma vascular endothelial growth factor, angiopoietin-2, and soluble angiopoietin receptor tie-2 in diabetic retinopathy: effects of laser photocoagulation and angiotensin receptor blockade. *Br J Ophthalmol.* 88:1543-1546.

Lisanti MP, Scherer PE, Tang Z, Sargiacomo M. (1994). Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *Trends Cell Biol.* **4**:231-5.

Liu P, Anderson RG. Compartmentalized production of ceramide at the cell surface. (1995). *J Biol Chem.* **270**:27179-27185.

Liu J, Razani B, Tang S, Terman BI, Ware JA *et al.* (1999). Angiogenesis activators and inhibitors differentially regulate caveolin-1 expression and caveolae formation in vascular endothelial cells. Angiogenesis inhibitors block vascular endothelial growth factor-induced down-regulation of caveolin-1. *J Biol Chem.***274**:15781-15785.

Liu H, Ren J, Cooper WL, Hawkins CE, Cowan MR. (2004). Identification of the antivasopermeability effect of pigment epithelium-derived factor and its active site. *Proc Natl Acad Sci USA.* **101**:6605-6610

Liu P, Rudick M, Anderson RG. (2002). Multiple functions of caveolin-1. *J Biol Chem.*277:41295-41298

Liu P, Ying Y, Anderson RG. (1997). Platelet-derived growth factor activates mitogen-activated protein kinase in isolated caveolae. *Proc Natl Acad Sci U S A.* **94:**13666-13670

Liu P, Ying Y, Ko YG, Anderson RG. (1996). Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. *J Biol Chem.* **271:**10299-303.

Lo WK, Zhang W. (1989). Endocytosis of macromolecules in the lenses of guinea pig and rabbit. *Lens Eye Toxic Res.* **6:**603-612.

Lo WK, Zhou CJ, Reddan J. (2004). Identification of caveolae and their signature proteins caveolin 1 and 2 in the lens. *Exp Eye Res.* **79:**487-498.

Lobov IB, Brooks PC, Lang RA. (2002). Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. *PNAS.* **99:**11205-11210

Loike JD, Brett L, Cao J, Ogawa S, Silverstein SC *et al.* (1994). Hypoxia induces glucose transporter expression in endothelial cells. *Am J Physiol.* **263**:C326-333

Loughna S, Sato TN. (2001). Angiopoietin and Tie signaling pathways in vascular development. *Matrix Biol.* **20:**319-325

Lu M, Kuroki M, Amano S, Tolentino M, Keough K, Kim I *et al.* (1998). Advanced glycation end products increase retinal vascular endothelial growth factor expression. *J Clin Invest.* **101**:1219-1224

Luez I, Creanier L, Audigier S, Gensac MC, Prats AC *et al.* (1998). Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. *Mol Cell Biol.* **18:**6178-90

Luckman SP, Hughes DE, Coxon DE, Graham FP, Russell G *et al.* (1998). *J Bone Miner Res.* **13:**581-589

Luna JD, Chan CC, Derevjanik NL, Mahlow J, Chiu C *et al.* (1997). Blood-retinal barrier (BRB) breakdown in experimental autoimmune uveoretinitis:comparison with vascular endothelial growth factor, tumor necrosis factor alpha, and interleukin-1 beta-mediated breakdown. *J Neurosci.* **49:**268-280

Lundbaek K, Christensen NJ, Jensen NJ. (1970). Diabetes, diabetic angiopathy and growth hormone. *Lancet.* **2**:131-133

Luo JC, Yamaguchi S, Shinkai A, Shitara K, Shibuya M. (1998). Significant expression of vascular endothelial growth factor/vascular permeability factor in ascites tumors. *Cancer Res.* **58:**2652-2660

Lutty GA, Cao J, Mcleod DS. (1997). Relationship of polymorphonuclear leukocytes to capillary dropout in the human diabetic choroids. *Am J Pathol.* **151:**707-714

Lutty GA, Mathews MK, Merges C, Mcleod DS. (1998). Adenosine stimulates canine retinal microvascular endothelial cell migration and tube formation. *Curr Eye Res.* **17**:594-607

Lutty GA, McLeod DS, Merges C, Diggs A, Plouët J. (1996). Localization of vascular endothelial growth factor in human retina and choroid. *Arch Ophthalmol.* **114**:971-977

Lyden D, Hattori K, Dias S, Costa C, Blaikie P *et al.* (2001). Impaired recruitment of bone-marrow-derived endothelial and haematopietic precursosr clls blocks tumor angiogenesis and growth. *Nat Med.* **7:**1194-1201

Lymboussaki A, Partanen TA, Olofsson B, Thomas-Crusells J *et al.* (1998). Expression of the vascular endothelial growth factor C receptor VEGFR-3 in lymphatic endothelium of the skin and in vascular tumors. *Am J Pathol.* **153**:395-403

Lynch JJ, Ferro TJ, Blumenstock FA, Brockenauer AM, Malik AB. (1990). Increased endothelial albumin permeability mediated by protein kinase C activation. *J Clin Invest.* **85**:991-998

Madan A, Curtin PT. (1993). A 24-base pair sequence 3′ to the human erythropoietin contains a hypoxia-responsive transcriptional enhancer. *Proc Natl Acad Sci USA*. **90**:3928-3932

Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG. (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci USA*. **88**:9267-9271

Maisonpierre PC, Suri C, Jones PF, Bartunkova S *et al.* (1997). Angiopoietin-2, a natural antagonist of tie2 that disrupts in vivo angiogenesis. *Science*. **277**:55-60

Majka S, McGuire PG, Das A. (2002). Regulation of matrix metalloproteinase expression by tumour necrosis factor in a murine model of retinal neovascularization. *Invest Ophthalmol Vis Sci.* **43:**260-266

Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI *et al.* (2001). Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF-receptor-3. *Nat Med.* **7:199-205**

Malecaze F, Clamens S, Simorre-Pinatel V, Mathis A, Chollet P *et al.* (1994). Detection of vascular endothelial growth factor messenger RNA and vascular endothelial growth factor-like activity in proliferative diabetic retinopathy. *Arch Ophthalmol.* **112**:1476-1482

Mandriota SJ, Seghezzi G, Vassalli J, Ferrara N, Wasi S *et al.* (1995). Vascular endothelial growth factor increases urokinase receptor expression in vascular endothelial cells. *J Biol Chem*. **270**:9709-9716

Mamputu G, Renier G. (2002). Advanced glycation end products, through a protein kinase C-dependent pathway, vascular endothelial growth factor expression in endothelial cells inhibitory effect of gliclazide. *J Diabetes Complic.* **16:**284-293

Mandriota SJ, Pepper MS. (1998). Regulation of Angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ Res.* **83**:852-859

Marconcini L, Marchio S, Morbidelli L, Cartocci E, Albini A *et al.* (1999). c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro. *Proc Natl Acad Sci USA*. **96:**9671-9676

Marion MS, Carlson EC. (1989). Ultrastructural analyses of acellular glomerular basement membranes and mesangial matrix in a spontaneously diabetic rhesus monkey. *Acta Anat (Basel).* **135:**119-128.

Mark K, Miller D. (1999). Increased permeability of primary cultured brain microvesssel endothelial cell monolayres following TNF-α exposure. *Life Sci.* **64:**1941-1953

Mark K, Trickler W, Miller D. (2001). Tumor necrosis factor-α induces cyclooxygenase-2 expression and prostaglandin release in brain microvessel endothelial cells. *J Pharmacol Exp Ther.* **297:**1051-1058

Martin RE, Elliott MH, Brush RS, Anderson RE. (2005). Detailed characterization of the lipid composition of detergent-resistant membranes from photoreceptor rod outer segment membranes. *Invest Ophthalmol Vis Sci.* **46:**1147-1154.

Maruo N, Morita I, Shirao M, Murota S. (1992). IL-6 increases endothelial permeability in vitro. *Endocrinology.* **131:**1710-1714

Matsumoto K, Ishikawa H, Nishimura D, Hamasaki K, Nakoa K *et al.* (2004). Antiangiogenic property of pigment epithelium-derived factor in hepatocellular carcinoma. *Hepatology.* **40:**252-259

Matsuoka M, Ogata N, Otsuji T, Nishimura T, Takehashi K *et al.* (2004). Expression of pigment epithelium derived factor and vascular endothelial growth factor in choroidal neovascular membranes and polypoidal choroidal vasculopathy. *Br J Ophthalmol.* **88:**809-815

Mattei M, Borg J, Rosnet O, Marmé D, Birnbaum D. (1995). Assignment of vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) genes to human chromosome 6p12-p21 and 14q24-q31 regions, respectively. *Genomics.* **32:**168-169

Matthews W, Jordan CT, Jenkins GM, Copeland NG, Lemischka IR. (1991). A receptor tyrosine kinase cDNA isolated from a population of enriched primitive haematopoietic cells and exhibiting close genetic linkage to c-kit. *Proc Natl Acad Sci USA*. **88**:9026-9030

Mayhan WG. (2002). Cellular mechanisms by which tumor necrosis factor-α produces disruption of the blood-brain barrier. *Brain Res*. **927:**144-152

McKay A. (1993). Types of growth factor activity: detection and characterization of new growth factor activities. In *Growth factors: a practical approach.* I McKay, I Leigh [eds] Oxford University Press, Oxford. pp 1-11

Meldrum DR. (1998a). Tumor necrosis factor in the heart. *Am J Physiol.* **274:**R577-R595

Meldrum DR, Meng X, Dinarello CA, Ayala A, Cain BS *et al.* (1998b). Human myocardial tissue TNFalpha expression following acute global ischemia in vivo. *J Mol Cell Cardiol.* **30:**1683-1689

Menon C, Ghartey A, Canter R, Fieldman M, Fraker DL. (2006). Tumour necrosis factor-alpha damages tumor blood vessel integrity by targeting VE-Cadherin. *Ann Surg.* **244:**781-791

Merrill JE. (1992). Tumor necrosis factor α, interleukin 1 and related cytokines in brain development: normal and pathological. *Dev Neurosci.* **14:**1-10

Metheny-Barlow LJ, Tian S, Hayes AJ, Li L. (2004). Direct chemotactic action of angiopoietin-1 on mesenchymal cells in the presence of VEGF. *Microvasc Res.* **68:**221-230

Meyer-Schwickerath R, Pfeiffer A, Blum WF, Freyberger H, Klein M *et al.* (1993). Vitreous levels of insulin-like growth factors I and II, and the insulin-like growth factor binding proteins 2 and 3 increase in neovascular disease. *J Clin Invest.* **92**:2620-2625

Michaelson IC. (1948). The mode of development of the vascular system of the retina, with some observations on its significance for certain retinal disease. *Trans Ophthalmol Soc UK.* **68**:137-180

Migita K, Eguchi K, Kawabe Y, Ichinose Y, Tsukada T *et al.* (1996). TNF-alpha-mediated expression of membrane-type matrix metalloproteinase in rheumatoid synovial fibroblasts. *Immunology.* **89:**553-557

Milici AJ, Watrous NE, Stukenbrok H, Palade GE. (1987). Transcytosis of albumin in capillary endothelium. *J Cell Biol.* **105:**2603-2612

Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Mϕler NPH *et al.* (1993). High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell.* **72**:835-846

Miller JW, Adamis AP, Shima D. (1994). Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with retinal angiogenesis in an animal model. *Am J Pathol.* **145**:574-584

Miller RF. (1994). The physiology and morphology of the vertebrate retina. In *Retina.* TE Ogden [ed]. Mosby, St Louis. pp 83-95

Minchenko A, Bauer T, Salceda S, Caro J. (1994b) Hypoxic stimulation of vascular endothelial growth factor expression *in vitro* and *in vivo*. *Lab Invest.* **71**:374-379

Minchenko A, Salceda S, Bauer T, Caro J. (1994a). Hypoxia regulatory elements of the human vascular endothelial growth factor gene. *Cell Mol Biol Res.* **40**:35-39

Mineo C, James GL, Smart EJ, Anderson RG. (1996). Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. *J Biol Chem.* **271:**11930-11935.

Minshall RD, Niles WD, Tiruppathi C, Vogel SM, Gilchrist A *et al.* (2000). Association of endothelial cell surface gp60 with caveolin-1 mediates vesicle formation and trafficking by activation of G_i-coupled *Src* kinase pathway. *J Cell Biol.* **150:**1057-1069

Mitamura Y, Tashimo A, Nakamura Y, Tagawa H, Ohtsuka K *et al.* (2002). Vitreous levels of placenta growth factor and vascular endothelial growth factor in patients ith proliferative diabetic retinopathy. *Diabetes care.* **25:**2352

Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T *et al.* (1999). Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. *Proc Natl Acad Sci USA.* **96:**10836-10841

Miyamura N, Bhutto IA, Amemiya T. (1999). Retinal capillary changes in Otsuka Long-Evans Tokushima fatty rats (spontaneously diabetic strain). Electron-microscopic study. *Ophthalmic Res.* **31:**358-366.

Mochizuki Y, Nakamura T, Kanetake H, Kanda S. (2002). Angiopoietin-2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells thorugh c-Fes and c-Fyn. *J Cell Sci.* **115:**175-183

Monier S, Parton RG, Vogel F, Behlke J, Henske A *et al.* (1995). VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes in vivo and in vitro. *Mol Biol Cell.* **6:**911-927

Montesano R, Vassali JD, Baird A, Guillemin R, Orci L. (1986). Basic fibroblast growth factor induces angiogenesis in vitro. *Proc Natl Sci USA.* **83**:7297-7301

Montrucchio G, Lupia E, Battaglia E, Passerini G, Bussolino F *et al.* (1994). Tumor necrosis factor alpha-induced angiogenesis depends on in situ platelet-activating factor biosynthesis. *J Exp Med.* **180:**377-382

Mora R, Bonilha VL, Marmorstein A, Scherer PE, Brown D *et al.* (1999). Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1. *J Biol Chem.* **274:**25708-25717.

Mora RC, Bonilha VL, Shin BC, Hu J, Cohen-Gould L, Bok D, Rodriguez-Boulan E. (2006). Bipolar assembly of caveolae in retinal pigment epithelium. Am J Physiol Cell Physiol. 2006 Mar;290(3):C832-43.

Morbidelli, Chang C-H, Douglas JG, Granger HJ, Ledda F *et al.* (1995). Nitric oxide mediates the mitogenic effect of VEGF on coronary venular endothelium. *Am J Physiol.* **270**:H411-H415

Mori K, Duh E, Gehlbach P, Ando A, Takahashi K. (2001). Pigment epithelium-derived factor inhibits retinal and choroidal neovascularization. *J Cell Physiol.* **188:**253-263

Mori K, Gehlbach P, Ando A, McVey D, Wei L *et al.* (2002c). Regression of ocular neovascularization in response to increased expression of pigment epithelium-derived factor. *Invest Ophthalmol Vis Sci.* **43:**2428-2434

Mori K, Gehlbach P, Ando A, Wahlin K, Gunther V. (2002a). Intraocular adenoviral vector-mediated gene transfer in proliferative retinopathies. *Invest Ophthalmol Vis Sci.* **43:**1610-1615

Mori K, Gehlbach P, Yamamoto S, Duh E, Zack *et al.* (2002b).AAV-mediated gene transfer of pigment epithelium-derived factor inhibits choroidal neovasculariztion. *Invest Ophthalmol Vis Sci.* **43:**1994-2000.

Morigiwa K, Quan M, Murakami M, Yamashita M, Fukuda Y. (2000). P2 purinoceptor expression and functional changes of hypoxia-activated cultured rat retinal microglia. *Neurosci Letts.* **282:**153-156

Moscatelli D, Rifkin DB. (1988). Membrane and matrix localisation of proteinases: a common theme in tumor cell invasion and angiogenesis. *Biochimica et Biophysica Acta.* **948**:67-85

Munoz-Fernandez MA, Fresno M. (1998). The role of tumour necrosis factor, interleukin 6, interferon-γ and inducible nitric oxide synthase in the development and pathology of the nervous system. *Prog Neurobiol.* **56:**307-340

Murata T, Ishibashi T, Khalil A, Hata Y, Yoshikawa H *et al.* (1995). Vascular endothelial growth factor plays a role in hyperpermeability of diabetic retinal vessels. *Ophthalmic Res.* **27**:48-52

Murata T, Nakagawa K, Khalil A, Ishibashi T, Inomata H, Sueishi K. (1996). The temporal and spatial vascular endothelial growth factor expression in retinal vasculogenesis of rat neonates. *Lab Invest.* **74**:68-77

Murohara T, Horowitz JR, Silver M, Tsurumi Y, Chen D *et al.* (1998). Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation.* **97**: 99-107

Murthy KS, Makhlouf GM. (2000). Heterologous desensitization mediated by G protein-specific binding to caveolins. *J Biol Chem.* **275:**30211-30219

Nair KS, Balasubramanian N, Slepak VZ. (2002). Signal-dependent translocation of transducin, RGS9-1-Gbeta5L complex, and arrestin to detergent-reistant membranes in photoreceptors. *Curr Biol.* **12:**421-425

Nakao S, Kuwano T, Ishibashi T, Kuwano M, Ono M. (2003). Synergistic effect of TNF-α in soluble VCAM-1-induced angiogenesis through α_4 integrins. **170:**5704-5711

Nambu H, Nambu R, Oshima Y, Hackett SF, Okoye G *et al.* (2004). Angiopoietin 1 inhibits ocular neovascularization and breakdown of the blood-retinal barrier. *Gene Ther.* **11:**865-73

Nambu H, Umeda N, Kachi S, Oshima Y, Akiyama H *et al.* (2005). Angiopoietin 1 prevents retinal detachment in an aggressive model of proliferative retinopathy, but has no effect on established neovascularization. *J Cell Physiol.* **204:**227-35

Nakayama T, Yoshizaki A, Kawahara N, Ohtsuru A, Wen CY, Fukuda E, Nakashima M. (2004). Expression of Tie1 and 2 receptors, and angiopoietin-1, 2 and 4 in gastric carcinoma;

immunohistochemical analyses and correlation with clinicopathological factors. *Histopathol.* **44:**232-239

Natarajan R, Bai W, Lanting L, Gonzales N, Nadler J. (1997). Effects of high glucose on vascular endothelial growth factor expression in vascular smooth muscle cells. *Am J Physiol.* **273**:H2224-H2231

Nathan DM (1996). The pathophysiology of diabetic complications: How much does the glucose hypothesis explain. *Ann Inter Med.* **124**:86-89

Naumann GOH, Apple DJ. (1986). Microscopic anatomy of the eye. *In Pathology of the eye.* Naumann GOH, Apple DJ [eds] Springer-Verlag, New York. pp19-58

Neely KA, Quillen DA, Schachat AP, Gardner TW, Blankenship G.W. (1998). Diabetic retinopathy. *Med Clin N Am.* **82**:4:847-876

Neufield G, Cohen T, Gengrinovitch S, Poltorak Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* **13:**9-22

Nguyen TT, Wong TY. (2006). Retinal vascular manifestations of metabolic disorders. *Trends Endocrinol Metab.* **17:**262-268.

Niles WD, Malik AB. (1999). Endocytosis and exocytosis events regulate vesicle traffic in endothelial cells. *J Membr Biol.* **167:**85-101

Nishimura F, Iwamoto Y, Mineshiba J, Shimizu A, Soga Y *et al.* (2003). Peridontal disease and diabetes mellitus: the role of tumor necrosis factor-alpha in a 2-way relationship. *J Peridontol.* **74:**97-102

Nissen NN, Polverini PJ, Kock AE, Volin MV, Gamelli RL *et al.* (1998). Vascular endothelial growth factor mediates angiogenic activity during the proiferative phase of wound healing. *Am J Pathol.* **152**:1445-1452

Nomura M, Yamagishi S, Harada S, Hayashi Y, Yamashima T *et al.* (1995). Possible participation of autocrine and paracrine vascular endothelial growth factors in hypoxia-induced proliferation of endothelial cells and pericytes. *J Biol Chem.* **270**:28316-28324

Notari L, Baladron V, Aroca-Aguilar JD, Balko N, Heredia R *et al.* (2006). Identification of a lipse-linked cell-membrane receptor for pigment epithelim-derived factor (PEDF). *J Biol Chem.* In press

Notari L, Miller A, Martinez A, Amaral J, Ju M *et al.* (2005). Pigment epithelium-derived factor is a substrate for matrix metalloproteinase type 2 and type 9: implications for downregulation in hypoxia. *Invest Ophthalmol Vis Sci.* **46:**2736-2747

Nourhaghighi N, Teichert-Kuliszewska K, Davis J, Stewart DJ, Nag S. (2003). Altered expression of angiopoietins during blood-brain barrier breakdown and angiogenesis. *Lab Invest.* **83:**1211-1222

Ogata N, Nishikawa M, Nishmura T, Mitsuma Y, Matsumura M. (2002a). Unbalanced vitreous levels of pigment epithelium-derived factor and vascular endothelial growth factor in diabetic retinopathy. *Am J Ophthalmol.* **134:**348-353

Ogata N, Nishikawa M, Nishimura T, Mitsuma Y, Matsumura M. (2002b). Inverse levels of pigment epithelium-derived factor and vascular endothelial growth factor in the vitreous of eyes with rhegmatogenous retinal detachment and proliferative vitreoretinopathy. *Am J Ophthalmol.* **133:**851-852

Ogata N, Tombran-Tink J, Jo N, Mrazek D, Matsumura M. (2001b). Upregulation of pigment epithelium-derived factor after laser photocoagulation. *Am J Ophthalmol.* **132:**427-429

Ogata N, Tombran-Tink J, Nishikawa M, Nishimura T, Mitsuma Y *et al.* (2001a). Pigment epithelium-derived factor in the vitreous is low in diabetic retinopathy and high in rhegmatogenous retinal detachment. *Am J Ophthalmol.* **132:**378-382

Ogata N, Wada M, Otsuji T, Jo N, Tombran-Tink J *et al.* (2002c). Expression of pigment-derived factor in normal adult rat eye and experimental choroidal neovascularization. *Invest Ophthalmol Vis Sci.* **43:**1168-1175

Ogata N, Wang L, Jo N, Tombran-Tink J, Takehashi K *et al.* (2001c). Pigment epithelium derived factor as a neuroprotective agent against ischemic retinal injury. *Curr Eye Res.* **22:**1994-2000

Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y. (1999). Hypoxia and vascular endothelial growth factor selectively upregulate angiopoietin-2 in bovine microvascular endothelial cells. *J Biol Chem.* **274**:15732-15739

Oh P, McIntosh DP, Schnitzer JE. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J Cell Biol.* **141:**101-114

Ohno-Matsui K, Morita I, Tombran-Tink J, Mrazek D, OnoderaM. (2001). Novel mechanism for age-related macular degeneration: an equilibrium shift between the angiogenesis factors VEGF and PEDF. *J Cell Physiol.* **189:**323-333

Okamoto T, Schlegel A, Scherer PE, Lisanti MP. (1998). Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem.* **273:**5419-5422

Okamoto N, Tobe T, Hackett SF, Ozaki H, Vinores MA *et al.* (1997). LaRochelle W, Zack DJ, Campochiaro PA. Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neovascularization. *Am J Pathol.* 151:281-291

Olgemoller B, Schleicher E. (1993). Alterations of glomerular matrix proteins in the pathogenesis of diabetic nephropathy. *Clin Invest.* **71** (5 Suppl):S13-19

Oliner J, Min H, Leal J, Yu D, Rao S *et al.* (2004). Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2. *Cell.* **6:**507-516

Olofsson B, Pajusola K, Kaipainen A, Von Euler G, Joukov V *et al.* (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA*. **93**:2576-2581

Orlidge A, D'Amore PA. (1987). Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol*. **105**:1455-1462

Ortego J, Escribano J, Beccera SP, Coca-Prados M. (1996). Gene expression of the neurotrophic pigment epithelium-derived factor in the human ciliary epithelium. Synthesis and secretion into the aqueous humor. *Ophthalmol Vis Sci.* **37**:2759-2767

Ortega N, Jonca F, Vincent S, Favard C, Ruchoux MM, Plouet J. *et al.* (1997). Systemic activation of the vascular endothelial growth factor receptor KDR/flk-1 selectively triggers endothelial cells with an angiogenic phenotype. *Am J Pathol.* **151:**1215-1224

Oshima Y, Deering T, Oshima S, Nambu H, Reddy PS *et al.* (2004). Angiopoietin-2 enhances retinal vessel sensitivity to vascular endothelial growth factor. *J Cell Physiol.* **199:**412-417

Oshima Y, Oshima S, Nambu H, Kachi S, Takahashi K *et al.* (2005). Different effects of angiopoietin-2 in different vascular beds: new vessels are most sensitive. *FASEB J.*

Osterby R, Bangstad HJ, Nyberg G, Rudberg S. (2001). On glomerular structural alterations in type-1 diabetes. Companions of early diabetic glomerulopathy. *Virchows Arch.* **438:**129-135.

Osterby R, Schmitz A, Nyberg G, Asplund J. (1998). Renal structural changes in insulin-dependent diabetic patients with albuminuria. Comparison of cases with onset of albuminuria after short or long duration. *APMIS.* **106:**361-370.

Otani A, Takagi H, Oh H, Koyama S, Matsumura M *et al.* (1999). Expression of angiopoietins and Tie-2 in human choroidal neovascular membranes. *Invest Ophthalmol Vis Sci.* **40:**1912-1920

Otani A, Takagi H, Suzuma K, Honda Y. (1998). Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. *Circ Res.* **82**:619-628

Ozaki H, Hayashi H, Vinores SA, Moromizato Y, Campochiaro PA *et al.* (1997). Intravitreal sustained release of VEGF causes retinal neovascularization in rabbits and primates. *Exp Eye Res.* **64:**505-517

Ozaki H, Seo M, Ozaki K, Yamada H, Yamada E *et al.* (2000). Blockade of vascular endothelial cell growth factor receptor signalling is sufficient to completely prevent retinal neovascularization. *Am J Pathol.* **156:**697-707

Ozaki H, Yu A, Della N, Ozaki K, Luna JD *et al.* (1999). Hypoxia inducible factor-1α is increased in ischaemic retina: Temporal and spatial correlation with VEGF expression. *Invest Ophthalmol Vis Sci.* **40**:182-189

Padro T, Bieker R, Ruiz S, Steins M, Retzlaff S *et al.* (2002). Overexpression of vascular endothelial growth factor (VEGF) and its cellular receptor KDR (VEGFR-2) in the bone marrow of patients with acute myeloid leukemia. *Leukemia.* **16:**1302

Pajusola K, Aprelikova O, Armstrong E, Morris S, Alitalo K. (1993). Two human FLT4 receptor tyrosine kinase isoforms with distinct carboxy terminal tails are produced by aternative processing of primary transcripts. *Oncogene.* **8**:2931-2937

Pajusola K, Aprelikova O, Korhonen J, Kaipainen A, Pertovara L *et al.* (1992). FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. *Cancer Res.* **52**:5738-5743

Pajusola K, Aprelikova O, Pelicci G, Weich H, Claesson-Welsh L *et al.* (1994). Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene.* **9**:3545-3555

Palade, GE. (1953). *J Appl Physiol.* **24:**1424-1436

Palade GE, Simionescu M, Simionescu N. (1979). Structural aspects of the permeability of the microvascular endothelium. *Acta Physiol Scand Suppl.* **463:**11-32

Pallares J, Rojo F, Iriarte J, Morote J, Armadans LI *et al.* (2006). Study of microvessel density and the expression of the angiogenic factors VEGF, bFGF and the receptors Flt-1 and FLK-1 in benign, premalignant and malignant prostate tissues. *Histol Histopathol.* **21:**857-865

Palmberg PF. (1977). Diabetic retinopathy. *Diabetes.* **26:**703-709.

Petrovic V, Bhisitkul RB. (1999). Lasers and diabetic retinopathy: the art of gentle destruction. *Diabetes Technol Ther.* **1:**177-187

Palmieri D, Watson J, Rinehart C. (1999). Age-related expression of PEDF/EPC-1 in human endometrial stromal fibroblasts: implications for interactive senescence.*Exp Cell Res.* **247:**142-147

Pan W, Zadina JE, Harlan RE, Weber JT, Banks WA *et al.* (1997).
Tumor necrosis factor-α: a neuromodulator in the CNS. *Neurosci Biobehav Rev.* **603-613**

Panek RB, Lee YJ, Itoh-Lindstrom Y, Ting JP, Benveniste EN. (1994). Characterization of astrocyte nuclear proteins involved in IFN-γ-and TNF-α-mediated class II MHC gene expression. *J Immunol.* **153:**4555-4564

Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS *et al.* (2000). Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/surviving pathway. *J Biol Chem.* **275:**9102-9105

Papapetropoulos A, Garcia-Cardena G, Dengler TJ, Maisonpierre PC, Yancopoulos GD *et al.* (1999). Direct actions of angiopoietins-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. *Lab Invest.* **79:**213-223

Paria BC, Maliki AB, Kwiatek AM, Rahman A, May MJ *et al.* (2003). Tumor necrosis factor-α induces nuclear factor-κB-dependent TRPC1 expression in endothelial cells. *J Biol Chem.* **278:**37195-37203

Park D, Rhee SG. (1992). Phosphorylation of Nck in response to a variety of receptors, phorbol myristate acetate, and cyclic AMP. *Mol Cell Biol.* **12**:5816-5823

Park DS, Woodman SE, Schubert W, Cohen AW, Frank PG *et al.* (2002). Caveolin-1/3 double-knockout mice are viable, but lack both muscle and non-muscle caveolae, and develop a severe cardiomyopathic phenotype. *Am J Pathol.* **160:**2207-2217

Park JE, Chen HH, Winer J, Houck KA, Ferrara N. (1994). Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem.* **269**:25646-25654

Parolini I, Sargiacomo M, Galbiati F, Rizzo G, Grignani F *et al.* (1999). Expression of caveolin-1 is required for the transport of caveolin-2 to the plasma membrane. Retention of caveolin-2 at the level of the golgi complex. *J Biol Chem.* **274:**25718-25

Partanen J, Armstrong E, Makela TP, Korhonen J *et al.* (1992). A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. *Mol Cell Biol.* **12**:1698-1707

Partanen TA, Alitalo K, Miettinen M. (1999). Lack of lymphatic vascular specificity of vascular endothelial growth factor receptor 3 in 185 vascular tumors. *Cancer.* **86:**2406-2412

Partanen TA, Arola J, Saaristo A, Jussila L, Miettinen M *et al.* (2000).VEGF-C and VEGF-D expression in neuroendocrine cells and their receptors, VEGFR-3, in fenestrated blood vessels in human tissues. *FASEB J.* **14:**2087-2096

Partanen R, Koskinen H, Hemminki K. (1995). Tumour necrosis factor-alpha (TNF-alpha) in patients who have asbestosis and develop cancer. *Occup Environ Med.* **52:**316-319

Parton RG. (1996). Caveolae and caveolins. *Curr Opin Cell Biol.* **8:**542-548

Patel JI, Hykin PG, Gregor ZJ, Boulton M, Cree IA. (2005). Angiopoietin concentrations in diabetic retinopathy. *Br J Ophthalmol.* **89:**480-483.

Patel N, Sun L, Moshinsky D, Chen H, Leahy KM *et al.* (2003). Aselective and oral small molecule inhibitor of vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-1 inhibits neovascularization and vascular permeability. *J Pharmacol Exp Therap.* **306:**838-845

Pe'er J, Folberg R, Itin A, Gnessin H, Hemo I, Keshet E. (1996). Upregulated expression of vascular endothelial growth factor in proliferative diabetic retinopathy. *British J Ophthalmol.* **80**:241-245

Pe’er J, Shweiki D, Itin A, Hemo I, Gnessin H, Keshet E. (1995). Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases. *Lab Invest.* **72**:638-645

Pekala P, Marlow M, Heuvelman D, Connoly D.(1990). Rgulation of hexose transport in aortic endothelial cells by vascular permeability factor and tumour necrosis factor-alpha but not by insulin. *J Biol Chem.* **265**:18051-18054

Penn JS, Thum LA, Rhem MN, Dell SJ. (1988). Effects of oxygen rearing on the electrocardiogram and GFA-protein in the rat. *IOVS.* **29**:1623-1630

Pepper MS, Ferrara N, Orci L, Montesano R. (1991). Vascular endothelial growth factor (VEGF) iniuces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res comm.* **181**:902-906

Pepper MS, Mandriota SJ, Jeltsch M, Kumar V, Alitalo K. (1998). Vascular endothelial growth factor (VEGF)-C synergizes with basic fibroblast growth factor and VEGF in the induction of angiogenesis in vitro and alters endothelial cell extracellular proteolytic activity. *J Cell Physiol.* **177:**439-452.

Pepper MS, Montesano R. (1990). Proteolytic balance and capillary morphogenesis. *Cell Diff Dev.* **32**:319-331

Persaud K, Tille J, Liu M, Zhu Z, Jimenez X *et al.* (2004). Involvement of the VEGF receptor 3 in tubular morphogenesis demonstrated with a human anti-human VEGFR-3 monoclonal antibody that antagonizes receptor activation by VEGF-C. *J Cell Sci.* **117**:2745-2756

Petak I, Houghton JA. (2001). Shared pathways: death receptors and cytotoxic drugs in cancer therapy. *Pathol Oncol Res.* **7**:95-106

Peters KG, De Vries C, Williams LT. (1993). Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proc Natl Acad Sci USA.* **90**:8915-8919

Petersen SV, Valnickova Z, Enghild JJ. (2003). Pigment-epithelium-derived factor occurs at a physiologically relevant concentration in human blood: purification and characterization. *Biochem J.* **374**:199-206

Palmberg PF. (1977). Diabetic retinopathy. *Diabetes.* **26**:703-709.

Petrovic V, Bhisitkul RB. (1999). Lasers and diabetic retinopathy: the art of gentle destruction. *Diabetes Technol Ther.* **1**:177-187

Pfeiffer A, Schatz H. (1992). Pathophysiological aspects of non-tyrosine kinase signal transduction. *Horm Metab Res.* **24**:219-224

Pfeiffer A, Schatz H. (1995). Diabetic microvascular complications and growth factors. *Exp Clin Endocrinol.* **103**:7-14

Pfeiffer K. (2003). Biological functions of tumor necrosis factor cytokines and their receptors. *Cytokine Growth Factor Rev.* **14**:185-191

Pfitzenmaier J, Vessella R, Higano CS, Noteboom JL, Wallace D Jr *et al.* (2003). Elevation of cytokine levels in cachectic patients are discontinuous and correlate with weight loss. *Eur J Clin Invest.* **30:**1107-1112

Pichiule P, Chavez JC, LaMann JC. (2004). Hypoxic regulation of angiopoietin-2 expression in endothelial cells. *J Biol Chem.* **279:**12171-12180

Pierce EA, Avery RL, Foley ED, Aiello LP, Smith LEH. (1995). Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc Natl Acad Sci USA*. **92**:905-909

Pignolo R, Rotenberg M, Cristofalo V. (1995). Analysis of EPC-1 growth state-dependent expression, specificity, and conservation of related sequences. *J Cell Physiol.* **162:**110-118

Pizurki L, Zhou Z, Glynos K, Roussos C, Papapetropoulos A. (2003). Angiopoietin-1 inhibits endothelial permeability, neutrophil adherence and IL-8 production. *Br J Pharmacol.* **139:**329-336

Phillips CJ, Clark CV, Tsukahara S. (1994). Ophthalmology. A primer for medical students and practitioners. Baillière Tindall, London

Phillips GD, Stone AM, Jones BD, Schultz JC, Whitehead RA *et al.* (1993). Vascular endothelial growth factor (rhVEGF165) stimulates direct angiogenesis in the rabbit cornea. *In Vivo*. **8**:961-965

Phillips HS, Hains J, Leung DW, Ferrara N. (1990). Vascular endothelial growth factor is expresed in rat corpus luteum. *Endocrinology*. **127**:965-967

Pike LJ, Casey L. (1996). Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains.J Biol Chem. 1996 Oct 25;271(43):26453-6.

Pizurki L, Zhou Z, Glynos C, Roussos A, Papapetropoulos A. (2003). Angiopoietin-1 inhibits endothelial permeability, neutrophil adherence and IL-8 production. *Br J Pharmacol.* **139:**329-336

Plate KH, Breier G, Weich HA, Risau W. (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature*. **359**:845-846

Plouët J, Schilling J, Gospodarowicz. (1989). Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. *EMBO J*. **8**:3801-3806

Praloran V, Mirshahi S, Favard C, Moukadari H, Plouët J. (1991). Mitogenic activity of vasculotropin for peripheral human lymphocytes. *C R Acad Sci III*. **313**:21-26

Predescu D, Horvat R, Predescu S, Palade GE. (1994). Transcytosis in the continuous endothelium of the myocardial microvasculature is inhibited by N-ethylmaleimide. *Proc Natl Acad Sci USA*. **91**:3014-3018

Predescu D, Palade GE. (1993). Plasmalemmal vesicles represent the large pore system of continuous microvascular endothelium. *Am J Physiol Heart Circ Physiol*. **265**:H725-H733

Predescu D, Predescu S, McQuistan T, Palade GE. (1998). Transcytosis of alpha1-acidic glycoprotein in the continuous microvascular endothelium. *Proc Natl Acad Sci U S A*. **95**:6175-6180.

Predescu SA, Predescu DN, Palade GE. (2001). Endothelial transcytotic machinery involves supramolecular protein-lipid complexes. *Mol Biol Cell*. **12**:1019-33.

Procopoio WN, Pelavin PI, Lee WM, Yeilding NM. (1999). Angiopoietin-1 and -2 coiled coil domains mediate distinct homo-oligomerization patterns, but fibrinogen-like domains mediate ligand activity. *J Biol Chem*. **274**:30196-30201

Puyraimond A, Fridman R, Lemesie M, Arbeille B, Menashi S. (2001). MMP-2 colocalizes with caveolae on the surface of endothelial cells. *Exp Cell Res*. **262**:28-36

Quam T, Xu Q, Joussen AM, Clemens MW, Qin W *et al.* (2001). VEGF-initiated blood-retinal barrier breakdown in early diabetes. *Invest Ophthalmol Vis Sci*. **42**:2408-2413

Quest AF, Leyton L, Parraga M. (2004). Caveolins, caveolae, and lipid rafts in cellular transport, signaling, and disease. *Biochem Cell Biol.* **82:**129-44.

Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT. (1993). Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci USA*. **90**:7533-7537

Rahimi N, Dayanir V, Lashkari K. (2000). Receptor chimeras indicate that the vascular endothelial growth factor receptor-1 (VEGFR-1) modulates mitogenic activity of VEGFR-2 in endothelial cells.*J Biol Chem.* **275:**16986-16992

Rahimi N, Kazlauskas A. (1999). A role for cadherin-5 in regulation of vascular endothelial growth factor receptor 2 activity in endothelial cells. *Mol Biol Cell.* **10:**3401-3410

Raisler BJ, Berns KI, Grant MB, Beliaev D, Hauswirth WW. (2002). Adeno-associated virus type-2 expression of pigmented epithelium-derived factor or kringles 1-3 of angiostatin reduce retinal neovascularization. *Proc Natl Acad Sci USA.* **99:**8909-8914

Rajah TT, Grammas P. (2002). VEGF and VEGF receptor levels in retinal and brain-derived endothelial cells. *Biochem Biophys Res Communic.* **293:**710-713

Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S *et al.* (1995). Mutant ras oncogene upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res.* **55:**455-4580

Rashid G, Benchetrit S, Fishman D, Bernheim J. (2004). Effect of advanced glycation end-products on gene expression and synthesis of TNF-α and endothelial nitric oxide synthase by endothelial cells. *Kidney International*. **66:**1099-1106

Raskin P, Pietri AO, Unger R, Shannon WA Jr. (1983). The effect of diabetic control on the width of skeletal-muscle capillary basement membrane in patients with Type I diabetes mellitus. *N Engl J Med.* **309:**1546-1550.

Rasmussen H, Chu KW, Campochiaro P, Gehlbach PL, Haller JA *et al.* (2001). Clinical protocol. An open-label phase 1 single administration, dose-escalation study of ADGVPEDF:11D(ADPEDF) in neovascular age-related macular degeneration (AMD). *Hum Gene Ther.* **12:**2029-2032

Raymond L, Jacobson B. (1982). Isolation and identification of stimulatory and inhibitory cell growth factors in bovine vitreous. *Exp Eye Res.* **53:** 34(2):267-86

Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL *et al.* (2001). Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem.***276:**38121-38

Razani B, Lisanti MP. (2001). Caveolin-deficient mice: insights into caveolar function and human disease. *J Clin Invest.* **108:**1553-1561

Razani B, Schlegel A, Lisanti MP. (2000). Caveolin proteins in signaling, oncogenic transformation and muscular dystrophy. *J Cell Sci.* **113:**2103-9.

Razani B, Woodman SE, Lisanti MP. (2002). Caveolae: from cell biology to animal physiology. *Pharmacol Rev.* **54:**431-67.

Reich SJ, Fosnot J, Kuroki A, Tang W, Yang X *et al.* (2003). Small interfering RNA (SiRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol Vis.* **9**:210-216

Rich RM, Rosenfield PJ, Puliafito CA, Dubovy SR, Davis JL *et al.* (2006). Short-term safety and efficacy of intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. *Retina.* **26:**495-511

Ridley SH, Sarsfield SJ, Lee JC, Bigg HF, Cawston TE *et al.* (1997). Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. *J Immunol.* **158:**3165-3173

Rink L, Kirchner H. (1996). Recent progress in the tumor necrosis factor-α field. *Int Arch Allergy Immunol.* **111:**199-209

Risau W. (1997). Mechanisms of angiogenesis. *Nature.* **386**:671-674

Robbins SG, Conoway JR, Ford BL, Roberto KA, Penn JS. (1997). Detection of vascular endothelial growth factor (VEGF) protein in vascular and non-vascular cells of the normal and oxygen-injured rat retina. *Growth Factors.* **14**:229-241

Robinson GS, Pierce EA, Rook SL, Foley E, Webb R *et al.* (1996). Smith LE. Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. *Proc Natl Acad Sci U S A.* 1996 **93:**4851-4856.

Rogers MJ, Brown RJ, Hodkin V, Blackburn GM, Russell RG *et al.* (1996*). Biochem Biophys Res Commun.* **224:**863-869

Romero LI, Tatro JB, Field JA, Reichlin S. (1996). Roles of IL-1 and TNF-α in endotoxin-induced activation of nitric oxide synthase in cultured rat brain cells. *Am J Physiol.* **270:**R326-R332

Rosenfield PJ, Rich RM, Lalwani GA. (2006). Ranibizumab:phase II clinical trial results. *Ophthalmol* Clin North Am. **19:**361-372

Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR *et al.* (1992). Caveolin, a protein component of caveolae membrane coats. *Cell.* **68:**673-682

Roviezzo F, Tsigkos S, Kotanidou A, Bucci M, Brancaleone V *et al.* (2005). Angiopoietin-2 causes inflammation in vivo by promoting vascular leakage. *J Pharmacol Exp Ther.* **314:**738-744

Roybal CN, Hunsaker LA, Barbash O, Dander Jagt DL. (2005). The oxidative stressor arsenite activates vascular endothelial growth factor mRNA transcription by an ATF4-dependent mechanism. *J Biol Chem.* **280:**20331-20339

Rujoi M, Jin J, Borchman D, Tang D, Yappert MC. (2003). Isolation and lipid characterization of cholesterol-enriched fractions in cortical and nuclear human lens fibers. *Invest Ophthalmol Vis Sci.* **44:**1634-1642

Russelakis-Carneiro M, Hetz C, Maundrell K, Soto C. (2004). Prion replication alters the distribution of synaptophysin and cavelin 1 in neuronal lipid rafts. *Am J Pathol.* **165:**1839-1848

Saharan P, Tammela T, Karkkainen MJ, Alitalo K. (2004). Lymphatic vasculature: development, molecular regulation and role in tumor metastasis and inflammation. *Trends Immunol.* **25:**387-395

Saito M, Hamasaki M, Shibuya M. (2003). Induction of tube formation by angiopoietin-1 in endothelial cell/fibroblast co-culture is dependent on endogenous VEGF. *Cancer Sci.* **94:**782-790

Samaniego F, Markham PD, Gendelman R, Watanabe Y, Kao V *et al.* (1998). Vascular endothelial growth factor and basic fibroblast growth factor present in kaposi's sarcoma (KS) are induced by inflammatory cytokines and synergize to pomote vascular permeability and KS lesion development. *Am J Pathol.* **154**:1433-1443

Sargiocomeo M, Scherer PE, Tang ZL, Lisanti MP. (1995). Oligomeric structure of caveolin: implications for caveolae membrane organization. *Proc Natl Acad Sci USA.* **92:**9407-9411

Sato N, Fukuda K, Nariuchi H, Sagara N. (1987). Tumor necrosis factor inhibiting angiogenesis in vitro. *J Natl Cancer Inst.* **79:**1383-1391

Sato A, Iwama A, Takakuru N, Nishio H *et al.* (1998). Characterization of TEK receptor tyrosine kinase and its ligands, angiopoietins, in human haematopoietic progenitor cells. *Int J Immunol.* **10**:1217-1227

Sato TN, Qin Y, Kozak CA, Audis KL. (1993). Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc Natl Acad Sci USA*. **90**:9355-9358

Sato TN, Tozawa Y, Deutsch U, Wolberg-Buchholz K, Fujiwara Y *et al.* (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature.* **376**:70-73

Satoh J, Yagihashi S, Toyota T. (2003). The possible role of tumor necrosis factor-alpha in diabetic polyneuropathy. *Exp Diabesity Res.* **4:**65-71

Satoh H, Yoshida MC, Matsushime H, Shibuya M, Sasaki M. (1987). *Jpn J Cancer Res (Gann).* **78**:772-775

Sawano A, Takahashi T, Yamaguchi S, Aonuma M, Shibuya M. (1996). Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. *Cell Growth and Differentiation.* **7**:213-221

Sawano A, Takahashi T, Yamaguchi S, Shibuya M. (1997). The phosphorylated 1169-tyrosine containing region of Flt-1 kinase (VEGFR-1) is a major binding site for PLCγ. *Biochem Biophys Res Comm.* **238**:487-491

Scharpfenecker M, Fielder U, Reiss Y, Augustin HG. (2004). The Tie-2 ligand angiopietin-2 destabalizes quiescent endothelium through an internal autocrine loop mechanism. *J Cell Sc.* **118:**771-780

Scheiffarth OF, Kampik A, Günther H, von der Mark K. (1988). Proteins of the extracellular matrix in vitreoretinal membranes. *Graefe's Arch Clin Exp OPhthalmol.* **226:**357-361
Scheiffele P, Verkade P, Fra AM, Virta H, Simons K et al. (1998). Caveolin-1 and -2 in the exocytic pathway of MDCK cells. *J Cell Biol.* **140:**795-806.

Schmidt CM, McKillop IH, Cahill PA, Sitzmann JV. (1997). Increased MAPK expression and activity in primary human hepatocellular carcinoma. *Biochem Biophys Res Commun.* **236:**54-58

Schmidt-Erfurth U, Schlötzer-Schrehard U, Cursiefen C, Michels S, Beckendorf A. (2003). Influence of photodynamic therapy on expression of vascular endothelial growth factor (VEGF), VEGF receptor 3, and pigment epithelium-derived factor. *Invest Ophthalmol Vis Sci.* **44:**4473-4480

Schneeberger SA, Hjelmeland LM, Tucker RP, Morse LS. (1997).Vascular endothelial growth factor and fibroblast growth factor 5 are colocalized in vascular and avascular epiretinal membranes. *Am J Ophthalmol.* **124:**447-54.

Schnitzer J. (1988a). Astrocytes in the guinea pig, horse, and monkey retina: their occurence coincides with the presence of blood vessels. *Glia.* **1**:74-89

Schnitzer J. (1988b). The development of astrocytes and blood vessels in the postnatal rabbit retina. *J Neurocytol.* **17:**433-49.

Schnitzer JE, Liu J, Oh P. (1995). Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, annexins, and GTPases. *J Biol Chem.* **270:**14399-14404.

Schnurch H, Risau W. (1993). Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage. *Development.* **119**:957-968

Schwartz EA, Reaven E, Topper JN, Tsao PS. (2005). Transforming growth factor-beta receptors localize to caveolae and regulate endothelial nitric oxide synthase in normal human endothelial cells. *Biochem J.* **390:**199-206

Schwencke C, Braun-Dullaeus RC, Wunderlich C, Strasser RH. (2006). Caveolae and caveolin in transmembrane signaling: Implications for human disease. *Cardiovasc Res.* **70:**42-49.

Schwesinger C, Yee C, Rohan RM, Joussen AM, Fernandez A *et al.* (2001). Intrachoroidal neovascularization in transgenic mice overexpresing vascular endothelial growth factor in the retinal pigment epithelium. *Am J Pathol.* **158:**1161-1172

Scott BB, Zaratin PF, Colombo A, Hansbury MJ, Winkler JD *et al.* (2002). Constitutive expression of angiopoietin-1 and -2 and modulation of their expression by inflammatory cytokines in rheumatoid arthritis synovial fibroblasts. *J Rheumatol.* **29:**230-239

Scott BB, Zaratin PF, Gilmartin AG, Hansbury MJ, Colombo A *et al.* (2005). TNF-α modulates angiopoietin-1 expression in rheumatoid synovial fibroblasts via the NF-κB signalling pathway. *Biochem Biophys Res Comm.* **328:**409-414

Sedgwick JB, Menon I, Gern JE, Busse WW. (2002). Effects of inflammatory cytokines on the permeability of human lung microvascular endothelial cell monolayers and differential eosinophil transmigration. *J Allergy Clin Immunol.* **110:**752

Seetharam L, Gotoh N, Maru Y, Neufield G, Yamaguchi S, Shibuya M. (1995). A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Oncogene.* **10:**135-147

Segawa Y, Shirao Y, Yamagishi S, Higashide T, Kobayashi M *et al.* (1997. Upregulation of retinal vascular endothelial growth factor mRNAs in spontaneously diabetic rats without ophthalmoscopic retinopathy. *Ophthalmic Res.* **30**:333-339

Seigel GM, Tombran_tink J, Becerra SP. (1994). Differentiation of Y79 retinoblastoma cell with pigment epithelium-derived factor and in the interphotoreceptor matrix wash: effects on tumorigenicity. *Growth Factors.* **10:**289-297

Selmaj KW, Farooq M, Norton WT, Raine CS, Brosnan CF. (1990). Proliferation of astrocytes in vitro in response to cytokines. A primary role for tumor necrosis factor. *J Immunol.* **144:**129-135

Senger DR, Connolly DT, Van De Water L, Feder J, Dvorak HF. (1990). Purification and NH-terminal amino acid sequence of guinea pig tumour-secreted vascular permeability factor. *Cancer Res.* **50**:1774-78

Senger DR, Galli SJ, Dvorak AM, Peruzzi CA, Harvey VS *et al.* (1983). Tumour cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science.* **219**:983-985

Senger DR, Perruzzi CA, Feder J, Dvorak HF. (1986). A highly conserved vascular permeability factor secreted by human and rodent tumour cell lines. *Cancer Res.* **46**:5629-32

Senger DR, Van De Water L, Brown LF *et al.* (1993). Vascular permeability factor (VPF, VEGF) in tumour biology. *Cancer Metastasis Rev.* **12**:303-24

Seno K, Kishimoto, M, Abe M, Higuchi Y, Mieda M. (2001). Light – and guanosine 5'-3-O-(thio)triphosphate-sensitive localization of a G protein and its effector on detergent-resistant membrane rafts in rod photoreceptor outer segments. *J Biol Chem.* **276:**20813-20816

Sexton PS, Neely AR, Cenedella RJ. (2004). Distribution of caveolin-1 in bovine lens and redistribution in cultured bovine lens epithelial cells upon confluence. *Exp Eye Res.* **78:**75-82.

Sharp P.S. (1995). The role of growth factors in the development of diabetic retinopathy. *Metabolism.* **44**:0:72-75

Shaul PW, Pace MC, Chen Z, Brannon TS. (1999). Developmental changes in prostacyclin synthesis are conserved in cultured pulmonary endothelium and vascular smooth muscle. *Am J Respir Cell Mol Biol.* **20**:113-21.

Sheta EA, Harding MA, Cenaway MR, Theodorescu D. (2000). Focal adhesion kinase, Rap1, and transcriptional induction of vascular endothelial growth factor. *J Natl Cancer Inst.* **92:**1065-1073

Shibuya M. (2003). Vascular endothelial growth factor receptor-2 its unique signalling and specific ligand, VEGF-E. *Cancer Sci.* **94:**751-756

Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M. (1990). Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene.* **5**:519-524

Shima DT, Adamis AP, Ferrara N, Yeo K, Yeo T, Allende R, Folkman J, D'Amore PA. (1995). Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. *Molec Med.* **1**:182-193

Shima DT, Gougos A, Miller JW, Tolentino M, Robinson G *et al.* (1996). Cloning and mRNA expression of vascular endothelial growth factor in ischemic retinas of *Macaca Fascicularis. Invest OPhthalmol Vis Sci.* **37:**1334-1340

Shweiki D, Itin A, Neufield G, Gitay Goren H, Keshet E. (1993). Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest.* **91**:2235-2243

Shweiki D, Itin A, Soffer D, Keshet E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature.* **359**:843-845

Simionescu M, Simionescu N, Palade GE. (1974). Morphometric data on the endothelium of blood capillaries. *J Cell Biol.* **60:**128-152

Simó R, Lecube A, Segura RM, García Arumí, Hernández C. (2002). Free insulin growth factor-1 and vascular endothelial growth factor in the vitreous fluid of patients with proliferative diabetic retinopathy. *Am J Ophthalmol.* **134:**376-382

Simionescu M, Simionescu N, Palade GE. (1974). Morphometric data on the endothelium of blood capillaries. *J Cell Biol.* **60:**128-152

Simonovic M, Gettins PG, Volz K. (2001). Crystal structure of human PEDF, a potent antiangiogenic and neurite growth-promoting factor. *Proc Natl Acad Sci USA.* **98:**11131-11135

Simorre-Pinatel V, Guerrin M, Chollet P, Penary M, Clamens S *et al.* (1994). Vasculotropin-VEGF stimulates retinal capillary endothelial cells through an autocrine pathway. *Ophthalmol Vis Sci.* **35**:3393-3400

Shibuya M. (2003). Vascular endothelial growth factor receptor-2 its unique signalling and specific ligand, VEGF-E. *Cancer Sci.* **94**:751-756

Sirajy HM, Awad A, Abadir P, Webb R. (2003). The angiotensin II type 1 receptor mediates renal interstitial content of tumor necrosis factor-alpha in diabetic rats. *Endocrinology.* **144**:2229-2233

Skobe M, Hamberg LM, Hawighorst T, Shirner M, Wolf GL. (2001a). Concurrent induction of lymphangiogenesis, angiogenesis and macrophage recruitment by VEGF-C in melanoma. *A J Pathol.* **159**:893-903

Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L. (2001b). Induction of tumor lymphangiogensis by VEGF-C promotes breast cancer metastasis. *Nat Med.* **7**:192-198

Smart EJ, Ying YS, Mineo C, Anderson RG. (1995). A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc Natl Acad Sci U S A.* **92**:10104-10108.

Soldi R, Mitola S, Strasly M, Defilippi P, Tarone G. (1999). Role of $\alpha_v\beta_3$ in the activation of vascular endothelial growth factor receptor-2. *EMBO J.* **18**:882-892

Song KS, Scherer PE, Tang Z, Okamoto T, Li M *et al.* (1996). Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J Biol Chem.* **271**:15160-15165

Sonveaux P, Martinive P, DeWever J, Batova Z, Daneau G *et al.* (2004). Caveolin-1 expression is critical for vascular endothelial growth factor-induced ischemic hindlimb collateralization and nitric oxide-mediated angiogenesis. *Circ Res.* **95**:154-161

Sosenko JM, Miettinen OS, Williamson JR, Gabbay KH. (1984). Muscle capillary basement-membrane thickness and long-term glycemia in type I diabetes mellitus. *N Engl J Med.* **311**:694-698.

Sowa G, Pypaert M, Fulton D, Sessa WC. (2003). The phosphorylation of caveolin-2 on serines 23 and 36 modulates caveolin-1-dependent caveolae formation. *Proc Natl Acad Sci U S A.* **100**:6511-6516

Spirin KS, Saghizadeh M, Lewin SL, Zardi L, Kenney MC *et al.* (1999). Basement membrane and growth factor gene expression in normal and diabetic human retinas. *Curr Eye Res.* **18**: 490-499

Sporn M, Roberts A. (1990). The multifunctional nature of peptide growth factors. In *Handbook of Experimental Pharmacology, Peptide Growth Factors and Their Receptors I.* Sporn M, Roberts A [eds]. Springer-Verlag, Berlin. pp3-15

Spranger J, Meyer-Schwickerath R, Klein M, Schatz H, Pfeiffer A. (1995). TNF-alpha level in the vitreous body. Increase in neovascular eye diseases and proliferative diabetic retinopathy. *Med Clin.* **90**:134-137

Spritz RA, Strunk KM, Lee ST, Lu-Kuo JM, Ward DC *et al.* (1994). A YAC contig spanning a cluster of human type III receptor protein kinase genes (PDGFRA-KIT-KDR) in chromosome segment 4q12. *Genomics.* **22**:431-436

Sreekumar PG, Kannan R, de Silva AT, Burton R, Ryan SJ. (2006). Thiol regulation of vascular endothelial growth factor-A and its receptors in human retinal pigment epithelial cells. *Biochem Biophys Res Commun.* **346**:1200-1206

Stacker SA, Caeser C, Baldwin ME, Thornton GE, Williams RA *et al.* (2001). VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med.* **7**:186-191

Stadelman C, Lassman H. (2000). Detection of apoptosis in tissue sections. *Cell tissue Res.* **301**:19-31

Stavri GT, Zachary IC, Baskerville PA, Martin JF, Erusalimisky JD. (1995). Basic fibroblast growth factor upregulates the expresssion of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. *Circ.* **92**:11-14

Steeghs N, Nortier JW, Gelderblom H. (2007). Small molecule tyrosine kinase inhibitors in the treatment of solid tumours: an update of recent developments. Ann Surg Oncol. **14:**942-953

Steele FR, Chader GJ, Johnson LV, Tombran-Tink J. (1993). Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. *Proc Natl Acad Sci USA.* **90:**1526-1530

Stellmach V, Crawford SE, Zhou W, Bouck N. (2001). Prevention of ischemia-induced retinopathy by the natural ocular angiogenic agent pigment epithelium-derived factor. *Proc Natl Acad Sci USA.* **98:**2593-2597

Stewart J, Siavash H, Hebert C, Norris K, Nikitakis NG *et al.* (2003). Phenotypic switching of VEGF and collagen XVIII during hypoxia in head and neck squamous carcinoma cells. *Oral Oncol.* **39:**862-9.

Stitt AW, Burke GA, Chen F, McMullen BT, Vlassara H. (2000). Advanced glycation end-product receptor interactions on microvascular cells occur within caveolin-rich membrane domains. *FASEB J.* **14:**2390-2392

Stone J, Chan-Ling T, Pe'er J, Itin A, Gnessin H, Keshet E. (1996). Roles of vascular endothelial growth factor and astrocyte degeneration in the genesis of retinopathy of prematurity. *Invest Ophthalmol Vis Sci.* **37**:290-299

Stone J, Dreher Z. (1987). Relationship between astrocytes, ganglion cells and vasculature of the retina. *J Comp Neurol.* **255**:35-49

Stone J, Itin A, Alon T, Pe'er J, Gnessin H *et al.* (1995). Development of retinal vascularature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J Neurosc.* **15**:4738-4747

Stratmann A, Risau W, Plate KH. (1998). Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am J Pathol.* **153**:1459-1466

Suarez S, Ballmer-Hofer K. (2001). VEGF transiently disrupts gap junctional communication in endothelial cells. *J Cell Sci.* **114:**1229-1235

Sueshi K, Hata Y, Murata T, Nakagawa K, Ishibashi T, Inomata H. (1996). Endothelial and glial cell interaction in diabetic retinopathy via the function of vascular endothelial growth factor (VEGF). *Polish J Pharmacol.* **48**:307-316

Sun Y, Jin K, Xie L, Childs L, Mao XO *et al.* (2003). VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J Clin Invest.* **111:**1843-1851

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC *et al.* (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell.* **87**:1171-1180

Suto K, Yamazaki Y, Morita T, Mizuno H. (2005). Crystal structures of novel vascular endothelial growth factors (VEGF) from snake venoms. *J Biol Chem.* **280:**2126-2131

Suzuki H, Watabe T, Kato M, Miyazawa K, Miyazono K. (2005). Roles of vascular endothelial growth factor receptor 3 signaling in differentiation of mouse embryonic stem cell-derived vascular progenitor cells into endothelial cells. *Blood.* **105:**2372-2379

Suzuma K, Takagi H, Otani A, Suzuma I, Honda Y. (1998). Increased expression of KDR/Flk-1 (VEGFR-2) in a murine model of ischaemia-induced retinal neovascularization. *Microvasc Res.* **56**:183-191

Takagi H, King GL, Aiello LP. (1996b). Identification and Characterization of vascular endothelial growth factor receptor (FLT) in bovine retinal pericytes. *Diabetes.* **45**:1016-1023

Takagi H, Koyama S, Seike H, Oh H, Otani A *et al.* (2003). Potential role of the angiopoietin/Tie2 system in ischaemia-induced retinal neovascularization. *Invest Ophthalmol Vis Sci.* **44:**393-402

Takagi H, King GL, Ferrara N, Aiello LP. (1996a). Hypoxia regulates vascular endothelial growth factor receptor KDR/Flk gene expression through adenosine A2 receptors in retinal capillary endothelial cells. *Invest Ophthalmol Vis Sci.* **37**:1311-1321

Takahama M, Tsutsami M, Tsujiuchi T, Nezu K, Kushibe K. (1999). Enhanced expression of Tie2, its ligand angiopoietin-1, vascular endothelial growth factor, and CD31 in human non-small cell lung carcinomas. *Clin Cancer Res.* **5:**2506-2510

Takekoshi K, Isobe K, Yashiro T, Hara H, Ishii K *et al.* (2004). Expression of vascular endothelial growth factor (VEGF) and its cognate receptors in human pheochromacytomas. *Life Sci.* **74:**863-871

Takita H, Yoneya S, Gehlbach PL, Duh EJ, Wei LL *et al.* (2003). Retinal neuroprotection against ischemic injury mediated by intraocular gene transfer of pigment epithelium-derived factor. *Invest OPhthalmol Vis Sci.* **44:**4497-4504

Tammela T, Enholm B, Alitalo K, Paavonen K. (2005). The biology of vascular endothelial growth factors. *Cardiovasc Res.* **65:**550-563

Tanaka S, Mori M, Sakamoto Y, Makuuchi M *et al.* (1999). Biologic significance of angiopoietin-2 expression in human hepatocellular carcinoma. *J Clin Invest.* **103**:341-345

Tang J, Mohr S, Du YD, Kern TS. (2003). Non-uniform distribution of lesions and biochemical abnormalities within the retina of diabetic humans. *Curr Eye Res.* **27:**7-13.

Tang Z, Scherer PE, Okamoto T, Song K, Chu C. (1996). Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J Biol Chem.* **271:**2255-2261

Teichert-Kuliszewska K, Maisonpierre PC, Jones N, Campbell AI, Master Z *et al.* (2001). Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. *Cardiovasc Res.* **49:**659-670

Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL *et al.* (1991). Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene.* **6**:1677-1683

Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC *et al.* (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Comm.* **187**:1579-1586

Tezel G, Li L, Patil RV, Wax MB. (2001). TNF-α and TNF-α receptor-1 in the retina of normal and glaucomatous eyes. *Invest Ophthalmol Vis Sci.* **42:**1787-1794

Tezel G, Wax MB. (2000). Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischaemia or elevated hydrostatic pressure induces apoptosis in cocultured retinal ganglion cells. *J Neurosci.* **20:**8693-8700

The Diabetic Retinopathy Study Research Group (1979): DRS No 3. Four risk factors for severe visual loss in diabetic retinopathy. **Arch** *Ophthalmol.* **97:**654-655

Thieme H, Aiello LP, Takagi H, Ferrara N, King GL. (1995). Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. *Diabetes.* **44**:98-103

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L *et al.* (2000). Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med.* **6:**460-463

Thurston G, Suri C, Smith K, McCain J, Sato TN *et al.* (1999). Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science.* **286:**2511-2514

Tian H, McKnight SL, Russell DW. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes & Development.* **11**:72-82

Tille J, Wang X, Lipson KE, McMahon G, Ferrara N *et al.* (2003). Vascular endothelial growth factor (VEGF) receptor-2 signaling mediates VEGF-$C_{\Delta N \Delta C}$ – and VEGF-A-induced angiogenesis in vitro. *Exp Cell Res.* **285:**286-298

Tischer E, Gospodarowicz D, Mitchell R, Silva M, Schilling J *et al.* (1989). Vascular endothelial growth factor: A new member of the platelet-derived growth factor gene family. *Biochem Biophys Res Comm.* **165**:1198-1206

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D *et al.* (1991). The human gene for vascular endothelial growth factor. *J Biol Chem.* **266**:11947-1954

Tobe T, Okamoto N, Vinores MA, Derevjanik NL, Vinores SA *et al.* (1998). Evolution of neovascularization in mice with overexpression of vascular endothelial growth factor in photoreceptors. *Invest Ophthalmol Vis Sci.* **39:**180-8.

Tolentino MJ, Mcleod DS, Taomoto M, Otsuji T, Adamis AP *et al.* (2002). Pathologic features of vascular endothelial growth factor-induced retinopathy in the nonhuman primate. *Am J Ophthalmol.* **133:**373-385

Tolentino MJ, Miller JW, Gragoudas ES, Jakobiec FA, Flynn E *et al.* (1996). Intravitreous injections of vascular endothelial growth factor produce retinal ischaemia and microangiopathy in an adult primate. *Ophthalmol.* **103**:1820-1828

Tombran-Tink, Barnstable CJ. (2003). PEDF: a multifaceted neurotrophic factor. *Nat Rev Neurosci.* **4:**628-636

Tombran-Tink J, Barnstable CJ. (2004). Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms and VEGF receptors: possible mediators of angiogenesis and matrix remodeling in the bone. *Biochem Biophys Res Commun.* **316:**573-579

Tombran-Tink J, Chader GG, Johnson LV. (1991). PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Exp Eye Res.* **53:**411-414

Tombran-Tink J, Johnson LV. (1989). Neuronal differentiation of retinoblastoma cells induced by medium conditioned by human RPE cells. *Invest Ophthalmol Vis Sci.* **30:**1700-1707

Tombran-Tink J, Mazuruk K, Rodriguez IR, Chung D, Linker T *et al.* (1996). Organization, evolutionary conservation, expression ans unusual Alu density of the human gene for pigment epithelium-derived factor, a unique neurotrophic serpin. *Mol Vis.* **2:**11

Tombran-Tink J, Shivaram SM, Chader GG, Johnson LV, Bok D. (1995). Expression, secretion, and age-related downregulation of pigment epithelium-derived factor, a serpin with neurotrophic activity. *J Neurosc.* **15:**4992-5003

Tout S, Chan-Ling T, Holländer H, Stone J. (1993). The role of Müller cells in the formation of the blood retinal barrier. *Neuroscience.* **55**:291-301

Trahey M, McCormick F. (1987). A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does no affect oncogenic mutants. *Science.* **238**:542-545

Tresini M, Pignolo R, Allen R, Cristofalo V. (1999). Effects of donor age on the expression of a marker of replicative senescence (EPC-1) in human dermal fibroblasts. *J Cell Physiol.* **179:**11-17

Trickler WJ, Mayhan WG, Miller DW. (2005). Brain microvessel endothelial cell responses to tumor necrosis factor-alpha involve a nuclear factor kappa B (NF-κB) signal transduction pathway. *Brain Res.* **1048:**24-31

Tsanou E, Ioachim E, Stefaniotou M, Gorezis S, Charalabopoulos K *et al.* (2005). Immunohistochemical study of angiogenesis and proliferative activity in epiretinal membranes. *Int J Clin Pract.* **59:**1157-1161.

Tsurumi Y, Murohara T, Krasinski K, Dongfen C, Witzenbichler B *et al.* (1997). Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. *Nat Med.* **3:**879-876

Uehara and Miyoshi. (2002). Localization of caveolin-3 in the sinus endothelial cells of the rat spleen. *Cell Tissue Res.* **307**:329-336

Uemura A, Ogawa M, Hirashima M, Fujiwara T, Koyama S *et al.* (2002). Recombinant angiopoietin-1 restores higher-order architecture of growing vessels in mice in the absence of mural cells. *J Clin Invest.* **110**:1619-1628

Ullrich A, Schlessinger J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell.* **61**:203-212

Umeda N, Ozaki H, Hayashi H, Kondo H, Uchida H, Oshima K. (2002). Non-paralled increase of hepatocyte growth factor and vascular endothelial growth factor in the eyes with angiogenic and nonangiogenic fibroproliferation. *Ophthalmic Res.* **34**:43-47

Unemori E, Ferrara N, Bauer EA, Amento EP. (1992). Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J Cell Physiol.* **153**:557-562

Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF. (1999). Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. *Proc Natl Acad Sci USA.* **96**:1904-1909

Valius M, Kazlauskas A. (1993). Phospholipase C-gamma 1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell.* **73**:321-334.

van Deurs B, Roepstorff K, Hommelgaard AM, Sandvig K. (2003). Caveolae: anchored, multifunctional platforms in the lipid ocean. *Trends Cell Biol.* **13**:92-100

Vasile E, Simionescu M, Simionescu N. (1983).Visualization of the binding, endocytosis, and transcytosis of low-density lipoprotein in the arterial endothelium in situ. *J Cell Biol.* **96**:1677-89.

van Eeden PE, Tee LB, Lukehurst S, Lai CM, Rakoczy EP *et al.* (2006). Early vascular and neuronal changes in a VEGF transgenic mouse model of retinal neovascularization. *Invest Ophthalmol Vis Sci.* **47:**4638-4645.

Vandamme W, Braet K, Cabooter L, Leybaert L. (2004). Tumour necrosis factor alpha inhibits purinergic calcium signaling in blood-brain barrier endothelial cells. *J Neurochem.* **88:**411-421

Valtola R, Salven P, Heikkila P, Taipale J, Joensuu H *et al.* (1999). VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol.* **154:**1381-1390

Varley MP, Frank E, Purnell EW. (1988). Subretinal neovascularization after focal argon laser for diabetic macular edema.*Ophthalmol.* **95:**567-573.

Vasile E, Simionescu M, Simionescu N. (1983). Visualization of the binding, endocytosis, and transcytosis of low-density lipoprotein in the arterial endothelium in situ. *J Cell Biol.* **96:**1677-1689.

Veikkola T, Jussila L, Makinen T. (2001), Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J.* **20:**1223-1231

Veikkola T, Karkkainen M, Claesson-Welsh K, Alitalo K. (2000). Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res.* **60:**203-212

Venkiteswaran K, Xiao K, Summers S, Calkins CC, Vincent PA *et al.* (2002). Regultion of endothelial barrier function and growth by VE-cadherin, plakoglobin and beta-catenin. *Am J Physiol Cell Physiol.* **283:**C811-C821

Viemann D, Goebeler M, Scmid S, Klimmek K, Sorg C *et al.* (2004). Transcriptional profiling of IKK2/NF-κB- and p38 MAP kinase-dependent gene expression in TNF-α-stimulated primary human endothelial cells. *Blood.* **103:**3365-3373

Virgintino D, Robertson D, Errede M, Benagiano V, Tauer U. (2002). Expression of caveolin-1 in human brain microvessels. *Neurosci.* **115:**145-152

Visconti RP, Richardson CD, Sato TN. (2002). Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). *Proc Natl Acad Sci USA.* **99:**8219-8224

Vogel SM, Minshall RD, Pilipovic M, Tiruppathi C, Malik AB. (2001). Albumin uptake and transport in endothelial clls in vivo induced by albumin-binding protein. *Am J Physiol Lung Cell Mol Physiol.* **281:**L1512-L1522

Volonte D, Zhang K, Lisanti MP, Galbiati F. (2002). Expression of caveolin-1 induces premature cellular senescence in primary cultures of murine fibroblasts. *Mol Biol Cell.* **13:**2502-2512

Volpert OV; Zaichuk T, Zhou W, Reiher F, Ferguson TA *et al.* (2002). Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. *Nat Med.* **8:**349-357

Von Ruhland CJ, Campbell L, Gumbleton M, Jasani B, Newman GR. (2004). Immunolocalization of caveolin-1 in rat and human mesothlium. *J Histochem Cytochem.* **52:**1415-1425

Wajant H, Scheurich P. (2001). Tumor necrosis factor receptor-associated factor (TRAF) 2 and its role in TNF signaling. *Int J Biochem Cell Biol.* **33:**19-32

Waltan SR, Oestrich C, Krupin T, Hanish S, Patzan S *et al.* (1978). Quantitative vitreous fluorophotometry. A sensitive technique for measuring early breakdown of the blood-retinal barrier in young diabetic patients. *Diabetes.* **27**:85-87

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin C. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem.* **269**:26988-26995

Waltenberger J, Mayr U, Pentz S, Hombach V. (1996). Functional upregulation of the vascular endothelial growth factor receptor KDR by hypoxia. *Circulation.* **94**:1647-1654

Wang H, Keiser JA. (1998). Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells. *Circ Res.* **83**:832-840

Wang M, Crisotomo PR, Herring C, Meldrum KK, Meldrum DR. (2006). Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-1 in response to TNF by a p38 MAPK-dependent mechanism. *Am J Physiol Regul Integr Comp Physiol.* **291**:R880-R884

Wang Y, Pampu S, Fujikawa K, Varticovski L. (2004). Oppsong effect of angiopoietin-1 on VEGF-mediated disruption of endothelial cell-cell interactions requires activation of PKCβ. *J Cell Physiol.* **198**:53-61

Wang Y, Pampou S, Varticovski L. (2000). The mechanism by which angiopoietin-1 inhibits VEGF-mediated vascular permeability. *Blood.* **96**:Abstract

Ware CF, Santee S, Glass A. (1998). Tumor necrosis factor related ligands and receptors. The cytokine handbook, New York: Academic Press. 549-592

Wartivaara U, Salven P, Mikkola H, Lassila R, Kaukonen J *et al.* (1998). Peripheral blood platelets express VEGF-C and VEGF which are released during platelet activation. *Thromb Haemost.* **80**:171-5

Watanabe M, Hitomi M, Van Der Wee K *et al.* (2002). The pros and cons of apoptosis assays for use in the study of cells, tissues, and organs. *Microsc Microanal.* **8**:375-391

Way M, Parton RG. (1996). M-caveolin, a muscle-specific caveolin-related protein. *FEBS Lett.* **378**:108-112

Weindel K, Marme D, Weich HA. (1992). AIDS-associated Kaposi's sarcomacells in culture express vascular endothelial growth factor. *Biochem Biophys ResCommun.* **3**:1167-1174

Weindel K, Moringlane JR, Marme D, Weich HA. (1994). Detection and quantification of vascular endothelial growth factor/vascular permeability factor in brain tumour tissue and cyst fluid: the key to angiogenesis?. *Neurosurgery.* **45**:439-449

Weiss S, Shintani S, Weber A. (2004). Src blockade stabalizes a Flk/cadherin complex, reducing edema and tissue injury following myocardial infarction. *J Clin Invest.* **113:**885-894

Wen Y, Edelman JL, Kang T, Zeng N, Sachs G. (1998). Two functional forms of vascular endothelial growth factor receptor-2/Flk-1 mRNA are expressed in normal rat retina. *J Biol Chem.* **273**:2090-2097

White RR, Shan S, Rusconi CP, Shetty G, Dewhirst MW *et al.* (2003). Inhibition of rat corneal angiogenesis by a nuclease-resistant RNA aptamer specific for angiopoietin-2. *Proc Natl Acad Sci USA.* **100:**5028-5033

Whiteside CI, Thompson J. (1989). The role of angiotensin-II in progressive diabetic glomerulopathy in the rat. *Endocrinology.* **125:**1932-1940

Wiedemann MD. (1992). Growth factors in retinal diseases: proliferative vitreoretinopathy, proliferative diabetic retinopathy, and retinal degeneration. *Survey of Ophthalmol.* **36**:373-384

Wilkinson-Berka JL. Jones D, Taylor G, Jaworski K, Kelly DJ *et al.* (2006). SB-267268, a nonpeptidic antagonist of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, reduces angiogenesis and VEGF expression in a mouse model of retinopathy of prematurity. *Invest OPhthalmol Vis Sci.* **47:**1600-1605

Willam C, Koehne P, Jurgensen JS, Grafe M, Wagner KD *et al.* (2000). Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells. *Circ Res.* **87:**370-377

Williams TM, Lisanti MP. (2005). Caveolin-1 in oncogenic transformation, cancer, and metastasis. *Am J Physiol Cell Physiol.***288:**494-506.

Williamson JR, Chang K, Frangos M. (1993). Hyperglycemic pseodohypocia and diabetic complications. *Diabetes*.**42**:801-813

Williamson JR, Tilton RG, Chang K. (1988). Basement membrane abnormalities in diabetes mellitus: Relationship to clinical micrangiography. *Diabetes Metab Rev.* **4**:339-370

Wilting J, Christ B, Bokeloh M, Weich HA. (1993). *In vivo* effects of vascular endothelial growth factor on the chicken chorioallantoic membrane. *Cell Tissue Res.* **274**:163-172

Wise LM, Veikkola T, Mercer AA, Savory LJ, Fleming SB *et al.* (1999). The vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. *Proc Natl Acad Sci USA.* **96:**3071-3076

Witmer AN, Blaauwgeers HG, Weich HA, Alitalo K, Vrenson GFJM *et al.* (2002). Altered expression patterns of VEGF receptors in human diabetic retina and in experimental VEGF-induced retinopathy in monkey. *Invest Ophthalmol Vis Sci.* **43:**849-857

Witmer AN, van Blijswijk BC, Dai J, Hofman P, Partanen TA *et al.* (2001). VEGFR-3 in adult angiogenesis. *J Pathol.* **195:**490-497

Witmer AN, van Blijswijk BC, van Noorden CFJ, Vrensen GFJM, Schlingemann RO. (2004). In vivo angiogenic phenotyes of endothelial cells and pericytes induced by vascular endothelial growth factor-A. *J Histochem Cytochem.* **52:**39-52

Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulas I *et al.* (1998b). Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischaemia. *Am J Pathol.* **153**:381-394

Witzenbichler B, Maisonpierre PC, Jones P, Yancopoulos GD *et al.* (1998a). Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J Biol Chem.* **273**:18514-18521

Wong AL, Haroon ZA, Werner S, Dewhirst MW *et al.* (1997). Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ Res.* **81**:567-574

Wong CG, Rich KA, Liaw LH, Hsu HT, Berns MW. (2001). Intravitreal VEGF and bFGF produce florid retinal neovscularization and hemorrohage in the rabbit. *Curr Eye Res.* **22:**140-147

Wong HY, Boulton ME, Clark P, Bayley M, Marshall J. (1987). Retinal pigment epithelial cells produce mitogenic factors for retinal microvascular cells in culture: a preliminary report. *Eye*. **1**:754-756

Woodman SE, Ashton AW, Schubert W, Lee H, Williams TM *et al.* (2003). Medina FA, Wyckoff JB, Combs TP, Lisanti MP. Caveolin-1 knockout mice show an impaired angiogenic response to exogenous stimuli. *Am J Pathol.* **162:**2059-68.

Wu HM, Huang Q, Yuan Y, Granger HJ. (1996). VEGF indiuces NO-dependent hyperpermeability in coronary venules. *Am J Physiol*. **271**:H2735-H2739

Wu YQ, Becerra SP. (1996). Proteolytic activity directed toward pigment epithelium-derived factor in vitreous of bovine eyes: implications of proteolytic processing. *Invest Ophthalmol Vis Sci.* **37:**1984-1993

Wu YQ, Notario V, Chader GJ, Becerra SP. (1995). Identification of pigment epithelium-derived factor in the interphotoreceptor matrix of bovine eyes. *Protein Expr Purif.* **6:**446-456

Xiao Q, Zeng S, Ling S, Lv M. (2006). Up-regulation of HIF-1alpha and VEGF expression by elevated glucose concentrations and hypoxia in cultured human retinal pigment epithelial cells. *J Huazhong Univ Sci Technolog Med Sci.* **26:**463-465

Yamade E. (1955). The fine structure of the gall bladder epithelium of the mouse. *J Biophys Biochem Cytol.* **1:**445-458

Yamagishi S, Amano S, Inagaki Y, Okamoto T, Koga K *et al.* (2002). Advanced glycation end products-induced apoptosis and overexpression of vascular endothelial growth factor in bovine retinal pericytes. *Biochem Biophys Res Commun.* **290:**973-978

Yamagishi S, Kawakami T, Fujimori H, Yonekura H, Tanaka N *et al.* (1999). Insulin stimulates the growth and tube formation of human microvascular endothelial cells through autocrine vascular endothelial growth factor. *Microvasc Res.* **57**:329-339

Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J. (1993). flk-1, a flt-related receptor tyrosine kinase, is an early marker for enothelial cell precursors. *Devel.* **118**:489-498

Yamagishi S, Amano S, Inagaki Y, Okamoto T, Koga K *et al.* (2002). Advanced glycation end products-induced apoptosis and overexpression of vascular endothelial growth factor in bovine retinal pericytes. *Biochem Biophys Res Commun.* **290:**973-8.

Yamagishi S, Amano S, Inagaki Y, Okamoto T, Takeuchi M *et al.* (2003). Pigment epithelium-derived factor inhibits leptin-induced angiogenesis by suppressing vascular endothelial growth factor gene expression through anti-oxidative properties. *Microvasc Res.* **65:**186-190

Yamagishi S, Hsu CC, Taniguchi M, Harada S, Yamamoto Y *et al.* (1995). Receptor-mediated toxicity to pericytes of advanced glycosylation end products: a possible mechanism of pericyte loss in diabetic microangiopathy. *Biochem Biophys Res Commun.***213:**681-687.

Yamagishi S, Inagaki Y, Amano S, Okamoto T, Takeuchi M *et al.* (2002). Pigment epithelium-derived factor protects cultured retinal pericytes from advanced glycation end product-induced injury through its antioxidative properties. *Biochem Biophys Res Commun.* **296:**877-882

Yamagishi S, Inagaki Y, Amano S, Okamoto T, Takeuchi M. (2002). Up-regulation of vascular endothelial growth factor and down-regulation of pigment epithelium-derived factor messenger ribonucleic acid levels in leptin-exposed cultured retinal pericytes. *Int J Tiss Reactions.* **24:**137-142

Yamagishi S, Nakamura K, Matsui T, Inagaki Y, Takenaka K. (2006). Pigment epithelium-derived factor inhibits advanced glycation end product-induced retinal vascular hyperpermeability by blocking reactive oxygen species-mediated vascular endothelial growth factor expression. *J Biol Chem.* **281:**20213-20220

Yamakawa M, Liu LX, Date T, Belanger AJ, Vincent KA *et al.*(2003). Hypoxia-inducible factor-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors. *Circ Res.* **93:**664-673

Yamamoto M, Toya Y, Jensen RA, Ishikawa Y. (1999). Caveolin is an inhibitor of platelet-derived growth factor receptor signaling. *Exp Cell Res.***247:**380-8.

Yamamoto M, Toya Y, Schwencke C, Lisanti MP, Myers MG Jr *et al.* (1998). Caveolin is an activator of insulin receptor signaling. *J Biol Chem.* **273:**26962-26968.

Yamamoto T, Yamashita H, Hori S. (1989). Electron microscopic observation of preretinal membranes. *Jpn J Ophthalmol.* **33:**151-65.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ *et al.* (2000). Vascular-specific growth factors and blood vessel formation. *Nature.* **407:**242-248

Yang S, Graham J, Kahn JW, Schwartz EA, Gerritsen ME. (1999). Functional roles for PECAM-1 (CD31) and VE-cadherin (CD144) in tube assemble and lumen formation in three-dimensional collagen gels. *Am J Pathol.* **155:**887-895

Yang H, Li M, Chai H, Yan S, Zhang R *et al.* (2004). Expression and regulation of neuropilins and VEGF receptors by TNF-alpha in human endothelial cells. *J Surg Res.* **122:**249-55.

Yang P, McKay BS, Allen JB, Roberts WL, Jaffe GJ. (2003). Effect of mutant on cytokine-induced activation of NF-κB in cultured human RPE cells. *Invest Ophthalmol Vis Sci.* **44:**1339-1347

Yang R, Thomas GR, Bunting S, Ko A, Keyt B *et al.* (1996). Effects of haemodynamics and cardiac performance. *J Cardiovasc Pharmacol.* **27**:838-844

Yao JS, Zhai W, Young WL, Yang G. (2006). Interleukin-6 triggers human cerebral endothelial cells proliferation and migration: The role for KDR and MMP-9. *Biochem Biophys Res Commun.* **342:**1396-1404

Yatoh S, Mizutani M, Yokoo T, Kozawa T, Sone H *et al.* (2006). Antioxidants and an inhibitor of advanced glycation ameliorate death of retinal microvascular cells in diabetic retinopathy. *Diabetes Metab Res Rev.* **22:**38-45.

Yilmaz A, Kliche S, Mayr U, Fellbrich G, Waltenberger J. (2003). P38 MAPK inhibition is critically involved in VEGFR-2-mediated endothelial cell survival. *Biochem Biophys Res Commun.* **306:**730-736

Yokoi M, Yamagishi S-I, Takeuchi M, Ohgami K, Okamoto T *et al.* (2005). Elevations of AGE and vascular endothelial growth factor with decreased total antioxidant status in the vitreous fluid of diabetic patients with retinopathy. *Br J Ophthalmol.* **89:**673-675

Yoon M, Cho C, Lee CS, Jang I, Ryu SH *et al.* (2003). Localization of Tie-2 and phospholipase D in endothelial caveolae is involved in angiopoietin-1-induced MEK/ERK phosphorylation and migration in endothelial cells. *Biochem Biophys Res Commun.* **308:**101-105

Yoshida N, Ikemoto S, Narita K, Sugimura K, Wada S *et al.* (2002). Interleukin-6, tumor necrosis factor alpha and interleukin-1beta in patients with renal cell carcinoma. *Br J Cancer.* **86:**1396-1400

Yoshida S, Yoshida A, Ishibashi T. (2004). Induction of IL-8, MCP-1, and bFGF by TNF-α in retinal glial cells:implications for retinal neovascularization during post-ischemic inflammation. *Graefe's Arch Clin Exp Ophthalmol.* **242:**409-413

Yossuck P, Tadesse Y, Higgins RD. (2001). Dexamethasone alters TNF-α expression in retinopathy. *Mol Gen Metabol.* **72:**164-167

Young TA, Wang H, Munk S, Hammoudi DS, Young DS *et al.* (2005). Vascular endothelial growth factor expression and secretion by retinal pigment epithelial cells in hugh glucose and hypoxia is protein kinase C-dependent. *Exp Eye Res.* **80:**51-662

Yu J, Bergaya S, Murata T, Alp IF, Bauer MP *et al.* (2006). Direct evidence for the role of caveolin-1 and caveolae in mechanotransduction and remodeling of blood vessels. *J Clin Invest.* 116:1284-1291.

Yu Q, Stamenkovic I. (2001). Angiopoietin-2 is implicated in the regulation of tumor angiogenesis. *Am J Pathol.* **158:**563-570.

Yu Y, Varughese J, Brown LF, Mulliken JB, Bischoff J. (2001). Increased Tie2 expression, enhanced response to angiopoeitin-1, and dysregulated angiopoietin-2 expression in hemangioma-derived endothelial cells. *Am J Pathol.* **159:**2271-2280

Zachary I, Gliki G. (2001). Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res.* **49:**568-581

Zebrowski BK, Yano S, Liu W. (1999). Vascular endothelial growth factor levels and induction of permeability in malignant pleural effusions. *Clin Cancer Res* **5:**3364-3368

Zhang Z, Liu Z, Sun Q. (2006). Expression of angiopoietins, Tie-2 and vascular endothelial growth factor in angiogenesis and progression of hepatocellular carcinoma. *World J Gastroenterol.* **12:**4241-4245

Zhang Y, McCluskey K, Fujii K, Wahl LM. (1998). Differential regulation of monocyte matrix metalloproteinase and TIMP-1production by TNF-alpha, granulocyte-macrophage CSF and IL-1 beta through prostaglandin-dependent and independent mechanisms. *J Immunol.* **161:**3071-3076

Zhang SX, Wang JJ, Gao G, Shao C, Mott R *et al.* (2005). Pigment epithelium-derived factor (PEDF) is an endogenous anti-inflammatory factor. *FASEB J.*

Zhao B, Smith G, Cai J, Ma A, Boulton ME. (2006). VEGF-C Promotes Survival of Retinal Vascular Endothelial Cells via VEGFR-2. *Br J Ophthalmol.* [In press.]

Zhao YY, Liu Y, Stan RV, Fan L, Gu Y et al. (2002). Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice. *Proc Natl Acad SciUSA* 99:11375-11380.

Zhou J, Callapina M, Goodall GJ, Brüne. (2004). Functional integrity of nuclear factor κB, phosphatidylinositol 3'-kinase, and mitogen-activated protein kinase signaling allows tumor necrosis factor α-evoked Bcl-2 expresion to provoke internal ribosome entry site-dependent translation of hypoxia-inducible factor 1α. *Cancer res.* **64:**9041-9048

Zhu WH, MacIntyre A, Nicosia RF. (2002). Regulation of angiogenesis by vascular endothelial growth factor and angiopoietin-1 in the rat aorta model: distinct temporal patterns of intracellular signaling correlate with induction of angiogenic sprouting. *Am J Pathol.* **161:**823-830.

Zhu T, Sennlaub F, Beauchamp MH, Fan L, Joyal JS *et al.,* (2006). Proangiogenic effects of protease-activated receptor 2 are tumor necrosis factor-α and consecutively Tie2 dependent. *Arterioscler Thromb Vac Biol.* **26:**744-750

Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S *et al.* (1997). Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast factor-induced angiogenesis. *J Clin Invest.* **99**:2625-2634

Zundel W, Swiersz LM, Giaccia A. (2000). Caveolin-1-mediated regulation of receptor tyrosine kinase-associated phosphatidylinositol 3-kinase activity by ceramide. *Mol Cell Biol.* **20:**1507-1514

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VEGF localisation in diabetic retinopathy

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Abstract

***Aim*—To determine the staining pattern of vascular endothelial growth factor (VEGF) at different stages of diabetic retinopathy (including post-laser photocoagulation) and to compare staining in excised fibrovascular and fibrocellular (non-diabetic) preretinal membranes.**

***Methods*—Immunohistochemical localisation of VEGF, using antibodies raised against $VEGF_{165}$ and $VEGF_{121,165,189}$, was carried out on specimens of normal human retina (n=15), diabetic retinas ((a) with no overt retinopathy (n=19), (b) with intraretinal vascular abnormalities but no proliferative retinopathy (n=6), (c) with active proliferative retinopathy (n=6), (d) with no residual proliferative retinopathy after photocoagulation therapy (n=15)), excised diabetic fibrovascular membranes (n=19), and non-diabetic fibrocellular membranes (n=7). The degree and pattern of immunostaining was recorded.**

***Results*—In general, VEGF was absent from the majority of normal retinas. VEGF staining was apparent in most diabetic tissues but the staining pattern was dependent on both the specificity of the antibody used and the category of tissue. Staining with the $VEGF_{165}$ antibody was generally confined to endothelial cells and perivascular regions while the $VEGF_{121,165,189}$ antibody was also associated with extravascular components of the inner retina. Intensity of immunostaining of diabetic eyes was dependent on the severity of retinopathy being least in diabetics with no overt retinopathy and greatest in retinas with proliferative retinopathy. Interestingly, the intensity of immunostaining in diabetic retinas which had undergone laser surgery for proliferative retinopathy was reduced to basal levels. Moderate to intense immunostaining was observed in all fibrovascular and fibrocellular membranes examined.**

***Conclusions*—This study supports a circumstantial role for VEGF in the pathogenesis of both the preclinical and proliferative stages of diabetic retinopathy.**
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Among the microvascular manifestations of diabetic retinopathy are pericyte dropout, basement membrane thickening, microaneurysm formation, and capillary non-perfusion resulting in inner retinal ischaemia and hypoxia.[1] The precise stimulus for the subsequent preretinal neovascularisation that characterises proliferative diabetic retinopathy (PDR) remains uncertain, but historically retinal hypoxia has been proposed as the stimulus for release of a diffusible angiogenic factor which promotes neovascularisation from adjacent retinal vessels and rubeosis.[2] While a number of candidate molecules have been suggested for this role, including basic fibroblast growth factor (bFGF),[3] platelet derived growth factor (PDGF),[4] and insulin-like growth factor-1 (IGF-1),[5] recent evidence has supported vascular endothelial growth factor (VEGF) as an important modulator of PDR.

VEGF is a mitogen for endothelial cells whose expression both in vivo and in vitro can be induced by hypoxia.[6] Reports have demonstrated high affinity, membrane bound VEGF receptors located on vascular endothelial cells[7–9] and recently on retinal pigment epithelial cells.[10] These receptors, which are autophosphorylating tyrosine kinases, are also upregulated by hypoxia.[11 12] In situ hybridisations against VEGF mRNA have shown increased retinal expression in human vasoproliferative retinopathies and also in animal models of ocular neovascularisation.[13–17] Furthermore, VEGF levels are elevated in vitreous samples from patients with active PDR when compared with (a) diabetic eyes without retinopathy, (b) diabetic eyes with background retinopathy,[18–20] and (c) diabetic eyes with quiescent PDR and after successful laser photocoagulation.[18] The aim of this study was to compare the pattern and intensity of VEGF immunostaining in (a) normal human retina, (b) diabetic retina (with and without non-proliferative retinopathy or PDR), (c) laser photocoagulated diabetic retina with presumed neovascular regression, and (d) surgically excised diabetic preretinal membranes, to determine whether there is a correlation between VEGF protein distribution and PDR. In addition, fibrocellular membranes were included as a comparative control for the fibrovascular membranes and two antibodies with differing specificities for VEGF were tested.

Materials and methods

DONOR EYES

Donor human eyes, fixed in 10% neutral buffered formalin (NBF) within 12 hours post mortem, were provided by the National Disease Research Interchange (NDRI), Philadelphia, USA. The anterior segment was removed and biomicroscopy of the posterior segment performed (a) to note overt features of retinopathy (for example, preretinal membranes, cotton wool spots, haemorrhages) and (b) to determine the extent of any scatter photocoagulation. Eyes were categorised as follows:

Normal—15 human eyes with no known ophthalmic disease and no history of diabetes.

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Donor age ranged from 20 to 92 years (mean 56 years).

Diabetic with no overt retinopathy—19 human eyes from diabetics with no clinical history of PDR and no overt features of retinopathy or retinal photocoagulation. Donor age ranged from 44 to 89 years (mean 74 years). A complete medical history was unavailable for all donors but, where available (7/18), the duration of diabetes was between 6 and 25 years.

Diabetic with obvious intraretinal vascular changes but no evidence of PDR—six human eyes from diabetics with no clinical history of PDR and no overt features of PDR or retinal photocoagulation. Retinas exhibited cotton wool spots, haemorrhages, and/or obvious microaneurysms. Donor age ranged from 55 to 96 years (mean 71 years). A complete medical history was unavailable for all donors but, where available (3/6), the duration of diabetes was between 3 and 21 years.

Diabetic with PDR—six human eyes from diabetics defined clinically as having PDR and exhibiting preretinal membranes. Donor age ranged from 37 to 76 years (mean 58 years). Duration of diabetes ranged from 3 to 18 years (mean 9 years).

Diabetic with scatter laser photocoagulation but no evidence of active PDR—15 human eyes from diabetics specified clinically as having had PDR and having received scatter laser photocoagulation (no details were available as to time post laser). No preretinal membranes were observed. Donor age ranged from 41 to 82 years (mean 63 years). Duration of diabetes ranged from 3 to 35 years (mean 17 years).

METHODS

The posterior segment of each eye was cut in the sagittal plane through the centre of the optic nerve head. Cuts were then made perpendicular to this line (a) on the horizontal midline on the nasal side and (b) two cuts were made at approximately 5 mm above and below the midline on the temporal side. A final vertical cut was made parallel to the initial cut and approximately 3 mm lateral to the macula. Five μm sections were cut from a portion of retina/choroid/sclera (a) approximately 3 mm lateral to the macula and perpendicular to the horizontal plane (this region was chosen owing to its susceptibility to retinal changes associated with diabetes) and (b) other representative areas across the retina (for example, areas of neovascularisation).

FIBROVASCULAR MEMBRANES

Nineteen fibrovascular preretinal membranes were obtained from 19 eyes during closed microsurgery for sequelae of PDR at the Manchester Royal Eye Hospital. Membranes were fixed in 10% NBF immediately upon removal for a minimum of 12 hours before paraffin wax embedding.

FIBROCELLULAR MEMBRANES

Seven non-vascularised epiretinal membranes were obtained from seven eyes of non-diabetic patients during closed microsurgery for elimination of retinal traction at the Manchester Royal Eye Hospital. Membranes were fixed in 10% NBF immediately upon removal for a minimum of 12 hours before paraffin wax embedding.

IMMUNOHISTOCHEMISTRY

Five μm sections (1–1.3 cm in length in the case of retinal specimens) were cut and mounted on APES coated slides. Deparaffinised sections were digested with 0.01% chymotrypsin for 20 minutes at 37°C. The sections were incubated in 10% milk proteins (Marvel)/10% normal rabbit serum (Dako) in TRIS buffered saline (TBS) for 60 minutes at room temperature. Excess blocking solution was removed and the sections incubated overnight at 4°C in either (a) polyclonal goat anti-human $VEGF_{165}$ (R&D Systems) raised against purified insect cell line *Sf* 21 derived recombinant human $VEGF_{165}$ or (b) polyclonal goat anti-human $VEGF_{121,165,189}$ (Santa Cruz Biotechnology) raised against a peptide corresponding to amino acids 1–20 mapping at the amino terminus of VEGF of human origin and which recognises the 121, 165, and 189 amino acid splice variants of VEGF; both were diluted in TBS to 5 μg/ml. Following two 3 minute washes, the sections were incubated with biotinylated rabbit anti-goat IgG (Dako) diluted to 1/600 in TBS for 30 minutes at room temperature. The sections were subsequently incubated with an avidin-biotin alkaline phosphatase reaction complex (Dako) and antibody binding visualised by incubation in fast red substrate solution (Sigma) resulting in the formation of a red product. Immunostained sections were counterstained with haematoxylin.

CONTROLS FOR IMMUNOSTAINING

Negative controls included (1) omission of the primary antibody, (2) substitution of the primary antibody with an inappropriate goat antibody (goat anti-human colostrum whey (Sigma) at the same concentration as the primary antibody), and (3) incubation of 100 μl (0.5 μg) of anti-$VEGF_{165}$ antibody with 1 μg of recombinant human $VEGF_{165}$ (R&D systems) overnight at 4°C before use in the above protocol.

ASSESSMENT OF IMMUNOSTAINING

The degree and pattern of immunostaining both within and between specimens as observed by standard light microscopy was assessed and recorded by two independent observers but pathological status of the specimens was obvious. The intensity of staining was graded qualitatively as background (corresponding to the level of staining seen in the negative controls), weak, moderate, or intense (corresponding to the highest level of immunoreactivity observed). These intensities were recorded as 0, 1, 2, and 3 respectively. For each retinal specimen staining intensity was recorded for the choroid, RPE, photoreceptor inner and outer segments, outer nuclear layer, inner nuclear layer, retinal vessels, ganglion cell layer, and internal limiting membrane. For

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fibrovascular and fibrocellular membranes staining intensity was recorded for vessels and surrounding matrix.

Results

VEGF staining was apparent in most diabetic tissue but the staining pattern was dependent on both the specificity of the antibody used and the category of tissue (see Figs 1 and 2 and Tables 1 and 2). Staining with the anti-$VEGF_{165}$ antibody was generally confined to endothelial cells (whether retinal or choroidal) and perivascular regions whereas staining with the $VEGF_{121,165,189}$ antibody was also associated with non-vascular components of the inner retina. No correlation was found between staining intensity/distribution and either donor age, postmortem time, or duration of diabetes (where known).

NORMAL

Immunostaining with the anti-$VEGF_{121,165,189}$ antibody was absent in the majority of retinas examined while retinal staining with the anti-$VEGF_{165}$ antibody, although generally absent, did result in weak staining associated with retinal vessels (7/15) and in the RPE (3/15) (Fig 1A; Tables 1 and 2). Weak to moderate staining with the anti-$VEGF_{165}$ antibody was also observed in the choroidal vessels (12/15). Staining was absent from the inner and outer retina in all but one specimen.

DIABETIC WITH NO OVERT RETINOPATHY

Staining intensity with the anti-$VEGF_{165}$ antibody was not elevated above that observed in non-diabetic retinas. Twelve of 19 specimens exhibited some positive staining of endothelial cells of inner retinal vessels, while 9/19 were VEGF positive in vessel basement membranes (generally those diabetic retinas with thickened endothelial basement membranes). By contrast, immunostaining with the anti-$VEGF_{121,165,189}$ antibody, albeit weak to moderate, was elevated in all tissue layers examined compared with that observed in normal retinas (Fig 1B; Table 2). Immunostaining was most intense in the inner retinal and ganglion cell layers (13/19).

DIABETIC WITH OBVIOUS INTRARETINAL VASCULAR CHANGES BUT NO EVIDENCE OF PDR

Intensity of immunostaining with the anti-$VEGF_{165}$ antibody was increased in the retinal vessels, the choroid, and the ganglion cell layer compared with that observed in normal retinas and diabetic retinas with no overt retinopathy (staining in the other retinal layers remained unchanged) (Figs 1C and 2A). Variable immunostaining ranging from weak to intense was observed in both the retinal vessels and choroid with a close correlation between intense choroidal staining and moderate to intense staining around retinal vessels. Intensity of immunostaining with the anti-$VEGF_{121,165,189}$ antibody in this group was elevated compared with that observed in non-diabetic retinas but not increased compared with that observed for diabetic retina with no overt retinopathy (Fig 1D).

DIABETIC WITH PDR

Intense immunostaining with the anti-$VEGF_{165}$ antibody of the inner retinal vessels was seen in all diabetic retinas (6/6) with active neovascular PDR membranes on their surfaces (Fig 1E). Equally intense staining was observed within the membranes themselves (Fig 1E). While immunoreactivity for VEGF was found around choroidal vessels in all these PDR retinas, with staining ranging from weak to intense, the RPE layer and outer retina were generally $VEGF_{165}$ negative. The immunostaining pattern with the anti-$VEGF_{121,165,189}$ antibody showed a similar moderate to high intensity staining of retinal vessels but, in addition, there was increased staining in the outer nuclear layer, inner retina, ganglion cell layer, and internal limiting membrane (ILM) compared with the other tissue groups (Fig 1F).

Table 1 Mean (SD) intensity of immunostaining of the retina/choroid using an anti-$VEGF_{165}$ antibody

Tissue category	Tissue layer: Choroidal vessels	RPE	Outer retina	Inner retina	Retinal vessels	Ganglion cell layer	ILM
Normal (n=12)	1.6 (0.3)	0.3 (0.2)	0	0.3 (0.2)	0.9 (0.2)	0.1 (0.1)	0
Diabetic:							
No overt retinopathy (n=19)	1.6 (0.2)	0.1 (0.1)	0	0.4 (0.2)	1.2 (0.2)	0.2 (0.1)	0.1 (0.1)
Vascular abnormalities (n=6)	2.3 (0.3)	0.5 (0.3)	0.2 (0.2)	0.3 (0.2)	2.2 (0.5)	1.0 (0.4)	0.3 (0.2)
PDR (n=6)	2.2 (0.4)	0.4 (0.3)	0.6 (0.2)	0.6 (0.2)	3.0 (0)	0.6 (0.2)	0.6 (0.2)
Laser, no PDR (n=15)	1.7 (0.3)	0.1 (0.1)	0.3 (0.1)	0.3 (0.1)	1.0 (0.3)	0.2 (0.1)	0.1 (0.1)

0 = background, 1 = weak, 2 = moderate, 3 = intense.

Table 2 Mean (SD) intensity of immunostaining of the retina/choroid using an anti-$VEGF_{121,165,189}$ antibody

Tissue category	Tissue layer: Choroidal vessels	RPE	Outer retina	Inner retina	Retinal vessels	Ganglion cell layer	ILM
Normal (n=12)	0.3 (0.1)	0.5 (0.4)	0.2 (0.1)	0.2 (0.1)	0.3 (0.1)	0.4 (0.2)	0.3 (0.2)
Diabetic:							
No overt retinopathy (n=19)	0.7 (0.2)	1.3 (0.2)	0.8 (0.2)	1.6 (0.2)	1.0 (0.2)	1.9 (0.2)	1.5 (0.2)
Vascular abnormalities (n=6)	0.5 (0.3)	0.8 (0.4)	0.3 (0.3)	1.2 (0.5)	1.1 (0.4)	1.7 (0.4)	1.3 (0.4)
PDR (n=6)	1.0 (0.5)	1.8 (0.4)	1.5 (0.4)	1.8 (0.3)	2.2 (0.3)	2.0 (0.4)	1.8 (0.4)
Laser, no PDR (n=15)	0.7 (0.2)	1.2 (0.3)	1.2 (0.3)	1.1 (0.2)	1.0 (0.2)	1.2 (0.2)	1.0 (0.2)

0 = background, 1 = weak, 2 = moderate, 3 = intense.

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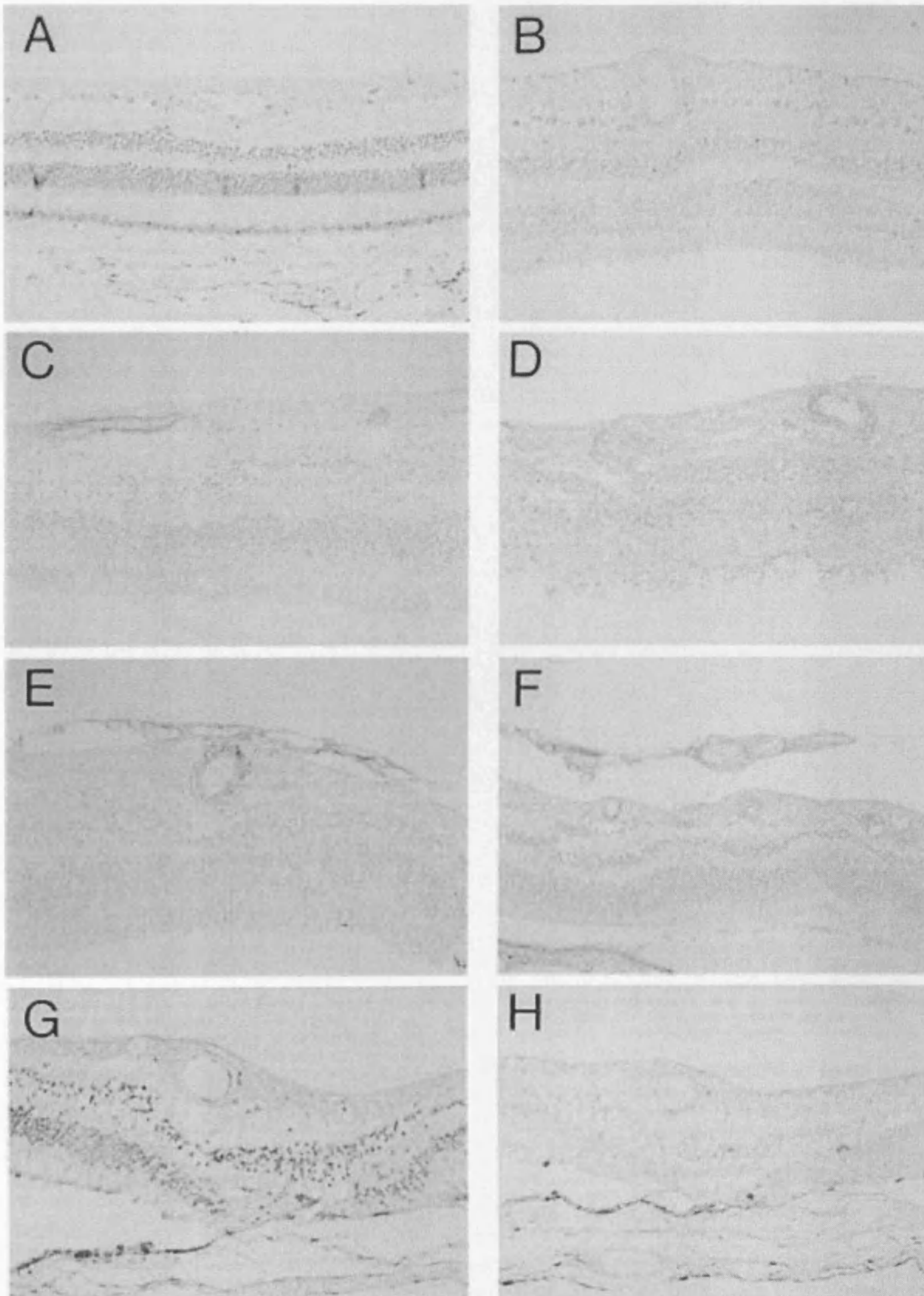

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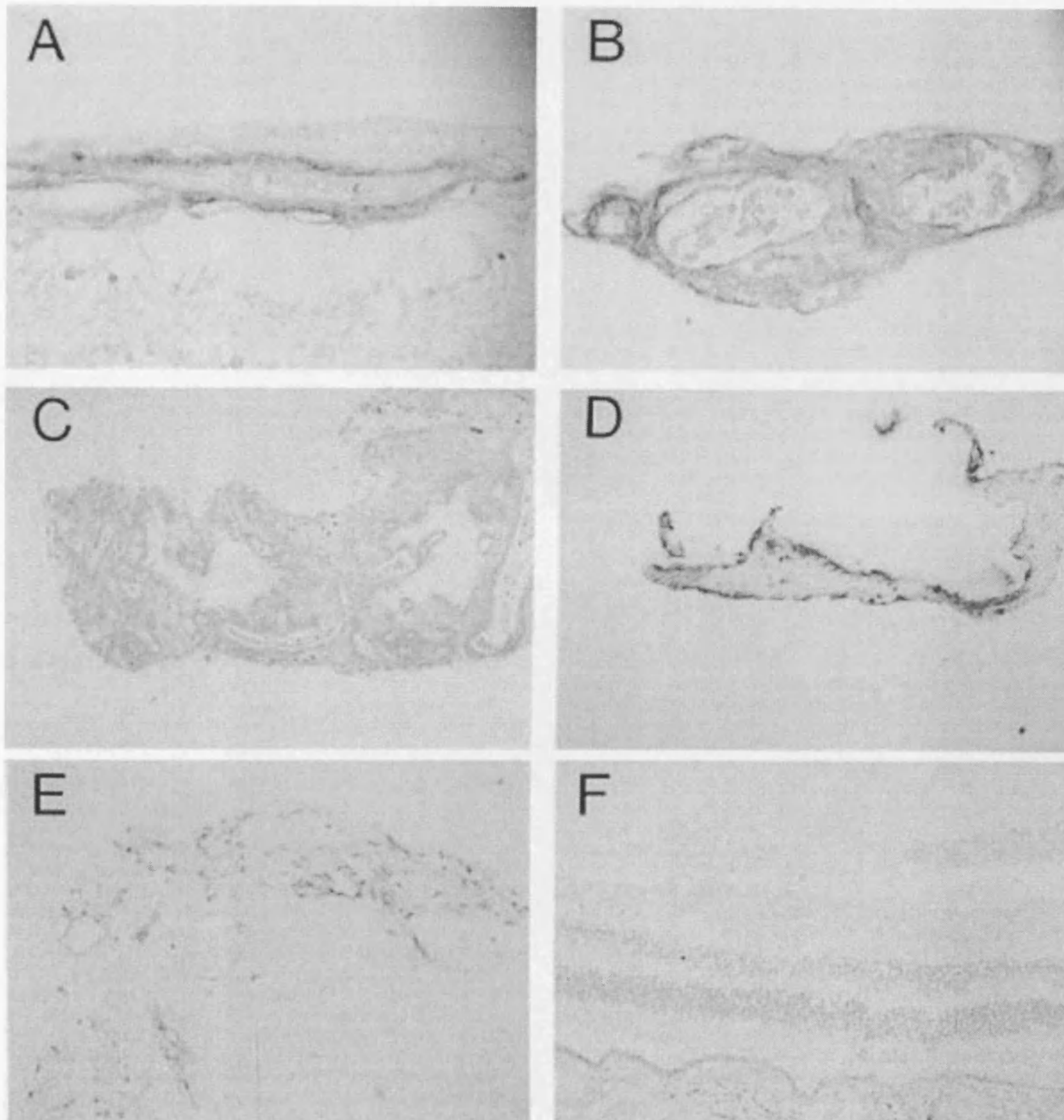

Figure 2 Photomicrographs demonstrating VEGF immunostaining of PDR retina and excised membranes. Intense immunostaining for $VEGF_{165}$ is localised to the vasculature (A) while $VEGF_{121,165,189}$ immunostaining is observed in both vascular and extravascular tissue (B). Moderate to intense staining can be observed in all specimens of excised fibrovascular (C) and fibrocellular (D) epiretinal membranes. Immunoreactivity for VEGF was abolished in control sections of PDR retina and membranes processed with omission of the primary antibody (E) or prior incubation of the antibody with VEGF. Magnification, A, B ×200; C–F ×70.

No correlation was observed between VEGF positive glial staining and active neovascularisation.

DIABETIC WITH SCATTER LASER PHOTOCOAGULATION BUT NO EVIDENCE OF PDR

The intensity of immunostaining in diabetic retinas that had undergone apparently successful laser therapy (that is, those with no preretinal neovascularisation) was similar to that observed for diabetic retinas with no overt retinopathy (Fig 1G, H). This was true for both antibodies used. In many laser treated retinas (11/15) a total absence of immunoreactivity was observed within inner retinal vessels, even those with thickened basement membranes. This was especially apparent in those vessels located near laser burns. Staining

Figure 1 Photomicrographs demonstrating VEGF immunostaining of normal retina (A), diabetic retina with no obvious retinopathy (B), diabetic retina with obvious intraretinal vascular changes but no evidence of PDR (C, D), diabetic retina with PDR (E, F), and diabetic retina after laser treatment for PDR (G, H). Sections were immunostained with either an antibody raised against $VEGF_{165}$ (A, C, E, G) or $VEGF_{121,165,189}$ (B, D, F, H). Immunostaining was greatest in diabetic retinas with PDR for both antibodies tested, minimal in normal retinas, and intermediate in diabetic retinas without PDR. It was interesting to note that immunostaining in lasered diabetic retinas with no current evidence of PDR was greatly reduced compared with the staining intensity in retinas with PDR. Magnification, A–E, G ×90; F, H ×70.

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intensity was also reduced/absent in the outer nuclear layer, inner retina, ganglion cell layer, and ILM compared with diabetic retinas with PDR.

FIBROVASCULAR MEMBRANES

A similar staining pattern was observed for both anti-VEGF antibodies used. In general, two immunostaining profiles for VEGF were observed in the preretinal diabetic membranes; those in which staining was essentially confined to the vascular component (9/19) and those in which moderate to intense staining was also found in areas of acellular matrix (10/19) (Fig 2B, C). Staining of vessels was variable both between and within specimens, with those vessels at the periphery generally staining more intensely than those in the centre of the membrane. In addition, staining of extracellular matrix was variable but also tended to be highest at the periphery. Staining of non-vascular cells was weak and variable, being present in only 7/19 membranes.

FIBROCELLULAR MEMBRANES

VEGF staining was observed in all seven fibrocellular membranes; the staining profile was similar for both anti-VEGF antibodies. Staining was associated with pigmented cells, non-pigmented cells and surrounding matrix (Fig 2D). The intensity of staining was highly variable both within and between membranes and ranged from weak to intense. Intensity of staining in the matrix was generally greater than that found in the cellular areas.

CONTROLS

Immunoreactivity for VEGF was abolished in sections processed with omission or substitution of the primary antibody (Fig 2E) and considerably reduced by prior incubation of the antibody with recombinant $VEGF_{165}$ (Fig 2F).

Discussion

We have demonstrated changes in the profile and intensity of VEGF staining dependent upon the severity of diabetic retinopathy. The most intense staining correlated with active neovascularisation and was present in both preretinal vessels and associated inner retinal vessels. Interestingly, the elevation of immunostaining for $VEGF_{121,165,189}$ in diabetic retinas with no overt retinopathy infers that VEGF may play a role in both the preclinical and the proliferative stages of diabetic retinopathy.

Lutty and co-workers detected VEGF immunoreactivity within some smooth muscle cells of retinal arterioles and in the pericytes and some endothelial cells of retinal capillaries in non-diabetic human retinas.[21] Furthermore, they noted a significantly increased immunoreactivity in the contractile elements of retinal vessels, in the endothelium of human diabetic retina and in and around the choroidal vessels, results consistent with those presented here. Immunohistochemical staining for VEGF has also been shown to be markedly increased in the retina of streptozotocin treated diabetic rats, with positive staining located in and around the inner retinal capillaries.[22] However, in areas of non-perfused human diabetic retina and in rabbit and primate models of ocular angiogenesis, in situ hybridisation studies have shown hypoxia induced VEGF expression not in the component cells of retinal vessels but in the inner and outer nuclear layers or the ganglion cell layers.[13 14] This distribution is in agreement with immunostaining of VEGF within Muller cells and in the ganglion cell layer in oxygen induced neovascularisation models in rat retina[23 24] and in diabetic retina.[25] We could only observe significant staining in these extravascular regions with one of the anti-VEGF antibodies used—that is, $VEGF_{121,165,189}$. This difference in specificity may reflect either (a) the availability of the appropriate epitope or (b) differential expression of VEGF isoforms within the retina. The generation of antibodies against the 121 and 189 VEGF isoforms may help address this question.

Rather than defining the sites of potential VEGF synthesis, the increase in staining for VEGF protein in diabetic retinal vessels demonstrated in our study, especially in eyes with neovascularisation, may reflect the localisation of sites of action or accumulation of the factor. Since VEGF binds to hypoxia inducible receptors found on endothelial cells, such cells surrounding non-perfused areas of diabetic inner retina may be likely to manifest increased VEGF binding. In addition, VEGF, especially the larger splice variants ($VEGF_{189,206}$), binds to heparin[26] and increased staining in and around retinal vessels may to some extent reflect binding to heparan sulphate associated with basement membrane thickening in diabetes.

We have demonstrated that VEGF protein increases in diabetic eyes and that staining is greatest in PDR. The role of VEGF in preclinical retinopathy is unclear but may relate to vascular permeability. Raised VEGF levels are known to raise vascular permeability,[27-29] a feature prominent in background and preproliferative diabetic retinopathy.[1] These observations suggest that VEGF is elevated in the diabetic retina in the absence of hypoxia and extensive retinal ischaemia inferring that other mechanisms (for example, hyperglycaemia) may upregulate VEGF. However, it should be noted that in this, as in other,[25] studies it is difficult to determine the degree of retinal ischaemia, if any, in postmortem diabetic retinas with preproliferative retinopathy. While vascular permeability has been suggested as being a critical step in angiogenesis,[30] VEGF itself is also likely to directly affect other cellular events associated with neovascularisation.[31 32] These observations are supported by Tolentino *et al* who reported that intravitreal injection of VEGF into primates resulted in leaky vessels, progressively dilated and tortuous vessels, microaneurysms, haemorrhage, and capillary closure.[33]

Our observation that high levels of VEGF are associated with the periphery of preretinal vessels is further support that VEGF plays an

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important role in the progression of preretinal neovascularisation. The intense staining of acellular matrix in many of the membranes may also reflect the matrix binding properties of VEGF. VEGF present in PDR membranes may derive from the retina but may also be synthesised by the cells within the membrane. We observed staining for VEGF within non-vascular cells in some membranes, while mRNA for VEGF has been found in diabetic membranes.[20] Vitreous fluid from patients with active retinal neovascularisation has previously been shown to contain high concentrations of VEGF.[18] Independent of its origin, membrane and vitreous associated VEGF may propel the neovascular response resulting in membrane growth.

VEGF immunoreactivity in epiretinal membranes is in agreement with Chen and colleagues.[34] It is difficult to explain the presence of VEGF but it may reflect the hypoxic nature of this avascular tissue or that VEGF is modulating non-vascular cells; RPE cells[10] and fibrocellular membranes[34] are known to express VEGF receptors.

Scatter laser photocoagulation induces regression of active diabetic neovascularisation. We have shown that immunostaining for VEGF is reduced in diabetic retinas that have no overt preretinal neovascularisation following laser therapy. This is in close agreement with Aiello *et al* who found decreased vitreous levels of VEGF in patients after laser therapy.[18] It is possible that a reduction in retinal ischaemia after laser treatment reduces the production of VEGF suppressing neovascularisation and leading to regression and quiescence.

An expanding body of evidence, to which these results contribute, now suggests that VEGF may be Michaelson's ischaemia induced ocular angiogenic factor. However, it is unlikely that VEGF functions in isolation. Other angiogenic factor levels have been found to be elevated in diabetic retina, fibrovascular membranes, and vitreous including bFGF, IGF-1, TGF-β, PDGF, and placenta growth factor (PlGF).[35 36] Thus, VEGF may work synergistically with other factors; repeated intravitreal injection of VEGF into primates produces vascular abnormalities associated only with background retinopathy[33] inferring that additional growth factors are required to initiate preretinal neovascularisation. Furthermore, VEGF may play a largely ignored, but important, role in modifying vascular permeability during the early stages of preproliferative retinopathy. Studies which determine the biological effects of VEGF and its interactions with other growth factors should increase our understanding of the vasoproliferative retinopathies and provide opportunities for new therapeutic interventions for these blinding conditions.

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1 Garner A. Vascular diseases. In: Garner A, Klintworth GK, eds. *Pathobiology of ocular disease. A dynamic approach.* 2nd ed. New York: Marcel Dekker, 1994:1625–710.
2 Michaelson IC. The mode of development of the vascular system of the retina with some observations on its significance for certain retinal disorders. *Trans Ophthalmol Soc UK* 1948;**68**:137–80.
3 Hanneken A, de Juan E, Lutty GA, *et al.* Altered distribution of basic fibroblast growth factor in diabetic retinopathy. *Arch Ophthalmol* 1991;**109**:1005–11.
4 Robbins SG, Mixon RN, Wilson DJ, *et al.* Platelet-derived growth factor ligands and receptors immunolocalized in proliferative retinal disorders. *Invest Ophthalmol Vis Sci* 1994;**35**:3649–63.
5 Dills DG, Moss SE, Klein R, *et al.* Association of elevated IGF-1 levels with increased retinopathy in late-onset diabetes. *Diabetes* 1991;**40**:1725–30.
6 Minchenko A, Bauer T, Salceda S, *et al.* Hypoxic stimulation of vascular endothelial growth factor expression in vivo and in vitro. *Lab Invest* 1994;**71**:374–9.
7 deVries C, Escobedo JA, Ueno H, *et al.* The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992;**255**:989–91.
8 Terman BI, Vermazen MD, Carrion ME, *et al.* Identification of the KDR tyrosine kinase as a receptor for vascular endothelial growth factor. *Biochem Biophys Res Commun* 1992;**34**:1578–86.
9 Quinn T, Peter KG, deVries C, *et al.* Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci USA* 1993;**90**:7533–7.
10 Guerrin M, Moukadiri H, Chollet P, *et al.* Vasculotropin/vascular endothelial growth factor is an autocrine growth factor for human retinal pigment epithelial cells cultured in vitro. *J Cell Physiol* 1995;**164**:385–94.
11 Thieme H, Aiello LP, Takagi H, *et al.* Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. *Diabetes* 1995;**44**:98–103.
12 Tuder RM, Flook BE, Voelkel NF. Increased gene expression for VEGF and the VEGF receptor KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia. *J Clin Invest* 1995;**95**;1798–807.
13 Miller JW, Adamis AP, Shima DT, *et al.* Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angogenesis in a primate model. *Am J Path* 1994;**145**:574–84.
14 Pe'er J, Shweiki D, Itin A, Hemo I, *et al.* Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularising ocular diseases. *Lab Invest* 1995;**72**:638–45.
15 Pe'er J, Folberg R, Itin A, *et al.* Upregulated expression of vascular endothelial growth factor in proliferative diabetic retinopathy. *Br J Ophthalmol* 1996;**80**:241–5.
16 Stone J, Pe'er J, Chan-Ling T, *et al.* The roles of vascular endothelial growth factor and neuroglia in the pathogensis of retinopathy of prematurity. *Invest Ophthalmol Vis Sci* 1995;**36(suppl)**:871.
17 Pierce E, Foley ED, Smith LEH. Regulation of retinal vascular endothelial growth factor (VEGF/VPF) levels by hyperoxia and hypoxia: a possible etiology for ROP. *Invest Ophthalmol Vis Sci* 1995;**36(suppl)**:871.
18 Aiello LP, Avery RL, Arrigg PG, *et al.* Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 1994;**331**:1480–7.
19 Adamis AP, Miller JW, Bernal M-T, *et al.* Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol* 1994;**118**:445–50.
20 Malecaze F, Clamens S, Simorre-Pinatel V, *et al.* Detection of vascular endothelial growth factor messenger RNA and vascular endothelial growth factor-like activity in proliferative diabetic retinopathy. *Arch Ophthalmol* 1994;**112**:1476–82.
21 Lutty GA, McLeod DS, Merges C, *et al.* Localization of vascular endothelial growth factor in human retina and choroid. *Arch Ophthalmol* 1996;**114**:971–7.
22 Murata T, Isibashi T, Khalil M, *et al.* Vascular endothelial growth factor plays a role in hyperpermeability of diabetic retinal vessels. *Ophthalmic Res* 1995;**27**:48–52.
23 Robbins SG, Penn JS, Conaway JR, *et al.* Distribution of vascular endothelial growth factor (VEGF) in normal and oxygen-injured rat retinas. *Invest Ophthalmol Vis Sci* 1995;**36(suppl)**:871.
24 Youssri AI, Luma J, Vinores S, *et al.* Immunohistochemical localisation of vascular endothelial growth factor (VEGF) in retinas with oxygen-induced ischemic retinopathy and non-ischemic retinas. *Invest Ophthalmol Vis Sci* 1995;**36(suppl)**:401.
25 Amin R, Frank R, Kennedy A, *et al.* Vascular endothelial growth factor is present in glial cells of the retina and optic nerve of human subjects with non-proliferative diabetic retinopathy. *Invest Ophthalmol Vis Sci* 1997;**38**:36–47.
26 Houck KA, Ferrara N, Winer J, *et al.* The vascular endothelial growth factor family: four molecular species and characterisation of alternative splicing of RNA. *Mol Endocrinol* 1991;**5**:1806–14.
27 Senger DR, Van De Walter L, Brown LF, *et al.* Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev* 1993;**12**:303–24.

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28 Connolly DT, Olander JV, Heuvelman D, *et al.* Human vascular permeability factor. *J Biol Chem* 1989;**264**:20017–24.
29 Murata T, Nakagawa K, Khalil A, *et al.* The relation between expression of vascular endothelial growth factor and breakdown of the blood retinal barrier in diabetic rat retinas. *Lab Invest* 1996;**74**:819–25..
30 Dvorak HF. Tumors: wounds that do not heal. Similarity between stroma generation and wound healing. *N Engl J Med* 1986;**315**:1650–6.
31 Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 1989;**161**:851–9.
32 Leung DW, Cachianes G, Kuang W-J, *et al.* Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989;**246**:1306–9.
33 Tolentino M, Miller J, Gragoudas E, *et al.* Intravitreous injections of vascular endothelial growth factor produce retinal ischaemia and microangiopathy in an adult primate. *Ophthalmology* 1996;**103**:1820–8.
34 Chen Y-S, Hackett S, Schoenfeld C-L, *et al.* Localisation of vascular endothelial growth factor and its receptors to cells of vacular and avascular epiretinal membranes. *Br J Ophthalmol* 1997;**81**:919–26.
35 Boulton ME, Foreman D, McLeod D. Vascularised vitreoretinopathy: the role of growth factors. *Eye* 1996;**10**: 691–6.
36 Khaliq A, Foreman D, Ahmed A, *et al.* Increased expression of placenta growth factor (PlGF) in proliferative diabetic retinopathy. *Lab Invest* 1998; (in press).

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Br J Ophthalmol 1999;83:486–494

Immunolocalisation of the VEGF receptors FLT-1, KDR, and FLT-4 in diabetic retinopathy

Gillian Smith, David McLeod, David Foreman, Mike Boulton

Abstract

***Aim*—To determine the spatial and temporal changes in the staining pattern of the VEGF receptors FLT-1, KDR, and the putative receptor FLT-4 during the pathogenesis of diabetic retinopathy.**

***Methods*—Immunohistochemical localisation of VEGF receptors, using antibodies against FLT-1, FLT-4, and KDR, was carried out on specimens of normal human retina (n=10), diabetic retinas (a) with no overt retinopathy (n=12), (b) with intraretinal vascular abnormalities but no proliferative retinopathy (n=5), (c) with active proliferative retinopathy (n=6), and (d) with no residual proliferative retinopathy after scatter photocoagulation therapy (n=14), and surgically excised diabetic fibrovascular membranes (n=11). The degree and pattern of immunostaining was recorded.**

***Results*—FLT-1 staining was apparent in the retinas from both non-diabetic and diabetic retinas; weak to moderate staining was generally confined to the inner nuclear layer, the ganglion cell layer, and the retinal vessels during all stages of the disease process. Staining of the retinal vessels was raised in diabetic tissue compared with non-diabetic tissue. The preretinal vessels of the diabetic subjects stained moderately to intensely for FLT-1. In contrast with FLT-1 staining minimal immunostaining for KDR was demonstrated in the non-diabetic eyes and the unlasered eyes; however, weak staining for KDR was observed in the inner nuclear layer and the ganglion cell layer of the unlasered eyes with diabetic changes. In those retinas with preretinal neovascularisation KDR immunoreactivity was moderate to intense in the intra- and preretinal vessels. However, in the excised membranes, where the vessels may have been in a quiescent state, the levels of KDR were weak to moderate. After apparently successful laser treatment KDR staining was reduced in the intraretinal vessels. Minimal FLT-4 staining was observed throughout normal eyes while weak to moderate FLT-4 staining was generally confined to the inner nuclear layer and the ganglion cell layer of the unlasered diabetic eyes. Weak to moderate levels of FLT-4 staining were observed in the intraretinal vessels except after apparently successful laser treatment where reduced levels of staining were observed. Weak to moderate staining was observed in the preretinal vessels.**

***Conclusions*—This study supports a role for FLT-1, KDR, and possibly FLT-4 in the pathogenesis of diabetic retinopathy; however, their specific roles in the progression of the disease may differ.**

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Proliferative diabetic retinopathy (PDR), the archetypical vasoproliferative retinopathy (VPR), is characterised by preretinal neovascularisation and fibrosis, ultimately leading to vitreous haemorrhage and traction retinal detachment.[1] A number of growth factors have been implicated in PDR of which vascular endothelial growth factor (VEGF) is considered to be of major importance since (a) it is a diffusible factor,[2–4] (b) it increases vascular permeability,[2–5] (c) it modulates angiogenesis,[2–4] (d) it stimulates endothelial cell proliferation[2–4 6] and migration,[6] (e) it is upregulated in response to hypoxia,[7–9] and (f) agents which inhibit the binding of VEGF to its receptors have been demonstrated to reduce neovascularisation.[10 11] In situ hybridisation, northern blotting, and immunohistochemistry have demonstrated increased expression of VEGF in animal models for VPRs[8 9 12] and in diabetic human retinas.[8 13–16]

VEGF is believed to act through high affinity receptors located on endothelial cells.[2 3 6] These receptors are autophosphorylating type III tyrosine kinases and consist of KDR (FLK-1 in mouse, TKrC in rats, Quek1 and 2 putative avian[5]) and FLT-1 receptors.[2 3] Both receptors are characterised by the presence of seven immunoglobulin-like domains in their extracellular region[2] and are expressed during embryogenesis where they appear to play an important role in endothelial growth and differentiation during vasculogenesis and angiogenesis.[17 18] FLT-1 is believed to regulate metabolic activity including vascular permeability while KDR is considered to modulate angiogenic responses (for example, endothelial cell migration and proliferation). The importance of FLT-1 is further inferred by the recent demonstration that placenta growth factor (PlGF) is associated with diabetic retinopathy;[19] PlGF acts through the FLT-1 receptor.[20] A third tyrosine kinase receptor may be important in VEGF recognition by endothelial cells; FLT-4, which has a similar structure to FLT-1 and KDR, is expressed in the placenta and in several mouse tissues during embryogenesis.[21 22]

Although there are a large number of reports documenting upregulation of VEGF mRNA

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and protein in the VPRs there is very little information on the profile of VEGF receptors. In this study we used immunohistochemistry to detect FLT-1, KDR, and FLT-4 protein in (a) normal human retinas, (b) diabetic retinas with various stages of retinopathy, and (c) in preretinal fibrovascular membranes excised during diabetic vitrectomy.

Materials and methods

DONOR EYES

A total of 47 eyes enucleated and fixed in 10% neutral buffered formalin, within 10 hours post mortem, were obtained from the National Disease Research Interchange (NDRI), Philadelphia, USA. Each eye was dissected into an anterior and posterior segment. A complete medical history was not available for all donors but details were available regarding glycaemic management. Of the 37 diabetic donors 25 had been injecting insulin for at least 6 months (mean age 62 years) and 12 were not receiving insulin treatment but did use oral hyperglycaemic drugs (mean age 65 years). Examination of the posterior segment was performed by an ophthalmologist (DM) using a Zeiss Stemi SV8 zoom dissecting microscope with Schott light source (a) to note overt features of retinopathy (for example, the presence of preretinal membranes, cotton wool spots, microaneurysms,

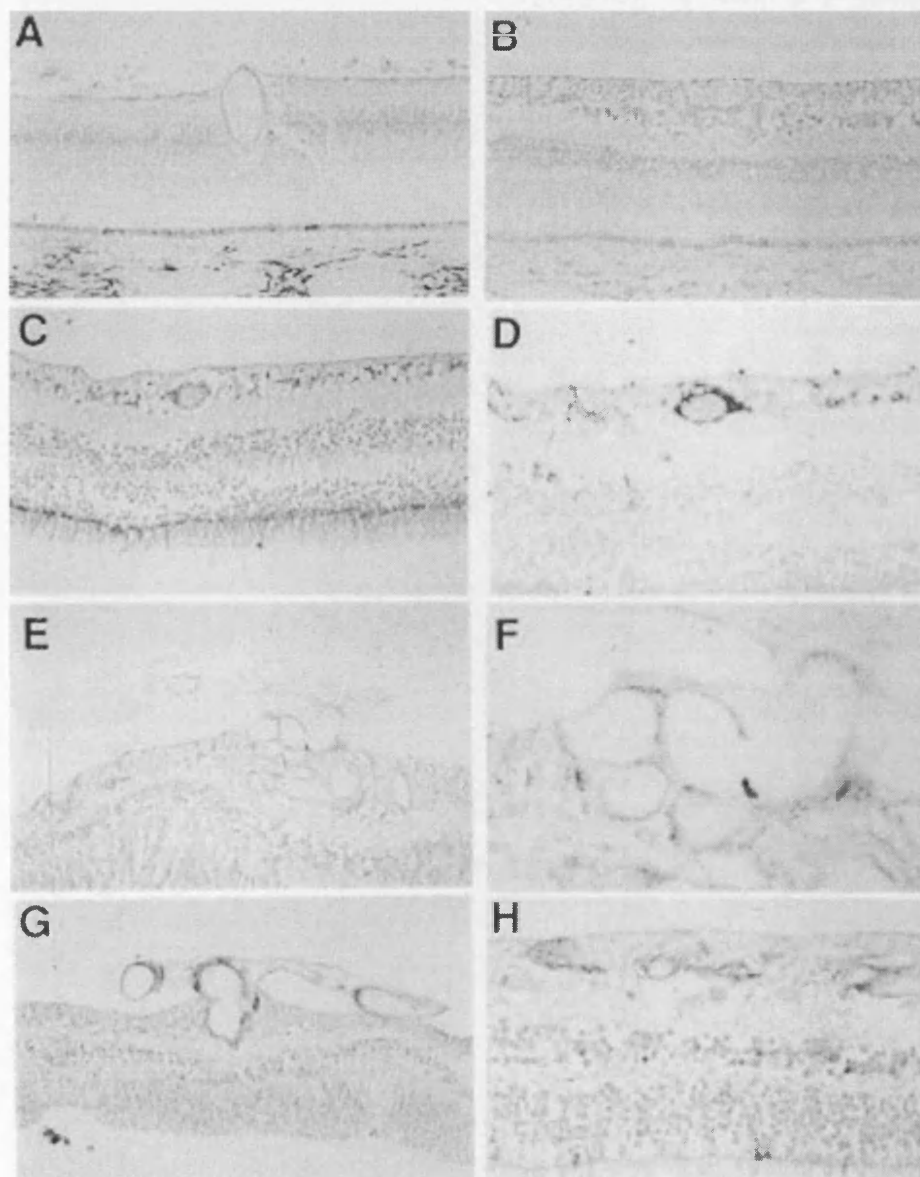

Figure 1 Photomicrographs demonstrating FLT-1 immunostaining of non-diabetic retina (A), diabetic retina with no obvious retinopathy (B), diabetic retina with obvious intraretinal vascular changes but no evidence of PDR (C), the same retina stained with GFAP (D), diabetic retina with PDR (E, F, G), diabetic retina post laser but with no residual PDR (H). Immunostaining for FLT-1 was greatest in the diabetic tissue compared with non-diabetic tissue. Increased staining was generally confined to the inner nuclear layer and the ganglion cell layer. Magnification A, G ×94; B, C, E ×118; D ×156; F ×378; H ×236.

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etc) and (b) to determine the extent of any scatter photocoagulation.

Eyes were categorised as follows:

Normal—10 human eyes with no known ophthalmic disease, no history of diabetes, and no abnormalities on biomicroscopy. Donors ranged in age from 34 to 89 years (mean 69 years).

Diabetic with no overt retinopathy—12 human eyes from diabetic donors with no clinical history and no overt biomicroscopic features of retinopathy or retinal photocoagulation. Donors ranged in age from 57 to 89 years (mean 77 years), five had been injecting insulin and seven had not. A complete medical history was unavailable for all donors but, in those where medical histories were known (10/12), the duration of diabetes was between 6 and 10 years (mean 7.3 years).

Diabetic with intraretinal changes but no evidence of PDR—five human eyes from diabetic donors with intraretinal changes on biomicroscopy but no clinical history or overt features of PDR or retinal photocoagulation. Retinas exhibited cotton wool spots and/or obvious microaneurysms or haemorrhages. Donors ranged in age from 62 to 96 years (mean 74 years), three had been injecting insulin and two had not. A complete medical history was known for four donors, the duration of diabetes being between 3 and 21 years (mean 13 years).

Diabetic with preretinal PDR—six human eyes from diabetic donors defined clinically as having PDR and exhibiting preretinal membranes when examined by biomicroscopy. All eyes had previously received laser photocoagulation. Donors ranged in age from 37 to 76 years (mean 58 years), all had been injecting insulin. Duration of diabetes ranged from 3–29 years (mean 14.3 years).

Diabetic with scatter laser photocoagulation but no evidence of residual PDR—14 human eyes from diabetic donors defined clinically as having had PDR and having received scatter laser photocoagulation. No preretinal membranes could be observed when retinas were examined by biomicroscopy. Donors ranged in age from 40 to 82 years (mean 57 years), 11 had been injecting insulin and three had not. A complete medical history was known for 13 donors, the duration of diabetes being between 10 and 35 years (mean 20 years).

The posterior segment of each eye was cut in the sagittal plane through the centre of the optic nerve head. Cuts were then made perpendicular to this line (a) on the horizontal midline on the nasal side and (b) at approximately 5 mm above and below the midline on the temporal side. A final vertical cut was made parallel to the initial cut and approximately 3 mm temporal to the macula. For this study tissue was wax embedded and 5 μm sections were cut from a portion of retina/choroid/sclera (a) approximately 3 mm lateral to the macula and perpendicular to the horizontal plane (this region was chosen because of its susceptibility to retinal changes associated with diabetes) and (b) other representative areas across the retina (for example, areas of neovascularisation).

Fibrovascular membranes—11 fibrovascular preretinal membranes excised at vitreous surgery from eyes with PDR were obtained from the Manchester Royal Eye Hospital. Membranes were fixed in 10% neutral buffered formalin immediately upon removal and for a minimum of 12 hours before wax embedding.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was undertaken as previously described.[16] The 5 μm sections were cut and mounted on APES (Sigma) coated slides. Sections were dewaxed and rehydrated. They were blocked for 60 minutes with 10% milk protein (Marvel)/normal goat serum (Sigma) before incubation overnight at 4°C

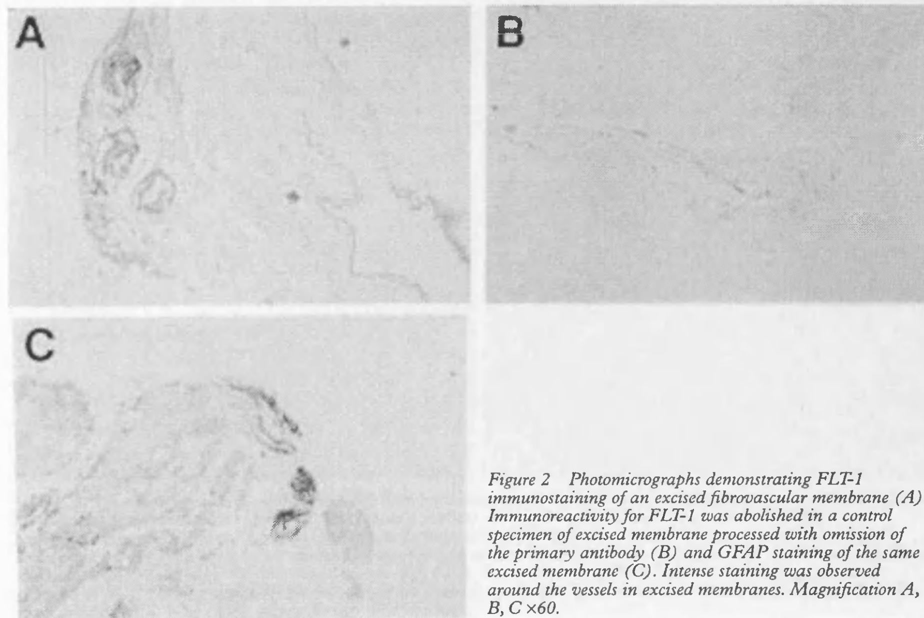

Figure 2 Photomicrographs demonstrating FLT-1 immunostaining of an excised fibrovascular membrane (A). Immunoreactivity for FLT-1 was abolished in a control specimen of excised membrane processed with omission of the primary antibody (B) and GFAP staining of the same excised membrane (C). Intense staining was observed around the vessels in excised membranes. Magnification A, B, C ×60.

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with either (a) a polyclonal rabbit antibody raised against a peptide corresponding to amino acids 1312–1328 mapping at the carboxy terminus of FLT of human cell origin and reacting with FLT of mouse, rat, and human cell origin (R&D Systems) diluted to 1 µg/ml in TRIS buffered saline (TBS), (b) a polyclonal rabbit antibody raised against a GST fusion protein containing FLK-1 sequences corresponding to amino acids 1158–1345 mapping at the carboxy terminal of FLK-1 of mouse origin (that is, the murine form of KDR) and reacting with FLK-1 of mouse, rat, and human cell origin (R&D Systems) diluted to 2 µg/ml in TBS, or (c) a polyclonal rabbit antibody raised against a peptide corresponding to amino acids 1279–1298 mapping at the carboxy terminus of FLT-4 of human origin and reacting with FLT-4 of human origin (R&D Systems) diluted to 1 µg/ml in TBS. A selection of slides were also stained for polyclonal rabbit antiglial fibrillary acidic protein (GFAP) antibody isolated from human spinal cord, directed against the 56 kD GFAP protein and reacting with GFAP of bovine, rat, and human origin (Euro-Diagnostica), diluted 1/50 in TBS. Negative controls were incubated with 0.2% goat serum in place of the primary antibody or substitution of the primary antibody with an inappropriate rabbit IgG at the same concentration as the primary antibody. Sections were washed three times with TBS and then incubated for 30 minutes with biotinylated goat anti-rabbit IgG (Sigma) and then incubated for 30 minutes with an avidin-biotin alkaline phosphatase reaction mixture (Dako Ltd). The sections were washed three times with TBS and then incubated with Fast Red TR/naphthol AS-MX substrate (Sigma). When the red colour had sufficiently developed the slides were washed in distilled water and counterstained with Mayer's haematoxylin.

ASSESSMENT OF IMMUNOSTAINING

The degree and pattern of immunostaining both within and between specimens was assessed by standard light microscopy by two masked observers (both of whom obtained similar results). The intensity of staining was

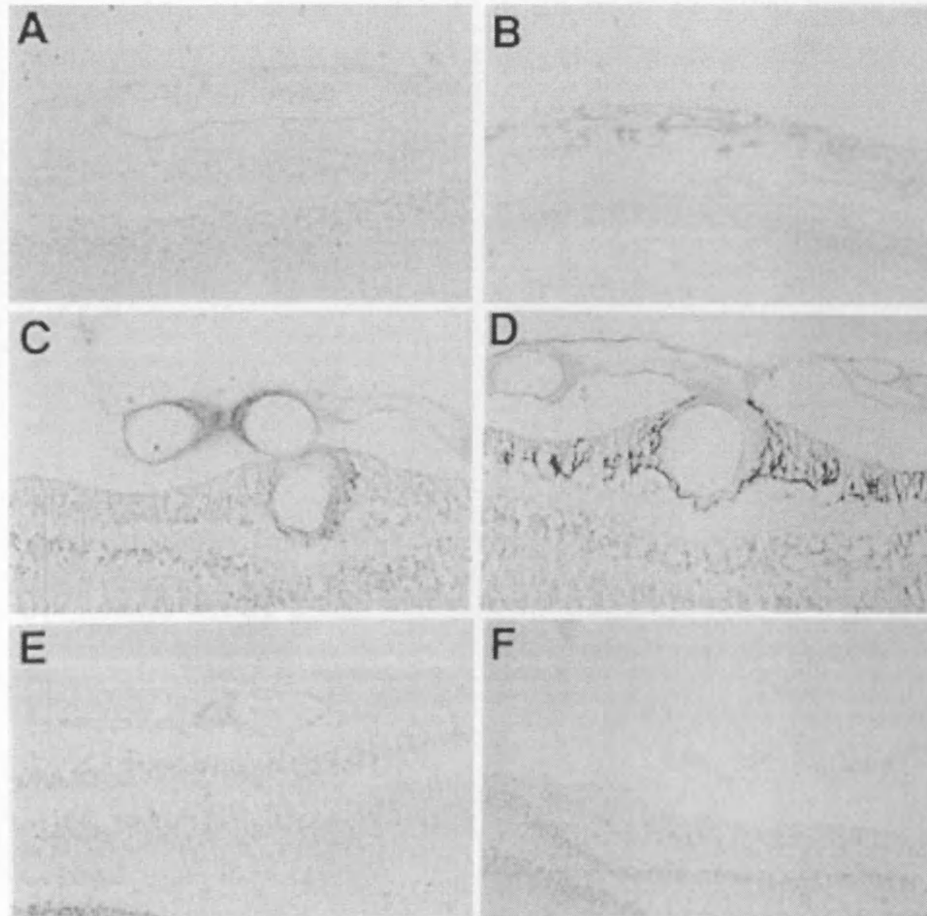

Figure 3 Photomicrographs demonstrating KDR immunostaining of non-diabetic retina (A), diabetic retina with PDR (B, C), the same retina stained with GFAP (D), and diabetic retina post laser but with no residual PDR (E). Immunostaining for KDR was greatest in the diabetic tissue with PDR and was minimal in most other diabetic tissue. Interestingly, immunostaining in the diabetic retinas which had undergone apparently successful laser treatment was reduced compared with the staining intensity in the retinas with PDR. Immunoreactivity for KDR was abolished in a control specimen of PDR retina processed with omission of the primary antibody (F). Magnification = A, C, D ×156; B ×60; E ×118; F ×78.

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graded qualitatively as background (corresponding to the level of staining seen in the negative controls), weak, moderate, or intense (corresponding to the highest level of immunoreactivity), each of these being recorded as 0, 1, 2, and 3 respectively. For each retinal specimen staining intensity was recorded for choroid, RPE, photoreceptors, outer retina, inner retina, ganglion cell layer, and retinal vessels. For the fibrovascular membranes staining intensity was recorded for the vessels and the surrounding matrix. An average score was then calculated for each retinal layer within each group.

Results

Staining was observed in both non-diabetic and diabetic vascular and extravascular retinal tissue; increased immunostaining was observed in preretinal and intraretinal vessels of diabetic tissue compared with non-diabetic tissue (see Figs 1–4 and Tables 1–3). For all receptors variable staining of the vessels within each retina was observed with some vessels staining positive and some staining negative. In some instances staining was associated with both endothelial cells and the perivascular region of the vessels. The variability in staining within retinas of the same group did not show a correlation with donor age, the type of glycaemic control in the case of the diabetic groups, or time post mortem.

FLT-1 IMMUNOREACTIVITY

Staining intensity for FLT-1 was generally weak or absent in the choroidal vessels, the RPE, and the photoreceptors. Weak staining was observed in the outer nuclear layer of most tissue categories but staining intensity tended to be elevated in diabetic eyes with vascular abnormalities and in those which had been successfully lasered. Weak to moderate staining was observed in the inner nuclear layer and weak to intense staining was observed in the ganglion cell layer of all the tissue categories including the non-diabetic eyes (Fig 1A–C; E–H; Table 1). The pattern of staining in the ganglion cell layer appeared to be associated with the Müller cell feet as it co-localised with positive GFAP staining (Fig 1D). While weak staining was observed in the retinal vessels of the non-diabetic eyes and the diabetic eyes

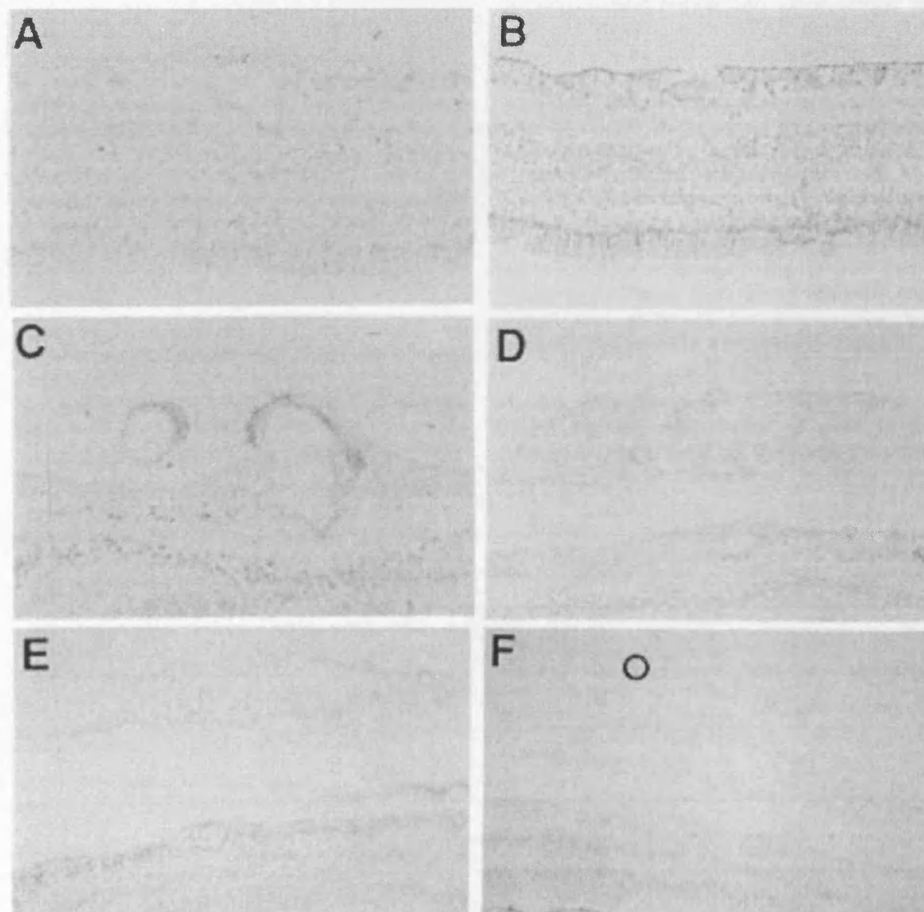

Figure 4 Photomicrographs demonstrating FLT-4 staining of normal retina (A), diabetic retina with obvious vascular intraretinal changes but no evidence of PDR (B), diabetic retina with PDR (C, D) and the same retina stained with GFAP (E). Immunostaining for FLT-4 was raised in diabetic tissue compared with non-diabetic tissue. Immunostaining was intermediate in the PDR specimens. Immunoreactivity for FLT-4 was abolished in a control specimen of PDR retina processed with omission of the primary antibody (F). Magnification A, D ×156; B ×118; C ×94; E, F ×60.

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Table 1 Mean intensity (SD) of FLT-1 staining in the retina/choroid

	Choroid	*RPE*	*Photo receptors*	*Outer nuclear layer*	*Inner nuclear layer*	*Ganglion cell layer*	*Retinal vessels*	*Membrane vessels*	*Membrane extravascular matrix*
Non-diabetic (n=10)	0.70 (0.46)	1.10 (0.83)	0.60 (0.80)	0.20 (0.40)	1.10 (0.94)	1.50 (1.12)	1.0 (1.0)		
No overt retinopathy (n=12)	0.92 (0.64)	0.58 (0.86)	0.42 (0.49)	0.33 (0.47)	1.42 (0.49)	1.92 (0.86)	1.25 (0.92)		
Intraretinal changes (n=5)	0.60 (0.80)	0.80 (0.75)	0.80 (1.17)	1.20 (1.17)	1.60 (0.80)	2.20 (0.75)	1.60 (1.02)		
PDR (n=6)	1.0 (0)	0.17 (0.37)	0 (0)	1.0 (0.82)	1.17 (0.69)	2.0 (0.82)	1.50 (1.12)	2.50 (0.50)	1.17 (0.37)
Laser, no residual PDR (n=14)	0.57 (0.49)	0.93 (1.03)	0.21 (0.56)	1.50 (0.63)	1.50 (0.63)	1.86 (0.74)	1.71 (0.45)		
PDR excised membranes (n=11)								1.55 (0.78)	1.09 (0.79)

0=background staining; 1=weak staining; 2=moderate staining; 3=intense staining.

without vascular changes, staining was moderate in all the other categories of diabetic tissue (Fig 1A–C; E–H; Table 1). The highest intensity of FLT-1 staining in the intraretinal vessels was associated with successful laser treatment with most (13/14) retinas staining. In all tissue categories staining tended to be confined to small and venous vessels in the superficial layers, although in 5/14 lasered retinas and in 2/6 retinas with PDR (both of which had previously been lasered) staining of the arterial vessels was observed. The most intense staining for FLT-1 was observed in the vessels of preretinal membranes of the diabetic subjects who had PDR (Table 1). In this tissue category staining of the intraretinal vessels was associated both with the membranes and across the retina (Fig 1E, F, G) Staining was moderate in the excised membranes but staining tended to be confined to a proportion of the vessels within each membrane with 4/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix (Fig 2A). Weak staining was associated with the non-vascular components of the membranes and staining with GPAP antibody confirmed that some of the perivascular and extravascular staining was glial cell in origin (Fig 2C).

KDR IMMUNOREACTIVITY

KDR immunoreactivity was generally minimal or absent in the choroidal vessels, the RPE, and the retinal layers of all the categories (Fig 3A); however, weak staining was observed in the inner nuclear layer and the ganglion cell layer of the unlasered eyes with obvious diabetic changes. Minimal to weak staining of the retinal vessels was observed in most categories but it became moderate to intense in the retinal vessels of diabetics with PDR with all (6/6) of the retinas staining (Fig 3B, C; Table 2). In 4/6 diabetic retinas with PDR staining of the intraretinal vessels was associated with the membranes but in 2/4 of these staining was also observed in vessels across the retina. In all categories staining tended to be associated with small and venous vessels (with one exception which was a non-diabetic eye) and was always observed in the superficial retinal layers. Moderate staining of the preretinal vessels was observed in most of the membranes (Fig 3B, C). In some instances staining was observed in the perivascular region and extravascular region and staining with GFAP antibody confirmed this to be glial cell in origin (Fig 3D). In those retinas which had undergone apparently successful laser treatment staining was reduced (Fig 3E; Table 2). In the excised membranes staining tended to be confined to a proportion of the vessels within each membrane with 2/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Weak or absent staining was associated with the non-vascular components of the membranes and staining with GFAP antibody confirmed that some of the perivascular and extravascular staining was glial cell in origin.

FLT-4 IMMUNOREACTIVITY

FLT-4 staining was absent or weak in the choroidal vessels, the RPE, the photoreceptors, and the outer nuclear layer in both non-

Table 2 Mean intensity (SD) of KDR staining in the retina/choroid

	Choroid	*RPE*	*Photo receptors*	*Outer nuclear layer*	*Inner nuclear layer*	*Ganglion cell layer*	*Retinal vessels*	*Membrane vessels*	*Membrane extravascular matrix*
Non-diabetic (n=10)	0.40 (0.49)	0.20 (0.40)	0.10 (0.30)	0.10 (0.30)	0.60 (0.66)	0.40 (0.49)	0.20 (0.40)		
No overt retinopathy (n=12)	0.08 (0.28)	0 (0)	0.25 (0.43)	0 (0)	0.75 (0.60)	0.75 (0.43)	0.75 (0.92)		
Intraretinal changes (n=5)	0.20 (0.40)	0 (0)	0.40 (0.80)	0 (0)	1.40 (1.20)	1.40 (0.80)	0.40 (0.49)		
PDR (n=6)	0.67 (0.75)	0 (0)	0 (0)	0 (0)	0.50 (1.12)	0.17 (0.37)	2.33 (0.75)	2.0 (1.15)	0.67 (1.11)
Laser, no residual PDR (n=14)	0.07 (0.26)	0.07 (0.26)	0.07 (0.26)	0 (0)	0.71 (0.45)	0.29 (0.45)	0.50 (0.82)		
PDR excised membranes (n=11)								1.55 (1.23)	0.55 (0.66)

0=background staining; 1=weak staining; 2=moderate staining; 3=intense staining.

Table 3 Mean intensity (SD) of FLT-4 staining in the retina/choroid

	Choroid	*RPE*	*Photo receptors*	*Outer nuclear layer*	*Inner nuclear layer*	*Ganglion cell layer*	*Retinal vessels*	*Membrane vessels*	*Membrane extravascular matrix*
Non-diabetic (n=10)	0.30 (0.46)	0 (0)	0 (0)	0.10 (0.30)	0.90 (0.94)	0.50 (0.67)	0.70 (1.0)		
No overt retinopathy (n=12)	0.42 (0.49)	0.08 (0.28)	0.33 (0.75)	0 (0)	1.50 (0.76)	1.83 (0.80)	1.0 (0.71)		
Intraretinal changes (n=5)	0.20 (0.40)	0.20 (0.40)	0.60 (0.80)	0 (0)	1.80 (1.17)	2.0 (1.10)	1.20 (1.47)		
PDR (n=6)	1.0 (0.82)	0.17 (0.37)	0 (0)	0 (0)	0.33 (0.47)	1.0 (0.82)	1.50 (1.12)	1.50 (1.26)	0.33 (0.47)
Laser, no residual PDR (n=14)	0.57 (0.62)	0.29 (0.45)	0.21 (0.56)	0.21 (0.41)	0.86 (0.64)	0.79 (0.56)	0.93 (0.80)		
PDR excised membranes (n=11)								1.73 (0.96)	0.18 (0.39)

0=background staining; 1=weak staining; 2=moderate staining; 3=intense staining.

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diabetic retinas (Fig 4A) and diabetic retinas (Fig 4B, C, D). In the inner nuclear layer and the ganglion layer FLT-4 immunoreactivity was only raised in the unlasered eyes, after laser treatment the levels reduced (Table 3). Staining with GFAP antibody confirmed that FLT-4 staining was associated with glial cells of the retina (Fig 4E). In the retinal vessels FLT-4 staining was low except in the PDR specimens where staining was weak to moderate (Fig 4C, D). In this tissue category staining in the intraretinal vessels was associated with the membranes in 3/6 retinas but staining in 2/3 of these was also observed in vessels across the retina. In all tissue categories staining tended to be associated with small and venous vessels of the superficial retinal layers although arterial staining was demonstrated in a small number of retinas. Weak to moderate staining was also observed in the preretinal vessels of the excised membranes. Staining tended to be associated with a proportion of vessels within each membrane with 2/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Minimal staining was associated with the non-vascular components of the membranes and staining with GFAP antibody confirmed that some of the perivascular and extravascular staining was glial cell in origin.

Discussion

The data presented in this study demonstrate (a) immunolocalisation of FLT-1, KDR, and FLT-4 receptors to retinal tissue and (b) upregulation of these receptors in diabetic retinopathy. These observations add further support for a role for VEGF family members in the initiation and progression of PDR.

Binding sites for VEGF have previously been demonstrated to be associated with vascular endothelial cells during the development of the vasculature,[17 23–25] during pathological angiogenesis—for example, in healing wounds, in skin diseases, in hypersensitivity reactions, and in carcinomas,[24 26–29] and from in vitro studies.[7 30–37] These observations advance a regulatory role for VEGF and its receptors in angiogenesis occurring both during normal vascular development and in various pathologies.

The observation in this study that KDR is greatly elevated in both intra- and preretinal vessels in PDR tissue and minimal in normal retina and the quiescent vessels of lasered diabetic retina with no evidence of PDR is in agreement with the view that KDR is involved in pathological angiogenesis. These findings correlate with the findings of various workers[38–40] who reported high levels of VEGF in the vitreous of patients with active PDR. By contrast, FLT-1 was observed in both non-diabetic and diabetic vascular and avascular retinal tissue. The presence of FLT-1 in non-diabetic tissue may reflect its involvement in metabolic control—for example, control of vessel permeability and endothelial cell maintenance. Upregulation of FLT-1 in diabetic vessels, particularly those undergoing active neovascularisation, indicates that the receptor plays a role in PDR. Firstly, it may induce vascular leakage; FLT-1 is known to promote vascular permeability.[5] Secondly, it has been suggested that it may participate in VEGF induced mitogenesis by heterodimer formation with KDR.[41] Thirdly, FLT-1 may regulate VEGF induced angiogenesis; a soluble form of FLT-1 can complex with the extracellular region of KDR and act as a negative regulator of VEGF action.[37] Fourthly, PlGF which is associated with PDR acts through the FLT-1 receptor.[20] FLT-4 represents a third putative receptor for the VEGF family which shares structural similarities with FLT-1 and KDR; it is believed to be a receptor for VEGF-C.[42] FLT-4 immunolocalisation was minimal in non-diabetic eyes but was upregulated in diabetic tissue, especially in the inner nuclear layer, the ganglion cell layer, and intraretinal and preretinal vessels. These observations suggest that FLT-4 may have a role in the pathogenesis of diabetic retinopathy.

Several ocular cell types, in addition to vascular endothelial cells[33 43 44] and pericytes,[7 45] express VEGF receptors. VEGF receptors have been identified on cultured corneal cells,[46] cultured lens epithelial cells,[47 48] and cultured RPE cells.[48 49] Increased levels of VEGF mRNA and protein have previously been demonstrated in retinal disorders in the cell bodies of the inner nuclear layer, the ganglion cell layer, and the outer nuclear layer.[8 9 13 15 16] Studies on the developing retinal vasculature have also demonstrated VEGF mRNA and protein in the retinal glial cells.[50 51] Chen and co-workers demonstrated intense VEGF staining in both vascular and extravascular epiretinal membranes.[49] They also demonstrated FLT-1 but not KDR expression by glial cells in the epiretinal membranes and in cultured retinal glial cells. In our study we also demonstrated increased immunoreactivity for FLT-1, FLT-4, and, to a lesser extent, KDR in the glial cells of the retina which was particularly associated with the end feet of the Müller cells. Thus, these observations demonstrate that VEGF may act through its receptors via both autocrine and paracrine mechanisms. It may be that one of the functions of the retinal glial cells is as early detectors of the hypoxic environment occurring during the earlier stages of diabetic retinopathy. This could explain why in our study FLT-1, FLT-4, and to a lesser extent KDR, were associated with the glial cells before proliferation had occurred. These cells may respond to hypoxia by upregulating their receptors and secreting VEGF which acts on the endothelial cells. The sustained production of VEGF would eventually lead to an angiogenic response. Sustained production of VEGF may be maintained by a positive feedback mechanism to the receptors on the glial cells and the endothelial cells which could explain why increased levels of FLT-1 were observed in the glial cells of the eyes with PDR. An interesting observation was that in some of the membranes GFAP staining was observed both around the vessels and in the surrounding matrix which corresponded to receptor immunoreactivity. It may be that these particular membranes were undergoing active

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neovascularisation or that there may have been hypoxic regions within these membranes.

VEGF receptor expression appears to be regulated by various stimuli including growth factors and cytokines[5] and, as mentioned above, hypoxia.[7 33 34 44 45] Takagi *et al* suggested that hypoxia may be responsible for increasing KDR/FLK expression indirectly via adenosine receptors on endothelial cells[44]; adenosine is hypoxia inducible in some tissues and it is known to stimulate angiogenesis and cellular proliferation.

In conclusion, this study confirms the presence of VEGF family receptors in the diabetic retina and indicates that while KDR appears to be involved principally with the angiogenic process (that is, PDR), FLT-1 may have a role in both normal endothelial cell homeostasis and in all stages of diabetic retinopathy. Therefore, any agent directed against VEGF or FLT-1 could have a detrimental effect on the normal structure and functioning of endothelial cells and vessels. A more attractive alternative would be to produce anti-angiogenic molecule(s) with low toxicity directed against KDR. One study by Strawn and co-workers found anti-angiogenesis compounds that can inhibit FLK-1/KDR tyrosine kinase activity as well as endothelial cell mitogenesis and blood vessel formation in the chorioallantoic membrane.[52] Further studies are necessary (a) to determine whether the receptors are active, (b) to ascertain the stimulus for upregulation of the receptors, and (c) to determine whether inhibition of receptor activation is the therapy of choice in preretinal angiogenesis.

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1 Boulton M, McLeod D, Garner A. Vasoproliferative retinopathies: clinical, morphogenetic and modulatory aspects. *Eye* 1988;2(**Suppl**):S124–39.
2 Ferrara N. The role of vascular endothelial growth factor in pathological angiogenesis. *Breast Cancer Research and Treatment* 1995;36:127–37.
3 Ferrara N. Vascular endothelial growth factor. The trigger for neovascularization in the eye. *Lab Invest* 1995;72:615–18.
4 Thomas KA. Vascular endothelial growth factor, a potent and selective angiogenic agent. *J Biol Chem* 1996;271:603–6.
5 Kolch W, Martiny-Baron G, Kieser A, *et al.* Regulation of the expression of the VEGF/VPS and its receptors: role in tumor angiogenesis. *Breast Cancer Research and Treatment* 1995;36:139–55.
6 Ferrara N, Houck KA, Jakeman LB, *et al.* The vascular endothelial growth factor family of polypeptides. *J Cell Biochem* 1991;46:211–18.
7 Nomura M, Yamagishi S, Harada S, *et al.* Possible participation of autocrine and paracrine vascular endothelial growth factors in hypoxia-induced proliferation of endothelial cells and pericytes. *J Biol Chem* 1995;270:28316–24.
8 Pe'er J, Shweiki D, Itin A, *et al.* Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases. *Lab Invest* 1995;72:638–45.
9 Pierce EA, Avery RL, Foley ED, *et al.* Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc Natl Acad Sci USA* 1995;92:905–9.
10 Aiello LP, Pierce EA, Foley ED, *et al.* Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc Natl Acad Sci USA* 1995;92:10457–61.
11 Adamis AP, Shima DT, Tolentino MJ, *et al.* Inhibition of vascular endothelial growth factor prevents retinal ischaemia-associated iris neovascularization in a non-human primate. *Arch Ophthalmol* 1996;114:66–71.
12 Murata T, Ishibashi T, Khalil A, H *et al.* Vascular endothelial growth factor plays a role in hyperpermeability of diabetic retinal vessels. *Ophthalmic Res* 1995;27:48–52.
13 Pe'er J, Folberg R, Itin A, *et al.* Upregulated expression of vascular endothelial growth factor in proliferative diabetic retinopathy. *Br J Ophthalmol 1996;80:241–5.*
14 Lutty GA, McLeod DS, Merges C, *et al.* Localization of vascular endothelial growth factor in human retina and choroid. *Arch Ophthalmol* 1996;114:971–7.
15 Amin RH, Frank RN, Kennedy A, *et al.* Vascular endothelial growth factor is present in glial cells of the retina and optic nerve of human subjects with non proliferative diabetic retinopathy. *Invest Ophthalmol Vis Sci* 1997;38:36–47.
16 Boulton M, Foreman D, Williams G, *et al.* VEGF localisation in diabetic retinopathy. *Br J Ophthalmol* 1998;82:561–8.
17 Millauer B, Wizigmann-Voos S, Schnurch H, *et al.* High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993;72:835–46.
18 Breier G, Clauss M, Risau W. Coordinate expression of vascular endothelial growth factor receptor-1(flt-1) and its ligand suggests a paracrine regulation of murine vascular development. *Developmental Dynamics* 1995;204:228–39.
19 Khaliq A, Foreman D, Ahmed A, *et al.* Increased expression of placenta growth factor (PLGF) in proliferative diabetic retinopathy. *Lab Invest* 1998;78:109–16.
20 Sawano A, Takahashi T, Yamaguchi S, *et al.* Flt-1 but not KDR/FLK-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. *Cell Growth Differ* 1996;7:213–21.
21 Galland F, Karamysheva A, Pebusque M, *et al.* The FLT4 gene encodes a transmembrane tyrosine kinase related to the vascular endothelial growth factor receptor. *Oncogene* 1993;8:1233–40.
22 Borg J, deLapeyriere O, Tetsuro N, *et al.* Biochemical characterization of two isoforms of FLT4, a VEGF receptor-related tyrosine kinase. *Oncogene* 1995;10:973–84.
23 Oelrichs RB, Reid HH, Bernard O, *et al.* NYK/FLK-1: a putative receptor protein tyrosine kinase isolated from E10 embryonic neuroepithelium is expressed in endothelial cells of the developing embryo. *Oncogene* 1993;8:11–18.
24 Peters KG, De Vries C, Williams LT. Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proc Natl Acad Sci USA* 1993;90:8915–19.
25 Quinn TP, Peters KG, De Vries C, *et al.* Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci USA* 1993;90:7533–7.
26 Detmar M, Brown LF, Claffey KP, *et al.* Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J Exp Med* 1994;180:1141–6.
27 Boocock CA, Charnock-Jones DS, Sharkey AM, *et al.* Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Natl Cancer Inst* 1995;87:506–16.
28 Brown LF, Olbricht SM, Berse B, *et al.* Overexpression of vascular permeability factor (VPF/VEGF) and its endothelial cell receptors in delayed hypersensitivity skin reactions. *J Immunol* 1995;154:2801–7.
29 Christofori G, Naik P, Hanahan D. Vascular endothelial growth factor and its receptors, flt-1 and flk-1, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. *Mol Endocrinol* 1995;9:1760–70.
30 Vaisman N, Gospodarowicz D, Neufield G. Characterization of the receptors for vascular endothelial growth factor. *J Biol Chem* 1990;265:19461–6.
31 Jakeman LB, Winer J, Bennet GL, *et al.* Binding sites for vascular endothelial growth factor are localised on endothelial cells in adult rat tissues. *J Clin Invest* 1992;89:244–53.
32 Terman BI, Khandke L, Dougher-Vermazan M, *et al.* VEGF receptor subtypes KDR and FLT-1 show different sensitivities to heparin and placenta growth factor. *Growth Factors* 1994;11:187–95.
33 Thieme H, Aiello LP, Takagi H, *et al.* Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. *Diabetes* 1995;44:98–103.
34 Brogi E, Schatteman G, Wu T, *et al.* Hypoxia induced paracrine regulation of vascular endothelial growth factor receptor expression. *J Cell Invest* 1996;97:469–76.
35 Gitay-Goren H, Cohen T, Tessler S, *et al.* Selective binding of VEGF121 to one of three vascular endothelial growth factor receptors of vascular endothelial cells. *J Biol Chem* 1996;271:5519–23.
36 Hewett PW, Murray JC. Coexpression of flt-1, flt-4 and KDR in freshly isolated and cultured human endothelial cells. *Biochem Biophys Res Comm* 1996;221:697–702.
37 Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Comm* 1996;226:324–8.
38 Adamis AP, Miller JW, Bernal M, *et al.* Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol* 1994;118:445–50.
39 Aiello LP, Avery RL, Arrigg PG, *et al.* Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 1994;331:1480–7.
40 Malecaze F, Clamens S, Simorre-Pinatel V, *et al.* Detection of vascular endothelial growth factor messenger RNA and vas-

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cular endothelial growth factor-like activity in proliferative diabetic retinopathy. *Arch Ophthalmol* 1994;112:1476–82.

41 Waltenberger J, Claesson-Welsh L, Siegbahn A, *et al*. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 1994;269:26988–95.

42 Joukov V, Pajusola K, Kaipainan A, *et al*. A novel vascular endothelial growth factor, VEGF-C, is a ligand for Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996;15:290–8.

43 Simorre-Pinatel V, Guerrin M, Chollet P, *et al*. Vasculotropin-VEGF stimulates retinal capillary endothelial cells through an autocrine pathway. *Ophthalmol Vis Sci* 1994;35:3393–400.

44 Takagi H, King GL, Ferrara N, *et al*. Hypoxia regulates vascular endothelial growth factor receptor KDR/Flk gene expression through adenosine A2 receptors in retinal capillary endothelial cells. *Invest Ophthalmol Vis Sci* 1996;37:1311–21.

45 Takagi H, King GL, Aiello LP. Identification and characterization of vascular endothelial growth factor receptor (FLT) in bovine retinal pericytes. *Diabetes* 1996;45:1016–23.

46 Gitay-Goren H, Soker S, Vlodavsky I, *et al*. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J Biol Chem* 1992;267:6093–8.

47 Neufield G, Tessler S, Gitay-Goran H, *et al*. Vascular endothelial growth factor and its receptors. *Progress Growth Factor Res* 1994;5:89–97.

48 Guerrin M, Moukadiri H, Chollet P, *et al*. Vasculotropin/vascular endothelial growth factor is an autocrine growth factor for human retinal pigment epithelial cells cultured in vivo. *J Cell Physiol* 1995;164:385–94.

49 Chen Y, Hackett SF, Schoenfeld C, *et al*. Localisation of vascular endothelial growth factor and its receptors to cells of vascular and avascular epiretinal membranes. *Br J Ophthalmol* 1997;81:919–26.

50 Murata T, Nakagawa K, Khalil A, *et al*. The temporal and spatial vascular endothelial growth factor expression in retinal vasculogenesis of rat neonates. *Lab Invest* 1996;74:68–77.

51 Stone J, Itin A, Alon T, *et al*. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J Neurosc* 1995;15:4738–47.

52 Strawn LM, McMahon G, App H, *et al*. Flk-1 as a target for tumour growth inhibition. *Cancer Res* 1996;56:3540–5.

Rapid Publication

Loss of the Antiangiogenic Pigment Epithelium-Derived Factor in Patients With Angiogenic Eye Disease

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Retinal neovascularization characterizes proliferative diabetic retinopathy (PDR). Pigment epithelium-derived factor (PEDF) has been shown to be a major antiangiogenic growth factor in the mammalian eye. PEDF expression is suppressed by hypoxia, and changes in PEDF have been correlated to the development of retinal neovascularization in animal models of hypoxic eye disease. However, whether this concept of a reduced angiogenesis inhibitor holds true in humans is as yet unclear. In this study, we analyzed the in vivo regulation of PEDF in patients with and without hypoxic eye disease. We used immunoblots to measure PEDF in ocular fluids obtained from 64 nondiabetic and diabetic patients. In addition, immunohistochemistry of PEDF was carried out in specimens of normal human retinas and retinas with various degrees of diabetic retinopathy. The PEDF concentrations in patients with PDR ($P < 0.001$) or extensive nondiabetic retinal neovascularization caused by retinal-vein occlusion ($P < 0.001$) were lower than in control patients. Levels of PEDF were replenished in PDR patients with previous retinal scatter photocoagulation compared with PDR patients without previous photocoagulation ($P = 0.01$). Immunohistochemistry revealed an interstitial staining pattern as expected for a secreted protein, with an intense staining in retinas of patients without proliferative eye disease. However, in patients with PDR, little or no staining was detectable. Our data strongly support the concept that retinal angiogenesis is induced by loss of the major angiogenesis inhibitor in the eye, PEDF, in combination with an increased expression of angiogenic growth factors such as vascular endothelial growth factor. Our findings suggest that substitution of angiogenesis inhibitors may be an effective approach in the treatment of PDR. ***Diabetes*** **50:2641–2645, 2001**

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EPC-1, early population doubling level cDNA-1; NPDR, nonproliferative diabetic retinopathy; NVD, new vessels on the disk; NVE, new vessels elsewhere; PDR, proliferative diabetic retinopathy; PEDF, pigment epithelium-derived factor; PRP, previous retinal photocoagulation; VEGF, vascular endothelial growth factor.

The control of retinal angiogenesis is of critical importance for the preservation of vision. Retinal neovascularization characterizes proliferative diabetic retinopathy (PDR), which is still one of the most common causes of blindness worldwide. Retinal ischemia induces intraocular neovascularization, presumably by stimulating the expression of angiogenic growth factors and by inhibiting the release of antiangiogenic cytokines (1,2). Vitreal levels of angiogenic growth factors have been shown to be directly associated with the degree of retinal angiogenesis (3,4). The ability to monitor and grade retinal angiogenesis within the eye as well as the ability to aspirate vitreous, which is known to contain retina-derived growth factors in direct association to the stage of retinal angiogenesis, makes the eye an ideal setting in which to investigate the delicate balance of new vessel growth and the influence of specific growth factors in vivo in humans.

Pigment epithelium-derived factor (PEDF) protects cerebellar granule cells against neurotoxic agents (5) and is also called early population doubling level cDNA-1 (EPC-1), reflecting its upregulation during cell cycle arrest (G_0) in young but not in senescent cultured fibroblasts (6). Recently, PEDF has been shown to be a highly effective inhibitor of angiogenesis in animal and cell culture models. The production of PEDF was decreased by hypoxia (7), which is also a central pathogenic stimulus in PDR. Immunoneutralization of PEDF diminished the ability of cadaveric human vitreous to inhibit migration of endothelial cells, thereby demonstrating that a loss of PEDF is functionally important in mediating angiogenic properties of human vitreous ex vivo. Most importantly, systemically administered PEDF prevented aberrant blood vessel growth in a murine model of ischemia-induced retinopathy (8). However, no information is yet available about the presence and regulation of PEDF in vivo in humans, particularly in hypoxia-induced proliferative retinopathy. If PEDF is involved in the control of retinal angiogenesis in humans, one would expect that PEDF is decreased in the ocular fluids of patients with hypoxia-induced proliferative retinopathy and that PEDF levels increase after at least

partially successful therapy, such as retinal photocoagulation. In this study, we attempted to ascertain whether intraocular concentrations of PEDF correlated with the gree of retinal neovascularization by measuring PEDF in the ocular fluid of 64 patients. We also investigated whether retinal scatter photocoagulation is capable of replenishing PEDF in the ocular fluid of patients with PDR. Spatial and temporal changes in the expression of retinal PEDF were determined by immunohistochemical localization of PEDF in the human retinas of patients with different degrees of diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Vitreous was obtained from 64 patients (32 women and 32 men). Patients without proliferative retinal disease (control subjects: n = 19, 6 women and 13 men, mean age 70 ± 3 years) were compared with patients with PDR (n = 37, 17 women and 20 men, mean age 61 ± 2 years, 6 patients with type 1 diabetes, 31 with type 2 diabetes, HbA_{1c} 7.8 ± 0.1%) and patients with extensive nondiabetic neovascularizing eye disease caused by central-vein occlusion (Rubeosis; n = 8, 2 women and 6 men, mean age 71 ± 3 years, no diabetes). A total of 27 patients with PDR had retinal photocoagulation before vitrectomy (PDR + previous retinal photocoagulation [PRP]), whereas 10 patients with PDR had no previous photocoagulation (PDR − PRP). PDR was considered to be active if there was extensive retinal neovascularization represented by perfused, multibranching preretinal capillaries and to be quiescent if mainly nonperfused or gliotic vessels were present. Altogether, 15 patients with PDR had active neovascularization, whereas 22 patients had quiescent retinal angiogenesis. A total of 13 patients with PDR + PRP had new vessels elsewhere (NVE), 3 had new vessels on the disk (NVD), and 11 had NVE + NVD. Five patients with PDR − PRP had NVE, four had NVD, and one had NVE and NVD. Age, HbA_{1c}, and duration of diabetes did not differ significantly between patients with PDR + PRP and PDR − PRP (age 61 ± 4 and 61 ± 2 years, HbA_{1c} 7.6 ± 0.3 and 8.2 ± 0.4%, duration of diabetes 18.3 ± 2 and 17 ± 4 years, respectively). Undiluted samples of human vitreous were obtained during pars plana vitrectomy. Samples were aspirated under standardized conditions directly above the retina at the beginning of surgery and prepared as previously described (2). Ocular neovascular activity was determined by fluorescein photography, via slit lamp examination, or by the surgeon at the time of surgery.

Specimens for immunohistochemistry were obtained from the National Disease Research Interchange (NDRI), Philadelphia, Pennsylvania. Eyes were enucleated and fixed in 10% neutral buffered formalin within 10 h post mortem. Examination of the posterior segment was performed by an experienced ophthalmologist using a Zeiss Stemi SV8 zoom dissecting microscope. Eyes were categorized as follows (n = 5 for each group): normal (*A*); diabetic without ocular abnormalities (*B*); diabetic with intraretinal changes but no evidence of PDR (*C*); diabetic with PDR (*D*); and diabetic with scatter laser photocoagulation and no evidence of residual PDR (*E*). Samples were prepared, and criteria for categorization were chosen as previously described (9).

Classification of specimen was performed before the experimental part of the study. The study was approved by the Ethical Committee of the University of Bochum, and informed consent was obtained from all patients included.

Western blot. PEDF was quantified by Western blotting using polyclonal PEDF-specific antibodies (anti-PEDF), which were raised as previously described (10). Blots were analyzed automatically by a digital imaging system with standardized imaging values, thereby obtaining observer-independent quantification of the band intensities. The samples were compared with defined quantities of purified human PEDF, which was run as an internal standard on every gel. The internal standard was engineered by transfecting a human PEDF cDNA (with a 6xHis tag cloned into CEP4) (Invitrogen) into human embryonic kidney cells as previously described (7). Recombinant PEDF was enriched from the conditioned media with the QIAexpress system (Qiagen, Hildesheim, Germany) and quantified using the Bradford assay (11).

Immunohistochemistry. Primary antibody (anti-PEDF, 1:300 dilution) was incubated for 60 min. Detection was performed with an alkaline phosphatase–based system (LASB+; Dako, Glostrup, Denmark). Staining procedures were performed under standardized conditions, and sections were counterstained with Mayer's hematoxylin. Negative controls were incubated without primary antibody or with primary antibody after preabsorbtion with recombinant PEDF. The intensity of staining was graded qualitatively as background (*0*), weak (*1*), moderate (*2*), or intense (*3*) by a blinded investigator without knowledge of the clinical data. An average score of staining was calculated within each group.

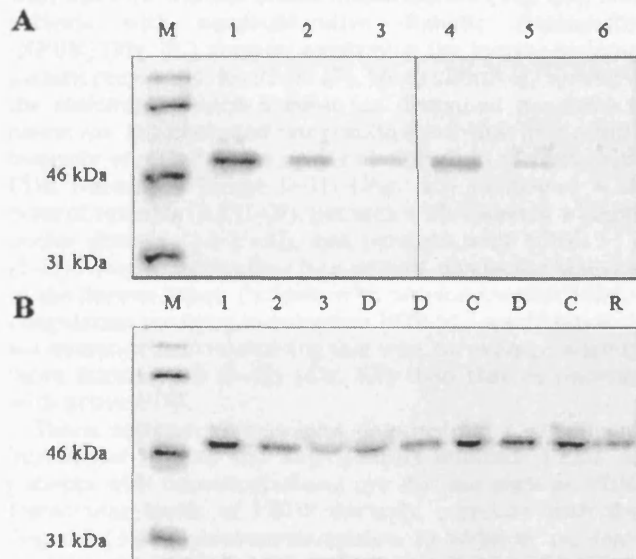

FIG. 1. Western blots with a polyclonal PEDF/EPC-1 antibody. *Lanes 1-3* represent a typical standard curve with a dilution of recombinant PEDF (*lane 1*, undiluted; *lane 2*, 1:2 dilution; *lane 3*, 1:4 dilution). M, molecular size marker. *A*: The PEDF bands in *lanes 1–3* were reduced or disappeared after preincubation of the antibody with recombinant PEDF (*lanes 4–6*), thereby demonstrating the specificity of the reaction. *B*: Western blot of vitreal samples of control subject (*C*), patients with PDR (*D*), and patients with severe intraocular neovascularization (Rubeosis iridis) caused by central-vein occlusion (*R*).

Statistical analysis. Data are reported as the mean ± SE. The Mann Whitney U test was used to compare quantitative data with unequal distributions. The correlation between variables was calculated by linear regression analysis of untransformed values. A level of $P < 0.05$ was considered significant.

RESULTS

Vitreal levels of PEDF were determined by immunoblot (Fig. 1). We detected a protein band of 50 kDa corresponding to the predicted molecular mass of PEDF. The band disappeared or was diminished after preincubation of the antibody with a previously enriched recombinant PEDF, thereby demonstrating specificity of the reaction. Recombinant PEDF occurred as a single band on a SDS-polyacrylamide gel as investigated by Ponceau S staining after immobilization on a nitrocellulose filter.

Vitreal PEDF levels are decreased in PDR. The intraocular levels of PEDF were determined by Western blot analysis and then quantified based on an internal standard of purified human recombinant PEDF (Fig. 2). The results suggest that the PEDF levels were significantly decreased in patients with PDR (20 ± 0.5 nmol/l, n = 37; $P < 0.001$) and patients with central-vein occlusion resulting in extensive neovascularization (17.6 ± 0.3 nmol/l, n = 8; $P < 0.0001$) compared with control subjects (23.7 ± 0.7 nmol/l, n = 19). Furthermore, patients with quiescent PDR had unchanged PEDF levels (22 ± 0.6 nmol/l, n = 22; P = 0.06) compared with control subjects, whereas patients with active PDR (17.2 ± 0.5 nmol/l, n = 15) had PEDF levels comparable with those of patients with Rubeosis. PEDF levels of patients with active PDR were significantly lower than those of control subjects ($P < 0.0001$) and patients with quiescent PDR ($P < 0.0001$).

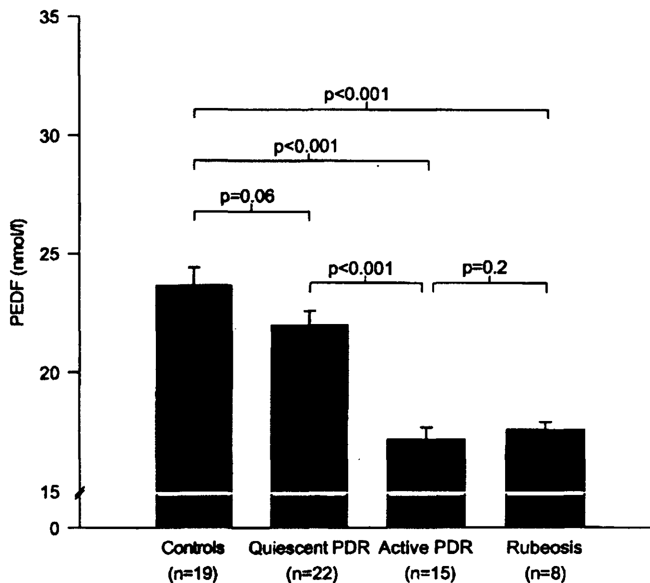

FIG. 2. Levels of vitreal PEDF in patients with proliferating eye disease. PEDF levels in intraocular samples from numerous patients were determined by Western blot analysis and compared with a standard concentration of purified human recombinant PEDF. The influence of intraocular activity was investigated by comparing levels of intraocular PEDF in patients with different degrees of neovascular activity: control subjects without angiogenesis, patients with quiescent PDR, patients with active PDR, and nondiabetic patients with extensive retinal neovascularization caused by central-vein occlusion (Rubeosis).

Photocoagulation replenishes intraocular levels of PEDF. Previous photocoagulation was associated with reduced neovascular activity (Fig. 3). Although 70% of the patients without prior photocoagulation (PDR − PRP) suffered from active angiogenesis, only 30% of the patients with PDR + PRP had active neovascularization. Patients with PDR + PRP had higher concentrations of PEDF (n = 27, 20.9 ± 0.7 nmol/l; P = 0.01) compared with patients with PDR − PRP (n = 10, 17.7 ± 0.3 nmol/l). However, PDR concentrations of patients with previous photocoagulation were still clearly below levels of control patients (P = 0.007).

PEDF levels are associated with the localization of retinal neovascularization. Taking all patients into account, levels of PEDF correlated significantly with the localization of retinal neovascularization. Patients with NVE and NVD (18 ± 0.4 nmol/l, n = 20) had decreased levels compared with control patients without proliferation (23.7 ± 0.7 nmol/l, n = 19; $P < 0.001$) and patients with NVE only (21 ± 1 nmol/l, n = 18; P = 0.02) (Fig. 4). Patients with NVE or NVD only (19 ± 1 nmol/l, n = 7) had lower levels than control patients (P = 0.053 and P = 0.002, respectively). We found no correlation between vitreal levels of PEDF and sex, duration of diabetes, HbA_{1c}, or age of the patients.

PEDF-specific immunohistochemistry of human retinas with different stages of diabetic retinopathy. To obtain data about spatial and temporal changes of PEDF expression in the course of diabetic retinopathy, 25 specimens of human retina were examined by immunohistochemistry. Our results revealed an interstitial accumulation of PEDF in the eyes of control subjects (Fig. 5*A*), patients with diabetes without ocular abnormalities (Fig. 5*B*), and patients with nonproliferative diabetic retinopathy (NPDR) (Fig. 5*C*), thereby confirming the murine staining pattern previously described (7). We qualitatively assessed the staining for each section (as described in RESEARCH DESIGN AND METHODS), and our results show that intraretinal intensity of staining was nearly abolished in patients with PDR (mean 0.4 [range 0–1]) (Fig. 5*D*) compared with control subjects (2.2 [1–3]), patients with diabetes without ocular disease (1.6 [1–2]), and patients with NPDR (1.2 [1–2]), despite unchanged intensity of unspecific staining of the fibrous tissue. Patients with previous scatter photocoagulation resulting in quiescent PDR had weak intraretinal immunochemical staining that was, on average, slightly more intense (1.0 [0–2]) (Fig. 5*E*) than that of patients with active PDR.

Taken together, our results demonstrate a significant intraocular loss of the angiogenesis inhibitor PEDF in patients with neovascularizing eye disease such as PDR. Intraocular levels of PEDF strongly correlate with the degree of retinal neovascularization. In addition, we demonstrate that retinal scatter photocoagulation, the treatment of choice for patients with diabetic retinopathy, replenishes concentrations of PEDF in the eye. Changes of vitreal levels are confirmed by immunohistochemistry, which reveals an interstitial staining pattern as expected for a secreted protein.

DISCUSSION

The switch to an angiogenic phenotype of proliferating tissues requires both upregulation of angiogenic stimulators and downregulation of angiogenesis inhibitors. An elevated expression of angiogenic growth factors such as vascular endothelial growth factor (VEGF) in patients with retinal neovascularization has been previously demon-

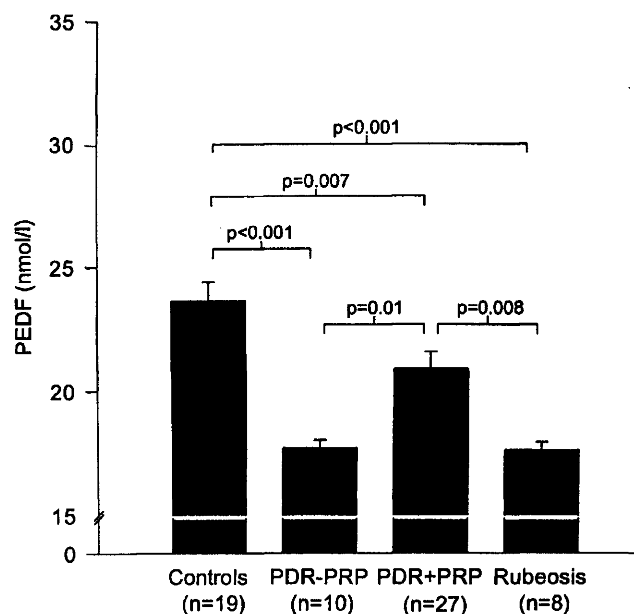

FIG. 3. Levels of vitreal PEDF depend on previous photocoagulation. PEDF levels were determined as described in Fig. 2 for patients with PDR − PRP, PDR + PRP, and Rubeosis, as well as nondiabetic patients with extensive retinal neovascularization due to central vein occlusion (control subjects).

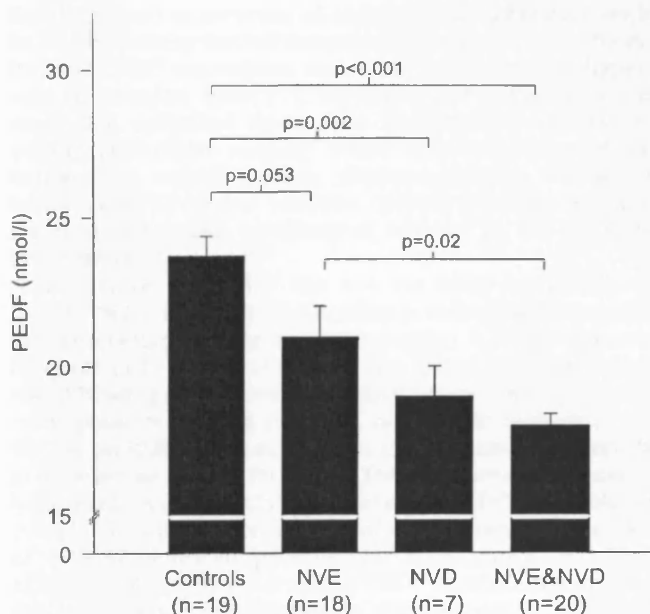

FIG. 4. Concentrations of PEDF in human vitreous depending on localization of neovasularization. PEDF levels were determined as described in Fig. 2. Levels of PEDF correlate with the localization of retinal neovascularization. We compared patients without retinal angiogenesis (control subjects), patients with NVD, those with NVE, and those with both NVE and NVD.

strated (4). Additionally, decreased expression of VEGF was observed in patients with reduced neovascular activity after panretinal photocoagulation (2). The question targeted by this study was whether a loss of angiostatic growth factors such as PEDF is critical in the development of retinal neovascularization in vivo in humans. We found PEDF concentrations in ocular fluid to be lower in patients with active neovascularization than in control subjects without retinal angiogenesis. The vitreal data are confirmed by the results of immunohistochemistry showing almost no staining in patients with active proliferation compared with a strong intraretinal staining in control patients. These results demonstrate regulation of the major intraocular angiogenesis inhibitor PEDF in vivo depending on the stage of retinal ischemia. The data support the concept that induction of angiogenesis in the human eye requires not only elevation of angiogenic growth factors such as VEGF (4) but also a decrease in angiogenesis inhibitors such as PEDF (1,7). PEDF has been proposed to be an age-dependent regulated protein (10). However, our data do not support this concept, although the number of control patients in our study may be too small to definitively answer this question.

We found that intraocular PEDF levels were reduced in nondiabetic patients with severe retinal ischemia caused by central-vein occlusion. Therefore, hypoxia rather than hyperglycemia promotes intraocular reduction of PEDF in humans. Our immunohistochemical findings show a small reduction in staining intensity in diabetic patients without retinal alterations and in diabetic patients with nonproliferative abnormalities (such as microaneurysms) compared with control subjects. These results suggest that glycemic control might also influence the expression of PEDF in the eye. Because of technical reasons in regard to quantification of immunohistochemistry in general, we cannot fully exclude small differences in the expression of PEDF in NPDR compared with control patients. Even such small differences might be relevant in the early stages of diabetic retinopathy, as suggested by data showing PEDF-dependent functional changes of retinal vessels (L.P. Aiello, Boston, MA; personal communication).

An important observation of this study was that patients with quiescent retinal neovascularization who mostly had retinal photocoagulation before intraretinal surgery had higher levels of PEDF compared with patients with active neovascularization without previous photocoagulation. Retinal photocoagulation induces regression of retinal neovascularization and has been shown to be associated with a reduction in the incidence of severe visual loss and retinal neovascularization (12). In our study group, patients with previous photocoagulation had reduced neovascular activity compared with patients without prior photocoagulation, suggesting that the positive effects of retinal photocoagulation are mediated at least in part by the reestablishment of near-normal PEDF levels. Presumably, a reduction in retinal ischemia after photocoagula-

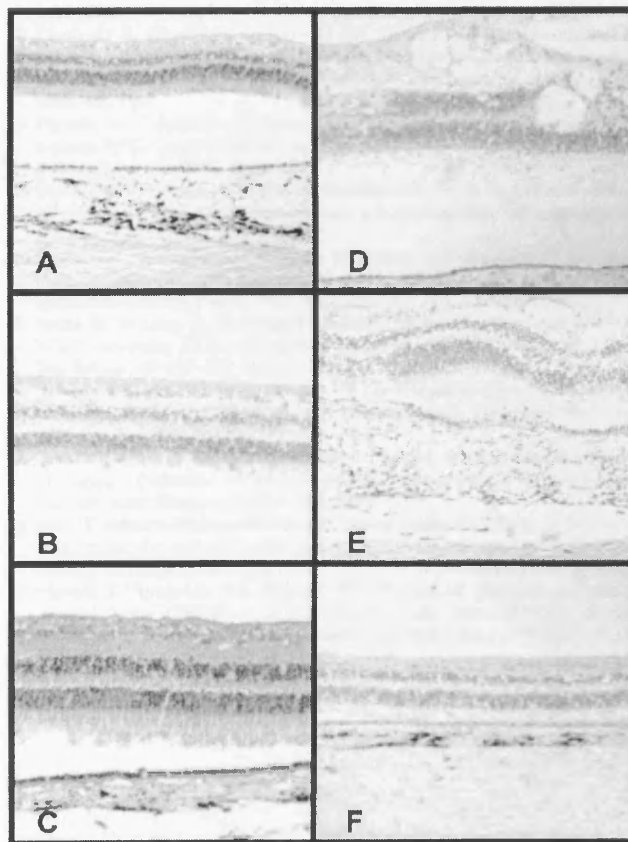

FIG. 5. PEDF protein expression in retinal samples from patients with different stages of diabetic retinopathy. Human retinas were examined by immunohistochemistry for PEDF expression. Staining patterns for representative sections are shown for control subjects (*A*), patients with diabetes without ocular abnormalities (*B*), and patients with NPDR (*C*) compared with patients with PDR (*D*) and patients with quiescent PDR after retinal photocoagulation (*E*). Specificity of reaction was demonstrated by the absence of staining after incubating sections with the primary antibody that was preabsorbed with the recombinant antigen (*F*).

tion increases expression of angiogenesis inhibitors such as PEDF, thereby further suppressing neovascular activity. Indeed, PEDF expression was initially induced by hyperoxia in neonatal mice (7). However, the patients in our study still exhibited intraocular proliferative activity requiring intraocular surgery. PEDF concentrations of patients after retinal scatter photocoagulation remained below those of control patients, thereby possibly explaining further existing proliferative activity in the subjects investigated.

A receptor for PEDF has not yet been identified, although radio-ligand binding studies in retinoblastoma cells and cerebellar granule neurons suggest a PEDF-specific receptor (13). Until now there has been no information about binding properties of putative receptors on vascular cells, putative binding proteins, or specific biological activities on different vascular cell types. Intraocular levels in mice are as high as 90 nmol/l. Despite these comparably high levels, systemically administered PEDF was able to completely inhibit aberrant retinal angiogenesis in a model of ischemia-induced proliferative retinopathy (8). This clearly indicates that increasing PEDF levels in the murine eye by systemic substitution is therapeutically effective. Our results with a loss of PEDF in humans strongly suggest that a similar PEDF-based treatment might be a promising therapeutic approach in patients with neovascularizing eye disease. Clearly, further investigations are needed to identify the exact mechanisms of PEDF release, PEDF-induced biological effects, and possible PEDF binding to putative binding proteins in the vitreous, such as that described for IGFs.

In conclusion, PEDF meets the criteria hypothesized for an ischemia-suppressed antiangiogenic factor (1). This principle, with obvious therapeutic impact, has been confirmed in animal studies (8). Here we suggest that the loss of a major angiogenesis inhibitor in the eye, PEDF, has a central role in vivo in humans in mediating the angiogenic response of retinal ischemia, such as that seen in PDR and other ischemic retinal disorders. In addition to the previously observed changes in angiogenic growth factors such as VEGF, our data support the hypothesis that an imbalance in the angiogenic ratio between angiogenic and antiangiogenic growth factors contributes significantly to the development of retinal neovascularization. Our data might potentially induce further investigations into the effectiveness of PEDF substitution in humans. Further characterization of ischemia-regulated PEDF expression and its biological effects should offer hopeful new therapeutic approaches to prevent blindness in patients with neovascularizing eye disease.

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REFERENCES

1. King GL, Kiyoshi S: Pigment-epithelium-derived factor: a key coordinator of retinal neuronal and vascular functions. *N Engl J Med* 342:349–351, 2000
2. Spranger J, Hammes H-P, Preissner KT, Schatz H, Pfeiffer AFH: Release of the angiogenesis inhibitor angiostatin in patients with proliferative diabetic retinopathy: association with retinal photocoagulation. *Diabetologia* 43:1404–1407, 2000
3. Meyer-Schwickerath R, Pfeiffer A, Blum WF, Freyberger H, Klein M, Losche C, Rollmann R, Schatz H: Vitreous levels of the insulin-like growth factors I and II, and the insulin-like growth factor binding proteins 2 and 3, increase in neovascular eye disease: studies in nondiabetic and diabetic subjects. *J Clin Invest* 92:2620–2625, 1993
4. Aiello LP, Avery RL, Arigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen HV, Aiello LM, Ferrara N, King GL: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331:1480–1487, 1994
5. Taniwaki T, Hirashima N, Becerra SP, Chader GJ, Etcheberrigaray R, Schwartz JP: Pigment epithelium-derived factor protects cultured cerebellar granule cells against glutamate-induced neurotoxicity. *J Neurochem* 68:26–32, 1997
6. Pignolo RJ, Cristofalo VJ, Rotenberg MO: Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G0 state. *J Biol Chem* 268:8949–8957, 1993
7. Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, Bouck NP: Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science* 285:245–248, 1999
8. Stellmach VV, Crawford SE, Zhou W, Bouck N: Prevention of ischemia-induced retinopathy by the natural ocular antiangiogenic agent pigment epithelium-derived factor. *Proc Natl Acad Sci U S A* 98:2593–2597, 2001
9. Smith G, McLeod D, Foreman D, Boulton M: Immunolocalisation of the VEGF receptors FLT-1, KDR, and FLT-4 in diabetic retinopathy. *Br J Ophthalmol* 83:486–494, 1999
10. DiPaolo BR, Pignolo RJ, Cristofalo VJ: Identification of proteins differentially expressed in quiescent and proliferatively senescent fibroblast cultures. *Exp Cell Res* 220:178–185, 1995
11. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
12. Early Treatment Diabetic Retinopathy Study Research Group: Early photocoagulation for diabetic retinopathy: ETDRS report number 9. *Ophthalmology* 98 (Suppl.):766–785, 1991
13. Alberdi E, Aymerich MS, Becerra SP: Binding of pigment epithelium-derived factor (PEDF) to retinoblastoma cells and cerebellar granule neurons: evidence for a PEDF receptor. *J Biol Chem* 274:31605–31612, 1999

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EXTENDED REPORT

Vascular endothelial growth factor C promotes survival of retinal vascular endothelial cells via vascular endothelial growth factor receptor-2

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Aim: To determine vascular endothelial growth factor C (VEGF-C) expression in retinal endothelial cells, its antiapoptotic potential and its putative role in diabetic retinopathy.
Method: Cultured retinal endothelial cells and pericytes were exposed to tumour necrosis factor (TNF)α and VEGF-C expression determined by reverse transcriptase-polymerase chain reaction. Secreted VEGF-C protein levels in conditioned media from endothelial cells were examined by western blotting analysis. The ability of VEGF-C to prevent apoptosis induced by TNFα or hyperglycaemia in endothelial cells was assessed by flow cytometry. The expression of VEGF-C in diabetic retinopathy was studied by immunohistochemistry of retinal tissue.
Result: VEGF-C was expressed by both vascular endothelial cells and pericytes. TNFα up regulated both VEGF-C and vascular endothelial growth factor receptor-2 (VEGFR)-2 expression in endothelial cells in a dose-dependent manner, but had no effect on VEGFR-3. Flow cytometry results showed that VEGF-C prevented endothelial cell apoptosis induced by TNFα and hyperglycaemia and that the antiapoptotic effect was mainly via VEGFR-2. In pericytes, the expression of VEGF-C mRNA remained stable on exogenous TNFα treatment. VEGF-C immunostaining was increased in retinal vessels in specimens with diabetes compared with retinal specimens from controls without diabetes.
Conclusion: In retinal endothelial cells, TNFα stimulates the expression of VEGF-C, which in turn protects endothelial cells from apoptosis induced by TNFα or hyperglycaemia via VEGFR-2 and thus helps sustain retinal neovascularisation.

Vascular endothelial growth factor A (VEGF-A) plays a key part in diabetic retinopathy by increasing retinal vascular permeability and inducing neovascularisation. However, the inhibition of VEGF-A only partially decreases neovascularisation and vessel hyperpermeability,[1] suggesting that other VEGF family members may also be involved in this process.[2 3]

VEGF-C is a member of the VEGF family that displays a high degree of homology with VEGF-A.[4] The VEGF-C precursor binds only vascular endothelial growth factor receptor (VEGFR)-3, whereas the fully processed VEGF-C ligand can bind and activate both VEGFR-2 and VEGFR-3.[5] VEGF-C stimulates proliferation and migration of blood vascular endothelial cells[5] and promotes release of nitric oxide and plasminogen activator from endothelial cells.[6 7] In animal models, VEGF-C induces angiogenesis and increases vascular permeability.[7 8] Furthermore, high expression of the VEGF-C protein and gene has been found in different vascularised tumour tissues.[9–11] The activation of both VEGFR-2 and VEGFR-3 has been implicated in angiogenesis,[12 13] and VEGFR-3 is present in different vascular beds including the retinal vasculature.[14 15]

The pathogenesis of diabetic retinopathy may be correlated with chronic subclinical inflammation,[16] and anti-inflammatory drugs have been shown to prevent early diabetic retinopathy via tumour necrosis factor (TNF)α suppression.[17] TNFα has been found in human retinas with proliferative eye diseases[18–20] and in animal models of retinal neovascularisation.[21] Furthermore, hyperglycaemia also plays an important part in the onset and progression of diabetic retinopathy by inducing apoptosis of vascular cells, advanced glycation end product deposition and up regulation of angiogenic factors.[22–24]

This paper reports that VEGF-C can promote survival of retinal endothelial cells and that this can be regulated by both TNFα and hyperglycaemia.

MATERIALS AND METHODS

Reagents

Recombinant TNFα, an anti-VEGFR-2 neutralising antibody, recombinant VEGF-C wild type (which binds both VEGFR-2 and VEGFR-3) and VEGF-C (Cys156Ser; a selective agonist of VEGFR-3) were obtained from R&D Systems Europe (Abingdon, UK). Anti-VEGF-C antibody was from Santa Cruz (UK). For immunohistochemistry, an affinity-purified goat polyclonal antibody raised against the carboxy terminus of the VEGF-C precursor of human origin (c-20) was obtained from Autogen Bioclear (Calne, Wiltshire, UK). TRIzol was from Invitrogen (Glasgow, UK), and polymerase chain reaction (PCR) Reddy Mix and Master Mix Kit were purchased from Abgene (UK). Vybrant apoptosis Assay Kit was from Molecular Probes (UK). All other materials were from Sigma unless otherwise stated.

Cell culture

Primary cultures of bovine microvascular retinal endothelial cells (MECs) and pericytes were isolated as described previously.[25] Endothelial cells were maintained in an endothelial

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEC, microvascular retinal endothelial cell; PCR, polymerase chain reaction; PDR, proliferative diabetic retinopathy; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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cell basal medium with growth supplement (TCS Works, Buckingham, UK). Cells were characterised by their cobblestone appearance and expression of factor VIII antigen.[25] Pericytes were cultured in Eagle's minimal essential medium (GibcoBRL, Paisley, UK) containing 10% fetal calf serum. Pericytes were identified and distinguished from endothelial cells by their size, irregular morphology and negative staining for factor VIII.[25] Both cell types were used between passages 1 and 3 for all experiments.

To ensure cross species recognition of VEGFRs, primary cultures from human donor eyes were obtained from the Bristol Eye Bank, Bristol, UK, and used in accordance with the tenets of the Declaration of Helsinki. The cultures were isolated and maintained as described above and used for the apoptosis studies within five passages.

TNFα treatment

For gene and protein expression studies, cells were treated with different concentrations of TNFα for up to 6 h. For time-dependent studies of TNFα treatment, cells were incubated with 10 ng/ml TNFα at different time points.

Reverse transcriptase-polymerase chain reaction

To investigate gene expression of VEGF-C and its receptors, total RNA was isolated from endothelial cells and pericytes exposed to different experimental conditions, using the isolation kit TRIzol, and then analysed by reverse transcriptase-polymerase chain reaction (RT-PCR) using the First Strand Synthesis Kit and PCR ReddyMix according to the manufacturer's protocol. Equal quantities of total RNA were used from different samples. The primers for VEGF-C were according to the sequences of bovine VEGF-C from GenBank. The oligonucleotide primers for amplification of VEGF-C cDNA were 5'-GAA CAA GGC TTA TGC AGG CAA AG -3' and 5'-CCA CAT CTG TAG ACG GAC ACA C-3'. The resultant PCR product was 348 bp long. The primers for VEGFR-2 were from Berisha *et al*[26] and VEGFR-3 was from Pepper *et al.*[6] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The sequences were 5'-TGT TCC AGT ATG ATT CCA CCC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3', and gave an 850 bp amplimer. The cDNA was amplified using the PCR Master Mix, each cycle consisting of 20 s at 94°C, 30 s at 55°C for amplifying VEGF-C and GAPDH cDNA, 51°C for VEGFR-2 cDNA, 56°C for VEGFR-3 cDNA and 60 s at 72°C. All the samples were amplified in a linear amplification range established using a serial cDNA dilution and varying the number of cycles. PCR products were electrophoresed on to a 1.2% agarose gel containing ethidium bromide and visualised under ultraviolet light. The relative intensities of the bands were quantified by densitometric analysis.

Immunoprecipitation and western blotting

To measure VEGF-C protein, preconfluent MECs were starved overnight in basal medium containing 1% fetal calf serum, after which either 1 or 10 ng/ml TNFα was added to the basal medium. De novo protein synthesis was blocked by the addition of 3.6 μM cycloheximide. Cells were exposed to different conditions for 24 h, the conditioned media was collected and centrifuged to remove cell debris. The protein concentrations were determined by the BCA protein assay (Pierce, UK). The medium with equal quantity of proteins was immunoprecipitated by incubation with an anti-VEGF-C antibody, and then protein A/G-agarose (Santa Cruz Biotechnology, USA). The immunoprecipitates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and proteins transferred on to nitrocellulose membranes. The membranes were probed with an anti-VEGF-C antibody followed by incubation with secondary antibody conjugated with horseradish peroxide. The enhanced chemiluminescence reaction system (Santa Cruz, UK) was used to visualise the bands.

Apoptosis assay

Apoptosis was evaluated using the Vybrant Apoptosis Assay Kit based on annexin-V binding to phosphatidylserine exposed on the outer leaflet of the plasma membrane lipid bilayer of cells. MECs were treated with either 100 ng/ml TNFα or 30 mM glucose in the presence or absence of 200 ng/ml VEGF-C for 48 h in culture medium. The cells from different treatments were subjected to the apoptosis assay according to the manufacturer's instructions. The samples were analysed using a fluorescent activated cell sorting 440 Flow Cytometer (Becton Dickinson, Oxford, UK). Viable cells were double-negative stained, early apoptotic cells stained positive for annexin V and negative for propidium iodide, whereas, late apoptotic/necrotic cells were double-positive stained for annexin V and propidium iodide. To define the role of VEGFR-2 in the anti-apoptotic effect of VEGF-C, 60 ng/ml anti-VEGFR-2 antibody was added to the culture medium for 1 h before incubation with 100 ng/ml TNFα and 200 ng/ml VEGF-C, or 30 mM glucose and 200 ng/ml VEGF-C. Anti-VEGFR-2 antibody alone acted as a control. To observe whether VEGFR-3 had an anti-apoptotic function, 200 ng/ml VEGF-C (Cys156Ser; a selective agonist of VEGFR-3) was administrated together with 100 ng/ml TNFα for 48 h.

To block the basal secretion of VEGF-C, cells were transfected with either VEGF-C small interfering RNA (siRNA) or scrambled siRNA for 72 h and then incubated with 100 ng/ml TNFα for 48 h. siRNA duplexes were designed and synthesised by Dharmacon Research (Lafayette, Colorado, USA) to target the bovine sequence of VEGF-C 5'-ACA GAG ATC TTA AGA AGT A-3'. The premade siRNA (scramble II; Dharmacon) was used as a negative control. Cells were transfected with siRNA duplexes using DharmaFECT 1 (Dharmacon) at a final RNA concentration of 100 nmol/l according to the manufacturer's protocol. To determine the efficiency of transfection, the medium from parallel samples was collected and subjected to immunoprecipitation and western blotting after 24-h of incubation.

Immunohistochemistry

A total of 47 eyes enucleated and fixed in 10% neutral-buffered formalin within 10 h after death were obtained from the National Disease Research Interchange, Philadelphia, USA. All procedures were performed according to the Declaration of Helsinki. Eyes were categorised by an ophthalmologist based on fundus appearance as normal (no known ophthalmic disease, no history of diabetes, no abnormalities on biomicroscopy), diabetic with no overt retinopathy, diabetic with intraretinal changes but no evidence of proliferative diabetic retinopathy (PDR), diabetic with preretinal PDR and diabetic with scatter laser photocoagulation but no evidence of residual PDR.[14]

Immunohistochemistry was performed on 5 μm sections as described previously.[14] Sections were incubated overnight at 4°C with a polyclonal VEGF-C antibody (2 μg/ml). The negative control was the substitution of the primary antibody with an inappropriate rabbit IgG. After washing, sections were incubated for 30 min with biotinylated rabbit anti-goat IgG, then for a further 30 min with alkaline phosphatase reaction mixture (Dako) and incubated with Fast Red TR/naphthol AS-MX substrate. Slides were counterstained with Mayer's haematoxylin. The degree and pattern of immunostaining was assessed by two blinded observers. The intensity of staining was graded qualitatively as background, weak, moderate or intense (corresponding to the highest level of immunoreactivity).

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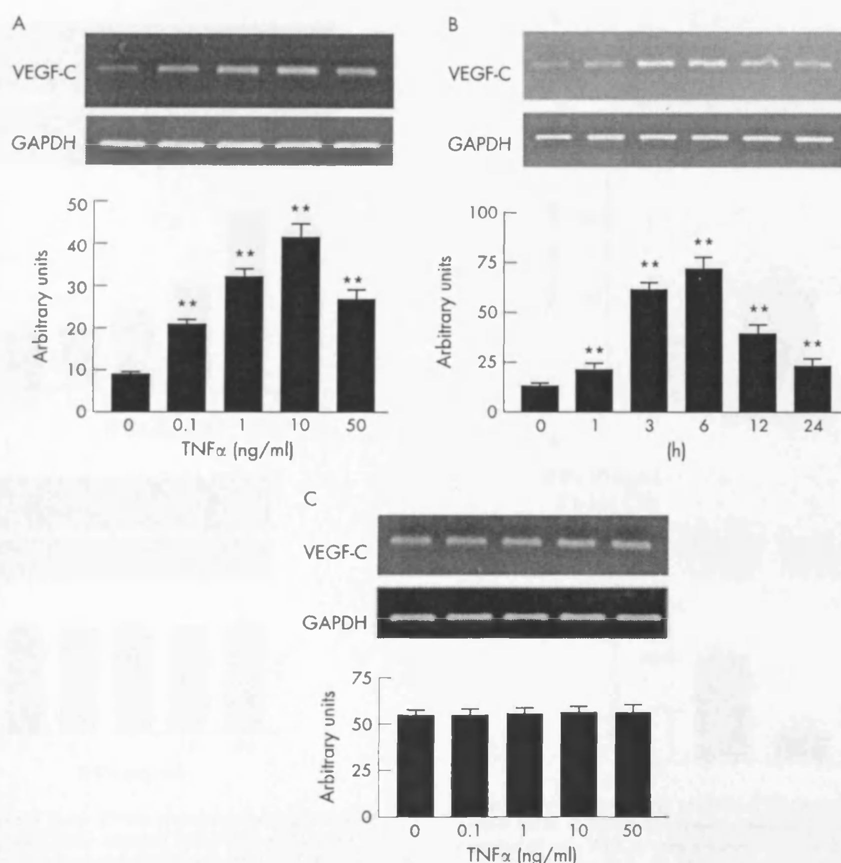

Figure 1 The regulation of vascular endothelial growth factor-C (VEGF-C) mRNA expression by tumour necrosis factor (TNF)α in microvascular endothelial cells and pericytes. (A) Dose–response of mRNA induction of VEGF-C by TNFα in microvascular retinal endothelial cells (MECs). MECs were stimulated with the indicated concentrations of TNFα for 6 h. (B) Time dependence of VEGF-C mRNA induction by TNFα. MECs were stimulated with TNFα (10 ng/ml) for 0–24 h. (C) Expression of VEGF-C mRNA in bovine retinal pericytes after exposure to different concentrations of TNFα for 6 h. The isolated total RNA from different treatments was subjected to reverse transcriptase-polymerase chain reaction and polymerase chain reaction products were analysed by agarose gel electrophoresis. Band intensities were quantified by laser densitometry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control. Representative results from three separate experiments are shown. Vertical bars represent mean (standard error of the mean); **$p<0.01$, indicating significant difference between treatment and controls.

Statistical analysis

The results represent the mean of at least three separate experiments. Statistical analysis was carried out using an unpaired Student's t test. Significance was defined as $p<0.05$. All numerical results are expressed as mean (standard error of the mean).

RESULTS

Regulation of VEGF C mRNA expression by TNFα in microvascular endothelial cells and pericytes

RT-PCR analysis showed that VEGF-C mRNA was expressed in MECs. TNFα stimulated the expression of VEGF-C mRNA in a dose-dependent manner, with a maximal 4.6 (0.5)-fold increase with 10 ng/ml TNFα (fig 1A). Stimulation of cells with 10 ng/ml TNFα increased VEGF-C mRNA expression in a time-dependent manner with a maximum at 6 h. Beyond 6 h, VEGF-C expression decreased, but even after 24 h stimulation, the expression of VEGF-C mRNA was still higher than that with no stimulation (fig 1B). Pericytes expressed VEGF-C mRNA, but TNFα had no regulatory effect on this expression (fig 1C).

Increased expression of VEGFR-2, but not VEGFR-3 mRNA in MECs challenged with TNFα

RT-PCR results showed that MECs expressed both VEGFR-2 and VEGFR-3 mRNA (fig 2). TNFα induced an increase of VEGFR-2 mRNA in a dose-dependent manner. The levels of VEGFR-2 mRNA began to increase at 1 ng/ml TNFα and reached a maximum level at 50 ng/ml TNFα (fig 2A). By contrast, the expression of VEGFR-3 was not modified by exposure to TNFα (fig 2B). The expression of VEGFR-2 and VEGFR-3 mRNA was not detectable in pericytes (data not shown).

TNFα increases VEGF C protein synthesis and secretion in MECs

Western blotting showed that the secreted peptide was present at high amounts, with a maximum 4.1 (0.4)-fold increase in the medium under TNFα conditions compared with the medium of control cultures (fig 3A). Treatment with cycloheximide considerably reduced the amount of VEGF-C in the conditioned medium. This result indicates that increased amounts of VEGF-C released by MECs in response to TNFα treatment are due to

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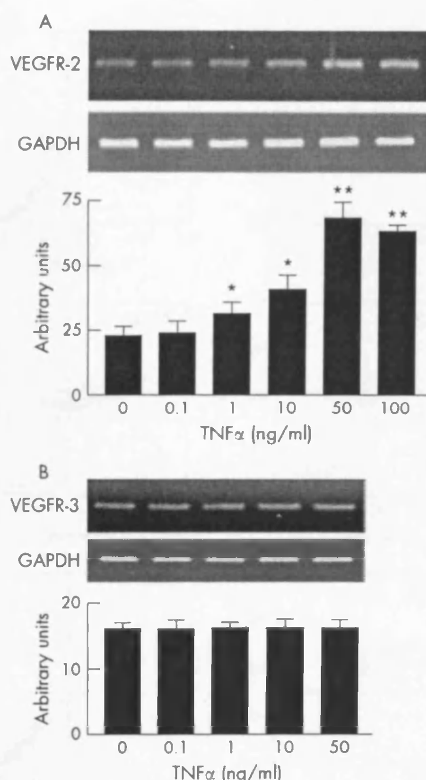

Figure 2 Tumour necrosis factor (TNF)α stimulates the expression of vascular endothelial growth factor receptor (VEGFR)-2 mRNA, but not VEGFR-3 in microvascular retinal endothelial cells (MECs). Total RNA was isolated 6 h after stimulation by different concentrations of TNFα. Reverse transcriptase-polymerase chain reaction was performed and polymerase chain reaction products were analysed by agarose gel electrophoresis. The signal intensity was determined by densitometry, and the amount of VEGFR-2/VEGFR-3 was normalised for the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) present. Representative results from at least three separate experiments are shown. Vertical bars represent mean (standard error of the mean); *$p<0.05$ and **$p<0.01$, indicating significant difference between treatment and controls. (A) Expression of VEGFR-2 mRNA; (B) expression of VEGFR-3 mRNA.

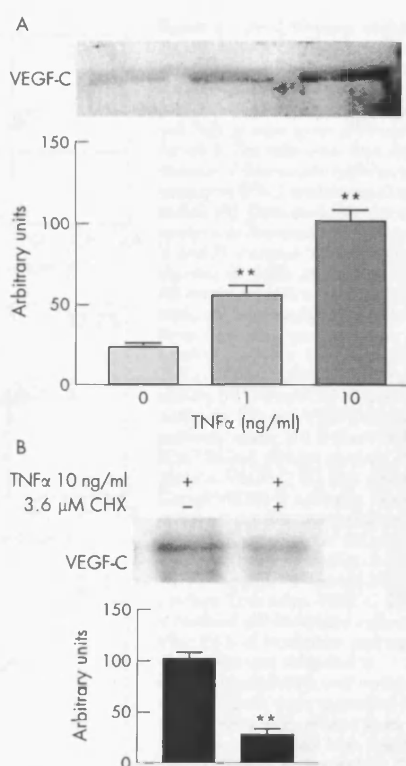

Figure 3 Western blotting analysis of de novo vascular endothelial growth factor-C (VEGF-C) protein synthesis. (A) Microvascular retinal endothelial cells (MECs) were treated with 1 or 10 ng/ml tumour necrosis factor (TNF)-α for 24 h. (B) MECs were treated with 10 ng/ml TNFα and 3.6 μM cyclohexamide (CHX) for 24 h, and 10 ng/ml TNFα alone was the control. Conditioned media were immunoprecipitated using a polyclonal antibody raised to VEGF-C and the immunoprecipitates electrophoresed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by blotting on a nitrocellulose membrane. Positive bands were visualised by an enhanced chemiluminescence reaction detection system. Band intensity for VEGF-C was quantified by laser densitometry from at least three separate experiments. Vertical bars represent mean (standard error of the mean); **$p<0.01$, indicating significant difference between treatment and controls.

increased protein synthesis rather than an increased release of VEGF-C from cell storage (fig 3B)

VEGF C prevents TNFα and hyperglycaemia-induced apoptosis in MECs and this effect occurs mainly via VEGFR-2

Flow cytometry showed that TNFα induced apoptosis/necrosis and that this was markedly inhibited by VEGF-C. The cell population at the late stage of apoptosis/necrosis reduced from 56.5% (1.38%) to 28.1% (0.7%) when VEGF-C was present (fig 4A–C). The apoptotic/necrotic population increased to 82.7% (2.8%) after exposure to TNFα in cells treated with VEGF-C siRNA compared with 57.9% (1.72%) in cells treated with scrambled siRNA (fig 4D,E). The efficiency of knockdown of VEGF-C expression in culture medium with RNAi was confirmed using immunoprecipitation and western blotting (fig 4L).

To identify which of the two VEGF-C receptors was responsible for the anti-apoptotic effect of VEGF-C, VEGFR-2 was blocked by neutralising antibody or cells were treated with a VEGFR-3 agonist. After neutralising VEGFR-2, the protective effect of VEGF-C on TNFα-induced apoptosis was considerably reduced. The population of apoptotic/necrotic cells was increased from 28.1% (0.7%) to 53.2% (1.41%) (fig 4C,F). VEGFR-2-neutralising antibody alone had no effect on promoting apoptosis/necrosis (fig 4G). After addition of VEGF-C (Cys156Ser), there was no protective effect on TNFα-induced apoptosis. The population of apoptotic/necrotic cells was 55.7% (2.1%), and showed no statistically significant change from cells treated with TNFα alone (fig 4B,H). After addition of VEGF-C, the percentage of apoptosis/necrosis induced by high glucose was reduced from 47.1% (1.6%) to 23.6% (1.2%) (fig 4I,J) and this rescue effect was abolished when VEGFR-2 was blocked by its neutralising antibody (fig 4K). Results were similar for both human and bovine MECs.

Expression of VEGF-C in diabetic retinopathy

Weak to moderate staining for VEGF-C was observed in the vessels of non-diabetic retinas and in retinas without overt retinopathy; staining was increased (moderate to intense) compared with non-diabetic retinas once intraretinal changes became obvious (4 of 5 eyes) and markedly increased in PDR retinas (6 of 6 eyes; table 1, fig 5). Intense staining was also

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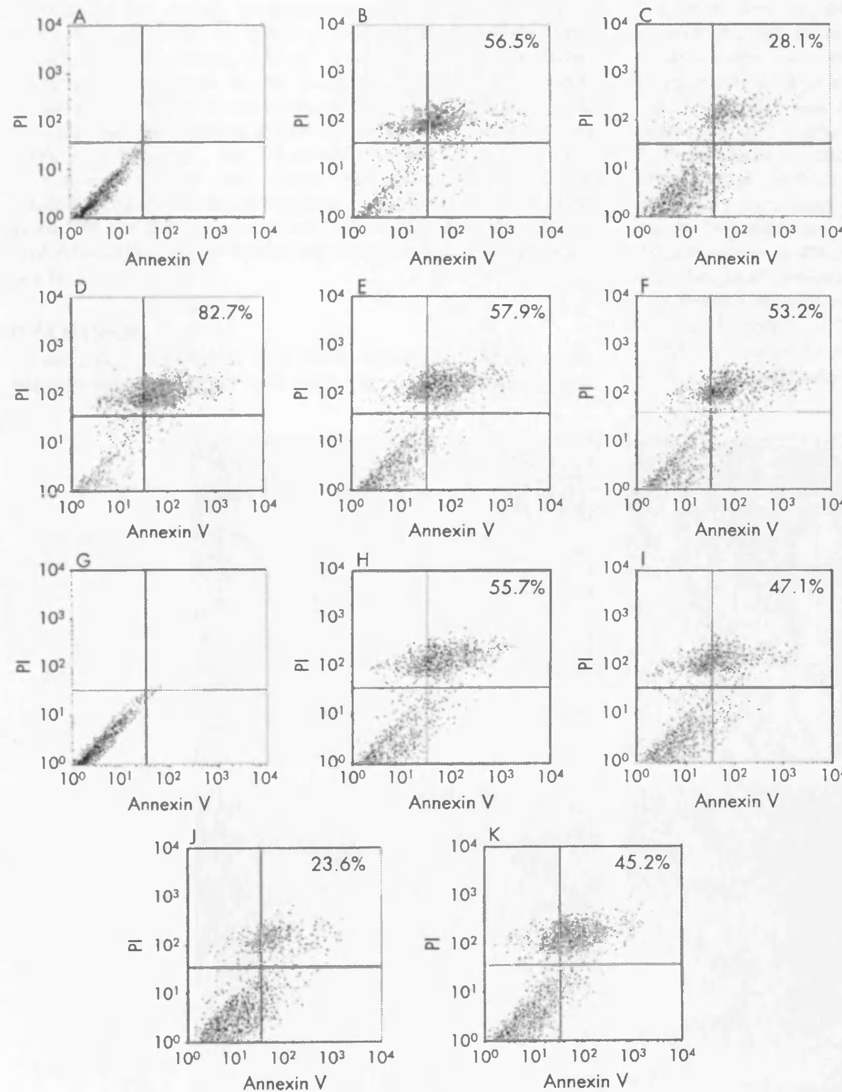

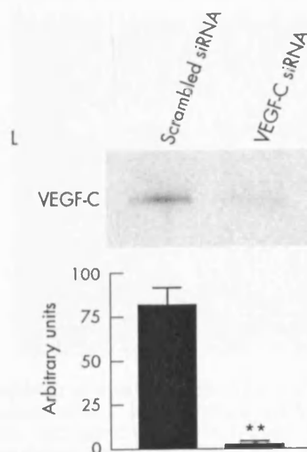

Figure 4 (A–K) Vascular endothelial growth factor (VEGF)-C prevents tumour necrosis factor (TNF)α-induced apoptosis via vascular endothelial growth factor receptor (VEGFR)-2. Microvascular retinal endothelial cell (MECs) were given different treatments for 48 h. The cells were then stained with annexin V-fluorescein isothiocyanate conjugate (FITC) containing propidium iodide (PI). Data show two-parameter analysis of fluorescence intensity of annexin V and PI. Annexin V/PI-negative cells were counted as viable cells (lower left quadrant). All measurements were performed in triplicate. Representative results from at least three separate experiments are shown. (A) Control; (B) TNFα; (C) TNFα+VEGF-C; (D) TNFα+VEGF-C siRNA; (E) TNFα+scrambled siRNA; (F) TNFα+VEGF-C+anti-VEGFR-2 antibody; (G) anti-VEGFR-2-neutralising antibody alone; (H) TNFα+VEGF-C (Cys156Ser); (I) high glucose; (J) high glucose+VEGF-C; (K) high glucose+VEGF-C+anti-VEGFR-2 antibody. Numbers in the quadrant are the percentage of $FITC^+/PI^+$ cells. (L) Knockdown of the expression of VEGF-C in culture medium from small interfering (si)RNA-treated MECs. Culture medium from either VEGF-C siRNA or scrambled siRNA-treated cells was collected after 24 h of incubation and equal quantity of proteins was subjected to immunoprecipitation and western blotting. Positive bands were quantified by laser densitometry from at least three separate experiments. Vertical bars represent mean (standard error of the mean); $^{**}p<0.01$, indicating significant difference between VEGF-C siRNA treatment and scrambled siRNA.

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observed in the vessels of preretinal membranes. After laser treatment, the levels of VEGF-C in the retinal vessels were reduced to weak staining in 11 of 14 eyes. In addition, increased staining was observed in the ganglion cell layer of diabetic retinas both with and without intraretinal changes as compared with the minimal staining in non-diabetic retinas. Staining was weak or absent in the choroidal vessels, the RPE, the photoreceptors, and the outer and inner retinal layers (table 1). The variability of staining within retinas of the same group did not show a correlation with donor age, the type of glycaemic control in the case of the diabetic groups or time after death.

DISCUSSION

In this study, we showed that TNFα strongly up regulates the expression of VEGF-C in MECs and that this induction is both time dependent and dose dependent. The dose–response study showed that the stimulatory effect of TNFα was produced at a concentration as low as 0.1 ng/ml, which is within the range of TNFα concentrations in vitreous fluid from patients with active PDR.[18] VEGFR-2 was also up regulated by TNFα, whereas the expression of VEGFR-3 mRNA remained stable with various TNFα treatments, suggesting that in our experimental system VEGF-C may exert its angiogenic effect mainly via increasing VEGFR-2 rather than VEGFR-3. This increase in VEGFR-2 may also be important in enhancing VEGF-A-induced angiogenesis. After blockade of VEGFR-2, the antiapoptotic effect of VEGF-C was abrogated, whereas the activation of VEGFR-3 by VEGF-C (Cys156Ser) did not attenuate TNFα-induced apoptosis showing that VEGFR-2 is the dominant receptor for VEGF-C action in MECs. Our data support the observations that VEGF-C, which was originally thought to be a potent inducer of

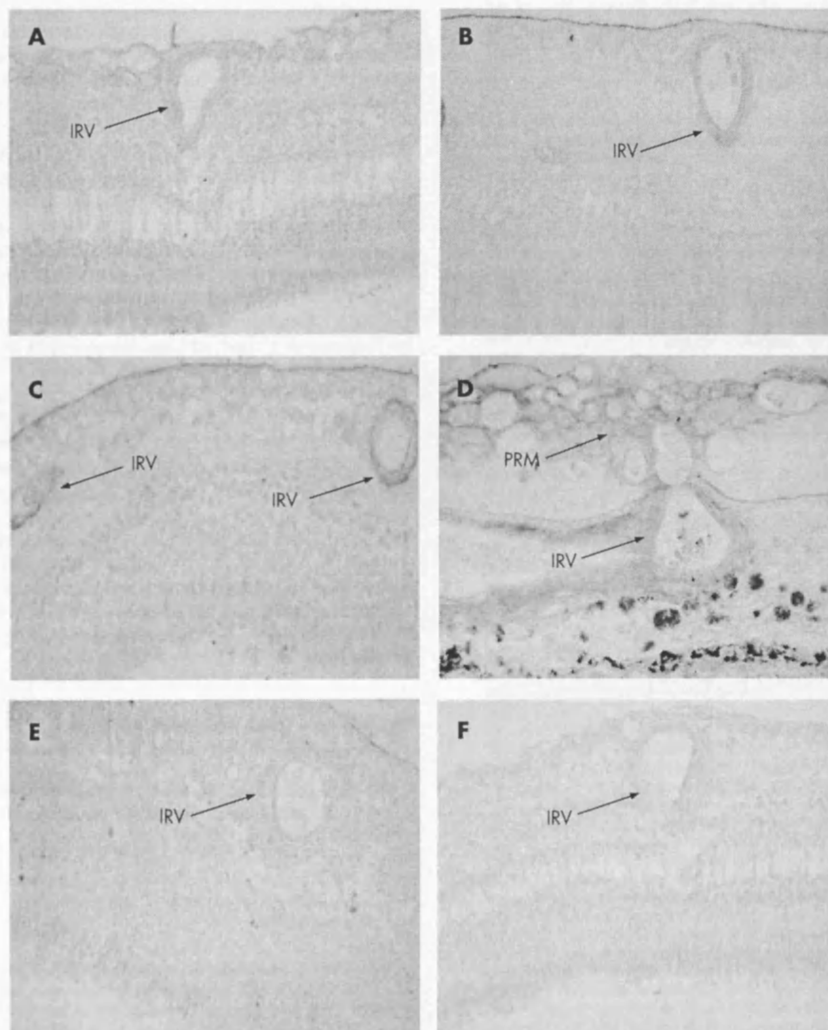

Figure 5 Immunolocalisation of vascular endothelial growth factor (VEGF)-C in the diabetic retina. VEGF-C staining is shown for representative retinal sections. Weak to moderate VEGF-C immunostaining was localised to non-diabetic retina (A) and diabetic retina with no obvious intraretinal vascular changes (B). By contrast, moderate to intense VEGF-C staining was observed in the diabetic retina with obvious intraretinal vascular changes but no evidence of proliferative diabetic retinopathy (PDR; C) and in the diabetic retina with PDR (D). After laser treatment, the only weak immunostaining for VEGF-C was observed (E). Immunostaining was raised in later-stage diabetic retinopathy and was generally confined to the intraretinal vessels (IRV). Immunoreactivity was abolished in the control retina processed with omission of primary antibody (F). Magnification ×200. PRM, polynomial regularisation method.

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Table 1 Mean (SD) intensity of vascular endothelial growth factor-C in the retina and choroids

	Choroid	RPE	Photoreceptors	Outer nuclear layer	Inner nuclear layer	Ganglion cell layer	Retinal vessels
Non-diabetic (n = 14)	0.6 (0.8)	0.1 (0.4)	0.4 (0.8)	0 (0)	0.1 (0.4)	0.3 (0.4)	1.1 (1.2)
No overt retinopathy (n = 12)	0.8 (0.6)	0.1 (0.3)	0.3 (0.7)	0 (0)	0.1 (0.3)	1.2 (1.0)	1.6 (1.1)
Intraretinal changes (n = 5)	0.8 (0.8)	0 (0)	0.6 (0.9)	0.2 (0.5)	0.6 (0.9)	1.2 (1.1)	2.4 (0.9)
PDR (n = 6)	0.8 (0.8)	0.3 (0.5)	0.3 (0.5)	0.2 (0.4)	0.3 (0.5)	0.3 (0.5)	2.5 (0.5)
Laser, no residual PDR (n = 14)	0.6 (0.6)	0 (0)	0.4 (0.6)	0.4 (0.9)	0.3 (0.5)	0.4 (0.5)	0.8 (1.2)

PDR, proliferative diabetic retinopathy; RPE,retinal pigment epithelium.
0, background; 1, mild; 2, moderate; 3, intense staining.

lymphangiogenesis,[27 28] may also act as a survival factor to suppress apoptosis in vascular endothelial cells. VEGF-C has been shown to be important in vascular angiogenesis in other vascular beds,[7 8 11 29] and the response can be robust and indistinguishable from that observed using VEGF-A.[30 31] The existence of VEGF-C in human retinas and the expression of VEGF-C in the retinal vasculature that increases in diabetic retinopathy further support a role for VEGF-C in diabetic retinopathy. Interestingly, laser photocoagulation, a proved treatment for reversing neovascularisation in PDR, resulted in a marked reduction in VEGF-C protein expression.

Our data showed that mRNA coding for VEGF-C was present not only in endothelial cells but also in pericytes. However, the regulatory effects differ between the two principal types of microvascular cells. The VEGF-C gene remained constitutively expressed in pericytes on adding various concentrations of TNFα, but VEGF-C is unlikely to signal in an autocrine fashion as only VEGFR-1 is expressed in pericytes[32 33]; TNFα is a macrophage/monocyte-derived pluripotent mediator. Whether TNFα plays a part in angiogenesis may be highly dependent on its concentration.[34] TNFα has been shown to be a powerful activator of angiogenesis in vivo in several animal models when used at appropriate doses.[15–17] Previous studies show that high glucose or advanced glycation end products induce the expression of proinflammatory cytokines, including TNFα from monocytes and macrophages,[38 39] and TNFα may have an important role in mediating angiogenesis in diabetic retinopathy.[18–20] The angiogenic effect of TNFα may be due to the generation of secondary mediators.[40 41]

In conclusion, increased expression of VEGF-C protein in the retinal vasculature of diabetic retinopathy suggests that VEGF-C may have an important role in its pathogenesis.

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REFERENCES

1 **Castellon R**, Hamdi HK, Sacerio I, *et al*. Effects of angiogenic growth factor combinations on retinal endothelial cells. *Exp Eye Res* 2002;**74**:523–35.
2 **Khaliq A**, Foreman D, Ahmed A, *et al*. Increased expression of placenta growth factor in proliferative diabetic retinopathy. *Lab Invest* 1998;**78**:109–16.
3 **Cai J**, Ahmad S, Jiang WG, *et al*. Activation of vascular endothelial growth factor receptor-1 sustains angiogenesis and Bcl-2 expression via the phosphatidylinositol 3-kinase pathway in endothelial cells. *Diabetes* 2003;**52**:2959–68.
4 **Lee J**, Gray A, Yuan J, *et al*. Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci USA* 1996;**93**:1988–92.
5 **Joukov V**, Sorsa T, Kumar V, *et al*. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *Embo J* 1997;**16**:3898–911.
6 **Pepper MS**, Mandriota SJ, Jeltsch M, *et al*. Vascular endothelial growth factor (VEGF)-C synergizes with basic fibroblast growth factor and VEGF in the induction of angiogenesis in vitro and alters endothelial cell extracellular proteolytic activity. *J Cell Physiol* 1998;**177**:439–52.
7 **Witzenbichler B**, Asahara T, Murohara T, *et al*. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am J Pathol* 1998;**153**:381–94.
8 **Cao Y**, Linden P, Farnebo J, *et al*. Vascular endothelial growth factor C induces angiogenesis in vivo. *Proc Natl Acad Sci USA* 1998;**95**:14389–94.
9 **Clarijs R**, Schalkwijk L, Hofmann UB, *et al*. Induction of vascular endothelial growth factor receptor-3 expression on tumor microvasculature as a new progression marker in human cutaneous melanoma. *Cancer Res* 2002;**62**:7059–65.
10 **Skobe M**, Hamberg LM, Hawighorst T, *et al*. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol* 2001;**159**:893–903.
11 **Valtola R**, Salven P, Heikkila P, *et al*. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 1999;**154**:1381–90.
12 **Meyer M**, Clauss M, Lepple-Wienhues A, *et al*. A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J* 1999;**18**:363–74.
13 **Dumont DJ**, Jussila L, Taipale J, *et al*. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 1998;**282**:946–9.
14 **Smith G**, McLeod D, Foreman D, *et al*. Immunolocalisation of the VEGF receptors FLT-1, KDR, and FLT-4 in diabetic retinopathy. *Br J Ophthalmol* 1999;**83**:486–94.
15 **Witmer AN**, Blaauwgeers HG, Weich HA, *et al*. Altered expression patterns of VEGF receptors in human diabetic retina and in experimental VEGF-induced retinopathy in monkey. *Invest Ophthalmol Vis Sci* 2002;**43**:849–57.
16 **Adamis AP**. Is diabetic retinopathy an inflammatory disease? *Br J Ophthalmol* 2002;**86**:363–5.
17 **Joussen AM**, Poulaki V, Mitsiades N, *et al*. Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. *FASEB J* 2002;**16**:438–40.
18 **Armstrong D**, Augustin AJ, Spengler R, *et al*. Detection of vascular endothelial growth factor and tumor necrosis factor alpha in epiretinal membranes of proliferative diabetic retinopathy, proliferative vitreoretinopathy and macular pucker. *Ophthalmologica* 1998;**212**:410–14.
19 **Limb GA**, Chignell AH, Green W, *et al*. Distribution of TNF alpha and its reactive vascular adhesion molecules in fibrovascular membranes of proliferative diabetic retinopathy. *Br J Ophthalmol* 1996;**80**:168–73.
20 **Spranger J**, Meyer-Schwickerath R, Klein M, *et al*. TNF-alpha level in the vitreous body. Increase in neovascular eye diseases and proliferative diabetic retinopathy. *Med Klin* 1995;**90**:134–7.
21 **Majka S**, McGuire PG, Das A. Regulation of matrix metalloproteinase expression by tumor necrosis factor in a murine model of retinal neovascularization. *Invest Ophthalmol Vis Sci* 2002;**43**:260–6.
22 **Cogan DG**, Toussaint D, Kuwabara T. Retinal vascular patterns. IV. Diabetic retinopathy. *Arch Ophthalmol* 1961;**66**:366–78.
23 **Wolffenbuttel BH**, Giordano D, Founds HW, *et al*. Long-term assessment of glucose control by haemoglobin-AGE measurement. *Lancet* 1996;**347**:513–15.
24 **Zhao B**, Cai J, Boulton M. Expression of placenta growth factor is regulated by both VEGF and hyperglycaemia via VEGFR-2. *Microvasc Res* 2004;**68**:239–46.
25 **Wong HC**, Boulton M, Marshall J, *et al*. Growth of retinal capillary endothelia using pericyte conditioned medium. *Invest Ophthalmol Vis Sci* 1987;**28**:1767–75.
26 **Berisha B**, Schams D, Kosmann M, *et al*. Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during the final growth of bovine ovarian follicles. *J Endocrinol* 2000;**167**:371–82.
27 **Jeltsch M**, Kaipainen A, Joukov V, *et al*. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* 1997;**276**:1423–5.
28 **Tsurusaki T**, Kanda S, Sakai H, *et al*. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. *Br J Cancer* 1999;**80**:309–13.
29 **Kubo H**, Fujiwara T, Jussila L, *et al*. Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. *Blood* 2000;**96**:546–53.
30 **Takeshita S**, Tsurumi Y, Couffinahl T, *et al*. Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor stimulates collateral development in vivo. *Lab Invest* 1996;**75**:487–501.

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31 **Takeshita S**, Zheng LP, Brogi E, *et al.* Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 1994;**93**:662–70.
32 **Takagi H**, King GL, Aiello LP. Identification and characterization of vascular endothelial growth factor receptor (Flt) in bovine retinal pericytes. *Diabetes* 1996;**45**:1016–23.
33 **Yamagishi S**, Yonekura H, Yamamoto Y, *et al.* Vascular endothelial growth factor acts as a pericyte mitogen under hypoxic conditions. *Lab Invest* 1999;**79**:501–9.
34 **Fajardo LF**, Kwan HH, Kowalski J, *et al.* Dual role of tumor necrosis factor-alpha in angiogenesis. *Am J Pathol* 1992;**140**:539–44.
35 **Frater-Schroder M**, Risau W, Hallmann R, *et al.* Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proc Natl Acad Sci USA* 1987;**84**:5277–81.
36 **Leibovich SJ**, Polverini PJ, Shepard HM, *et al.* Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. *Nature* 1987;**329**:630–2.
37 **Montrucchio G**, Lupia E, Battaglia E, *et al.* Tumor necrosis factor alpha-induced angiogenesis depends on in situ platelet-activating factor biosynthesis. *J Exp Med* 1994;**180**:377–82.
38 **Shanmugam N**, Reddy MA, Guha M, *et al.* High glucose-induced expression of proinflammatory cytokine and chemokine genes in monocytic cells. *Diabetes* 2003;**52**:1256–64.
39 **Vlassara H**, Brownlee M, Manogue KR, *et al.* Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science* 1988;**240**:1546–8.
40 **Bussolino F**, Camussi G, Baglioni C. Synthesis and release of platelet-activating factor by human vascular endothelial cells treated with tumor necrosis factor or interleukin 1 alpha. *J Biol Chem* 1988;**263**:11856–61.
41 **Okamura K**, Sato Y, Matsuda T, *et al.* Endogenous basic fibroblast growth factor-dependent induction of collagenase and interleukin-6 in tumor necrosis factor-treated human microvascular endothelial cells. *J Biol Chem* 1991;**266**:19162–5.