The Role of Decorin in Control of Endothelial Cell Behaviour

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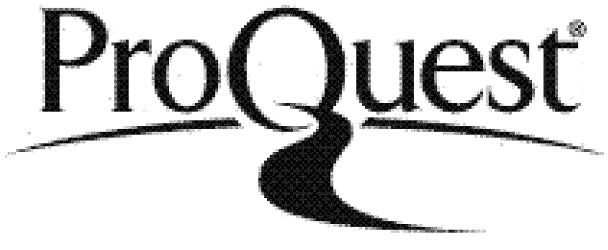
Lorna Fiedler, B.Sc. (Hons)
Dental Health and Biological Sciences
Dental School,
Cardiff University,
Cardiff



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Dedicated to the memory of

Dr. Elke Schönherr,

who always took the less travelled path

"Education is a progressive discovery of our own ignorance."

- Will Durant

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Summary

Angiogenesis is a complex process regulated by co-ordination of extracellular matrix (ECM) and growth factor signalling (*via* integrins and growth factor receptors respectively). Dysregulated angiogenesis contributes to disease pathology and progression, while an adequate blood supply is crucial for successful tissue repair and reconstruction. Thus an understanding of the mechanisms by which ECM controls angiogenesis would contribute to development of therapeutic strategies. Decorin is a small leucine-rich repeat proteoglycan consisting of a core protein and a single glycosaminoglycan. Decorin has been reported to contribute to ECM organisation through interaction with numerous ECM components, including collagen types I, VI, XII and XIV, fibronectin, thrombospondin, tropoelastin and tenascin-X. Further, decorin influences growth factor activity, interacts with α2β1 integrin, and can activate growth factor receptors. In the absence of decorin, angiogenesis is dysregulated however it is not known which of these interactions are responsible.

This thesis investigates the role of decorin interactions with collagen type I, α2β1 integrin (a collagen I receptor), and the IGF-I receptor in modulating endothelial cell behaviour. This thesis demonstrates that both collagen-bound and soluble decorin enhance endothelial cell adhesion and migration, and that the latter may involve activation of the IGF-I receptor, and the small GTPase Rac, by decorin. In accordance with this, decorin induced morphological changes consistent with activation of small GTPases. Further, decorin interacts with α2β1 integrin *via* the GAG moiety, and may influence integrin activity in an allosteric manner, although the intact proteoglycan is required for modulation of endothelial cell behaviour. It was also demonstrated that decorin supports α2β1 integrin activation in the presence of the specific inhibitor rhodocetin. Additionally, decorin activates transcription factors associated with long-term cell survival and quiescence. Together, these data implicate decorin as an important regulator of several aspects of angiogenesis pertinent to establishment of mature neo-vessels.

Abbreviations

BAE	Bovine aortic endothelial cells
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CBD	Cell binding domain
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CHO	Chinese hamster ovary
CS	Chondroitin sulphate
C-6S	Chondroitin-6 sulphate
C-4S	Chondroitin-4 sulphate (C-4S)
Dcn	Decorin
DNA	Deoxynucleic acid
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DS	Dermatan sulphate
ECM	Extracellular matrix
EDS	Ehlers-Danlos Syndrome
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACIT	Fibril associated collagen with interrupted triple helices
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GdnHCl	Guanidine hydrochloride

GDP	Guanosine diphosphate
GlcA	Glucuronic acid
Gal	Galactose
GalNAc	N-acetylgalactosamine
GDP	Guanosine diphosphate
GlcNAc	N-acetylglucosamine
GTP	Guanosine 5'-triphosphate
HA	Hyaluronan/ Hyaluronic acid
Hep	Heparin
HEK-293	Human embryonic kidney-293
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible transcription factor
HOX	Homeobox
HRP	Horseradish peroxidase
HS	Heparan sulphate
HUVEC	Human umbilical cord endothelial cells
IdoA	Iduronic acid
IFN-γ	Interferon gamma
IGF-BP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor - I
IGF-IR	Insulin-like growth factor - I receptor
IL	Interleukin
KS	Keratan sulphate
LRR	Leucine-rich repeat
MAPK	Mitogen activated protein kinase
MBP	Mannose binding protein
MES	2- (N-morpholino) ethanesulfonic acid
MEM	Minimum essential medium
MIDAS	Metal ion dependent adhesion site
MLC	Myosin light chain
MLCK	Myosin light chain kinase

MRCK	Myotonin-related Cdc42-binding kinase
MMP	Matrix metalloproteinase
MT-MMP	Membrane type – matrix metalloproteinase
NFDM	Non-fat dried milk
NO	Nitric oxide
PAGE	Polyacrylamide Gel Electrophoresis
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PI-3K	Phosphatidyl inositol – 3 kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulphonylfluoride
PTB	Phosphotyrosine binding
ROCK	Rho kinase
SDS	Sodium dodecyl sulphate
SLRP	Small leucine-rich repeat proteoglycan
TBS	Tris buffered saline
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitors of metalloproteinases
Tsp	Thrombospondin
TNF-α	Tumour necrosis factor-alpha
UPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

Chapter 1

General Introduction

1.1 General Introduction

The vascular system is responsible for maintaining tissue homeostasis. Vessels mediate blood-tissue exchange of oxygen, nutrients and waste products throughout the organism. This extensive vascular network is lined throughout with endothelial cells. While much of the mature endothelium is quiescent, it should not be considered to be inert. Remodelling and extension of the vascular system occurs to maintain tissue oxygen tension and meet changing metabolic demands, and is achieved through angiogenesis.

Angiogenesis can be defined as the formation of new vessels from pre-existing vasculature. Angiogenesis occurs in growth and development and postnatally under pathological and non-pathological conditions. When appropriately challenged, vessel permeability increases, and the endothelium presents a thrombotic surface, thereby allowing local adherence, activation and extravasation of inflammatory cells. The endothelium therefore controls immune surveillance and the inflammatory response.

Non-pathological angiogenesis occurs during bone maintenance and remodelling in response to natural mechanical stresses, while the high metabolic demand of exercise induces vascular remodelling in both bone and muscle. Pathologically, neo-angiogenesis occurs following injury to preserve tissue structure and function and promote tissue repair. However, aberrant angiogenesis and endothelial dysfunction contribute to the pathology of many conditions. For example, tumours meet the increased metabolic demands of exceeding a certain size through induction of angiogenesis, which also facilitates metastases. Diabetic patients commonly develop diabetic retinopathy, where vision is impaired by increased angiogenesis in the retina. Conversely, in these patients wound healing is often impaired elsewhere due to decreased angiogenesis. Angiogenesis occurs in many diverse diseases including periodontal disease, rheumatoid arthritis, osteoporosis, psoriasis, inflammatory bowel disease, asthma, primary pulmonary hypertension, atherosclerosis and macular degeneration of the eye (Carmeliet, 2003). The endothelium controls activation and extravasation of inflammatory cells, thus aberrant angiogenesis

and endothelial dysfunction are tightly linked to chronic inflammation. An understanding of control mechanisms in angiogenesis is essential in treating these conditions.

Angiogenesis is a complex process tightly controlled by a balance between pro- and anti-angiogenic factors derived from cells and the extracellular matrix (ECM). Cells regulate ECM synthesis, assembly and turnover. The structure and composition of ECM in turn influences and maintains cellular phenotype, thus ECM components play an essential role in neoangiogenesis. Cell-surface receptors for ECM include heterodimeric proteins called integrins. Integrin-mediated events are crucial to angiogenesis, and integrin expression alters throughout this process concomitant with matrix remodelling, allowing the cell to interact with, and respond to the changing environment. Integrins transduce signals from the ECM to intracellularly associated protein complexes involved in signalling pathways associated with angiogenesis, in adhesion, migration, tube formation, cell survival and proliferation (reviewed by (Davis and Senger, 2005; Ruegg et al., 2004; Stupack and Cheresh, 2003)).

Tissue-specific ECM composition occurs as a result of context-specific patterns of gene expression and post-translational modifications, fulfilling differential functional requirements. Extracellular matrix proteins contribute to structural integrity and confer differential tissue mechanical and biochemical properties. The matrix also provides a reservoir of growth factors. Moreover, matrix proteins themselves possess distinct signalling properties. During angiogenesis, the matrix microenvironment is continually altered and remodelled through *de novo* protein expression, deposition of plasma components and liberation of signalling protein fragments by protease activity. Signals from the extracellular matrix *via* integrins, and from growth factors to their receptors coordinate to control angiogenesis. Thus signals from the extracellular environment are crucial in control of this process.

Decorin is a multifunctional component of the extracellular matrix. This small leucine-rich repeat proteoglycan consists of a core protein and a single covalently attached glycosaminoglycan chain. Decorin is frequently associated with collagenous matrices (Bianco et al., 1990; Scott and Haigh, 1985; Scott and

Orford, 1981) and indeed regulates collagen fibrillogenesis both *in vitro* (Vogel et al., 1984) and *in vivo* (Danielson et al., 1997). However many studies strongly implicate an important role for decorin in control of inflammatory angiogenesis. Embryonic development is unaffected by the absence of decorin (Danielson et al., 1997). However, postnatal challenges requiring tissue repair and regeneration cannot be compensated, and neoangiogenesis in the cornea is reduced in the absence of decorin (Schönherr et al., 2004). Conversely, the absence of decorin results in enhanced neo-angiogenesis during dermal wound healing (Jarvelainen et al., 2006). Additionally, immunohistochemical studies reveal decorin expression in association with nascent blood vessels formed under inflammatory conditions *in vivo* (Nelimarkka et al., 2001; Schönherr et al., 1999). A role for decorin in angiogenesis is extended by *in vitro* studies. Quiescent endothelial cells do not express decorin, but expression is induced in cells spontaneously undergoing angiogenic morphogenesis (Jarvelainen et al., 1992), while the inflammatory mediators IL-6 and IL-10 induce endothelial decorin expression (Strazynski et al., 2004).

Decorin has also been shown to influence endothelial tube formation; however no clear pattern has emerged. Decorin can enhance endothelial tube formation (Schönherr et al., 1999), inhibit it (Davies et al., 2001; Sulochana et al., 2005) or have no influence (Grant et al., 2002). Endothelial tube formation is itself a complex process requiring extensive rearrangement of cell-matrix interactions. Modulation of cell adhesion and migration are therefore crucial in the control of this process. Indeed, decorin influences these events (for detailed discussion see Chapter 3). However many studies focus on decorin effects on fibroblasts rather than on endothelial cells. Additionally, a definitive mechanism for decorin influences on these integrin-mediated events does not exist. Decorin has been suggested to sterically interfere with integrin-matrix interactions and not to interact with matrix receptors themselves (Bidanset et al., 1992b; Ehnis et al., 1998; Merle et al., 1997; Winnemoller et al., 1991). However, decorin has been shown recently to signal directly through the growth factor receptor IGF-I in endothelial cells (Schönherr et al., 2005) while immobilised decorin supports platelet adhesion through interactions with, and signalling through the $\alpha_2\beta_1$

integrin (Guidetti et al., 2002). Signals from the extracellular matrix *via* integrins, and from growth factors to their receptors coordinate in control of angiogenesis. These more recent studies therefore indicate that the mechanism of decorin effects in angiogenesis could relate to its ability to signal directly through these cell-surface receptors. The role of decorin remains controversial, and the mechanisms unclear. Therefore re-evaluation of the mechanism of decorin effects on integrin-mediated events is warranted. Additionally, decorin effects specifically on endothelial cells have not been thoroughly investigated. This thesis aims to characterise and investigate the mechanisms of decorin effects on endothelial cells to elucidate the role of decorin in angiogenesis.

1.2 The Vascular System

1.2.1 Vessel Structure

The vertebrate vascular system distributes blood *via* an extensive connected network of branched vessels. The muscular arteries carry oxygenated blood under high pressure from the heart while veins transport de-oxygenated blood back to the heart. Larger vessels are composed of three layers, the *Tunica Intima* (endothelial lining and connective tissue), *Tunica Media* (smooth muscle) and *Tunica Adventitia* (outer connective tissue). The endothelium and smooth muscle are separated by the internal elastic lamina and the smooth muscle and outer connective tissue by the external elastic lamina (Figure 1.1). The largest arteries, e.g. the aorta, have the most distinct layers, and branch into smaller vessels called arterioles. Larger veins are characterised by the presence of valves, preventing backflow of blood, and similarly, these branch into venules. Arterioles and venules both branch into, and connect *via* the smallest, most extensive vessels; the capillary network or microvasculature. Capillaries consist of the *tunica intima*; endothelial cells surrounded by the basal lamina, interspersed with specialised supporting cells called pericytes. Enveloping capillaries is a thin layer of connective tissue, or *adventitia*, continuous with surrounding tissue (Figure 1.2A). The endothelium is one cell thick, allowing efficient blood-tissue exchange of nutrients, oxygen and waste products. Intercellular junctions between endothelial cells regulate endothelium permeability and integrity. There are three types of capillaries; continuous, where endothelium and basal lamina are continuous, conferring selective regulation of blood-tissue exchange e.g. in the blood-brain barrier; fenestrated, with continuous basal lamina, and regulated small openings (fenestrations) in the endothelium to allow controlled rapid passage of materials e.g. hormones in endocrine glands, or fluids e.g. in the kidneys (Figure 1.2B) and discontinuous; with highly fenestrated endothelium, and incomplete basal lamina. These large, irregular vessels allow free exchange of materials and cells in organs or tissues such as the liver, spleen and bone marrow.

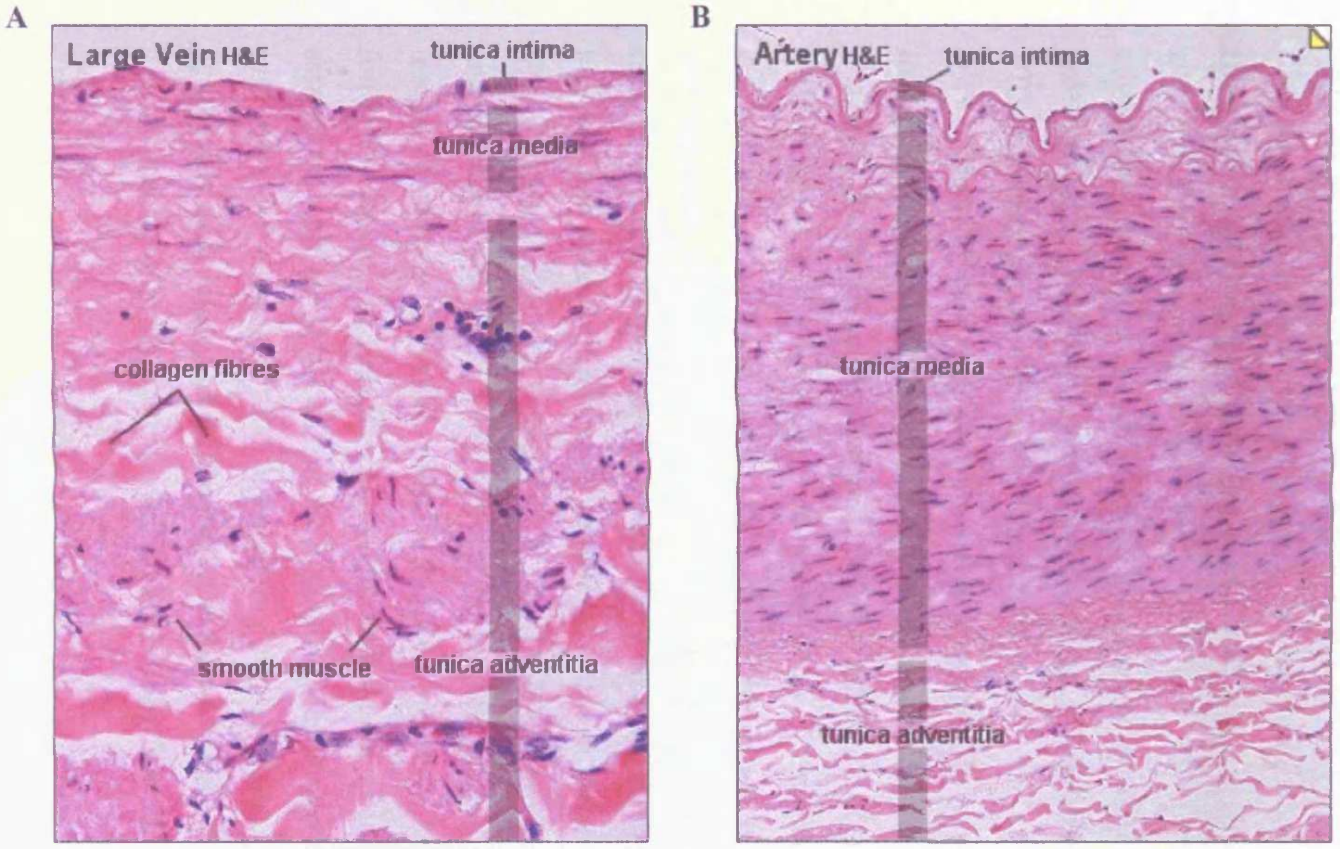


Figure 1.1: Hematoxylin and Eosin tissue staining of a cross-section through the lumen of a large vein (A) and a large artery (B). The major layers of these vessels are indicated. The tunica intima and tunica media are separated by the internal elastic lamina, and the outermost adventitia is continuous with the interstitial connective tissue (Professor J. McGeachie, http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Vascular/Vascular.htm).

The predominant vascular matrix components are the fibrillar collagen types I and III, which contribute to the structural integrity of the vessel, and are found throughout the vessel wall. The lamina (separating the major layers), are composed of elastic fibres, the major component of which is elastin, derived from the precursor, tropoelastin (Wight, 1996). Other minor collagens found in the vessel wall include collagen type V, which forms heterofibrils with type I (Birk et al., 1988), type VI, often associated with decorin (Bidanset et al., 1992a) and the FACIT collagen types XIV, XXI and XVIII (Wight, 1996). The adventitia particularly is rich in fibrillar collagens, while the intima is rich in proteoglycans, hyaluronan, and network forming collagens such as types IV and VIII (discussed in more detail in section 1.4.4).

Proteoglycans (see section 1.4.2) identified in vascular tissue matrix include those substituted with chondroitin sulphate e.g. versican (Yamagata et al., 1993), dermatan sulphate, such as decorin and biglycan (Bianco et al., 1990), keratan sulphate, e.g. lumican (Funderberg et al., 1991) and heparan sulphate e.g. perlecan (Couchman et al., 1993). While the intima is particularly rich in proteoglycans, they are also found in the media and adventitia, for example decorin is found in the latter in association with collagen types I and VI, while biglycan and versican/hyaluronan complexes occur in the media (Wight, 1996). The intima also contains cell-surface heparan sulphate proteoglycans; syndecans and glypicans (Wight, 1996). Glycoproteins (see section 1.4.3) are another matrix constituent of vessel walls, including fibronectin and thrombospondin, found throughout, and laminin, the major component of intima. Tenascin-X is associated with blood vessels in most tissues (Matsumoto et al., 1994), while expression of tenascin-C and osteopontin is more limited, and is associated particularly with injury and inflammation in the neointima; the latter particularly is often associated with areas of calcification (Giachelli et al., 1993).

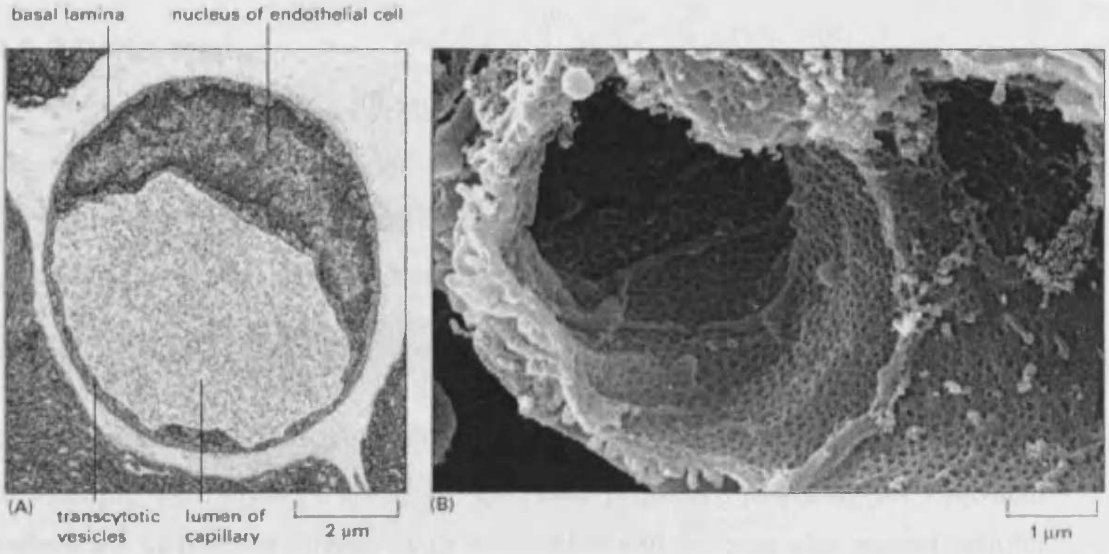


Figure 1.2: Cross-section of a capillary. An electron micrograph of a cross section of a small capillary in the pancreas illustrating a single endothelial cell surrounded by basal lamina (A). A scanning electron micrograph of the interior of a capillary in a glomerulus of the kidney is shown. Fenestrae are visible in the capillary wall (B). Part (A) is taken from (Bolender, 1974) and part (B) from (Gschmeissner, 2002).

1.2.2 Pericytes

Pericytes or mural cells are contractile cells associated with the abluminal surface of the endothelium (Figure 1.3). They become incorporated into the endothelial basement membrane, and extend numerous processes towards the vessel. Many of these processes are separated from the vessel by thin layers of basement membrane, while finer processes extend to interact directly with endothelial cells (Crocker et al., 1970). A single pericyte can extend processes to multiple endothelial cells (Orlidge and D'Amore, 1987), and to multiple capillaries (Weibel, 1974). These processes penetrate the basement membrane to interact directly with endothelial cells, which also extend cellular processes towards pericytes (Tilton et al., 1979a). Pericytes play a key role in control of vessel tone and maintenance of vascular integrity. Pericytes can originate from fibroblasts and further differentiate into true smooth muscle cells (Richardson et al., 1982), adipocytes (Rhodin and Fujita, 1989), osteoblasts (Diaz-Flores et al., 1992), and microglial cells (brain macrophages precursors) (Maxwell and Kruger, 1965). Pericytes can also be recruited by migrating and proliferating along existing vessels, specifically arterioles (Benjamin et al., 1998). In accordance with their contractile phenotype, pericytes regulate blood flow through modulation of vessel tone. Microvasculature endothelium compression and deformation correlates with points of contact between pericytes and capillaries in response to vasoactive agents (Tilton et al., 1979b) which can be secreted by endothelial cells.

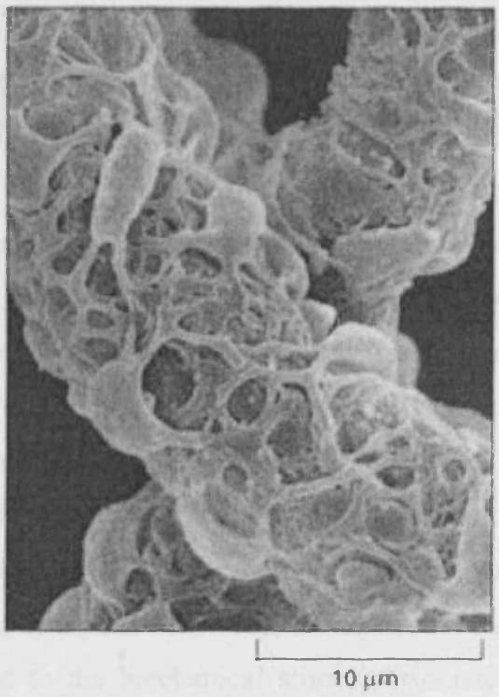


Figure 1.3: Scanning electron micrograph of a post-capillary venule of a cat mammary gland. Pericyte processes are wrapped around the blood vessel (Fujiwara and Uehara, 1984).

1.2.3 Vasculogenesis

Vasculogenesis is the *de novo* formation of the vasculature in the developing embryo. Haemangioblasts arise from the mesoderm, differentiating into both angioblasts (endothelial precursor cells) and haematopoietic cells (His, 1900; Sabin, 1920). The latter become surrounded by migrating angioblasts, forming blood islands (Gonzalez-Crussi, 1971; Kessel and Fabian, 1985). These differentiate into tube-like structures and fuse to form a primitive cardiovascular system (Evans, 1909; Hirakow and Hiruma, 1983; Sabin, 1920). This basic vasculature is remodelled and extended through angiogenesis.

The developing vascular system is also extensively remodelled through pruning, or vessel regression, where endothelial cells are removed by apoptosis. This occurs primarily during angiogenesis since proliferating endothelial cells are more susceptible to apoptosis and require sustained survival signals prior to vessel maturation (Brooks et al., 1994b). Pruning can also occur under conditions of hyperoxia, i.e. where oxygen supply exceeds demand (Ashton, 1966) and in immature, non-perfused vessels. Once circulation commences, the endothelium is exposed to the mechanical stimuli associated with blood flow, resulting in further modification of the basement membrane and pericyte recruitment and differentiation, which is implicated in vessel maturation (see section 1.5.3). For successful maturation, endothelial cells must exit the cell cycle, resulting in endothelium independence from sustained survival signals.

1.2.3.1 Primary Factors Involved in Vasculogenesis

Vasculogenesis occurs through coordinated signalling of a vast number of factors. Some key factors have emerged; VEGF (vascular endothelial growth factor), Tie receptors and ligands and ephrin receptors and their eph ligands. Disruption of a single allele of VEGF results in defects in blood island formation and decreased vascular complexity, i.e. angiogenesis, leading to embryonic lethality at E11 to E12 (Ferrara et al., 1996). Homozygous deletion of either of the VEGF receptors Flt-1 and Flk-1 results in early vascular defects, leading to embryonic lethality between E8.5 and E9.5 (Fong et al., 1995; Shalaby et al., 1995).

Endothelial cells express the receptors Tie-1 and Tie-2, each of which has distinct functions. The former is implicated in the maintenance of blood vessel integrity. Knockout mice die immediately after birth, exhibiting oedema and haemorrhaging. Tie-2 knockout mice die at E10.5, with reduced angiogenesis and a deficient vascular network (Sato et al., 1995). Mice lacking the Tie-2 ligand angiopoietin-1 die by E12.5 with reduced vascular complexity, and defects in pericyte recruitment (Suri et al., 1996).

Eph receptors are receptor tyrosine kinases which autophosphorylate upon ligand binding, known as 'forward signalling'. Their ligands, ephrins, are membrane bound rather than soluble, and are also capable of transmitting signals intracellularly upon binding to their receptors, referred to as 'reverse signalling'. Thus these ligands and receptors signal bidirectionally, mediating cell-cell communication, and controlling establishment and maintenance of boundaries. The endothelium does not comprise a homogenous population of endothelial cells. Endothelial cells differ in their expression of the Eph receptor and ephrin families, with ephrinB2 and its receptor EphB4 now recognised as being specific markers for arterial and venous endothelial cells respectively. Furthermore, the boundary between ephrinB2 positive and EphB4 positive cells correlates with the boundary between arteries and veins in capillaries. Reciprocal signalling between the two is essential for arterial-venous specification and remodelling of the capillary network during vasculogenesis (Wang et al., 1998).

1.2.4 Angiogenesis

The vasculature is extended and remodelled through angiogenesis. Once the vasculature is established, endothelial cells exit the cell cycle and the endothelium becomes quiescent. Nascent blood vessels (neo-angiogenesis) form from the existing vasculature only in response to specific stimuli. Under most circumstances this occurs in the microvasculature, although remodelling of arterial vessels (arteriogenesis) can occur postnatally.

Postnatal angiogenesis is critical for maintenance of tissue structure and function. A prime example can be considered in bone remodelling, which occurs in response to hormones, natural mechanical stresses, and in fracture repair. Net loss or gain of bone mass occurs when bone resorption (by osteoclasts) is enhanced, and/or deposition (by osteoblasts) is inhibited or *vice versa*. Vascular supply is coupled to bone remodelling. The pro-angiogenic factor VEGF is required for increased bone mass in response to exercise (Yao et al., 2004). Conversely, during inflammation, cytokines upregulate endothelial cell RANKL (receptor activator of NFkB ligand), which activates osteoclasts. Hence, inflamed blood vessels attract and activate osteoclasts, resulting in net bone loss (Collin-Osdoby et al., 2001). Cartilage is a non-vascular tissue, but becomes vascularised during endochondral bone formation. Proximal vessels invade cartilage in response to hypertrophic chondrocyte derived VEGF, facilitating resorption of cartilage and replacement with bone (ossification) (Gerber et al., 1999), while aberrant vessel invasion of cartilage occurs in the inflammatory condition rheumatoid arthritis (Autiero et al., 2003). Additionally to bone, exercise induces vascular remodelling in muscle. Angiogenesis is induced by increased shear stresses associated with increased blood pressure and by mechanical stretching of endothelium. Further, active muscle is hypoxic and hypoglycaemic; states which induce pro-angiogenic factors. Indeed, exercise-induced angiogenesis is dependent on VEGF, a hypoxic and hypoglycaemic response gene (Amaral et al., 2001). Sustained exercise is suggested to induce sequential remodelling of capillaries (increased capillarity), arterioles and larger vessels, resulting in increased large vessel diameter and blood flow capacity

(White et al., 1998). The metabolic demands of active tissue are thus met by enlarging the available surface area for blood-tissue exchange.

Angiogenesis can occur through non-sprouting (intussusception) and sprouting mechanisms. In intussusception, endothelial cells of existing vessels proliferate into the lumen, along with pillars of matrix. A single capillary splits into two without interrupting blood flow (Patan et al., 1996a). This mechanism of angiogenesis is particularly associated with pre-natal lung and heart development, but has been observed post-natally during tumour neoangiogenesis (Patan et al., 1996b) and during mechanically induced neo-angiogenesis in muscle. Shear stresses induce intussusception, while stretching is associated with abluminal sprouting mechanisms (Egginton et al., 2001) suggesting that these angiogenic mechanisms are differentially controlled. Additionally, there is evidence to suggest that the endothelial cells of new blood vessels do not arise only from proliferation of cells of pre-existing vessels, but also from incorporation of circulating endothelial precursor cells. CD34 positive cells circulating in adult blood can differentiate into endothelial cells (Asahara et al., 1997), and become incorporated into sites of physiological and pathological neoangiogenesis (Asahara et al., 1999).

Sprouting angiogenesis requires a more complex series of events, including extensive endothelial proliferation, and extracellular matrix degradation and remodelling, to allow abluminal sprouting and vessel extension. Indeed, the proteases matrix metalloproteinase-2 (MMP2) and membrane type 1-MMP (MT1-MMP) are associated only with sprouting angiogenesis (Rivilis et al., 2002) since this process requires ECM degradation whereas intussusception occurs without disruption of the basement membrane. In sprouting angiogenesis, quiescent endothelial cells are stimulated by pro-angiogenic factors to re-enter the cell cycle and proliferate, forming an abluminal sprout that migrates towards the angiogenic stimulus. Migration is aided by upregulation of proteinases that digest the extracellular matrix surrounding the cells. A provisional matrix is deposited from serum components, by *de novo* protein synthesis, and derived from matrix degradation products. A shift in the balance towards anti-angiogenic factors results in inhibition of angiogenesis. Endothelial cells exit the cell cycle,

and nascent vessels form a lumen. Pericytes are recruited, aiding in blood vessel stabilisation and maturation. New capillaries connect to the capillary network, forming loops, and supporting blood flow (known as inosculation or anastomosis). The quiescent matrix is deposited and re-established by the endothelium and adjacent cells.

1.4 Major ECM Components of Connective Tissues

Cells regulate ECM synthesis, assembly and turnover. Matrix assembly can occur spontaneously; some ECM proteins possess the ability to self-assemble into fibrils and networks. Others require, or are influenced by, interactions with cell-surface receptors (integrins). The structure and composition of the ECM in turn influences and maintains cellular phenotype, and ECM composition varies as a result of tissue-specific gene expression. The matrix also provides a reservoir of growth factors, and modulates the cell response to these factors. ECM proteins include collagens, hyaluronan, elastin, laminins, fibronectin, vitronectin, matricellular proteins and proteoglycans. The collagens are the most abundant family and are a major structural element of all connective and interstitial tissues. However, these and other matrix proteins are not simply structural components, but possess distinct signalling properties.

1.4.1 Collagens

Members of the collagen family are characterized by the presence of three polypeptide α chains associated in a triple helical structure (pro- or tropo-collagen). Each chain contains a gly-x-y repeating sequence, where X and Y are frequently proline and hydroxy-proline. Glycine residues allow close packing of chains, which are stabilised by hydrogen bonds between hydroxylated groups. Polypeptide chains are synthesized as precursor pro-peptides with proteolytically cleavable N- and C-terminal globular domains, removed extracellularly to yield collagen monomers consisting of a triple helical domain and short non-helical 'telopeptide' sequences at each terminus. Lysine residues in non-helical regions are involved in cross-links to other collagens or matrix components, stabilising the networks of the extracellular matrix. Collagens contain variable proportions

of collagenous regions, and non-collagenous domains contribute to differential structures, functions and associations of the various collagens.

The collagen family can be divided into fibrillar and non-fibrillar, the latter of which is further divided into various sub-groups summarised in Table I. Fibrillar collagens self-assemble into fibrillar structures which pack laterally in a staggered manner, resulting in a characteristic banding pattern (gap region, d-band or D-period) (Hulmes et al., 1981), occurring between the end of one molecule and the beginning of the next. Of the fibrillar collagens, type I is a principal component of interstitial matrix, while types V and XI may function as a core structure around which other collagens polymerise. Indeed, type V forms heterofibrils with collagens I and III, while collagen XI forms the core of collagen II heterofibrils (Birk et al., 1988; Petit et al., 1993). Mature collagen fibrils are thus composed of several collagen types.

FACIT (fibril associated collagens with interrupted triple helices) collagens consist of triple helical domains interrupted by short non-helical sequences. They cannot form fibrils alone, but interact with fibrillar collagens or project their N-termini into the matrix. FACIT collagens may serve to link fibrils or modulate interactions with other matrix components. Some members can be substituted with glycosaminoglycan chains (Watt et al., 1992; Yada et al., 1990). Other non-fibrillar collagens form networks, such as type IV, a primary component of basal lamina. Collagen VI forms microfibrils that contribute to a network of fine beaded filaments associated with other collagen fibrils (von der Mark et al., 1984), while collagens VIII and X form hexagonal networks.

Many collagens are capable of self-assembly, however non-collagenous matrix components participate in regulating formation of these structures. For example, the interstitial proteoglycan decorin plays a major role in collagen fibrillogenesis (Danielson et al., 1997), resulting in more ordered and regular fibrils. Decorin also interacts with non-fibrillar collagens including types VI (Bidanset et al., 1992a) and XIV (Font et al., 1993) and further, may bridge collagen types I and VI (Nareyeck et al., 2004). Decorin therefore may play an important role in regulating overall matrix organisation and stabilisation.

Sub-Group	Types	Localisation	Properties
Fibrillar	I, II, III, V, XI, XXIV, XXVII	Widespread; in dermis, bone, ligament, tendon, cornea, cartilage, blood vessels, intestine, lung, liver, spleen	I: main constituent of connective tissue, highly abundant. II: main fibrillar collagen of cartilage. III: abundant in extensible tissues, often associated with type I. V: widespread, often associated with type I. XI: often associated with type II.
FACIT	IX, XII, XIV, XV, XVI, XX, XXI, XVII, XVIII	IX: Cartilage, vitreous body XII: Ligament, tendon XIV: Skin, tendon, blood vessels, lungs, liver XV: fibroblasts, smooth muscle cells XVI: expressed by fibroblasts, keratinocytes XX: Tendon, cartilage corneal epithelium XXI: blood vessels XVII: Dermal-epidermal junction XVIII: vascular tissue, lungs, liver	IX: can contain a GAG, associated with type II. XII: associated with collagen type I XIV: fibril associated XVII: potential trans-membrane domain
Network-forming collagens	IV, VIII, X	IV: basement membranes VIII: Associated with endothelium X: hypertrophic, mineralising cartilage	IV: forms 2D networks. VIII: forms hexagonal lattices, short-chain length X: a short-chain collagen. Similar structure to type VIII
Transmembrane	XIII, XVII, XXIII, XXV	XIII : widespread in low quantity.	XIII: complex alternative splicing pattern
Multiplexins	XV, XVI, XVIII	Associated with basement membranes	proteins with multiple triple helix domains and interruptions
Others	VII, VI, XIX	VI: Widespread; dermis, cartilage, blood vessels, lungs. VII: Skin, oral mucosa, cervix XIX: expressed in small amounts by fibroblasts, tumour cells	VII: anchoring fibrils between basement membrane and dermis VI: forms periodic beaded structures XIX: Five triple-helical domains

Table I: Summary of the sub-groups of the collagen family, their distributions and properties. Adapted from Chapter 2, (Comper, 1996) and (Gelse et al., 2003).

1.4.2 Proteoglycans

Proteoglycans can be defined as molecules consisting of a protein core, covalently linked to one or more glycosaminoglycan chains (GAGs). GAGs are complex carbohydrates varying in length and composition, both between different proteoglycans and within the same proteoglycan. Any one proteoglycan therefore is capable of presenting alternative structures and properties in a context-dependent manner. Proteoglycans can be associated with the cell surface or the extracellular matrix. Their role in the matrix was originally considered to be in control of solute diffusion by virtue of the polyanionic GAGs, thereby maintaining tissue turgour, providing elasticity and mechanical support and influencing tissue permeability. However it is now recognised that proteoglycans are capable of direct and indirect influences on cell behaviour as well as participating in tissue structure, organisation and function.

It should be noted that to be strictly classed as a proteoglycan, the protein core should be covalently linked to a GAG. However, some members of the small leucine-rich repeat proteoglycan (SLRP) family for example, are in fact glycoproteins e.g. chondroadherin and opticin, rather than proteoglycans. By virtue of the similarities of the core proteins to other members of this family however, these glycoproteins are classed as SLRPs. Indeed, even the classical SLRP member decorin, can occur as a 'part-time' proteoglycan; the core protein moiety has been found without a GAG *in vivo* in human skin (Fleischmajer et al., 1991) and in porcine cartilage (Sampaio et al., 1988).

1.4.2.1 Proteoglycan Families

Proteoglycan families are grouped according to common core protein domains. Lecticans, heparan sulphate proteoglycans, small leucine-rich repeat proteoglycans (SLRPs) and 'part-time' proteoglycans are all proteoglycan families. The lecticans (also referred to as large, aggregating proteoglycans or hyalectins) consist of N- (G1) and C-terminal (G3) globular domains in between which is a variable domain containing GAG attachment sites. Lecticans interact with the matrix component hyaluronan *via* the N-terminal domain, and other

matrix proteins *via* a lectin-like domain within the C-terminus. This family includes aggrecan, brevican, neurocan and versican. The GAGs are principally chondroitin sulphate, or to a lesser extent, keratan sulphate.

A second proteoglycan family, the heparan sulphate proteoglycans can be membrane associated or located in the extracellular matrix. Examples of the latter include perlecan and agrin, while syndecans and glypicans are membrane-bound. These proteoglycans influence cell behaviour indirectly by binding growth factors e.g. bFGF, *via* their heparan sulphate moiety (Klagsbrun and Baird, 1991), functioning as co-factors by interactions with both the growth factor and its receptor (Kan et al., 1993). 'Part-time' proteoglycans, as the name implies, can exist either as the core protein, or may possess one or more GAG. This family includes CD44, CSF (colony stimulating factor), and some collagens. The SLRPs family will be discussed in detail in section 1.4.2.3.

1.4.2.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are unbranched polysaccharides composed of repeating disaccharide units. One monosaccharide is usually an uronic acid (glucuronate or iduronate), and the other a hexosamine (N-acetylgalactosamine or N-acetylglucosamine). These sugars are further modified by N-acetylation and N- and O-sulphation, the latter of which contributes to the polyanionic nature of glycosaminoglycans. There are several broad classifications of GAGs: chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparin (Hep), heparan sulphate (HS) and hyaluronan (HA). Glycosaminoglycan composition and modifications are summarised in Figure 1.4 and Table II.

Chondroitin sulphate consists of alternating glucuronate and N-acetylgalactosamine residues, which can be sulphated at positions 4 or 6 of the latter residue (termed chondroitin 4- or 6-sulphate, or chondroitin C and A respectively). Dermatan sulphate (chondroitin B) is an isomer of chondroitin sulphate. Chondroitin sulphate is post-translationally modified by epimerisation of the carboxyl group at C5 of the uronic acid to iduronic acid. Any GAG containing iduronic acid is classed as DS however the extent of epimerisation varies considerably. Thus DS represents a range of species containing regions of

both glucuronic and iduronic acid. Sulphation can occur at position 2 of the uronic acid, although this is more common in DS than CS (Hardingham and Fosang, 1992). HS has more N-acetyl groups and fewer N-sulphate and O-sulphate groups than heparin and is therefore less charged. The glucuronic acid of both heparin and HS can also be modified to iduronic acid by epimerisation, occurring to a greater extent in the former (Schönherr and Hausser, 2000). KS is the only GAG to not contain an uronic acid, consisting of galactose and N-acetylglucosamine. Monosaccharides can also be added to KS (NeuAc and Fuc).

GAGs are covalently linked to the protein core through a tetrasaccharide attachment unit, xylose-galactose-galactose-glucuronic acid. Xylose is linked to the core protein at a serine or threonine residue, and the final galactose to the first uronic acid of the GAG chain. CS, DS, heparin and HS are linked in this manner to serine, while KS can be linked *via* an O-glycosidic bond to serine or threonine, or alternatively *via* an N-glycosyl bond to asparagine.

Unlike the other GAGs, hyaluronan (or hyaluronate or hyaluronic acid) is not sulphated nor is it linked to a protein core. Hyaluronan (HA) is an unbranched linear polysaccharide of varying lengths consisting of repeating glucuronic acid - N-acetylglucosamine disaccharides. HA is synthesised on the cell surface by integral membrane proteins, hyaluronan synthases 1, 2 and 3 (Weigel et al., 1997). HA can serve as a backbone for large proteoglycan complexes and has a wide tissue distribution, including association with blood vessel connective tissue (Edelstam et al., 1991) and interstitial tissues. Isolated HA forms a hydrated gel, conferring viscoelastic and lubricant properties; it is abundant in cartilage and synovial fluid for example, and contributes to tissue and joint resistance to compressive forces (Fraser et al., 1997). HA also retards diffusion, playing an important role in osmotic regulation (Tammi et al., 2002). Further, HA can signal through two cell-surface receptors, receptor for hyaluronan mediated motility (RHAMM) (Hall and Turley, 1995) and CD44 (Lesley et al., 1993) to modulate cell responses in metastases, inflammation and wound healing. HA is anti-angiogenic while HA degradation products (generated by hyaluronidases) have pro-angiogenic capacity, and participate in regulating inflammatory responses (reviewed in (Slevin et al., 2007)).

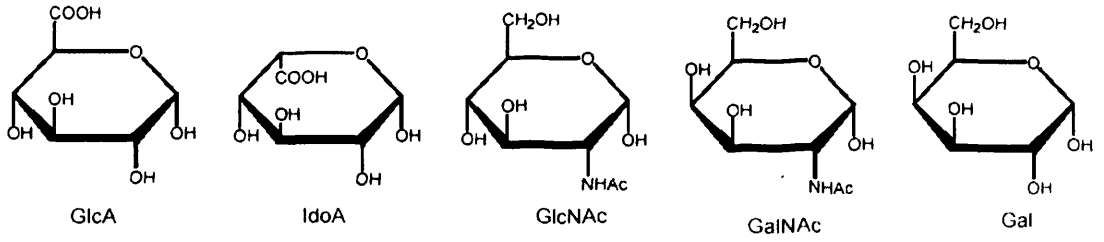


Figure 1.4: Monosaccharides occurring in glycosaminoglycan chains. One monosaccharide is an uronic acid (Glucuronic acid – GlcA or iduronic acid – IdoA), the exception being KS, which contains galactose – Gal. The second monosaccharide is a hexosamine (either GalNAc - N-acetylgalactosamine or GlcNAc - N-acetylglucosamine).

Glycosaminoglycan	Disaccharide	Further Modifications
Chondroitin Sulphate	GlcA β(1-4) – GalNAc β(1-3)	GlcA to IdoA (DS), Sulphated at C4, C6 or both of GalNAc
Dermatan Sulphate	IdoA β(1-4) – GalNAc β(1-3)	Sulphation on C2 of IdoA
Heparin	GlcA β(1-4) – GlcNAc α(1-4)	GlcA to IdoA. Sulphation on C2 of uronic acid, C3 and C6 of GlcNAc, acetyl groups of GlcNAc can be sulphated
Heparan Sulphate	GlcA β(1-4) – GlcNAc α(1-4)	GlcA to IdoA. Sulphation on C2 of uronic acid, C3 and C6 of GlcNAc, acetyl groups of GlcNAc can be sulphated
Keratan Sulphate	Gal β(1-3) – GlcNAc β(1-4)	C6 of GlcNAc always sulphated, Gal sometimes sulphated. Either monosaccharide can be modified by sialyation, fucosylation
Hyaluronan	GlcA β(1-4) – GlcNAc β(1-3)	None

Table II: Summary of glycosaminoglycan disaccharide components and potential modifications. Monomers can be further modified by N-acetylation and sulphation as indicated. KEY: GlcA - Glucuronic acid, IdoA - Iduronic acid, Gal – Galactose, GalNAc - N-acetylgalactosamine, GlcNAc - N-acetylglucosamine. Adapted from Chapter 2, (Comper, 1996) and (Schönherr and Hausser, 2000).

1.4.2.3 Small Leucine-rich Repeat Proteoglycans

The leucine-rich repeat (LRR) superfamily encompasses many diverse protein families, containing one or more leucine-rich repeat regions. Leucine-rich repeats vary in length from 20-29 residues long, with 24 being the most common. An eleven-residue segment, $LxxLxLxx^{N}/_{C}xL$ (where consensus leucines can be substituted with isoleucine, valine or other hydrophobic amino acids) is highly conserved while the remainder of the repeat is more variable. Each LRR consists of a β-strand parallel to an α-helix, tandem repeats of which result in a curved structure, where the outer surface presents α-helices and the inner surface consists of parallel β-pleated sheets. These regions function in protein-protein interactions in a wide range of systems, from ribonuclease inhibitor to neurotrophin receptors (Kajava, 1998; Kobe and Deisenhofer, 1994).

A division of the leucine-rich repeat super-family are the small leucine-rich repeat proteoglycans, or SLRPs. These extracellular matrix associated proteins contain multiple highly conserved leucine-rich repeat regions that, uniquely to this family, are flanked by cysteine rich regions involved in disulphide bond formation. The 24 amino acid LRR consensus sequence common to SLRPs is xx I/V/L xxxx F/P/L xx L/P xx L xx L/I x L xx N x I (where x is any amino acid) (Hocking et al., 1998). To date, there are at least 11 SLRP family members, classified as such by virtue of their similarities in structure and sequence to more established members. Each member has its own unique properties and functions, which may be at least partially dependent on temporal expression and localisation. The SLRPS can be sub-divided into classes, designated by phylogenetic analysis, sequence alignment and by the nature of the N-terminal cysteine-rich regions (summarised in table III) (Hocking et al., 1998). There are 3 distinct classes with chondroadherin and nyctalopin possibly representing a fourth. Podocan may represent an additional fifth class (Ross et al., 2003), although podocan, chondroadherin and nyctalopin have all been suggested not to be true proteoglycans since podocan has no C-terminal cysteines, while the others do not have a C-terminal 'ear' repeat common to all other SLRPs (McEwan et al., 2006).

Class	Cysteine Clusters	Proteoglycans
I	Cx_3CxCx_6C	Decorin, Biglycan, Asporin
II	Cx_3CxCx_9C	Lumican, Osteoadherin/Osteomodulin, Fibromodulin, Keratocan, PRELP
III	Cx_2CxCx_6C	PG-Lb/Epiphycan, Opticin/Oculoglycan, Mimecan/Osteoglycin/Osteoinductive factor
IV	Cx_3CxCx_8C	Chondroadherin, Nyctalopin
V	Cx_3CxCx_7C	Podocan (Ross et al., 2003)

Table III: The table shows the proteoglycan classes I to V and the cysteine clusters common to each class. Adapted from (Hocking et al., 1998; McEwan et al., 2006). Additional information to these references is indicated in the table.

1.4.2.4 Class I SLRPs

The ten leucine-rich repeat regions of decorin were recognised as being homologous motifs to numerous other apparently unrelated proteins (Krantz et al., 1991; Patthy, 1987); the LRR superfamily. Decorin and biglycan are different gene products; located on chromosomes 12q23 (Danielson et al., 1993) and Xq27-ter (Fisher et al., 1991) respectively. Despite this, they have 55% homology (Fisher et al., 1989). The protein cores of decorin and biglycan consist of 10 leucine-rich repeats and two or three consensus sites for N-linked oligosaccharides respectively. Decorin has a single CS or DS chain attachment site near the N-terminus, while biglycan has two such sites. The different moieties of decorin are responsible for differential interactions and functions. The DS glycosaminoglycan of decorin transiently increases in size during dermal wound healing, and is associated with altered collagen fibril packing (Kuwaba et al., 2002; Kuwaba et al., 2001). Furthermore, decorin core protein mediates binding of collagen type VI to collagen fibrils (Bidanset et al., 1992a), while the GAG moiety interacts with collagen type XIV (Font et al., 1993). In mineralisation, GAG composition is altered, whereby iduronic acid content is reduced from pre-mineralised to mineralised tissue respectively, thus the latter contains CS (Waddington et al., 2003), and DS-bearing decorin has been described as inhibiting calcification of soft tissues (Scott and Haigh, 1985). Thus decorin located in skin is primarily DS-bearing (Choi et al., 1989), in bone is CS-bearing (Franzen and Heinegard, 1984), while in cartilage, both forms of decorin exist (Choi et al., 1989). Of all the SLRPs, decorin has been particularly well studied and plays a major role in extracellular matrix structure and organisation. Decorin regulates collagen fibrillogenesis and additionally modulates fibronectin network formation (Kinsella et al., 2000). Additionally, fibronectin fibrillogenesis is dependent on collagen fibrillogenesis (Dzamba et al., 1993), and decorin associates with fibronectin fibrils at the cell surface (Schmidt et al., 1987). Consistent with a role in collagenous matrix organisation, decorin *in vivo* is associated with the connective tissue of dermis, cornea and cartilage, in particular, it is associated with collagen fibrils in these tissues

(Bianco et al., 1990; Poole et al., 1986; Volker et al., 1990; Voss et al., 1986). Decorin is also constitutively associated with larger blood vessels *in vivo*, in connective tissue of the *adventitia* and with smooth muscle-rich areas (Gutierrez et al., 1997a; Lin et al., 1996; Ungefroren et al., 1995). Further to its role in matrix organisation, decorin also directly influences cellular behaviour. Decorin signals through growth factor receptors such as the IGF-IR (Schönherr et al., 2005) and EGFR (Iozzo et al., 1999b) and through the α2β1 integrin on platelets (Guidetti et al., 2002). Decorin also influences TGF-β signalling through sequestration in the collagenous matrix or by influencing interactions with its receptors (Schönherr and Hausser, 2000).

The decorin knockout mouse phenotype confirms a relevant role for decorin in collagen fibril organisation, displaying loose and fragile skin attributed to irregular collagen fibril diameters (Danielson et al., 1997). Dysregulated collagen fibrillogenesis in the absence of decorin also results in defects in lung (Fust et al., 2005) and tendon function (Zhang et al., 2006). The knockout mouse also reveals a role for decorin in maintenance of tissue structure and function. In oral tissues, periodontal ligament organisation is adversely affected by enhanced fibroblast proliferation (Häkkinen et al., 2000), and angiogenesis in cornea and the dermis is dysregulated through unknown mechanisms (Jarvelainen et al., 2006; Schönherr et al., 2004).

The other class I member, biglycan, is constitutively expressed by cells such as epithelial and endothelial cells, and in contrast to decorin, in non-remodelling tissue, is located pericellularly (Bianco et al., 1990; Lin et al., 1996; Ungefroren et al., 1995). Decorin and biglycan expression is also differentially regulated; in fibroblasts FGF-2 decreases biglycan expression, but increases decorin expression (Tan et al., 1993), while endothelial cells in culture constitutively synthesise biglycan but not decorin (Jarvelainen et al., 1991). Despite many similarities in binding affinities, these proteoglycans consistently play differential roles. For example, both interact with TGF-β and fibrillar collagens, however only decorin appears to fulfil a function in modulation of TGF-β signalling and collagen fibrillogenesis. This may be dependent on different binding interactions. Decorin has been suggested to interact with

collagen *via* the core protein, while biglycan interacts through the GAG chain (Pogany et al., 1994). In contrast, biglycan has been shown to interact with collagen type I *via* the core protein with similar affinity to decorin, although biglycan-collagen interactions may be less stable (Schönherr et al., 1995). Different effects of decorin and biglycan likely also depend on differential temporal expression and localisation of these two proteoglycans.

Interestingly, the decorin-biglycan double knockout mouse illustrates an element of compensation, since the phenotype is more severe than either alone (Corsi et al., 2002). However, each of the separate knockout mice display features which cannot be compensated for. The biglycan knockout mouse has been less extensively studied however the phenotype illustrates a key role for this proteoglycan in regulating the structure and function of mineralised tissues (Xu et al., 1998).

A third member of this class, asporin, was recently identified. Asporin exists as a glycoprotein and was found most abundantly in adult human osteoarthritic articular cartilage, aorta, uterus, heart, and liver (Lorenzo et al., 2001) and in the skeleton of the developing mouse embryo (Henry et al., 2001).

1.4.2.5 Class II SLRPs

Fibromodulin has no classical GAG attachment site but instead contains four asparagine residues within the leucine-rich repeat (LRR) region for N-linked oligosaccharides, substituted with keratan sulphate. Towards the N-terminus there is also a sulphated tyrosine-rich region (Oldberg et al., 1989; Plaas et al., 1990). Fibromodulin is found in cartilage, tendon and sclera, in low amounts in skin and bone (Heinegard et al., 1986), and interacts with fibrillar collagen at sites distinct to that of decorin (Hedbom and Heinegard, 1993).

Analysis of the keratan sulfate-containing proteoglycans of bovine corneal stroma revealed three unique core proteins designated 37A, 37B, and 25 with approximately conserved 35% amino acid identity (Funderburgh et al., 1991b). In Lumican, protein 37B, two or three of five potential N-linked attachment sites are substituted with KS (Blochberger et al., 1992; Funderburgh et al., 1993). Lumican is strongly expressed in cornea (Blochberger et al., 1992)

and has been also found in intestine and placenta. In cartilage (Grover et al., 1995) and arteries (Funderburgh et al., 1991a) lumican exists predominantly as a glycoprotein. Lumican also interacts with fibrillar collagen (Rada et al., 1993), and abnormal collagen fibrils in the lumican (Chakravarti et al., 1998) and fibromodulin (Svensson et al., 1999) knockout mice also suggest a role for these proteoglycans in regulation of collagen fibrillogenesis. Keratocan, protein 37A, is found abundantly in cornea and sclera, and in smaller amounts in skin, ligament, cartilage, artery, and striated muscles (Corpuz et al., 1996). Keratocan knockout mice also display alterations in collagen fibrils in the cornea (Liu et al., 2003). The remaining members of class II SLRPs are osteoadherin and PRELP. Osteoadherin is a keratin sulphate substituted SLRP found in bone (Sommarin et al., 1998) while PRELP is found in cartilage (Bengtsson et al., 1995).

1.4.2.6 Class III SLRPs

Of the corneal keratan sulphate proteoglycans, protein 25 was designated mimecan. In a truncated form, mimecan is known as osteoglycin and is expressed in bone; mimecan and osteoglycin originate from the same gene product (Funderburgh et al., 1997), and the mimecan/osteoglycin knockout mouse also displays abnormalities in collagen fibrillogenesis (Tasheva et al., 2002). PG-Lb/Epiphycan/DSPG-3 is substituted with dermatan sulphate and at least one N-linked oligosaccharide in chick embryo (Shinomura and Kimata, 1992) and bovine epiphyseal cartilage, where it was also substituted with at least 3 N-linked oligosaccarides (Johnson et al., 1997). Opticin/oculoglycan is a glycoprotein of the eye (Friedman et al., 2000; Hobby P et al., 2000; Reardon et al., 2000) and was found associated with collagen fibrils in the eye. It is also found in ligament and skin (Reardon et al., 2000).

1.4.2.7 Class IV SLRPs

Two proteins have been described as potential members of this class; chondroadherin (Sommarin et al., 1998) and nyctalopin (Bech-Hansen et al., 2000) although both have been recently suggested not to be SLRPs (McEwan et al., 2006). Chondroadherin is a glycoprotein most strongly expressed in mouse

brain, eye, skeletal muscle, heart, lung, cartilage and embryo. Detailed analysis found that in most tissues it was associated with either peripheral nerves and/or blood vessels (Tasheva et al., 2004). Nyctalopin is found in the retina and kidney and mutations are linked to X-linked complete congenital stationary night blindness. It is most closely related to chondroadherin (Bech-Hansen et al., 2000; Pusch et al., 2000). Another potential member of this class is a glycoprotein called tsukushi which is most closely related to chondroadherin, and inhibits bone morphogenic protein (BMP) signalling in chick gastrulation (Ohta et al., 2004).

1.4.2.8 Other Potential SLRPS

The LRR region of extracellular matrix protein 2 (ECM2), a protein found in adipose tissue and female reproductive organs, is 34% identical to the corresponding domain of decorin, however ECM2 is much larger and structurally different from classic SLRPs (Nishiu et al., 1998). Periodontal ligament-associated protein-1 (PLAP-1) is a glycoprotein found in human periodontal ligament, which is suggested to be another class I member, since it has 51% homology to decorin and 48% to biglycan. However it has an N-terminal acidic stretch of aspartic acid residues which are not found in other class I members (Yamada et al., 2001) although the class II member osteoadherin has a C-terminal acidic domain (Sommarin et al., 1998). Synleurin represents another LRR protein which has closest homology to chondroadherin (Wang et al., 2003).

1.4.3 Glycoprotein Components

1.4.3.1 Fibronectin

Fibronectins exist as soluble glycoprotein dimers (Skorstengaard et al., 1986) in body fluids e.g. blood plasma and joint fluids, or as insoluble fibrillar networks in the ECM. Fibronectin mediates cell attachment, migration and differentiation. Accordingly, the knockout mouse displays a lethal phenotype, with severe vascular defects attributed to abnormal cell migration and behaviour (George et al., 1993). Fibronectin is abundant in ECM of mesenchymal tissues, particularly loose connective tissue (Stenman and Vaheri, 1978), and is a component of large vessel walls and stromal connective tissues of various organs. Fibronectin is frequently associated with collagen fibrils (Vaheri et al., 1978) and contains binding sites for fibrin, native and denatured collagens, heparin and heparan sulphate. The heparin and heparan sulphate binding region also contains a cell binding domain and a cryptic fibronectin binding site, both of which are required for fibronectin fibrillogenesis. Mesenchymal cells secrete and assemble fibronectin into fibrils pericellularly; interactions with cell-surface integrins and conformational changes in fibronectin expose cryptic fibronectin-binding sites. The cell-binding domain of fibronectin contains the sequence RGD; the smallest peptide required for interactions with RGD-dependent integrins (Pierschbacher and Ruoslahti, 1984). Proximal sites to this sequence contribute to binding affinities for different RGD-binding integrins (Aota et al., 1991; Mould et al., 1991), and a second site exists for $\alpha 4\beta 1$ integrin (Mould et al., 1991). Fibronectin is expressed during early angiogenesis (Chen et al., 1986; Nicosia and Madri, 1987), and in the event of vascular damage, blood extravasation causes plasma-derived fibrinogen and fibronectin to come into contact with sub-endothelial extracellular matrix, forming a provisional matrix. These proteins are the dominant components during wound repair (Bianco et al., 1990; Clark et al., 1982; Voss et al., 1986).

1.4.3.2 Laminin and Entactin/Nidogen

Laminins are trimeric glycoproteins, highly abundant in basement membrane. Laminin is composed of various isoforms of three disulphide-linked chains, α, β and γ, arranged in a cruciform or cross-shape structure (Engel et al., 1981). The α-chain has a unique C-terminal G-domain, with affinity for heparin. All three chains interact *via* alpha-helical segments that form a coiled-coil alpha-helix (Paulsson et al., 1985). There are currently 5α, 3β and 3γ chain isoforms known, resulting in a variety of possibly tissue-specific combinations. All three chains contain EGF-like repeats, which comprise the short arm of the cruciform, and which interact with nidogen. Nidogen has a dumbbell-like structure consisting of globular N and C-termini linked by a rod-like segment (Paulsson et al., 1986). Nidogen binds to laminin *via* the C-terminus globular domain (G3), and interacts with collagen type IV and the core protein of perlecan *via* the N-terminal globular domains, G1 and G2 (Fox et al., 1991)(Figure 1.5).

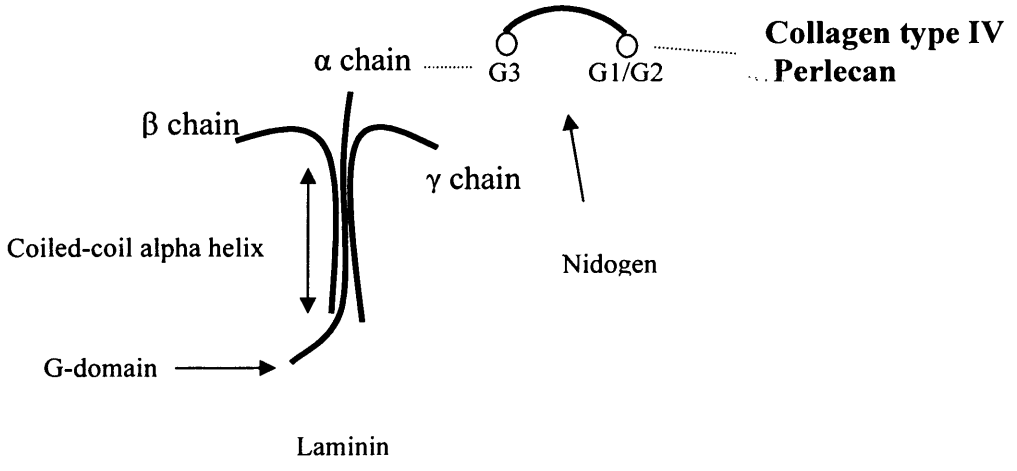


Figure 1.5: The cruciform structure and interactions of laminin. The alpha chain of laminin interacts with the G3 domain of nidogen/entactin, which interacts with collagen type IV and perlecan via the G1 and G2 N-terminal domains. The dotted lines denote interactions between proteins.

1.4.3.3 Vitronectin

Vitronectin is synthesised mainly by hepatocytes and secreted into the blood. It is involved in regulation of the complement system thus contributes to regulation of the inflammatory response. It has been detected in the extracellular matrix of blood vessels, although this could be blood derived as a consequence of increased vessel permeability or macrophage-derived, rather than synthesized by resident cells (Guettier et al., 1989). Like fibronectin, vitronectin interacts with RGD-dependent integrins through an RGD sequence (Cherny et al., 1993) and contains a heparin-binding domain (Suzuki et al., 1984). Vitronectin also binds to PAI-1 (a serine protease inhibitor), stabilising the active conformation and localising its activity in the matrix, resulting in activation of collagenases and fibrinolysis (Declerck et al., 1988; Mimuro and Loskutoff, 1989).

1.4.3.4 Elastic Fibres

Elastic fibres are components of the extracellular matrix that contribute to tissue flexibility and extensibility and can confer rubber-like properties. The structure of these fibres varies in a tissue-specific manner. For example, in elastic ligaments, fibres are arranged in rod-like fibres, while surrounding larger blood vessels they are arranged as concentric sheets. They occur as fine fibrillar networks in ear cartilage and the larynx, and combinations of the above in the skin and lungs. For a review, see Chapter 4, (Comper, 1996). Elastic fibres are predominantly composed of the highly hydrophobic protein elastin, which exists as an amorphous material surroundfed by microfibrils, generally composed of fibrillins (Sakai et al., 1991). These microfibrils are also associated with a variety of proteins including MAGP (microfibril associated glycoprotein), MP78, MP70, MP25 (Gibson et al., 1989), and AMP (associated microfibril protein) (Horrigan et al., 1992). Elastin associated microfibrils can also associate with other matrix components such as fibronectin and vitronectin (Dahlback et al., 1989).

1.4.3.5 Matricellular Proteins

Matricellular proteins are those associated with the matrix but not directly involved in its structure or organisation, for example, osteonectin, thrombospondins (Tsp) and tenascins. These proteins modulate cell-matrix interactions and often promote intermediate states of adhesion. For example, Tsp-1 and osteonectin disassemble pre-existing focal adhesions, and tenascin-C disrupts cell adhesion to fibronectin. While both pro- and anti-angiogenic roles for Tsp-1 have been suggested, it is generally considered anti-angiogenic. Tsp-1 enhances myofibroblast proliferation and migration (Nicosia et al., 1994a), and Tsp-1 counteracts VEGF promotion of endothelial cell survival (Jimenez et al., 2000; Nör et al., 2000), while in the absence of Tsp-1, endothelial cell proliferation and migration is enhanced (Wang et al., 2006). In the absence of Tsp-2, angiogenesis and wound healing is enhanced (Kyriakides et al., 1999), an observation also made in the absence of another glycoprotein, osteonectin (also termed SPARC or BM40) (Bradshaw et al., 2001), which is upregulated in remodelling tissues, and inhibits endothelial cell proliferation (Sage et al., 1989).

The three tenascins, C, R and X have restricted distributions and are generally anti-adhesive. Soluble tenascin-C disrupts endothelial cell focal adhesions (Murphy-Ullrich et al., 1991) and has also been reported to stimulate endothelial cell migration (Zagzag et al., 2002). Tenascin-C is highly abundant during embryonic development, but is absent from most adult tissues, although it is re-expressed during pathological angiogenesis (Mackie et al., 1988). Tenascin-R is expressed exclusively in the central nervous system (CNS) and regulates various functions of the CNS (Weber et al., 1999), while tenascin-X is the mostly widely and highly expressed tenascin in fetal tissues; abundantly in skeletal and cardiac tissues, smooth muscle, and surrounding blood vessels (Bristow et al., 1993). Tenascins can also associate with matrix CS-bearing proteoglycans, possibly versican or aggrecan and with fibronectin (Yamagata et al., 1993).

1.4.4 Structural Organisation of the Basal Lamina

The basal lamina (basement membrane) is a thin layer of specialised ECM surrounding parenchymal cells such as endothelium and epithelium. It separates cell layers from interstitial connective tissues, or from other cell types, e.g. the basal lamina of kidney glomerulus separates glomerular capillaries from podocytes. Basement membranes regulate diffusion of solutes and materials between cells and interstitial tissue, provide compartmentalisation, and provide mechanical support e.g. the basal lamina between skin epidermis and dermis. Cellular phenotype and behaviour is also modulated by basal lamina, through interactions with integrins. For example, laminin found in basal lamina maintains endothelial cells in a differentiated, quiescence state (Pauly et al., 1992) and laminin-1 induces persistent activation of Rac, which is implicated in control of endothelial permeability (Wojciak-Stothard et al., 2001). Laminin interacts with many ECM constituents (Laurie et al., 1982) and modulates cell-matrix interactions, thus influencing adhesion and migration (Aumailley and Smyth, 1998).

Basal lamina components vary with functional requirements. However they are rich in laminins, collagen type IV, nidogen/entactin and proteoglycans such as perlecan. Collagen type IV is secreted as heterotrimeric molecules that form flexible networks, and the N-terminus 7S region associates with four other type IV molecules (Timpl et al., 1981) while the C-terminal globular domain, NC1 has sites for end-to-end and lateral associations (Timpl et al., 1981; Tsilibary and Charonis, 1986). Laminin and nidogen/entactin are integral components (Form et al., 1986; Jerdan et al., 1991; Nicosia and Madri, 1987), which interact at the inner cross-region of laminin (Paulsson et al., 1987). Collagen type IV is linked to laminin *via* nidogen/entactin, and collagens IV and VIII form networks (Shuttleworth, 1997; Yurchenco and Schittny, 1990). Perlecan is also an integral component that assembles with other components so that vines of laminin-entactin and perlecan cover a lattice of type IV collagen (Paulsson, 1992; Yurchenco and Schittny, 1990) and interacts with endothelial β_1 and β_3 -containing integrins (Hayashi et al., 1992).

1.5 Control of Angiogenesis

1.5.1 Angiogenic Factors

Angiogenic factors could be considered to be those that play a role in angiogenesis, either in promotion or inhibition. Cells participating in, or neighbouring sites of angiogenesis, such as endothelial cells, fibroblasts and smooth muscle cells, express these factors. Blood serum components and immune cells are another source of angiogenic factors. Protease activation releases sequestered growth factors from ECM stores, while matrix degradation products themselves signal both positively and negatively during angiogenesis. Hypoxia, hypoglycaemia, mechanical stimulation, physical damage, inflammation and hormones, all of which result in upregulation and/or release of numerous pro- and anti-angiogenic factors, initiate sprouting angiogenesis.

1.5.1.1 Growth Factors

Growth factors influence all stages of angiogenesis and act on both endothelium and associated pericytes. Extracellular matrix components interact with, and sequester growth factors, providing a local store of factors that would otherwise be rapidly degraded or cleared by the blood. Local degradation of the matrix during angiogenesis releases growth factors, thereby controlling localisation of their activity. Additionally, the matrix microenvironment governs the cellular response to growth factors. It has been shown that cellular response to growth factors relies on coordinated adhesion signals from the matrix through integrin ligation. Thus the extracellular matrix continually modulates growth factor activity.

Growth factors stimulate quiescent cell re-entry into the cell cycle, stimulating growth, sprout formation, cell migration and proliferation, and enhancing cell survival. Growth factors are therefore pro-angiogenic factors. In support of this, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1) each promote rat aortic angiogenesis *in vitro* (Nicosia et al., 1994b). Growth factors particularly

associated with early angiogenesis are those upregulated by stimuli such as hypoxia, cellular damage or extensive shear stresses. For example, hypoxia and hypoglycaemia activate the transcription factor HIF-1 (hypoxia-inducible transcription factor) which regulates hypoxia-inducible genes like VEGF. Another growth factor involved in early angiogenesis is bFGF, released from damaged or necrotic endothelium (Muthukrishnan et al., 1991), or from stores in the extracellular matrix (Bashkin et al., 1989), where it is associated pericellularly with heparan sulphate (Rapraeger et al., 1991; Yayon et al., 1991). Excess shear stresses caused by increased blood flow induces endothelial cell expression of genes harbouring shear stress response elements (SSRE) in their promoters, for example PDGF (Resnick et al., 1997).

In general, growth factors stimulate migration and proliferation of endothelial cell sprouts. Many factors also act as chemotactic agents for pericytes, recruiting these cells to sites of angiogenesis and stimulating their proliferation and migration. In the latter stages of angiogenesis, pericyte recruitment supports a switch to vessel maturation and many growth factors additionally stimulate pericyte and endothelial cell differentiation. For example, hepatocyte growth factor (HGF) stimulates both endothelial cell growth and motility (Bussolino et al., 1992), while epidermal growth factor (EGF) enhances both endothelial cell proliferation and migration (Abramovitch et al., 1998). TGF-β acts co-ordinately with PDGF in control of vessel maturation. Shear stress induces endothelial cell expression of PDGF, recruiting PDGF-receptor-β positive pericytes (Westermark et al., 1990). Receptor ligation activates latent TGF-β which inhibits endothelial proliferation (Antonelli-Orlidge et al., 1989) and migration (Sato and Rifkin, 1989), and induces pericyte differentiation to smooth muscle-cell phenotypes (Hirschi et al., 1998; Verbeek et al., 1994). Control of growth factor localisation, availability, presentation and the cellular response is all controlled by the extracellular matrix and is crucial to regulation of angiogenesis.

1.5.1.2 Cytokines

Inflammatory cytokines also play a role in angiogenesis. For example, the pro-inflammatory mediator TNF-α activates endothelium which upregulates adhesion molecules and chemokines, attracting leukocytes and promoting their tethering and extravasation. TNF-α is suggested to be anti-angiogenic, through down-regulation of VEGF growth-factor receptors (Patterson et al., 1996), however TNF-α can stimulate angiogenesis *via* activation of the urokinase-type plasminogen activator (UPA) (Koolwijk et al., 1996), which generates the serine protease plasmin. Association of plasmin with the cell surface, results in restricted local areas of high proteolytic activity that may aid cell migration and tube formation. The endothelium also responds to TNF-α by releasing the vasodilator nitric oxide (NO), which relaxes associated pericytes and smooth muscle cells.

Since activated endothelium controls activation and extravasation of inflammatory cells, aberrant angiogenesis contributes to, and exacerbates many inflammatory conditions. Thus chronic inflammation, endothelial dysfunction and aberrant angiogenesis are tightly linked. This is illustrated by a study in which endothelial expression of a transmembrane form of the pro-inflammatory cytokine TNF-α was shown to activate endothelium, enhancing sprout formation independently of angiogenic growth factors (Rajashekhar et al., 2006). Interestingly, as the authors note, transmembrane TNF-α is upregulated *in vivo* in neovessels of atherosclerotic plaques (Barath et al., 1990) thus inflammatory mediators are important components of the angiogenic response.

1.5.1.3 MMPs

Matrix metalloproteinases are a family of zinc dependent proteases that degrade extracellular matrix components. MMPs are composed of a pro-peptide, a catalytic domain and a haemopexin-like C-terminal domain, which is linked to the catalytic domain by a flexible hinge region. Membrane type MMPs (MT-MMPs) are attached to the cell membrane *via* the C-terminus. MMPs are synthesized in an inactive form; the pro-peptide domain must be removed for activation. MMPs are grouped by substrate specificity (Table IV), although it should be noted that specificities overlap; MMPs-2, -6 and -9 degrade collagen IV, V and X, while MMP-7 degrades gelatin and collagen type IV. MMP activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs); a family of four inhibitors, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Visse and Nagase, 2003).

MMP activity is restricted through cell-surface interactions that also protect MMPs from TIMP activity. Integrins are MMP receptors; MMP-2 interacts with αvβ3 for example (Brooks et al., 1996). MT-MMPs activate latent MMPs at the cell surface, e.g. MT1-MMP activates latent MMP-2 (reviewed in (Ivaska and Heino, 2000). Cell surface MMPs also regulate growth factor activity, by locally liberating growth factors from the matrix and cleaving growth factor receptors and integrins. Perlecan digestion by MMP-3 and -13 releases bFGF (Whitelock et al., 1996), decorin is cleaved by MMPs-2, -3, -7 releasing TGF-β (Imai et al., 1997) and MMPs-1, -2 and -3 cleave IGFBP-3, to release IGF-1 (Fowlkes et al., 1994). MMPs also generate biologically active molecules as fragments of matrix proteins, e.g. degradation of collagen type XVIII generates endostatin-like fragments (Lin et al., 2001)

ProMMPs can also be activated *in vivo* by proteinases such as plasmin or chemically, in the case of proMMP-3 which is activated by NO during cerebral ischemia. MMP activity is therefore controlled at the level of transcription, by proteolytic activation, localisation of activity and specific inhibition by TIMPs (Visse and Nagase, 2003).

Class	MMP	Substrates
Collagenases	MMP-1 (Collagenase 1)	Collagens I, II, III, VII, VIII, X, XI, FN, VN, LM, gelatin, nidogen, tenascin, aggrecan, versican
	MMP-8 (Collagenase 2)	Collagens I, II, III, aggrecan
	MMP-13 (Collagenase 3)	Collagens I, II, III, IV, VI, IX, X, XIV, gelatin,FN,SPARC,aggrecan, perlecan
	MMP-18 (Collagenase 4, *Xenopus*)	Collagen type I
Stromelysins	MMP-3 (Stromelysin 1)	Collagens III, IV, V, VII, IX, X, XI, gelatin, elastin, FN, VN, LM, nidogen, tenascin, SPARC, aggrecan, decorin, perlecan, versican
	MMP-10 (Stromelysin 2)	Collagens III, IV, V, gelatin, elastin, FN, aggrecan
	MMP-11 (Stromelysin 3)	Gelatin, FN, collagen type IV, LM
Gelatinases	MMP-2 (Gelatinase A),	Collagens I, II, III, IV, V, VII, X, XI, gelatin, elastin, FN, VN, LM, nidogen, tenascin, SPARC, aggrecan, versican, decorin
	MMP-9 (Gelatinase B)	Collagens IV, V, XI, XIV, gelatin, elastin, VN, LM, SPARC, aggrecan, versican, decorin
Matrilysins	MMP-7 (Matrilysin 1)	Collagens I, IV, gelatin, elastin, FN, VN, LM, nidogen, tenascin, SPARC, aggrecan, decorin, versican
	MMP-26 (Matrilysin 2)	Collagen type IV, gelatin, FN, VN
Membrane type (MT) – Transmembrane	MMP-14 (MT1-MMP)	Collagens I,II,III, gelatin, FN, tenascin, VN, LM, nidogen, aggrecan, perlecan
	MMP-15 (MT2-MMP)	FN, tenascin, nidogen, LM, aggrecan, perlecan
	MMP-16 (MT3-MMP)	Collagen type III, gelatin, FN, VN, LM
	MMP-24 (MT5-MMP)	FN, gelatin, CS- and DS-proteoglycan
Membrane type (MT) – GPI anchored	MMP-17 (MT4-MMP)	Gelatin
	MMP-25 (MT6-MMP)	Collagen type IV, gelatin, FN, CS- and DS-proteoglycan
Others	MMP-12 (Macrophage elastase)	Collagens I,V,IV, gelatin, elastin, FN, VN,LM,nidogen,osteonectin, aggrecan
	MMP-19	Collagen type IV, gelatin, nidogen, FN, aggrecan, COMP
	MMP-20 (Enamelysin)	Amelogenin, aggrecan, COMP
	MMP-21 (XMMP, *Xenopus*)	Gelatin
	MMP-23 (CA-MMP)	Gelatin
	MMP-27 (CMMP, *Gallus*)	Gelatin
	MMP-28 (Epilysin)	No ECM substrates, digests casein

Table IV: Matrix metalloproteinase classification and their substrates. N.B. Only major extracellular matrix substrates are shown. MMPs also degrade a wide range of other proteins including growth factors, and cell surface receptors. Where appropriate, these are discussed in the text. FN – Fibronectin, VN – Vitronectin, LM – Laminin, CS – Chondroitin Sulphate, DS – Dermatan Sulphate. Adapted from (Visse and Nagase, 2003), Supplementary data.

Continuous signalling between the ECM and cells allows cells to 'sense' the changing microenvironment and respond accordingly by altering expression of MMPs and matrix components. For example, osteogenic cells cultured in a 3D collagen matrix upregulate MMP-1 expression *via* α2β1 integrin ligation with collagen, and this interaction results in down regulation of collagen expression (Riikonen et al., 1995). Further, migrating keratinocytes express MMPs only when in contact with collagen (Pilcher et al., 1997). Matrix degradation products also interact with integrins, for example endostatin interacts with αv and α5 containing integrins, and tumstatin interacts with αv (Maeshima et al., 2001), allowing the cell to sense changes in the microenvironment.

The role of MMP-2 exemplifies the role of MMPs in angiogenesis from initiation through to inhibition and maturation. During early angiogenesis, the serum component thrombin activates MMP-2 resulting in degradation of the basement membrane, which facilitates endothelial sprout interaction with interstitial collagen type I. Endothelial cells in contact with fibrillar collagen type I upregulate MT1-MMP, which further activates MMP-2. MMP-2 activates MMP-1, which degrades interstitial fibrillar collagen type I, promoting migration through interstitial tissues. MMP-2 also plays a role in tube formation, since microvascular endothelial cells in a collagen type I lattice upregulate MMP-2 and MT1-MMP, and inhibition of MMP-2 activity inhibits formation of capillary-like structures (Haas et al., 1998). MMP-2 also generates an anti-angiogenic fragment, angiostatin, from circulating plasminogen, resulting in inhibition of angiogenesis (O'Reilly et al., 1997). This provides a mechanism by which MMPs in the early stages of angiogenesis can act to promote angiogenesis however sustained signalling results in the formation of inhibitory signalling products. Thus feedback between cells and the local environment controls modification of the extracellular matrix throughout angiogenesis.

1.5.1.4 Additional Factors

Many factors key to vasculogenesis are also involved in postnatal angiogenesis, for example, VEGF, angiopoietins and their Tie receptors, and Eph receptors and their Eph ligands. Postnatally, angiopoietins regulate vessel stability. Ang-2 competes with Ang-1 for binding to the receptor Tie-2. Ang-1 binding to Tie-2 stabilises the vasculature, while Ang-2 interaction results in vessel destabilisation, leading to angiogenesis in the presence of VEGF, or apoptosis in its absence (Maisonpierre et al., 1997).

Integrin-mediated events play an essential role in angiogenesis, and the eph receptors and ephrin ligands can influence integrin-mediated adhesion, although in some cases this results in enhanced adhesion (Huai and Drescher, 2001), while in others results in focal adhesion disassembly (Miao et al., 2000). EphB4 inhibits post-natal vascular branching and network formation and activates the angiopoietin-1/Tie2 system resulting in reduced vessel permeability (Erber et al., 2006). The ligand Ephrin B2 signals through the EphB4 receptor in endothelial cells and promotes migration *in vitro* and neo-angiogenesis *in vivo* (Maekawa et al., 2003). In contrast, ephrin B2 inhibited endothelial migration and network formation while EphB4 enhanced endothelial cell migration and sprouting angiogenesis. Further, it was demonstrated that forward EphB4 signalling results in segregation from Eph B2 expressing cells, supporting a role for these proteins in post-natal regulation of arteriovenous microvasculature network organisation (Fuller et al., 2003). Indeed, EphB4 is preferentially expressed by venous endothelial cells, whereas ephrinB2 is expressed by arterial and angiogenic endothelial cells (Gale et al., 2001; Shin et al., 2001).

The homeobox (Hox) genes are implicated in control of angiogenesis through regulation of the endothelial cell cycle. Initiation of angiogenesis activates HoxD3 and HoxB3 which upregulates genes involved in angiogenesis including MMPs, UPA and $\alpha_v\beta_3$ integrin (Boudreau et al., 1997; Myers et al., 2000), while maturation and restoration of quiescence is associated with re-expression of HoxD10 which down-regulates $\alpha_v\beta_3$ integrin and upregulates TIMP (Myers et al., 2002). Many homeobox genes upregulate transcription

factors that act on extracellular matrix genes, thus the homeobox genes are linked closely to matrix regulation (Botas, 1993).

1.5.2 Angiogenesis and the Cell Cycle

In response to proliferative signals from growth factors, quiescent cells re-enter the cell cycle and undergo mitosis. There are four major phases to the cell cycle, with three restriction or check-points (summarised in Figure 1.6), allowing progression only when certain requirements have been met. In G_0, which occurs prior to the G_1 checkpoint cells are permitted to exit the cell cycle, and become quiescent (non-proliferative) or differentiate. This occurs in the absence of sufficient nutrients or proliferative signals (such as growth factors) or in response to differentiation signals.

The cell cycle is controlled by factors that are regulated at the level of transcription (initiated by specific transcription factors), at the protein level through activation or degradation induced by phosphorylation, and by localisation and associations with other factors. The major protein families are cyclin dependent kinases (CDKs), cyclin dependent kinase inhibitors (CDKIs) and cyclins. CDKs require cyclin association and phosphorylation on a conserved threonine residue for activation. Cyclin binding induces a conformational change in the CDK, resulting in partial activation. CAK (cdk activating kinase) phosphorylates threonine, inducing a further conformational change and increasing CDK kinase activity. Thus the cell cycle is controlled by sequentially fluctuating CDK activity, regulated by periodic synthesis and degradation (by ubiquitin-dependent proteolysis) of the regulatory cyclins (summarised in Figure 1.6). Progression through checkpoints requires activation of specific CDK-cyclin complexes at different stages of the cell cycle. CDKIs act by binding to cyclin-CDK complexes and inducing conformational changes that distort the active site, inhibiting CDK kinase activity.

Retinoblastoma tumour suppressor (pRb) is a growth suppressing protein, the activity of which is controlled by phosphorylation. pRb controls G_1 progression by suppressing G_1 to S transition. In its dephosphorylated active form (in G_0 and early G_1), pRb binds to and sequesters the transcription factor

E2F. For transition into S phase, pRb is inactivated by CDK-mediated phosphorylation, resulting in dissociation from E2F. E2F promotes expression of genes required for progression into S phase, for example cyclin E. E2F also upregulates itself, further increasing cyclin levels and active CDK complexes which further phosphorylate and inactivate pRb.

There are two families of cyclin kinase inhibitors, the INK and the Cip/Kip family. The first includes the proteins p15, p16, p18 and p20, which bind to complexes of cyclin D-CDK4 and cyclin D-CDK6, thus blocking cells in G_1. The Cip/Kip family includes the proteins p21, p27 and p57 which have binding affinity for all cyclin-CDK complexes found in G1 and during the transition from G1 to the S phase, but act preferentially to inhibit the activity of CDK2-containing complexes. CDKI activities are summarised in Table V.

In cells in G_0 or arrested in G_1, CDK activity is inhibited by accumulation of the CDKI p27 and by pRb mediated inhibition of cyclin gene transcription. Extracellular stimulation results in accumulation of active CDK complexes, which phosphorylate pRb and p27. The latter becomes a target for ubiquitination and degradation, relieving these inhibitory mechanisms. DNA damage in G_1 leads to activation of p53, stimulating transcription of another CDKI, p21, thus blocking progression through the G_1 checkpoint. Upregulation of p21 occurs in response to DNA damage or differentiation signals, through p53 dependent and independent mechanisms. Upregulation of p27 is associated with endothelial cell withdrawal from the cell cycle into G_0 (Chen et al., 2000), while adenoviral-mediated expression of either p21 or p27 in endothelial cells induces cell cycle arrest in G_0 (Solodushko and Fouty, 2006). Additionally, p21 and p27 may be involved in cell survival, since caspase-mediated cleavage of these proteins precedes endothelial cell apoptosis (Levkau et al., 1998).

Major transcription factors which regulate gene expression in endothelial cells include the AP, HIF, NF-kB, STATs and forkhead families, which are activated throughout angiogenesis by various stimuli including hypoxia, shear stress, vascular damage, growth factors, hormones, cytokines, chemokines, matrix components and pathogens. For a detailed review, see (Minami and Aird, 2005).

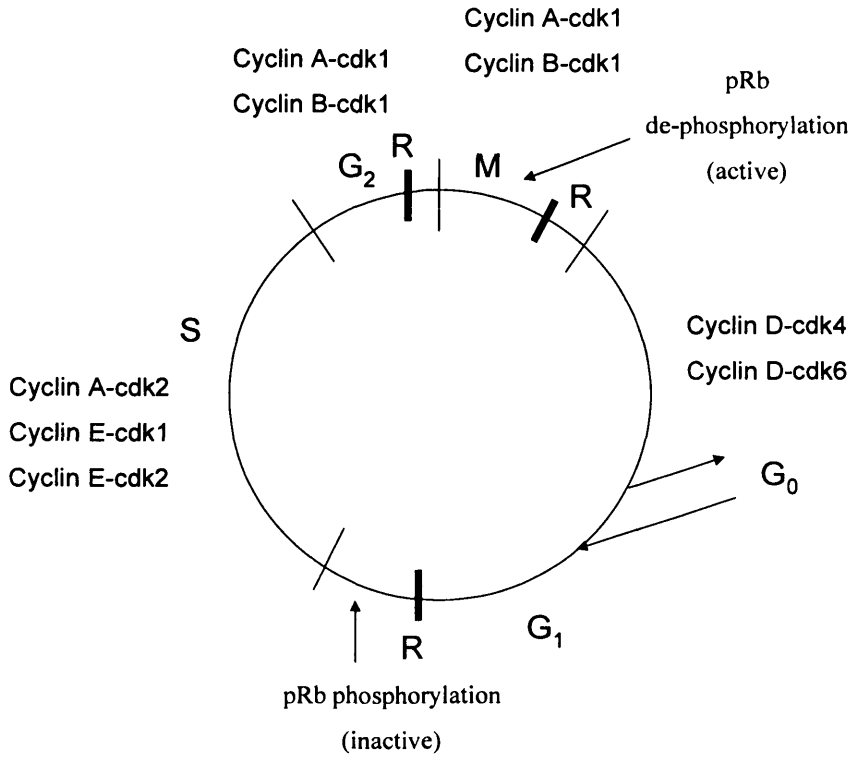


Figure 1.6: The major phases of the cell cycle, and associated cyclin complexes. During Gap 1 (G_1), cells prepare for DNA synthesis, which occurs during synthesis-phase (S-phase). Once this is complete, cells enter G_2 and prepare for chromosome segregation and cell division, before entering mitosis phase (M-phase) and undergoing cell division. Prior to the G_1 checkpoint there is an additional phase referred to as G_0, in which cells can exit the cell cycle and differentiate, or quiesce if conditions are unfavourable. Checkpoints allow control of the cell cycle, which can only progress if requirements are met. R denotes restriction or checkpoint, and the major cyclin-cdk complexes associated with each phase are indicated.

Phase	Cyclins Involved	CDK binding partners	CKI
G1	Cyclins D1, D2, D3	4/6	p16, p15, p21, p27
G1 to S	Cyclin E	2	p21, p27
G1 to S	Cyclin A	2	p21, p27
G2 to M	Cyclin A	1	p21, p27
G2 to M	Cyclin B	1	p21, p27
Exit M	Cyclin B destruction		

Table V: Summary of cyclins involved in each phase of the cell cycle, and their possible CDK and CKI binding partners.

1.5.3 Vessel Maturation

1.5.3.1 The Role of Pericytes

Pericytes play a key role in control of vessel tone, maintenance of vascular integrity and in the switch from immature to mature and quiescent blood vessels. For immature vessels to differentiate and become quiescent, cells must cease proliferating, exit the cell cycle and deposit basement membrane components. Associations between endothelial cells and pericytes are associated with arrest of vessel growth (Crocker et al., 1970), while pericyte 'drop-out' occurs in diabetic retinopathy before the onset of neo-vascularisation (Cogan et al., 1961). Similarly, haemangiomas, which are vascular abnormalities characterised by excessive endothelial cell proliferation have few pericytes associated (Feldman et al., 1978), suggesting a role for pericytes in promoting vessel maturity, stability and quiescence. During angiogenesis, endothelial cell survival requires specific signals for maintenance of immature, pericyte-free vessels (Benjamin et al., 1998). Unlike quiescent endothelial cells, those of immature vessels are susceptible to pruning and regression by apoptosis (Stupack and Cheresh, 2003). Prior to maturation, survival of angiogenic endothelium depends on sustained survival signals from ECM and growth factors such as VEGF; hyperoxia or unsustained VEGF signalling results in blood vessel pruning in the eye (Alon et al., 1995). VEGF is required for neo-angiogenesis during the female reproductive cycle (Ferrara et al., 1998). On ovum release, VEGF production stops and complete vascular regression occurs (Dickson et al., 2001). Similarly, on VEGF withdrawal, nascent vessels in the eye regress (Alon et al., 1995). Sustained VEGF signalling may be required for permanency of vessels; withdrawal prior to maturation results in regression (Dor et al., 2002), while after maturation, endothelium survival is independent of VEGF (Alon et al., 1995). Immature vessels therefore have a window of plasticity where they can be remodelled prior to pericyte association and maturation.

1.5.3.2 Lumen formation

Endothelial lumen formation arises from the formation and coalescence of pinocytic vesicles (Folkman and Haudenschild, 1980), the formation of which is dependent upon the actin cytoskeleton. Vacuole and lumen formation are completely dependent on the collagen-binding integrin α2β1, while other endothelial cell integrins do not appear to be involved (Davis and Camarillo, 1996). Endothelial lumen formation from vacuoles also requires the small GTPases Cdc42 and Rac-1 but not Rho (Bayless and Davis, 2002). Fibronectin and its receptors, the α5 and αV-containing integrins, could also be involved in maturation; fibronectin is found in junctions between pericyte processes and endothelial cells (Courtoy and Boyles, 1983), while αvβ3 ligation is required for survival of nascent vessels. Further, the RGD-dependent integrins are required for lumen formation in fibrin matrices (Bayless et al., 2000). MMP-2 also plays a role in tube formation, since microvascular endothelial cells in a collagen type I lattice upregulate MMP-2 and MT1-MMP, and inhibition of MMP-2 activity inhibits formation of capillary-like structures (Haas et al., 1998).

The basement membrane must also be re-established around maturing blood vessels. The endothelium has been shown to contribute to basement membrane formation in the developing kidney (Ekblom, 1981), and both endothelial and mural cells contribute to basement membrane formation (Mandarino et al., 1993) by *de novo* protein synthesis.

1.5.4 Cell-Matrix Interactions

For cell survival, growth, proliferation and migration, cells must be in contact with the ECM. The cell cycle is adhesion dependent during G_1 and in transition into the S phase. However after this point completion of the cell cycle is adhesion independent. Cells lose matrix attachments and adopt a more rounded morphology as they undergo mitosis and cell division. Thus cells undergo a cycle of extracellular matrix attachment and detachment, continually rearranging their contacts with both the ECM and with neighbouring cells.

1.5.4.1 Integrins

Heterodimeric proteins called integrins mediate contact between cells and the ECM. These proteins span the plasma membrane to contact ECM and cytoplasmic components simultaneously. Integrins are composed of one α and one β chain, associated non-covalently. There are currently 18 α and 8 β chains, of which 24 combinations are known. Binding affinities of some integrins depend on the sequence RGD, found in substrates such as fibronectin, and these are referred to as RGD dependent integrins. Nine of the 18α chains, including the collagen binding integrins α1, α2, α10 and α11, contain a highly conserved I (inserted) or A-domain responsible for ligand binding (see Chapter 4 for detailed discussion) and are termed RGD independent, instead recognising triple-helical structures of collagens. Integrins have overlapping functions (redundancy) and can have multiple binding partners. Integrin ligands and potential roles in angiogenesis are summarised in Table VI. Of the RGD-dependent integrins, αvβ3 has the largest number of ligands. Of the RGD-independent integrins, α2β1 has an increasingly large number of binding partners. Integrin expression alters throughout angiogenesis concomitant with matrix remodelling allowing the cell to interact with, and respond to the changing environment. Integrin mediated signalling is essential for angiogenesis since deletion of certain subunits result in embryonic lethality, while specific postnatal targeting with various inhibitors or blocking antibodies to other subunits inhibits postnatal angiogenesis.

Integrin	Ligand	Role in Angiogenesis
α1β1	Collagen IV, I, II, LM-1	Inhibitors and knockout of α1 reduces tumour angiogenesis. α1β1 promotes endothelial migration and proliferation
α2β1	Collagens I, II, IV, LM-1, tenascin, snake venom proteins, chondroadherin	α2 inhibitors reduce tumour angiogenesis. A2β1 promotes endothelial migration and lumen formation
α3β1	LM-5, LM-1, FN, tsp, nidogen	α3β1 suppresses endothelial cell proliferation
α4β1	FN, VCAM-1	α4 knockout has a lethal phenotype at E11-14 due to vascular defects
α5β1	FN, fibrin	α5 knockout is lethal at E10. Vasculogenesis occurs but no angiogenesis or maturation. α5β1 stimulates endothelial proliferation,migration and lumen formation
α6β1	LM	Expressed on endothelial cells
α7β1	LM	
α8β1	FN, tenascin, VN, osteopontin	
α9β1	VCAM-1, tenascin	
α10β1	Collagen type I and II in cartilage, FN	None known
α11β1	Collagen type I and II in muscle	None known
αvβ5	VN, osteopontin, bone sialoprotein	β5 knockout, enhanced angiogenesis, and inhibitors reduce tumour angiogenesis.
αvβ6	FN	αV knockout has lethal phenotype at E12 due to vascular defects
αvβ1	VN, FN	
αvβ3	VN, FN, LM, fibrinogen, fibrin, TSP, tenascin, vWF, denatured collagen, osteopontin, MMP-2, FGF-2, thrombin	β3 knockout has no defects in vasculogenesis but shows enhanced postnatal angiogenesis. Inhibitors to β3 reduce tumour angiogenesis.
αvβ8	VN, FN	β8 knockout has lethal phenotype at E12 due to vascular defects
α6β4	LM-5 in hemidesmosomes	
αIIbβ3	Fibrinogen, FN, vWF, VN	Platelet integrin

Table VI: A summary of integrin ligands and their known roles in angiogenesis. Adapted from (Ruegg et al., 2004; Wehrle-Haller and Imhof, 2003) key: VN vitronectin, FN fibronectin, LM laminin, vWF von willebrand factor, TSP thrombospondin

1.5.4.2 Integrin Signalling

Integrins are capable of bidirectional signalling, from the ECM to the inside of the cell and *vice versa*, often referred to as 'outside-in' and 'inside-out' signalling respectively. In the former case, integrin ligation from the extracellular matrix results in conformational changes, which are transduced to an intracellular protein complex, associated with the integrin cytoplasmic domain. 'Inside-out' signalling is less well understood, but signals originating intracellularly can modulate extracellular integrin conformation, thus influencing ligand-binding affinities (see Chapters 4 to 6 for detailed discussion). The receptor is converted from a low to a high affinity state, an event that may be regulated by small GTPases (Hynes, 2002). Talin interaction with the cytoplasmic domain of integrins can also switch integrins into an active conformation (Calderwood et al., 1999).

Signalling arising from ligand binding is linked to events such as proliferation and cell survival through effects on gene expression. Integrins do not possess intrinsic kinase capacity; however the intracellularly associated protein complexes include tyrosine kinases, thus creating docking sites for, and activating, proteins involved in diverse signalling pathways. Integrins are linked to the actin cytoskeleton *via* a number of adaptor proteins such as talin and vinculin (Nobes and Hall, 1995). Such sites of contact between cells and ECM are termed focal adhesions. Classically, integrins bound to their ligands move laterally in the plasma membrane, forming integrin clusters, influencing organisation of the cytoskeleton and recruiting protein complexes, thereby controlling cell migration.

A classic example of a focal adhesion protein is focal adhesion kinase (FAK). Integrin ligation induces FAK autophosphorylation (Burridge et al., 1992). Src kinases are recruited, which further phosphorylate and enhance FAK activity. Phosphorylation on multiple tyrosine residues creates multiple docking sites, recruiting further focal adhesion proteins. Thus FAK and subsequently integrins, are linked to numerous signalling pathways (Schlaepfer et al., 2004).

1.5.4.3 Integrins and Angiogenesis

Modulation of integrin expression is concomitant with changes in the extracellular matrix microenvironment. This is essential in allowing the endothelium to interact with the microenvironment, since survival signals from ECM are essential in preventing endothelial apoptosis. The integrin αvβ3 for example, is upregulated during angiogenesis (Brooks et al., 1994a), while antagonists of this integrin (Brooks et al., 1994b) and integrins α5β1 and α2β1 promote endothelial cell apoptosis (Kim et al., 2000; Senger et al., 1997). Soluble integrin ligands can induce apoptosis, while the same ligand promotes cell survival when immobilised within the ECM (Petitclerc et al., 2000; Rehn et al., 2001) underlining the importance of cell interactions with immobilised matrix ligands.

Many studies support an essential role for endothelial integrins in angiogenesis. Inhibition of a number of integrins with antibodies or peptides inhibits angiogenesis; blocking antibodies to αvβ3 inhibit angiogenesis despite the fact that mice lacking both this integrin and the related integrin αvβ5 have no deficiencies in either vasculogenesis or angiogenesis (although tumour angiogenesis was increased) (Reynolds et al., 2002). Further, integrin expression is modulated by angiogenic factors like VEGF, bFGF and TGF-β, and integrins in turn influence growth factor signalling by recruiting growth factor receptors to sites of adhesion (Miyamoto et al., 1996). VEGF signalling upregulates αvβ3 (Senger et al., 1996) and the collagen-binding integrins α2β1 and α1β1, and blocking these integrins *in vivo* inhibits VEGF-induced angiogenesis (Senger et al., 1997).

Appropriate signals from the ECM to integrins are also required for proper cellular response to growth factors; in the mitogen-dependent stage of the cell cycle, signals from both integrins and growth factors are required for cell cycle progression from G_0 to S-phase. VEGF promotes endothelial cell survival *via* Akt, in a mechanism requiring cell contact with extracellular matrix (Fujio and Walsh, 1999), while during cell migration, FAK can interact directly with the cytoplasmic domain of the EGF receptor, suggesting that cross-talk between

integrins and growth factor receptors occurs (Sieg et al., 2000). Moreover, VEGF-A signalling through its receptor Flt-1 promotes tubulogenesis in a FAK dependent manner (Maru et al., 2001), while integrin-association with PDGF receptors can enhance responses to PDGF (Schneller et al., 1997). Exemplifying the close relationship between adhesion and growth factor signals, growth factor receptors can, under some circumstances, be activated by adhesion signals in the absence of classic ligands. This occurs where the receptor is abundant, for example in tumour cells or primary fibroblasts. Furthermore, EGF receptor phosphorylation patterns differ in response to adhesion signals compared to the growth factor ligand, implying that adhesion regulates signals distinct from those of growth factors (Moro et al., 2002). Integrins can also directly regulate growth factor activity. TGF-β is inactive while associated with its latency- associated peptide, LAP-β. This peptide contains an RGD domain, recognised by αvβ8 on epithelial cells. In conjunction with MT1-MMP, this integrin promotes release of active TGF-β (Mu et al., 2002). Integrins are therefore key in mediating cell adhesion, migration, proliferation and survival responses.

1.5.4.4 Cell Migration

Cell migration requires cyclical modulation and turnover of focal adhesions. Cell movement is propelled by polymerising actin in protruding filopodia and lamellipodia at the leading edge of migrating cells, while at the rear focal contacts are disassembled for cell retraction, or reinforced into mature focal adhesions. Initial points of contact between matrix and cells are often referred to as focal complexes, or focal contacts. These can remain small and transient, or mature into larger, more adherent focal adhesions after recruitment and activation of numerous proteins.

The activity of the small Rho GTPases; Rac, Cdc42 and Rho, plays a central role in modulating the structure of the actin cytoskeleton. Rac activity modulates lamellipodia and Cdc42 is implicated in control of filopodia, while RhoA controls focal contacts in the main cell body. In migration, small focal complexes appear at the leading edge of the cell, in Rac induced lamellipodia (Ridley et al., 1992), which contain fine actin networks. These complexes exhibit slow turnover, and remain stationary, therefore as the cell migrates, they become located more in the main body of the cell. Increased tension on the actin network induces stress fibre formation. Fibres are anchored in focal contacts, which mature into focal adhesions under the control of Rho. Sliding focal contacts are located at the rear of the migrating cell, and are quickly turned over through Rho activity, which induces integrin turnover (Ballestrem et al., 2001) Integrins in turn, control and localise GTPase activity, for example, GTP associated Rac binds to integrins, which facilitates removal of the GDI (GDP-dissociation inhibitor) (Del Pozo et al., 2002).

1.6 The Role of Extracellular Matrix During Angiogenesis

During angiogenesis, the matrix microenvironment is continually altered and remodelled through *de novo* protein expression, deposition of plasma components and liberation of signalling protein fragments and growth factors by protease activity. Extracellular matrix composition also varies with localisation depending on specific tissue requirements.

Vitronectin, fibronectin, fibrin and osteopontin are present in the provisional matrix during VEGF-driven angiogenesis (Senger et al., 1996) as well as the interstitial collagens, the majority of which is type I. Many pro-angiogenic factors, such as VEGF-A increase vascular permeability, resulting in deposition of a provisional matrix with which the emerging endothelial sprouts come into contact (Van Hinsbergh et al., 2001). Components of provisional and interstitial matrix (fibronectin, collagen type I) support endothelial cell proliferation and migration, while basement membrane components such as laminin, maintain endothelium in a differentiated, quiescent state (Pauly et al., 1992). Consistent with an environment less permissive for proliferation, hepatocytes cultured on basement membrane downregulate genes associated with proliferation (Rana et al., 1994).

During the latter stages of angiogenesis, endothelial cell proliferation decreases through the arrest and exit of the cell cycle and induction of differentiation, i.e. tube formation. The microenvironment; local matrix composition and organisation and growth factor availability, influences formation of tubular structures. Differentiation may occur when proliferation is not supported (in the absence of growth factors), and is accelerated by certain matrix components (Maciag et al., 1982). For example, endothelial proliferation decreases when cells are cultured on matrigel, and vessel-like structures form even in the presence of growth factors. Laminin was shown to be a major factor in induction of differentiation. However laminin-coated dishes alone could not induce tube formation, nor could collagen-coated dishes (Kubota et al., 1988). In contrast, microvascular endothelial cells cultured inside collagen type I gels spontaneously formed capillary-like structures (Montesano et al., 1983),

suggesting that endothelial cell differentiation responses differ between two-dimensional and three-dimensional environments; further, interstitial matrix components can induce differentiation. In contrast, bovine aortic endothelial cells (BAE) cultured on or within collagen type I gels could form capillary-like structures (Schor et al., 1983). Further studies support differential roles for interstitial and basement membrane matrix components. Culturing microvascular endothelial cells on interstitial collagens (type I and III) supported proliferation but not tube formation, while basement membrane collagens (type IV and V) supported tube formation but not proliferation (Madri and Williams, 1983). Interestingly, on two-dimensional matrigel substrates, network formation was observed, but tube-like structures did not form when cells were in a 3D environment. In contrast, in three-dimensional collagen gels, endothelial cells formed tube-like structures in a manner dependent on MMP-2 activity (Haas et al., 1998). Combinations, structures and concentrations of matrix components are likely to be of particular importance. In a rat aortic explant model, the basal lamina components laminin and entactin had a biphasic effect on vessel formation in collagen type I gels; at lower concentrations vessel formation was enhanced, while higher concentrations inhibited this process. However vessels which formed in the latter case were more stable and were associated with organised laminin-entactin networks (Nicosia et al., 1994a). Tube formation therefore may initiate in a predominantly interstitial environment. However these structures may be immature and more plastic, i.e. undergoing remodelling. In a predominantly basement membrane environment, more mature, differentiated vessels are formed.

Despite the matrix being continually remodelled by MMP-mediated degradation, and deposition of new matrix from cells, there is some evidence to suggest that cells also continuously secrete matrix components, and may maintain the composition if not the organisation of their local environment. A study of neoangiogenesis in rabbit and guinea pig cornea suggested the continued presence of laminin, collagen type IV, heparan sulphate containing proteoglycans and nidogen surrounding developing sprouts and established vessels (Jerdan et al., 1991). The presence of laminin and collagen type IV in

vessel sprouts was confirmed in rat aortic tissue (Nicosia and Madri, 1987), while vessel sprouts in mouse cornea were associated with laminin but not collagen IV (Form et al., 1986) or heparan sulphate proteoglycans (Ausprunk et al., 1981). Difference between these studies may be explained by epitope availability and different experimental procedures (Jerdan et al., 1991). Interestingly, the continued presence of low concentrations of unorganised laminin and nidogen could facilitate initiate tube formation in an interstitial (collagen type I) environment (Nicosia et al., 1994a), while increasing concentrations and organisation of these proteins could then contribute to vessel maturation.

In an *in vivo* model of angiogenesis, laminin and collagen type IV were localised to the periphery of nascent vessels. Expression of collagen type IV peaked then declined, while laminin and entactin expression remained constant, suggesting that not only concentrations of basement membrane proteins (Nicosia et al., 1994a) but the ratios may also be important (Sephel et al., 1996). Endothelial cells adhere more efficiently to collagen type IV than laminin, and the former promotes cell migration, while laminin does not (Herbst et al., 1988). Increased expression of collagen type IV earlier in angiogenesis may promote migration and initial tube formation. As angiogenesis progresses, the ratio of collagen type IV to laminin decreases and cell migration is inhibited, contributing to tube stability and maturation. Endothelial expression of collagen is also associated with formation of tubular structures (Fouser et al., 1991) and endothelial cells require continual secretion of collagens for migration (Madri and Stenn, 1982) demonstrating a requirement for collagen in tube formation. It could be envisioned that cells engaged in tube formation might not be actively migrating, however early tube formation could require migratory signals for reorganisation of cell-cell and cell-matrix interactions. Thus an interstitial environment may support and promote proliferation, migration and early tube formation, while increased synthesis and organisation of basement membrane components inhibits migration and proliferation, supporting vessel maturation.

1.7 Matrix Signalling in Angiogenesis

The complex process of angiogenesis is mediated *via* multiple signalling pathways, which control events critical to all stages in angiogenesis. Signals from the extracellular matrix *via* integrins, and from growth factors to their receptors coordinate to exert effects on the cytoskeleton, the cell cycle and on gene expression.

1.7.1 Signalling Pathways in Angiogenesis

1.7.1.1 PI-3K/Akt Signalling

The PKB/Akt pathway is involved in promotion of angiogenesis, and endothelial migration and survival. For activation, Akt requires phosphatidyl inositol-3 phosphate (PIP3)-dependent translocation to the cell membrane and phosphorylation at two sites, 308T and 473S (Lawlor and Alessi, 2001). Activation of survival pathways is essential for vessels to be maintained through to maturity. PKB/Akt-dependent signalling promotes endothelial cell survival by phosphorylation and inactivation of the pro-apoptotic molecules Bad, Bax and caspases and inhibition of the stress-activated kinase p38 (Gratton et al., 2001). Active Akt rescues endothelial cell survival in the presence of integrin antagonists (Maeshima et al., 2002) and blocks apoptosis initiated by caspase 8 (Suhara et al., 2001) and caspase 9 (Cardone et al., 1998). Endothelial cell migration induced by VEGF involves integrin activation via a PI-3K and PKB/Akt-dependent signaling pathway (Byzova et al., 2000). A recent study in Akt-1 deficient mice suggests Akt-1 signalling *in vivo* to be anti-angiogenic, playing a role in vascular maturation and permeability through induction of Tsp-1 and Tsp-2 (Chen et al., 2005). In an *in vitro* model of angiogenesis, Akt was activated by decorin, and expression of this proteoglycan was also associated with enhanced cell survival and tube formation (Schönherr et al., 1999), supporting a role for Akt signalling in control of endothelial cell behaviour and angiogenesis.

1.7.1.2 MAPK Pathways

MAPKs (mitogen-activated protein kinases) are serine-threonine kinases, which include ERKs, JNKs, SAPKs (stress activated protein kinases) and p38 MAPK. The MAPK pathways involve a kinase cascade where the signal is amplified at each step, starting with activation of a MAPKKK (classically Raf) by signalling through cell-surface receptors. The MAPKKK (Raf) phosphorylates a MAPKK (MEK), which phosphorylates a MAPK (ERK), which moves into the nucleus and activates protein transcription. The MAPK pathway is involved in endothelial cell migration, proliferation and survival. Angiogenesis in the chorio-allantoid membrane (CAM) assay requires two waves of ERK activation, the first one being dependent on FGF-2, and the second on αVβ3 integrin ligation (Eliceiri et al., 1998).

1.7.1.3 Small Rho GTPases

Another family of proteins implicated in angiogenesis are the small Rho GTPases, Rac, Rho and Cdc42. These control cytoskeletal organisation and cell migration (discussed in section 1.5.4.3) as well as playing a role in lumen formation (discussed in 1.5.3.2). Additionally, Rac and Rho but not Cdc42 are implicated in regulation of endothelium permeability (Wojciak-Stothard et al., 2001). GTPases cycle between active, GTP-associated and inactive, GDP-associated conformations. This is regulated by guanine nucleotide exchange factors (GEFs), which promote release of GDP, allowing GTP to bind (since the local concentration of GTP exceeds that of GDP), and GTPase activating proteins (GAPs), which promote hydrolysis of GTP to GDP.

1.7.2 Matrix Degradation Products

If the extracellular matrix network is considered as a protein mesh encapsulating sequestered growth factors, then it follows that protease-mediated degradation releases this store of growth factors. Additionally, actively signalling matrix degradation products are generated. A large level of complexity can be envisioned; degradation products may retain signalling activities or binding properties of intact proteins, or exhibit new ones. Newly generated complexes of matrix degradation products and growth factors may also exhibit similar or alternative signalling properties to those of the intact binding partners. The ECM continually modulates associated growth factor activity, exemplified by the role of decorin in TGF-β signalling. This growth factor has a short half-life in serum but is sequestered in the matrix by decorin. A soluble complex of decorin and TGF-β may be rapidly cleared in the blood (Markmann et al., 2000), and in binding to decorin, collagen could be considered to sequester both proteins. TGF-β is thus protected from clearance and proteolysis, and maintained as an extracellular store. Upon MMP activation, decorin, TGF-β and fibrillar collagen degradation potentially generates a variety of differentially signalling complexes. For example, decorin degradation (by MMP-2, MMP-3 and MMP-7) *in vitro*, releases TGF-β from a decorin-TGF-β complex (Imai et al., 1997), while fibrillar collagen type I but not decorin, is a substrate for MMP-1 (Imai et al., 1997). These *in vitro* studies have strong implications for *in vivo* signalling; different spectrums of MMP activity could locally generate different degradation products.

Interactions between integrins and their ECM ligands directly influence cell behaviour. MMP activity exposes cryptic sites for alternative integrin recognition sites, encountered by cells only during remodelling, thereby influencing cell behaviour in a controlled manner (Streuli, 1999). For example, MMP cleavage of fibronectin enhances cell migration (Fukai et al., 1995) while MMP-1 cleavage of collagen type I enhances keratinocyte migration (Pilcher et al., 1997).

1.7.3 Decorin Expression in Angiogenesis

Decorin expression is associated with nascent vessels formed during inflammatory-induced neoangiogenesis *in vivo*. For example, decorin is associated with areas of artherosclerotic plaque neovascularisation (Gutierrez et al., 1997b), and co-localises with TGF-β in macrophage-rich plaque cores (Evanko et al., 1998). Decorin expression is also associated with endothelial cells involved in neo-angiogenesis *in vivo* in human granulomatous tissue (Schönherr et al., 1999). This observation was confirmed and extended (Nelimarkka et al., 2001), whereby decorin expression was demonstrated to correlate with the presence of macrophages in neovessels formed under inflammatory conditions; granulation tissue of healing dermal wounds, in pyogenic granulomas and in giant cell arteritis. In contrast, neoangiogenesis in ovarian tissue, commonly not associated with inflammation (Ross, 1999) correlated with few macrophages and an absence of decorin (Nelimarkka et al., 2001).

In a co-culture *in vitro* model of angiogenesis, fibroblast derived factors induced endothelial cell expression of decorin (Schönherr et al., 1999). It was subsequently shown that the inflammatory cytokines IL-6 and IL-10 could stimulate decorin expression in endothelial cells (Strazynski et al., 2004). Under these conditions, enhanced endothelial cell survival and tube formation was observed, and adenovirally induced decorin expression was sufficient for these angiogenic phenomena to occur (Schönherr et al., 1999). Further, endothelial cells undergoing spontaneous angiogenic phenomena *in vitro* were found to synthesise decorin which was associated with both tube-like structures and adjacent cells (Jarvelainen et al., 1992).

The decorin knockout mouse develops without deficiencies in either vasculogenesis or angiogenesis (Danielson et al., 1997). However neither of these events would be expected to be associated with inflammation. Under inflammatory conditions, reduced postnatal angiogenesis was observed in the decorin knockout mouse in response to wounding of the cornea (Schönherr et al., 2004). Conversely, enhanced angiogenesis was observed during dermal

wound healing in the absence of decorin (Jarvelainen et al., 2006). Interestingly, diabetic patients commonly develop retinopathy (enhanced angiogenesis in the eye) (Arfken et al., 1998; Gariano and Gardner, 2004) but defects in dermal wound healing, mediated at least in part, by decreased angiogenesis (Sivan-Loukianova et al., 2003). These studies indicate that angiogenesis is differentially regulated in these tissues. Endothelial tube formation is a key process in angiogenesis. Decorin expression enhances tube formation in collagen lattices (Schönherr et al., 1999). However, as a substrate, decorin inhibited collagen-induced tube formation (Davies et al., 2001). In contrast, exogenous decorin was found to have no influence on (Grant et al., 2002), or to inhibit, matrigel-induced tube formation (Sulochana et al., 2005). Tube formation is a complex process requiring extensive rearrangement of cell-matrix interactions. Therefore, investigation of decorin effects on cell adhesion and migration are crucial in understanding the role of decorin in angiogenesis. Decorin influences cell adhesion and migration however conflicting effects have been reported (discussed in detail in Chapter 3). Furthermore, many studies have focussed on decorin effects on fibroblasts rather than on endothelial cells and a definitive mechanism for decorin effects on these integrin-mediated events does not exist (discussed in detail in Chapter 3).

1.7.3.1 The Relevance of Decorin Effects on Endothelial Cells

A more specific role of decorin in inflammatory-mediated angiogenesis is illustrated by the differential roles of decorin and the related proteoglycan, biglycan. These proteoglycans exert many similar effects in vitro, for example on fibroblast migration (Tufvesson and Westergren-Thorsson, 2003) and adhesion (Bidanset et al., 1992b). However, differential spatial and contextual roles clearly exist. Increased synthesis of biglycan, but not decorin occurred in migrating endothelial monolayers. FGF released from wounded cells was responsible for upregulation of biglycan (Kinsella et al., 1997; Kinsella and Wight, 1986). This study does not preclude a role for decorin in cell migration in general, but indicates differential roles for these proteoglycans. Indeed, their expression is differentially regulated; endothelial cells constitutively express

biglycan, but not decorin (Jarvelainen et al., 1991). However, when endothelial cells undergo angiogenic morphogenesis, decorin is expressed (Jarvelainen et al., 1992) and decorin expression is associated with neovessels in inflammatory conditions in vivo (Nelimarkka et al., 2001; Schönherr et al., 1999). Localisation of these proteoglycans also differs; decorin is generally associated with fibrillar matrices, while biglycan is pericellular (Bianco et al., 1990). These data indicate roles for decorin specifically during matrix remodelling and in control of endothelial cell behaviour during inflammatory angiogenesis.

1.7.4 Decorin as a Signalling Molecule

Decorin has binding affinity for matrix components, growth factors, growth factor receptors and integrins. Thus decorin has the capacity to influence angiogenesis through multiple mechanisms. Moreover, decorin influence on matrix organisation also presents an indirect mechanism for decorin effects. This level of complexity may make it difficult to separate different effects of decorin, leading to some conflicting results in the literature.

1.7.4.1 Decorin, Cytokine and Growth Factor Signalling

Decorin can interact with various factors, including TNF-α (Tufvesson and Westergren-Thorsson, 2002), PDGF (Nili et al., 2003), FGF-2 (Penc et al., 1998) and IFN-γ (Brooks et al., 2000). Both decorin and biglycan bind to the active form of TGF-β *in vitro* (Hildebrand et al., 1994), inhibiting its activity (Kolb et al., 2001). However, in TGF-β-induced pulmonary fibrosis only decorin could decrease TGF-β levels in bronchoalveolar fluid, thereby inhibiting fibrosis (Kolb et al. 2001). The authors suggest that the localisation of decorin *in vivo* accounts for its anti-fibrotic effects (Kolb et al., 2001). Decorin sequesters TGF-β in a collagenous matrix, preventing it from interacting with its receptors, while biglycan is associated pericellularly or with the cell surface (Bianco et al., 1990), hence its binding to TGF-β might not prevent receptor interactions. The importance of decorin sequestration of TGF-β in the ECM is supported by a study on TGF-β-induced osteosarcoma cell-mediated contraction of collagen gels (Markmann et al. 2000). Exogenous decorin could not influence

contraction, while decorin expression inhibited TGF-β effects. Therefore, decorin bound to TGF-β in the media could not inhibit it from binding to its receptor. However when decorin was expressed and collagen-associated, TGF-β activity was inhibited by sequestration in the matrix (Markmann et al., 2000). In agreement with this hypothesis, it has also been shown that decorin has separate binding sites for collagen type I and TGF-β, therefore has the potential to bind both molecules simultaneously (Schönherr et al., 1998).

1.7.4.2 Decorin Signalling in Cancer – in vitro Studies

In transformed cells that over-express the ErbB family of receptors, decorin suppresses proliferation *via* a common mechanism. A number of studies together demonstrated that decorin signals through ErbB receptors, activating MAPK, and leading to upregulation of the CDKI p21, resulting in arrest of the cell cycle in G_1 and down-regulation of the receptor (Santra et al. 1995, DeLuca et al. 1996, Santra et al. 1997, Moscatello et al. 1998, Iozzo et al. 1999b, Santra et al. 2000, Csordas et al. 2000). For example, decorin expression in human colon cancer cells arrests cells in G_1 (Santra et al., 1995), *via* activation of EGFR (Santra et al., 1997). Decorin interactions with EGFR (ErbB1), induces receptor dimerisation and autophosphorylation (Iozzo et al., 1999b), activation of MAPK (Moscatello et al., 1998), calcium mobilisation (Patel et al., 1998) and p21 upregulation (De Luca et al., 1996; Santra et al., 1997). Long-term exposure down-regulates the receptor (Csordas et al., 2000). Similarly, in a breast cancer cell line over-expressing ErbB2, decorin activates ErbB4, which interacts with ErbB2, leading to inactivation and down-regulation of ErbB2, p21 up-regulation, and growth suppression (Santra et al., 2000). In a number of neoplastic cell lines, decorin also suppressed cell growth *via* up-regulation of p21 resulting in arrest of the cell cycle in G_1 (Santra et al., 1997). In these studies, it was shown that the decorin core is sufficient for these effects, and that signalling occurs irrespective of whether decorin is presented exogenously or endogenously.

1.7.4.3 Decorin Signalling in Cancer – in vivo Studies

Results obtained from the *in vitro* experiments discussed above are also relevant *in vivo*. When injected into nude mice, decorin-expressing breast cancer cells failed to form tumours compared to controls (Santra et al., 2000). In pre-established tumours, adenoviral decorin gene delivery decreased tumour proliferation and invasiveness, mediated at least in part through EGFR signalling (Reed et al., 2002). Similarly, in pre-established breast cancer tumours, decorin suppressed growth *via* long-term down-regulation and inactivation of ErbB2, and additionally decreased lung metastases (Reed et al., 2005). Further, decorin expression blocked squamous carcinoma tumour growth by down-regulation of the EGFR (Csordas et al., 2000). Tumours originating from three different backgrounds, induced to express decorin, exhibited reduced growth *in vivo* compared to wild-type, and reduced neovascularization was also observed in two of the three tumours. This effect was attributed to decorin-mediated reduction of VEGF expression (Grant et al., 2002). Interestingly, some evidence of cell differentiation was also observed in the presence of decorin (Reed et al., 2002), similarly, in mammary carcinoma cells some differentiation was also observed in the presence of decorin (Santra et al., 2000). Further to this, low levels of decorin expression are associated with larger tumours, increased recurrence and a poor outcome in invasive breast cancer (Troup et al., 2003). Decreased levels of decorin were also found in many ovarian tumours and ovarian tumour cell lines (Shridhar et al., 2001). The decorin knockout mouse does not spontaneously develop tumours; however the p53-decorin double knockout mouse has an increased rate of thymic lymphoma tumour development over the p53-null mouse alone, supporting an anti-oncogenic role for decorin (Iozzo et al. 1999a). The absence of decorin therefore, appears to be permissive for tumour development in a background of cancer predisposition (Iozzo et al., 1999a).

1.7.4.4 Decorin Signalling in non-cancer cell types

In fibroblasts, decorin may play a role in control of proliferation. Decorin inhibits fibroblast proliferation; indeed it becomes upregulated as fibroblasts become quiescent (Mauviel et al., 1995), while in the decorin knockout mouse, enhanced fibroblast proliferation occurred in periodontal ligament (Häkkinen et al., 2000). Interestingly, decorin restored normal phenotype independently of EGFR activation and p21 induction (Häkkinen et al., 2000). However, these effects may be attributed to decorin effects on TGF-β signalling, since decorin neutralises the proliferative effects of this growth factor in arterial smooth muscle cells (Fischer et al., 2001), and in chinese hamster ovary (CHO) cells (Yamaguchi et al., 1990).

Decorin does not exclusively signal through ErbB receptors since decorin is also an alternative ligand for the IGF-I receptor in endothelial cells (Schönherr et al. 2005). In a mechanism reminiscent of decorin signalling through EGFR, the IGF-IR is first activated by decorin, resulting in upregulation of p21 and in long-term down-regulation of the receptor (Schönherr et al., 2005). The related CDKI p27 was also induced by decorin. This phenomena has also been observed in other non-neoplastic cell lines such as aortic smooth muscle cells (Fischer et al., 2001), macrophages (Xaus et al., 2001) and neuronal progenitor cells (Santra et al., 2006).

Upregulation of p27 by decorin however appears to be more specific to non-neoplastic cells, since an investigation of a number of neoplastic cells demonstrated that decorin upregulated p21 but not p27 (Santra et al., 1997) with one exception (U-87 glioblastoma cells) (Santra et al., 2006). In endothelial cells decorin induced p21 through the IGF-IR and the serine/threonine kinase Akt (Schönherr et al 2005). However p27 was upregulated *via* an undetermined Akt independent pathway (Schönherr et al., 2001a). Similar to interactions with the EGFR, decorin interacts with the IGFR *via* the core protein, but possesses distinct binding sites for each receptor (Schönherr et al., 2005). In endothelial cells, macrophages and neuronal progenitor cells, decorin suppresses apoptosis (Santra et al., 2006; Schönherr et al., 1999; Xaus et al., 2001), while cancer cell

lines undergo apoptosis in response to decorin (Tralhao et al., 2003). Many tumours express high levels of IGF-IR (Haddad and Yee, 2006). Thus it does not seem unreasonable to suggest that decorin could also signal through IGF-IR in cancer cells. It is not known yet whether this mechanism of decorin signalling is relevant to tumours. Further, whether decorin signals preferentially through IGF-IR or EGFR on a particular cell type could depend on factors such as receptor expression, availability and relative binding affinities. However, decorin-mediated down-regulation of IGF-IR is relevant to *in vivo* situations. Increased expression of IGF-I receptor was observed in neovessels of wounded cornea in the decorin knockout mouse (Schönherr et al., 2005). Further, increased expression of IGF-IR was also observed in uninjured cornea in the absence of decorin. In a model of kidney disease, apoptosis of kidney tubular epithelial cells was enhanced in the absence of decorin, and increased levels of the IGF-I receptor were observed (Schönherr et al., 2005). Additionally, both IGF-I and decorin signal through the same pathway to upregulate fibrillin-1 in renal fibroblasts (Schaefer et al., 2007). Further, in a diabetes model induced in decorin-null mice, the IGF-I receptor was up-regulated in the kidney, however, this could not compensate for the lack of decorin in regulation of fibrillin-1 synthesis (Schaefer et al., 2007).

A major difference between signalling pathways activated by decorin appears to be the mechanism of p21 upregulation. In many neoplastic cell lines, p21 is upregulated through activation of MAPK *via* ErbB receptors (see section 1.7.4.2). In non-neoplastic cells such as endothelial cells, decorin activated the PI3-K dependent protein, Akt, through the IGF-I receptor to upregulate p21 (Schönherr et al., 2001a; Schönherr et al., 2005). Decorin also upregulated p21 in neural progenitor cells and neoplastic neural cells, but this was independent of PI3-K activation. The mechanism of p21 upregulation also did not involve signalling through ErbB receptors (Santra et al., 2006). Interestingly, in the same study, it was found that PI3-K was required for decorin-mediated enhancement of cell survival, but alternative pathways were required for upregulation of p21.

1.7.4.5 The Role of Decorin in Angiogenesis

A combination of different biological activities, including direct and indirect effects of decorin, could be responsible for influence of decorin on angiogenesis. For example, decorin regulates both collagen (Danielson et al., 1997; Vogel KG, 1984) and fibronectin fibrillogenesis (Kinsella et al., 2000) and influences matrix composition through modulation of MMP expression by fibroblasts (Al Haj Zen et al., 2003) and endothelial cells (Schönherr et al., 2001b). Matrix organisation influences cellular responses to growth factors, therefore decorin could modulate endothelial cell behaviour indirectly through these mechanisms. Decorin sequestration of the pro-fibrotic TGF-β (Schönherr and Hausser, 2000) could also play a role. Through manipulating the structure and composition of the extracellular matrix, decorin could alter integrin-ligand interactions, thus modulating integrin-mediated events such as cell adhesion and migration. However, much data exists to support a direct role for decorin in influencing these processes.

Decorin influences cellular proliferation, differentiation, survival, adhesion and migration, therefore has the capacity to influence multiple aspects of angiogenesis. Signals from the extracellular matrix *via* integrins, and from growth factors to their receptors coordinate to control all aspects of angiogenesis. Decorin can signal directly through the IGF-I receptor in endothelial cells (Schönherr et al., 2005). Immobilised decorin was also shown recently to support platelet adhesion through interactions with, and signalling through the collagen-binding α2β1 integrin (Guidetti et al., 2002). Therefore decorin could interact directly with both growth factor receptors and cell-surface matrix receptors to activate signalling pathways involved in control of endothelial cell behaviour.

1.8 Aims

Decorin expression is associated with endothelial cells undergoing angiogenic morphogenesis both *in vivo* and *in vitro*, and angiogenesis is dysregulated in the absence of decorin. However, many of the mechanisms by which decorin controls endothelial cell behaviour and contributes to regulation of angiogenesis are unknown. The overall objectives of this thesis are to investigate the role of decorin interaction with collagen type I, α2β1 integrin and IGF-I receptor in the control of endothelial cell behaviour.

The hypotheses to be tested are:

(i) Decorin influences endothelial cell behaviour by directly signalling through cell-surface receptors, rather than by sterically blocking integrin interaction with matrix ligands

(ii) The presentation of decorin to endothelial cells as either a soluble effector or as a matrix-associated component differentially contributes to control of endothelial cell behaviour (i.e. attachment, adhesion, migration)

The specific aims to achieve these objectives are:

1. To determine whether decorin influences endothelial cell behaviour by signalling through IGF-IR and α2β1 integrin
2. To determine how decorin interacts with α2β1 integrin i.e. where is the binding site located on the integrin, and which moiety of decorin is responsible for this interaction
3. To determine which signalling pathways downstream of IGF-IR and α2β1 integrin (or other cell-surface receptors) are activated by decorin in endothelial cells and how this relates to modulation of endothelial cell behaviour by decorin

Chapter 2

Decorin purification and characterisation

2.1 Introduction

The related class I proteoglycans decorin and biglycan were first isolated as a mixture from bovine tendon in 1975 by Anderson (Anderson, 1975) and were later isolated also from bovine sclera (Cöster and Fransson, 1981), calf skin (Fujii and Nagai, 1981), porcine skin (Damle et al., 1982), and bovine periodontal ligament (Pearson and Gibson, 1982). Two distinct populations of proteoglycan were observed in bovine sclera (Cöster and Fransson, 1981) and in cartilage (Rosenberg et al., 1985) and bone extracts (Fisher et al., 1987), and the presence of two distinct protein cores was confirmed (Fisher et al., 1987; Heinegård et al., 1985; Rosenberg et al., 1985). The larger of these proteoglycans was termed PG I and the smaller PG II (Cöster and Fransson, 1981); now known as biglycan and decorin respectively.

cDNAs encoding decorin have been isolated from human (Krusius and Ruoslahti, 1986), bovine (Day et al., 1987), chicken (Li et al., 1992) and rat (Abramson and Woessner, 1992), and the complete human decorin gene sequence was published in 1993 (Danielson et al., 1993). With the exception of chicken decorin which differs in the N-terminal region (Li et al., 1992), decorin is highly conserved between species (Abramson and Woessner, 1992). Decorin is composed of a small protein core (40-45KDa) to which a single glycosaminoglycan is covalently attached at serine 4 (Chopra et al., 1985). Decorin also contains six conserved cysteines (4 near the N-terminus and 2 near the C-terminus), participating in 3 disulphide bridges (Scott et al. 1986). Characterization of decorin expressed by human skin fibroblasts demonstrated two populations of decorin, with either 2 or 3 N-linked oligosaccharides (Glossl et al. 1984), in agreement with the protein sequence, which indicated three possible N glycosylation sites (Krusius and Ruoslahti, 1986).

Structurally, decorin was originally proposed to adopt a monomeric 'horseshoe-like' conformation (Weber et al., 1996), based on the crystal structure of another LRR-containing protein, ribonuclease inhibitor (Kobe and Deisenhofer, 1993). However the crystal structure of decorin revealed a more open 'banana-shaped' conformation, and further, indicated a dimeric structure

mediated by interactions between the concave surfaces of decorin (Scott et al., 2004). The considerably different structures seen for these two LRR proteins (decorin and ribonuclease inhibitor) is likely to be a consequence of the shorter LRR regions found in decorin (McEwan et al., 2006). However the nature of this methodology forces proteins into the most energetically favourable conformation to facilitate crystal formation. Thus whether decorin exists as a dimer under biologically relevant conditions is controversial.

Early light scattering studies on decorin in solution suggested the presence of large aggregates (Cöster et al. 1981, Zangrando et al. 1989). However, recent light and X-ray scattering analyses of decorin in solution (Scott et al., 2006; Scott et al., 2003), and analytical ultracentrifugation studies (Scott et al., 2004) detected dimers. The aggregated material seen previously was suggested to have been an artefact of decorin precipitation and/or lyophilisation during preparation. This material exhibited strong light scattering properties, and likely obscured light scattering by decorin dimers (Scott et al., 2003). Indeed, a small proportion of the decorin population had earlier been visualised as large aggregates by rotary shadowing electron microscopy, while the majority of the population was dimeric (Ward et al., 1987). Decorin dimers however, could not be cross-linked in solution, and multimers were only observed when decorin was lyophilised prior to analysis, thus were proposed to also be artefacts of this procedure (Goldoni et al., 2004). While decorin dimers were originally observed in lyophilised preparations (Scott et al. 2003), they could still be detected when decorin was purified in the absence of chaotropes and without lyopilisation (Scott et al., 2004). Interestingly, where decorin dimers could not be detected by use of cross-linking agents, a detergent, CHAPS, was present in decorin preparations (Goldoni et al., 2004). This may have interfered with protein-protein interactions. Whether decorin exists as a monomer or dimer could be dependent on decorin isolation procedures. It would certainly be relevant to consider whether decorin interacts with cell-surface receptors as a monomer or a dimer or whether these forms contribute differentially to different functions of decorin. Given the controversy of the current understanding however, these questions cannot be answered at this juncture.

Decorin has also been suggested to self-associate through the glycosaminoglycan (GAG) moiety. Free dermatan sulphate (DS) was shown to promote decorin aggregation (Cöster et al. 1981), and co-eluted with decorin after gel filtration (Fransson et al. 1982). Indeed, free GAG also self-associates (Fransson, 1976). Of the GAGs that can be attached to decorin, free DS has the highest propensity to self-associate, in an anti-parallel fashion (Scott, 1992; Scott, 1995). Chondroitin-6 sulphate (C-6S) can associate to some extent, while chondroitin-4 sulphate (C-4S) cannot (Scott, 1992; Scott, 1995). However, association of decorin with DS was later observed only when DS was prepared by tryptic digestion (yielding a polysaccharide linked to at least 14 amino acids) (Bittner et al., 1996). It was suggested that ionic interactions between intact decorin and DS-associated peptides could therefore be responsible for association under these conditions (Bittner et al., 1996). Visualisation of decorin as a monomer (Keene et al., 2000), dimer (Ward et al., 1987), or as large aggregates (Scott et al., 1990; Ward et al., 1987) by rotary shadowed electron microscopy also adds to the controversy. However, the different methods by which decorin was obtained in these studies; as a recombinant Histidine-tagged core protein (Keene et al., 2000) or as tissue-extracted proteoglycan (Ward et al., 1987; Scott et al., 1990) may have contributed to these conflicting observations. Interesingly, where aggregates were observed, these were mediated by core-protein rather than GAG interactions (Ward et al., 1987; Scott et al.,1990).

Decorin is traditionally extracted from tissues using strongly denaturing solutions of 4 M or 6 M guanidine hydrochloride (GdnHCl). Extracted proteins are commonly separated chromatographically in the presence of another chaotropic agent, urea. Many studies have used such preparations successfully to characterise proteoglycan composition in normal (Heinegard et al., 1986; Pearson and Gibson, 1982) or disease states (Hall et al., 1996; Vijayagopal et al., 1996) or in cell culture (Kinsella and Wight, 1988). However, when investigating the biological activities and functions of decorin, preservation of the native structure is imperative since differences in biological activities of decorin have been observed between decorin purified under non-denaturing and denaturing conditions. For instance, decorin extracted from cartilage with

GdnHCl could not effectively compete with native decorin for binding to collagen type I (Svensson et al., 1995), while tissue-derived decorin had lower TGF-β binding activity than recombinant decorin (Hildebrand et al., 1994). These different activities of decorin preparations could be explained by variations in oligosaccharide and/or GAG composition. However other studies indicate that denaturing conditions adversely influence decorin activity independently of these modifications. Natively purified recombinant decorin core protein had higher activity than tissue-derived proteoglycan in collagen fibrillogenesis assays. When core protein was denatured however, and denaturant removed, core protein activity was decreased to the same level as tissue-derived decorin proteoglycan (Gu et al., 1997). Further, decorin is endocytosed *via* the core protein (Hausser et al., 1989), but when purified under denaturing conditions decorin uptake was decreased (Truppe and Kresse, 1978). Decorin activities are also extremely sensitive to storage conditions, since decorin-mediated inhibition of fibroblast adhesion was abrogated by repeated freeze-thaw or by storage of decorin at less than 0.5mg/ml (Winnemoller et al., 1991).

Decorin activity could be dependent on the native structure of the proteoglycan, which may be compromised by use of denaturing reagents. In some studies, decorin purified under denaturing conditions could not refold to the native conformation following restoration of non-denaturing conditions. The circular dichroism (CD) spectra of cartilage-extracted or recombinant decorin purified in GdnHCl indicated irreversible loss of secondary structure after chaotrope removal (Ramamurthy et al., 1996). Denaturation of recombinant decorin core protein with GdnHCl also did not appear to be reversible in another study, since core protein activity in a collagen fibrillogenesis assay could not be regained (Gu et al., 1997). However, other studies suggest that decorin denaturation is reversible; urea-induced (Brown et al., 2002) and GdnHCl-induced (Scott et al., 2006) denaturation of decorin was reversible on chaotrope removal. Thermal denaturation was similarly reversible (Scott et al., 2006), but could not be reversed in another study (Brown et al., 2002). The presence of reducing agents abolished decorin refolding, confirming a critical role for

disulphide bridge retention in refolding of decorin (Scott et al., 2006). Indeed, the conformational stability of decorin was demonstrated previously to be dependent on disulphide bridges for collagen interactions (Scott et al., 1986). Recently, the N-terminal cysteine clusters of decorin have been suggested to form a disulphide knot and act as a capping motif, representing a structural element contributing to the stability of the LRR regions of the protein (McEwan et al., 2006). It has been suggested that BSA can act as a chaperone and is required for correct refolding of decorin peptides (Schönherr et al., 1995). However, GdnHCl-induced loss of secondary structure was reversible in the absence of BSA (Scott et al., 2006). Further, within another study, urea-induced unfolding was reversible while thermal denaturation was not (Brown et al., 2002). These studies indicate that multiple factors are likely to influence decorin refolding.

With the availability of decorin cDNA since 1986, decorin has been recombinantly expressed, eliminating the need for use of chaotropic agents. Decorin has thus been expressed in a number of mammalian systems. The vaccinia virus/T7 bacteriophage system was used to express a polyhistidine-decorin fusion protein (His-Dcn) in HT-1080 human fibrosarcoma cells, subsequently purified using metal affinity chromatography (Ramamurthy et al., 1996). Recombinant decorin proteoglycan has also been produced in the mammalian cell lines HeLa (Svensson et al., 1995, Sugars et al., 2002), HEK293 (Kresse et al., 1997) and CHO (Santra et al., 1997) by stable transfection with human decorin cDNA. Decorin core protein has also been recombinantly expressed as a maltose-binding protein fusion protein (MBP-Dcn) (Hering et al., 1996) and as a MBP and His-tagged fusion protein (Hildebrand et al., 1994) in *Escherichia coli*, and untagged, in insect cells (Gu et al., 1997).

Many studies however have continued to include chaotropic agents for efficient purification of recombinant decorin (Hildebrand et al., 1994; Svensson et al., 1995; Hering et al., 1996; Santra et al., 1997; Sugars et al., 2002). Decorin has also been purified from secretions of cells that naturally express this proteoglycan, for example myofibroblasts (Honda and Munakata, 2004) and fibroblasts (Glossl et al., 1984; Kresse et al., 1997). However decorin was

precipitated by ammonium sulphate (Glossl et al., 1984; Kresse et al., 1997) or was lyophilised (Honda et al., 2004) in these studies, and this may result in formation of oligomers with reduced activities (Goldoni et al., 2004). Such oligomers had reduced activity in collagen fibrillogenesis assays, and reduced ability to activate EGFR (Goldoni et al., 2004). Commercially available, natively purified and lyophilised recombinant decorin (EMP-genetech, Ingolstadt, Germany) also exhibited reduced activity in biological assays (Johannes Eble, personal communication). His-Dcn (Ramamurthy et al., 1996) has been isolated under non-denaturing conditions and without lyophilisation. However, histidine tags can interfere with His-Dcn activity in collagen fibrillogenesis assays (Claus Ruehland, personal communication). Few studies have utilised decorin proteoglycan which has been natively purified and not subjected to precipitation and/or lyophilisation.

Of relevance to this thesis, signalling properties of decorin may be sensitive to purification procedures. His-Dcn or decorin core protein suppressed carcinoma cell growth through EGFR-mediated signalling (Moscatello et al., 1998). However MBP-Dcn was completely ineffective in the same study, despite previous demonstration that both His- and MBP-tagged decorin could effectively bind to TGF-β (Hildebrand et al., 1994). This would suggest that different folding states of decorin could exhibit some expected properties but still be inactive in other functions of decorin. Further, thermal denaturation of decorin or the core protein prevented binding to EGFR (Iozzo et al., 1999), while freeze-thaw abrogated decorin interactions with EGFR and MAPK pathway activation by decorin (Grant et al., 2002), suggesting that signalling properties of decorin are particularly sensitive to treatment of preparations.

Purification of decorin under non-denaturing conditions has proved difficult due to losses during purification and is associated with low yields. Inclusion of either GdnHCl or Triton X-100 throughout purification increased yields 2-3 fold (Ramamurthy et al. 1996) and 0.1% Triton X-100 was critical for good recovery of decorin from anion-exchange media (Schaefer et al., 2000). Propylene glycol has also been suggested to improve recovery of decorin core protein (Brown et al., 2002). For ease of purification, many studies therefore use

tissue-derived decorin purified under denaturing conditions or natively purified His-Dcn. These sources of decorin are verified by demonstrating known binding properties of decorin. However, as discussed above, this material may not be suitable to study signalling properties of decorin. Indeed, different decorin preparations exhibit different properties in biological assays relevant to angiogenesis (Davies et al., 2001; Sulochana et al., 2005). In the latter study (Sulochana et al., 2005), decorin was recombinantly expressed and purified from insect cells and lyophilised (R and D systems Inc, Minneapolis, MN), or was purified under dentaturing conditions from cartilage and also lyophilised (Sigma-Aldrich), and the recombinant decorin preparation was less effective at inhibiting tube formation than cartilage-derived decorin (Sulochana et al., 2005). In the former study (Davies et al., 2001), natively purified decorin from human skin fibroblasts (obtained from Dr. Hans Kresse, and purified as in (Hausser et al., 1989)) was less effective at inhibiting tube formation than cartilage-derived decorin (Davies et al., 2001). These studies indicate that different treatments and/or origin of decorin may result in different activities, although contributions of differential decorin post-translational modifications cannot be excluded.

Given that certain biological properties of decorin are particularly sensitive to purification and storage conditions, it is imperative to use decorin purified under non-denaturing conditions for signalling studies. Also, recombinantly produced decorin may not only result in proteoglycan that is fully post-translationally processed, but also in many intermediates. For this reason, human skin fibroblasts were used as a source of naturally expressed decorin proteoglycan. Care was taken to preserve biological functions of decorin through omission of denaturation and precipitation steps during purification, although initial steps require inclusion of 0.1% Triton X-100 to prevent large losses in recovery. This Chapter details the purification procedures for decorin proteoglycan and core protein used throughout the study, and verifies the use of this decorin preparation in investigation of signalling properties of decorin.

2.2 Materials and Methods

2.2.1 Cell Culture and Maintenance

Human skin fibroblasts (Muenster University, Germany) were maintained in, and conditioned media collected in, MEM containing glutamine (minimum essential medium, Gibco, Invitrogen) supplemented with 10% FCS v/v (foetal calf serum, S 0113, Biochrom AG, Berlin, Germany or Gibco, Invitrogen, UK), NEAA (non-essential amino acids) and 100 units/ml penicillin, 100μg/ml streptomycin (both Sigma-Aldrich, UK). Cells were maintained in T-75 flasks (Greiner Bio-one, Gloucestershire, UK) and passaged on reaching 70% to 90% confluency (approximately 1:4 every 10 days). Conditioned media was collected at time of passage and mid-way between passages (every 4 to 7 days). Media was centrifuged at 2,175 x g for 10 minutes at room temperature to pellet cell debris and the supernatant was immediately frozen and stored at -20°C.

The human endothelial cell line EA.hy926 (Edgell et al., 1983) was maintained in MCDB 131 medium (Gibco, Invitrogen) supplemented with 10% FCS, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), 100 units/ml penicillin, 100 μg/ml streptomycin and 2mM glutamine (all Gibco, Invitrogen). Cells were passaged on attaining 70% to 90% confluency and split to 1 in 5 to 1 in 10 for re-seeding. Cells were detached with 2ml trypsin (Gibco, Invitrogen, UK) per T-75 flask (Greiner Bio-One, UK). Trypsin activity was stopped by addition of 8ml growth media, and cells collected by centrifugation at 725 x g for 5 min. Cells were resuspended in 10ml growth medium, and 1 to 2ml was re-seeded. The EA.hy926 cell line was generated by fusion of human umbilical vein endothelial cells (HUVEC) with the immortalised human lung epithelial carcinoma cell line A549, and retained the signalling characteristics of HUVEC rather than A549 cells. Decorin activated the same signalling pathways in both EA.hy926 and the parent HUVECs (Schönherr et al., 2001). Further, decorin induced apoptosis of A549 cells (Tralhao et al., 2003) but enhanced HUVEC or EA.hy926 cell survival (Schönherr et al., 1999). Further properties of the EA.hy926 cell line have been reviewed (Bouis et al., 2001).

2.2.2 Decorin Purification

2.2.2.1 Initial Purification using Anion Exchange, DEAE-Sepharose

Upon defrosting, fibroblast-conditioned media was supplemented with protease inhibitors to a final concentration of 100mM 6-aminohexanoic acid (a competitive inhibitor of various proteases including fibrinolysin, chymotrypsin) 10mM ethylene-diaminetetraacetic acid (EDTA, inhibits MMP activity by chelating metal ions required for activity), 1mM phenylmethylsulphonylfluoride (PMSF, irreversibly inhibits serine proteases e.g. plasmin, thrombin) and 5 mM benzamidine (a competitive inhibitor of serine proteases) (from a 10x protease inhibitor stock solution). Triton X-100 was added to give a final concentration of 0.1%, and the pH adjusted to pH7.5 using a solution of 1M Tris-HCl, pH8.0. Media was filtered by gravity through Whatman filter paper (Number 4, Whatman International, Maidstone, UK) and collected in a 1 litre duran immersed in ice. 1 litre of media was used for purification by anion-exchange chromatography (column 1.5 cm x 30 cm, DEAE-Sepharose Fast Flow, Amersham Biosciences, GE Healthcare, Bucks, UK). The column was equilibrated with 0.15M NaCl, 20mM Tris-HCl, pH7.4, 0.1% Triton X-100 and protease inhibitors. All steps were carried out under gravity at 4°C. Unbound proteins were removed by application of 150ml equilibration buffer, and bound contaminating proteins eluted with 150ml of 0.4M NaCl, 20mM Tris-HCl, pH7.4, 0.1% Triton X-100 and protease inhibitors, followed by 200ml of 0.4M NaCl, 20mM Tris-HCl, pH7.4 and discarded. Remaining bound proteins, including decorin, were eluted with 1M NaCl, 20mM Tris pH7.4. A fraction of 15ml was collected initially, and a further 5 fractions of 25ml were manually collected. Protein content within fractions was monitored spectrophotometrically at 280nm. DNA contamination was assessed by monitoring absorbance at 260nm. Fractions containing more protein than in fraction 1 were pooled, and typically contained decorin, BSA, and several unknown high molecular weight proteins. DEAE-Sepharose matrix was regenerated by application of 300ml 1M NaOH followed by 200ml ddH_2O. The column and apparatus was stored in 20% ethanol (v/v ddH_2O).

2.2.1.2 Further Purification using Anion Exchange, Source Q15

Partially purified decorin obtained from DEAE-Sepharose step was applied to a second anion-exchange matrix, Source Q15 (column 1 cm x 9.5 cm, Amersham Biosciences, GE Healthcare, UK), coupled to an Akta Prime chromatography system. Purification was carried out at 4°C and all buffers were degassed and filtered (0.22μm pore nitrocellulose discs, Millipore, UK) using a vacuum pump (Edwards RV5, BOC Ltd., Sussex, UK). Decorin purified from three litres of media on DEAE-Sepharose matrix was combined for application to Source Q15 matrix. To reduce salt content for binding to anion-exchange media, partially purified decorin preparation was diluted to less than 0.4M NaCl by addition of ddH_2O. Source Q15 matrix was equilibrated in 0.15M NaCl, 20mM Tris-HCl, pH7.4 at a flow rate of 1ml/min. The diluted decorin solution was applied overnight at a flow rate of 1ml/min. After loading, the column was washed with buffer A (0.4M NaCl, 20mM Tris-HCl, pH7.4) at a flow rate of 1ml/min until stable conductivity was reached. A gradient of 0 to 50% buffer B (1M NaCl, 20mM Tris-HCl, pH7.4, mixed with buffer A) was applied at a flow rate 0.8ml/min over 18ml and 0.8ml fractions collected. Absorbance at 280nm and conductivity were monitored and the values at the start of each fraction recorded. After elution was complete, a step elution of 100% buffer B at a flow rate of 1ml/min was applied and 1ml fractions collected. Decorin eluted in this step. After elution was complete, Source Q15 matrix was regenerated with 60ml 1M NaOH, followed by 60ml ddH_2O and finally 60ml 20% ethanol (v/v in ddH_2O). Fractions were analysed for the presence of decorin by dot blotting as described in section 2.2.4.5.

2.2.1.3 Decorin Storage

Purified decorin eluted from Source Q-15 matrix was pooled. Decorin was buffer exchanged into sterile PBS (phosphate buffered saline, Dulbecco A tablets, Oxoid, Basingstoke, UK) and concentrated by ultrafiltration (Centriprep-10 concentrators, Amicon, Millipore, UK) to no less than 0.5mg/ml. Briefly, ultrafiltration devices were rinsed with 20% ethanol (v/v in ddH_2O) and rinsed repeatedly with sterile water. Purified decorin was transferred to the ultrafiltration device and centrifuged twice at 3,000 x g for 40 min to reduce sample volume. Decorin was diluted from approximately 4ml to 16ml with sterile PBS and centrifuged repeatedly until sample volume was reduced to approximately 4ml. Decorin was similarly diluted with PBS and concentrated a further two times for buffer exchange into PBS, finally concentrated to 500μL, aliquoted and stored at -20°C to avoid repeated freeze-thaw.

2.2.3 Core Protein Preparation

2.2.3.1 Chondroitinase ABC Lyase Digestion

To obtain decorin core protein, the GAG moiety was removed by exhaustive digestion with chondroitin ABC lyase (protease free, EC 4.2.2.4, Seikagaku Corporation, Kogyo, Tokyo, Japan). Decorin was digested with 0.2 milliunits (mU)-2mU enzyme per μg decorin in 0.1M Tris-HCl, pH8.0, 0.03M sodium acetate at 37°C for a minimum of 90 minutes. For large-scale production of decorin core protein, 580μg of purified decorin in 400μL PBS was incubated with 100mU chondroitin ABC lyase. The total volume was adjusted to 600μL by addition of 120μL 5x buffer (0.5M Tris-HCl, pH8.0, 0.15M sodium acetate) and water. Decorin was digested for 2h at 37°C.

2.2.3.2 Core Protein Purification

Following digestion with chondroitin ABC lyase, the unwanted proteins/oligosaccharides present are the enzyme, BSA (contained in the enzyme preparation) and digested glycosaminoglycan. Decorin core protein was purified in one step using an anion exchange matrix (DEAE-Trisacryl M, Serva,

Heidelberg, Germany). Chondroitin ABC lyase and BSA bind to the anion-exchange media under low salt conditions, while the core protein cannot (Blochberger et al., 1992). 500µL DEAE-Trisacryl matrix was equilibrated in PBS by repeated centrifugation (2 min, 725 x g) and resuspension in 1ml PBS a total of 5 times. The matrix was incubated with the decorin digestion mixture by mixing continuously overnight at 4°C. The matrix and bound contaminants were collected by centrifugation and the supernatant retained as purified decorin core protein. The matrix was washed with a further 100µL of PBS, and the supernatant similarly collected and pooled with the first supernatant. Core protein was stored in aliquots at -20°C to avoid loss of activity due to repeated freeze-thaw.

2.2.4 Characterisation of Decorin

2.2.4.1 Protein Concentration Determination

Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce International) mixed with 4% $CuSO_4$. In the presence of protein, Cu^{2+} is reduced to Cu^{+}. This monovalent cation is chelated by BCA, forming a coloured product. This reaction is mediated by cysteine, tryptophan and tyrosine residues, by peptide bonds and additionally by overall protein structure (Wiechelman et al., 1988). 20µL samples of unknown concentration were mixed with 200µL protein assay reagent containing 4% $CuSO_4$ in a 96-well microtitre plate (Greiner Bio-One). Samples were incubated at 37°C for 30 min and the plate read at 540nm on a 96-well microplate reader (EL311, BioTek Instruments, Winooski, VT, USA). Protein concentration was calculated using known concentrations of BSA at 50, 250, 500 and 1000 µg/ml to generate a standard curve on the same plate.

2.2.4.2 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2μg or 4μg of decorin was digested with 0.005 units chondroitin ABC lyase as described in section 2.2.3.1. Digested decorin and equal concentration of undigested decorin were separated by SDS-PAGE. Samples were prepared by addition of sample buffer (NuPAGE 4x LDS sample buffer (Invitrogen, UK containing 10% β-mercaptoethanol) and heating to 70°C for 10 min. Samples of 10μL were loaded onto pre-cast 4-12% BisTris NuPAGE polyacrylamide gels, 1.5 mm x 15 well (Novex, Invitrogen, UK). Both reservoirs contained 1x MES buffer (20x MES SDS-PAGE running buffer, NuPAGE, Invitrogen) and proteins were separated by electrophoresis at a constant voltage (200 V, limit 120mA) for 45 min or until proteins had sufficiently separated using a Novex X-Cell SureLock Mini-Cell system, power supply Novex PowerEase 500' (both Invitrogen, UK). 7μL of a pre-stained molecular weight marker (SeeBlue Plus2 pre-stained standard, Invitrogen, UK) was also loaded to visualise protein separation. Proteins were visualised by silver staining (section 2.2.4.3) or were transferred to nitrocellulose for Western blotting (section 2.2.4.4).

2.2.4.3 Silver Staining

Following SDS-PAGE (section 2.2.4.2), protein was visualised by silver staining based on a previously published method (Nesterenko et al., 1994). All chemicals were obtained from Sigma-Aldrich, UK. Gels were fixed in 35% acetone, 8.7% TCA, 10% formaldehyde for 5 min followed by extensive washing with H_2O. Prior to addition of silver reagent, gels were prepared by soaking in 50% acetone for 5 min followed by incubation for 1 min in 0.016% Na_2SO_3. After washing twice with H_2O, gels were incubated in silver reagent for 8 min (0.27% $AgNO_3$, 0.78% formaldehyde). The gels were washed once with water and developed by incubation in 0.01% Na_2SO_3, 0.039% formaldehyde and 2% Na_2CO_3 for approximately 30 seconds or until bands had sufficiently developed. Development was stopped by incubation with 1% acetic acid for at least 2 min and gels were stored in water.

2.2.4.4 Western Blotting

Following SDS-PAGE (section 2.2.4.2), gels were placed onto Protran nitrocellulose membrane (Schleicher and Schuell, Germany) and proteins were transferred to the membrane in 1x transfer buffer (20x Transfer buffer, NuPAGE, Invitrogen, UK) containing 10% methanol and Antioxidant (NuPAGE, Invitrogen, UK) for 2 h at 25mV, 160mA using an X-Cell II blot module in the Novex X-Cell SureLock Mini-Cell system, power supply Novex PowerEase 500 (all Invitrogen, UK). The membrane was removed and blocked with 5% non-fat dried milk (NFDM, Marvel dried milk powder) in TBS (150mM NaCl, 20mM Tris-HCl, pH7.4) containing 0.1% Tween 20. Antibodies were diluted in 1% NFDM in TBS/0.1% Tween 20 unless otherwise indicated. The membrane was washed three times between incubations with TBS/0.1% Tween 20 for 5 min. The membrane was probed with antibodies to human decorin overnight at 4°C (1:500 in 1% BSA/PBS). Polyclonal rabbit antiserum to human decorin has been previously described (Hausser et al., 1992). The antiserum was raised to chondroitin ABC lyase digested decorin, and the IgG fraction isolated (Glossl et al., 1984). This antiserum also recognises the remaining dermatan sulphate stubs (Voss et al., 1986). Bound antibodies to decorin were detected with horseradish peroxidase (HRP) conjugated anti-rabbit IgG antibodies (1:2000, DakoCytomation, UK). Alternatively, for signal amplification, bound antibodies to decorin were detected with biotin-conjugated anti-rabbit IgG antibodies (1:1000, Sigma-Aldrich, UK) followed by HRP conjugated to streptavidin (1:1000, Sigma-Aldrich, UK). Binding was visualised by chemiluminescence using Supersignal (Pierce). The luminol-containing solution was mixed with an equal volume of a peroxide-containing solution. HRP catalyses the oxidation of luminol by peroxide, and the generated product emits light which can be visualised by exposure to photographic film (Hyperfilm ECL, Amersham Biosciences). Where required, densitometric analysis was performed using Quantity One software (Bio-Rad).

2.2.4.5 Detection of Decorin by Dot Blotting

For analysis of decorin contained in fractions during purification on Source Q15, 5µL of each fraction was transferred to nitrocellulose as follows. Two sheets of filter paper (Whatman filter paper Number 4, Whatman International, Maidstone, UK) were soaked in 1x transfer buffer (20x Transfer buffer, NuPAGE, Invitrogen, UK) containing 10% methanol and Antioxidant (NuPAGE, Invitrogen, UK) and placed onto multiple sheets of dry filter paper. Nitrocellulose membrane was marked with a pencil into approximately 1cm squares, soaked in transfer buffer and placed on top of the soaked filter paper. 5µL of each fraction was spotted onto each square using a pipette and the liquid drawn through by capillary action. The membrane was blocked with 5% NFDM (Marvel dried milk powder) in TBS (20mM Tris-HCL, 150mM NaCl, pH7.4), 0.1% Tween 20. Decorin was detected using three antibody steps; anti-decorin antibodies (1:500, 1% BSA/PBS), biotin-conjugated anti-rabbit IgG antibodies (1:1000, Sigma-Aldrich, UK), and HRP conjugated to streptavidin (1:1000, Sigma-Aldrich, UK) each in 1% NFDM/TBS/Tween 20. Binding was visualised by chemiluminescence and exposure to photographic film as described (section 2.2.4.4).

2.2.5 Collagen Isolation

In analysis of decorin functions and signalling properties, it is important to consider interaction of decorin with the matrix component collagen type I. Further, to mimic *in vivo* conditions, endothelial cells can be cultured on matrix substrates such as collagen. Collagen type I was purified from rat-tail tendons by acid extraction under sterile conditions. Tails were incubated in 70% ethanol for 20 min and the skin removed to reveal the tendons. Tails were washed with 70% ethanol and air-dried. Tendons were excised, cut into small pieces, sterilised in 70% ethanol for 10 min and air-dried. Collagen was extracted by placing tendons in 0.1% acetic acid (approximately 25ml per tail) and continuously agitating for 48 h at 4°C using a magnetic stir-bar. The extract was centrifuged for 30 min at 16,000 x g and the supernatant retained and stored at 4°C. Protein concentration was determined by BCA assay as described (section 2.2.4.1).

2.2.5.1 Collagen Gel Preparation

For preparation of collagen gels, collagen type I (final concentration 0.167mg/ml) fibrillisation was induced by neutralisation of the acid-extracted collagen. To prepare 1.5ml collagen-containing solution, 750µL 2-fold concentrated serum-free Waymouth MAB 87/3 medium was mixed with 2-fold concentrated antibiotics (200units/ml penicillin, 200µg/ml streptomycin), 70µL 0.5M NaOH and water. 100µL acid-extracted rat-tail collagen was added (1/15 dilution from stock concentration 2.5mg/ml) and the solution mixed by repeated pipetting. Hydrophilic tissue-culture plates or dishes (Greiner bio-one, Gloucestershire, UK) were coated thinly with the neutralised collagen mixture by addition of excess solution, and immediate removal of as much as possible. Plates or dishes were incubated at 37°C for 30 min to allow gelling.

2.2.6 Decorin Characterisation

To investigate whether decorin purified in this Chapter had retained expected binding characteristics, an ELISA-based assay was used to ascertain whether decorin could interact with immobilised collagen type I. It has been previously shown that decorin activates Akt in endothelial cells cultured on collagen within 10 min of incubation with decorin (Schönherr et al., 2001). This property of decorin was used to assess whether decorin had retained expected signalling characteristics.

2.2.6.1 Determination of Binding of Decorin to Collagen type I by ELISA

Wells of a 96-well microtitre plate were coated in duplicate overnight at 4°C by incubation with 100µL each of 1% BSA or 80µg/ml collagen in PBS. The following day, wells were washed three times with 100µL PBS and remaining binding sites blocked by incubation with 200µL per well 1% BSA/PBS for 1 h at room temperature. Wells were washed twice with PBS, and then 100µL per well of decorin at 2 or 10 µg/ml (diluted in blocking buffer) was added to each well, and the plate incubated for 2 h at room temperature. Unbound decorin was removed by washing three times with PBS, and bound decorin detected using polyclonal rabbit antibodies to human decorin (1:1000),

followed by HRP-conjugated anti-rabbit antibodies (1:5000, Dako Cytomation). Each antibody step was carried out for 1 h at room temperature and was followed by 3 washes with PBS/0.05% Tween. The ELISA was developed by addition of 50μL SureBlue tetramethylbenzidine dihydrochloride (TMB) Microwell Substrate (520003) for 2 min. The reaction was stopped with 50μL of TMB stop solution (508506, both KPL, Insight Biotechnology, Wembly, UK) and the absorbance at 450nm measured using a 96-well microplate reader (EL311, BioTek Instruments, Winooski, VT, USA).

2.2.6.2 Akt Activation by Decorin

Two 35mm tissue-culture dishes were thinly coated with fibrillar collagen type I by addition of 500μL neutralised collagen mixture per dish to cover the surface and immediate removal of as much liquid as possible. Endothelial cells, 500,000 per dish, were seeded in 2ml Waymouth medium (supplemented with 0.5% FCS, 100 units/ml penicillin, 100μg/ml streptomycin) per dish and cultured for 48 h. Medium was replaced with 500μL serum-free Waymouth medium (supplemented with antibiotics) containing 70μg/ml decorin or the equivalent volume of PBS. After 10 min, the medium was removed and cells harvested by scraping into 150μl cold extraction buffer (20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 150mM NaCl, 1% Nonidet-P40 (NP-40), 1mM sodium vanadate ($NaVO_4$), 20mM β-glycerophosphate, 20mM p-Nitrophenyl phosphate, 2mM sodium fluoride (NaF), 0.25% Na deoxycholate, 1mM ethyleneglycoltetraacetic acid (EGTA), 10μg/ml leupeptin, 10μg/ml aproteinin, 10% glycerol, 1mM phenylmethylsulphonylfluoride (PMSF)). Lysates were sonicated twice briefly at a setting of 4 with a Heat Systems/Ultrasonics Model W-220F Cell Disruptor (approximately 2 second bursts while on ice), and cleared from particulate matter by centrifugation at 16,000 x g for 10 min at 4°C and protein content estimated by BCA assay (section 2.2.4.1). Equal amounts (15μg) of protein were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions as described (section 2.2.4.2) and protein analysed by Western blotting as described (section 2.2.4.4). The membrane was probed with antibodies raised in

rabbit, to Akt phosphorylated at serine 473 (1:1000, Cell Signalling, Danvers, MA, USA) followed by HRP-conjugated antibodies to rabbit IgG (1:2000, DakoCytomation, UK). For normalisation, the membrane was stripped for 30 minutes at 50°C in stripping buffer (2% SDS, 60mM Tris-HCl, pH7.4, 0.7% 2-mercaptoethanol) and re-probed with antibodies to focal adhesion kinase (FAK, 1:100, PC314, Oncogene), visualised by HRP-conjugated antibodies to rabbit IgG (1:2000, DakoCytomation, UK). Densitometric analysis was performed using Quantity One software (Bio-Rad).

2.3 Results

2.3.1 Decorin Purification

2.3.1.1 Initial Purification using Anion Exchange, DEAE-Sepharose

Decorin was partially purified from fibroblast-conditioned media by anion-exchange chromatography. Media was fractionated one litre at a time by elution from DEAE-Sepharose matrix using a step-wise salt gradient. Partially purified decorin was eluted in high salt (1M NaCl) and fractions were collected. It has been previously shown by Western blot that decorin elutes in this step (Elke Schönherr, personal communication). Protein elution was monitored spectrophotometrically at 280nm until completion, and DNA contamination was assessed by monitoring absorbance at 260nm. Spectrophotometric analysis of a typical purification is shown in Figure 2.1. All fractions containing more protein than in fraction 1 were pooled.

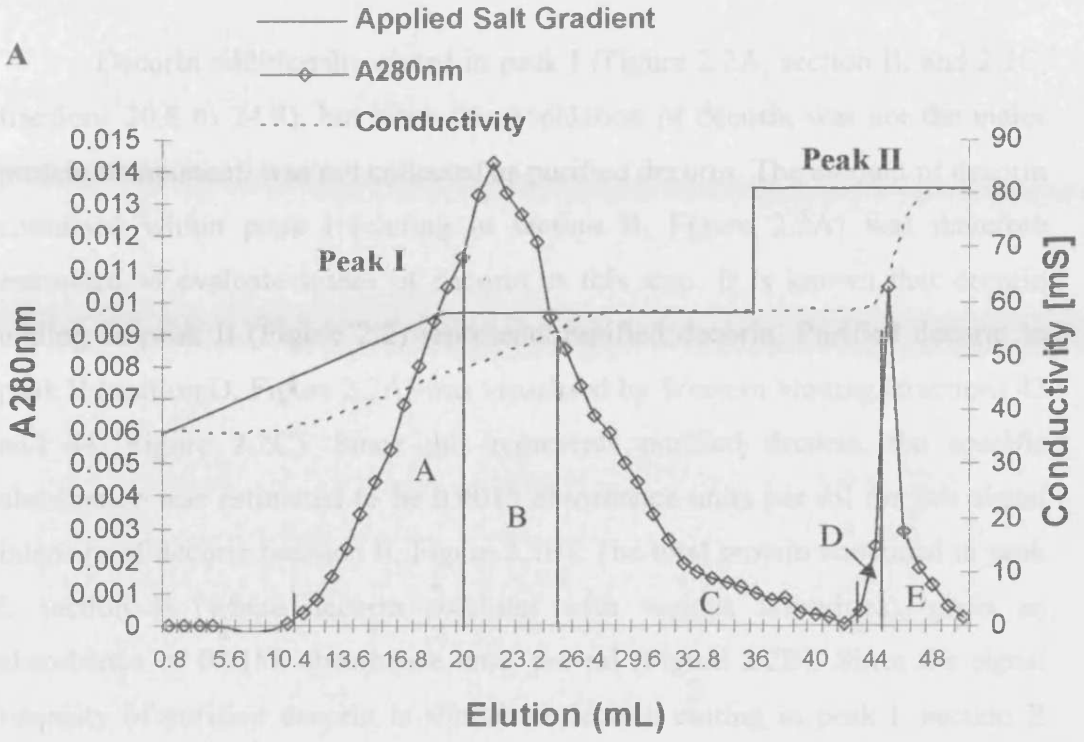
Fraction	A260nm	A280nm
1	0.012	0.015
2	0.026	0.033
3	0.048	0.059
4	0.043	0.061
5	0.017	0.019
6	0.009	0.001

Figure 2.1: A typical profile of decorin elution from DEAE-Sepharose media. Fibroblast conditioned media was prepared as described and one litre applied to DEAE-Sepharose matrix. After eluting contaminants with 0.4M NaCl, partially purified decorin was eluted with 1M NaCl, and protein elution followed by monitoring absorbance at 260 and 280nm. A typical result is shown.

2.3.1.2 Further Purification using Anion Exchange, Source Q15

Partially purified decorin obtained in step 1 was applied to a second anion-exchange matrix, Source Q15. Source Q15 is a stronger anion-exchange matrix, which facilitates further resolution of proteins that co-elute with decorin on elution from DEAE-Sepharose. Decorin partially purified by elution from DEAE-Sepharose was applied to Source Q15 matrix and decorin eluted using a salt gradient up to 0.7 M NaCl, followed by a high salt step elution (1 M NaCl). A typical elution profile (after purification of 3 litres of media on DEAE-Sepharose which were pooled before application to Source Q15) is shown in Figure 2.2A. The peaks eluted from the column were divided into sections (A-E). The total absorbance and volumes of these sections is shown in Figure 2.2B. 5μL of indicated fractions were analysed for decorin content by dot-blotting onto nitrocellulose membrane as described in materials and methods (Figure 2.2C). The greatest amounts of decorin were detected in peak II (Figure 2.2A, sections D and E). Decorin additionally elutes in peak I (Figure 2.2A, section B, and 2.2C, fractions 20.8 to 24.8). This pattern was consistently observed. Peak II was collected as purified decorin since this contains the purest preparation and represents the major population of decorin.

A



B

Fractions (Inclusive)	Area	Total Volume (ml)	A280nm	A280 per ml
10.4-20	A	9.6	0.0643	0.0067
20.8-24.8	B	4	0.0750	0.0188
25.6-42	C	16.4	0.0551	0.0034
43-44	D	2.0	0.0029	0.0015
45-50	E	6.0	0.0172	0.0029

C

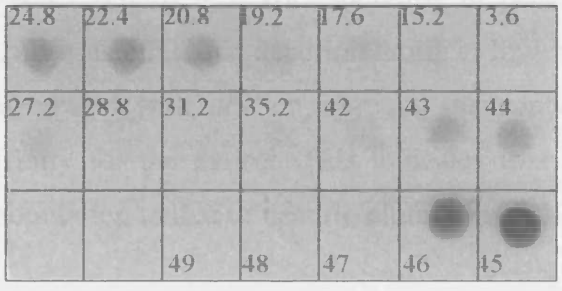


Figure 2.2: A typical profile of decorin elution from Source Q15 media. A pool of partially purified decorin obtained from three litres of fibroblast-conditioned media after purification on DEAE-Sepharose was adjusted to less than 0.4M NaCl and applied to Source Q15 matrix. Proteins were eluted with a gradient of 0.4M-0.7M NaCl and then with 0.7M NaCl until conductivity and A280nm had stabilised, decorin was eluted with 1M NaCl. Conductivity and absorbance at 280nm was recorded and is represented in (A). Two peaks were observed (labelled I and II). The elution profile was divided into areas A-E, and the total absorbance and volume of each area is shown in part (B). Decorin content in 5µL samples of indicated fractions were tested by dot blot with decorin-specific antibodies and visualised by chemiluminescence and exposure to photographic film (C).

Decorin additionally eluted in peak I (Figure 2.2A, section B, and 2.2C, fractions 20.8 to 24.8), but since this population of decorin was not the major protein component, was not collected as purified decorin. The amount of decorin contained within peak I (eluting in section B, Figure 2.2A) was therefore estimated to evaluate losses of decorin at this step. It is known that decorin eluting in peak II (Figure 2.2) represents purified decorin. Purified decorin in peak II (section D, Figure 2.2A) was visualised by Western blotting (fractions 43 and 44, Figure 2.2C). Since this represents purified decorin, the specific absorbance was estimated to be 0.0015 absorbance units per ml for this signal intensity of decorin (section B, Figure 2.2B). The total protein contained in peak I, section B (where decorin co-elutes with various impurities), gives an absorbance of 0.0188 absorbance units per ml (Figure 2.2B). Since the signal intensity of purified decorin is similar to decorin eluting in peak I, section B (fractions 20.8 to 24.8, figure 2.2C), it can be estimated that 0.0015 units per ml of the total 0.0188 units per ml represents purified decorin. Decorin therefore represents approximately 10% of the total protein eluting in peak I.

To investigate whether decorin in peak I represented an inherently different population of decorin, or a similar population to that of peak II, decorin-positive fractions from peak I were pooled and re-applied on Source Q15 matrix under identical conditions. Under these conditions, a minor peak was resolved with 1M NaCl (Figure 2.3, corresponding to peak II in the original separation, Figure 2.2) indicating that decorin eluting at lower salt concentration may be due to interaction with one or more of the contaminating proteins altering decorin affinity for the matrix. This indicates that decorin eluting in peak I is a similar population to that of decorin eluting in peak II.

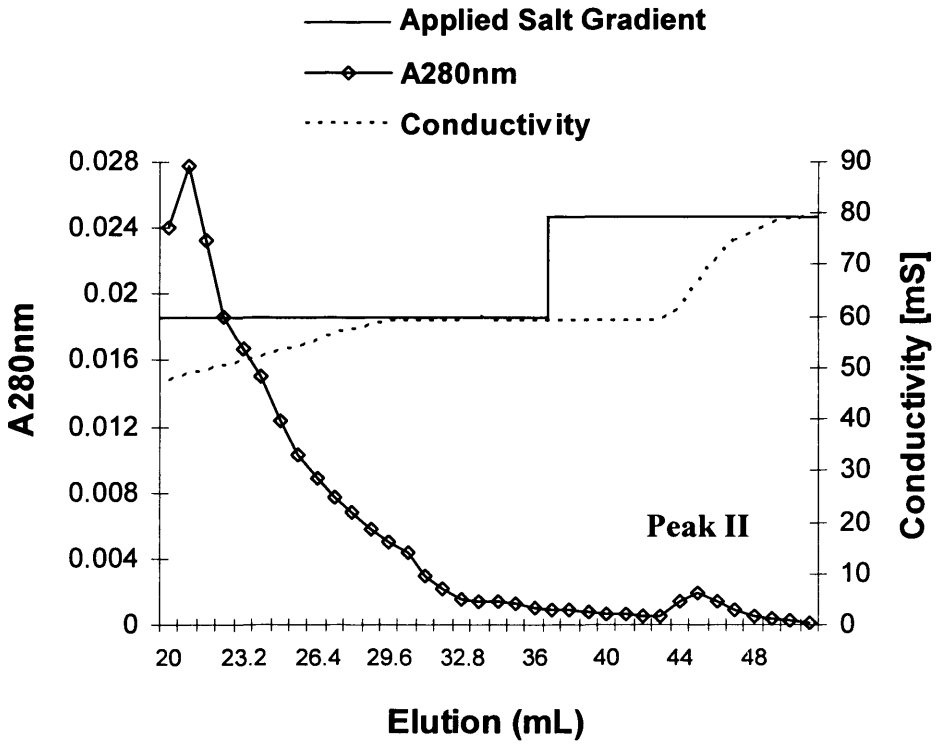


Figure 2.3: Re-purification of decorin eluting in peak I on Source Q-15 media. From Source Q15 matrix separations, decorin positive fractions from peak I were pooled (from three separate runs). The pool was adjusted to less than 0.4M NaCl and re-applied to Source 15Q matrix. Proteins were eluted as before; with a gradient of 0.4M-0.7M NaCl. Once conductivity and A280nm had stabilised, decorin was eluted with 1M NaCl. Conductivity and absorbance at 280nm was recorded and is represented graphically.

2.3.1.3 Decorin Purity

Decorin eluting in peak II (Figure 2.2A) was pooled. Decorin elutes from Source Q15 matrix in high salt (0.7M to 1M NaCl). Since this study will investigate decorin effects on endothelial cell behaviour and signalling, buffer exchange was necessary. It has also been previously shown that decorin storage at -20°C at less than 0.5mg/ml and repeated freeze-thaw was sufficient to abrogate effects of decorin on fibroblast adhesion to fibronectin (Winnemoller et al., 1991). Therefore, decorin was buffer exchanged into sterile PBS and concentrated by ultracentrifugation at 4°C as described in materials and methods (to more than 0.5mg/ml). Concentrated decorin was transferred into 100μL aliquots to avoid repeated freeze-thaw, and was stored at -20°C. The purity of the decorin preparation was checked by SDS-PAGE followed by silver staining (Figure 2.4A), and decorin identity was confirmed by Western blotting using antibodies directed against decorin (Figure 2.4B).

Lane 1 of Figure 2.4A demonstrates the presence of a proteoglycan (showing a characteristic smear of between 55 KDa and 100 KDa). On digestion with chondroitin ABC lyase (lane 2, Figure 2.4A), the smear was resolved into a doublet of approximately 43 KDa and 45 KDa, representing species with 2 or 3 N-linked oligosaccharides attached to the protein core (Glossl et al., 1984). Additional bands to this doublet originate from chondroitin ABC lyase (lane 2, Figure 2.4A), including BSA (62 KDa), and the enzyme itself (two subunits of 86 KDa and 32 KDa). This figure therefore demonstrates that decorin proteoglycan had been isolated (Figure 2.4A, lane 1). To further demonstrate the identity of decorin, chondroitin ABC lyase digested and undigested decorin was analysed by Western blotting (Figure 2.4B). The undigested proteoglycan was immunoreactive using antibodies directed against decorin and appeared as the characteristic smear. Upon digestion, the smear was resolved into a doublet similar to that shown in Figure 2.4A. A further band of approximately 85 KDa was also recognised by antibodies against decorin (Figure 2.4B, open arrow-head).

A similar band of between 80 and 90 KDa has also been detected in decorin preparations from other sources, after purification by anion-exchange (Figure 2.5). When decorin was purified from stably (Figure 2.5A) or transiently (Figure 2.5B) transfected cells, this band was also visualised by Western blotting. Further, when decorin was purified from human skin fibroblasts, both polyclonal and monoclonal antibodies to decorin could detect this band (Figure 2.5C, D). All bands (43, 45, 80-90kDa) were excised from Coomassie-stained SDS-PAGE gels by a member of our laboratory (Gregoire Martin), and confirmed to be derived from decorin by tryptic digestion and mass spectrometry (MALDI-TOF). Further, the only match with a significant MOUSE score from these bands was decorin, indicating that this band does not represent contamination from any known protein species. Further, at twice the molecular weight of monomeric decorin core protein, the band between 80 and 90 KDa could represent covalently cross-linked decorin dimer.

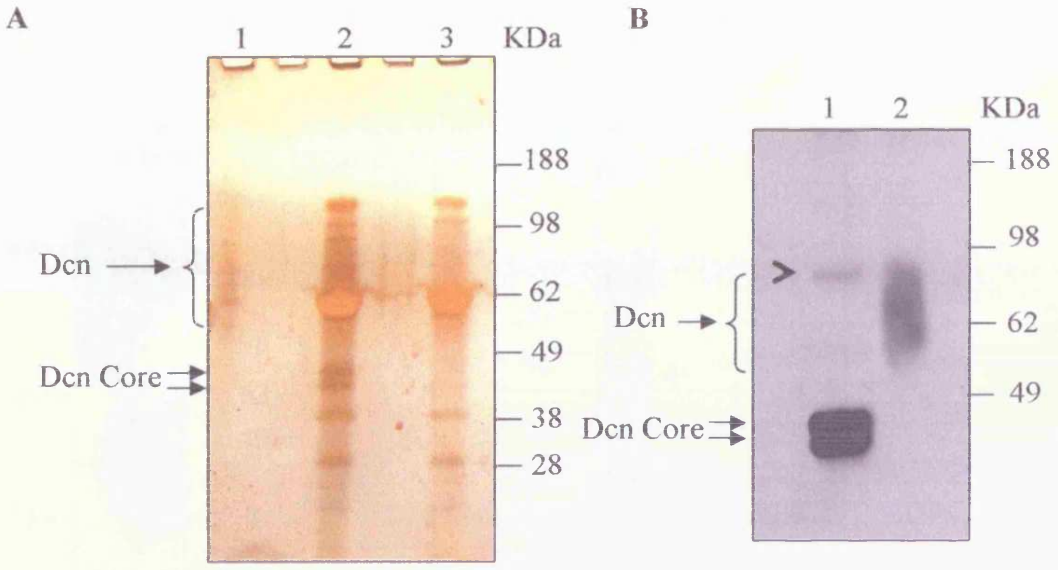


Figure 2.4: Decorin purity and immunoreactivity. A total of 4 litres of conditioned media were purified on DEAE-Sepharose and Source 15Q matrix. Decorin-positive fractions from peak II of 1M NaCl step elution from Source Q15 matrix were pooled, buffer exchanged into sterile PBS and concentrated by ultrafiltration. Purity was assessed by SDS-PAGE followed by silver staining (A). Samples were as follows. Lane 1: 4µg purified decorin, Lane 2: 4µg purified decorin digested with chondroitin ABC lyase, Lane 3: Chondroitin ABC lyase, equivalent amount to lane 2. To confirm identity of decorin, chondroitin ABC lyase digested (lane 1) and intact (lane 2) samples of 2µg each of purified decorin were separated by SDS-PAGE and transferred to nitrocellulose membrane (B). The membrane was probed with polyclonal antibodies directed against decorin and detected using HRP-conjugated secondary antibodies to rabbit IgG. Binding was detected by chemiluminesence and exposure to photographic film. Decorin core protein and proteoglycan of expected molecular weights are indicated. A further band recognised by antibodies to decorin is indicated by the unlabelled spear-headed arrow (B).

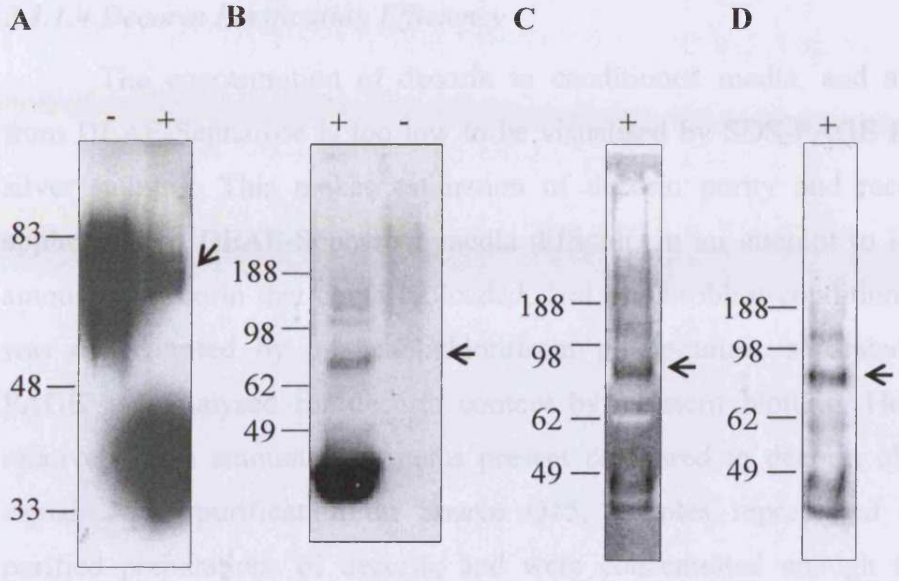


Figure 2.5 Immunoblotting of Decorin from different sources. Decorin was purified from culture medium of human embryonic kidney cells stably transfected with decorin cDNA (A), endothelial cells (EA.hy 926) transfected with decorin adenovirus (B), or non-transfected human skin fibroblasts (C,D). 10µg purified decorin was digested with chondroitin ABC lyase (+) and separated by SDS-PAGE along with 10µg undigested decorin (-). Decorin was detected by Western blotting using polyclonal antibodies raised in rabbit and directed against decorin, followed by HRP-conjugated secondary antibodies to rabbit IgG. Binding was detected by chemiluminesence and exposure to photographic film (A,B,C). Alternatively, in (D), the membrane was probed with monoclonal antibodies to decorin followed by HRP-conjugated secondary antibodies to mouse IgG (Schmidt et al., 1987). A further band to the expected molecular weight of undigested decorin and decorin core protein, but recognised by both polyclonal (A,B,C) and monoclonal (D) antibodies to decorin is indicated by unlabelled spear-headed arrows. The data shown in this Figure was supplied by Dr. Gregoire Martin.

2.3.1.4 Decorin Purification Efficiency

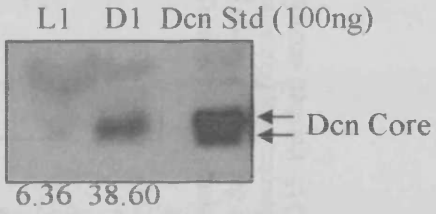
The concentration of decorin in conditioned media, and after elution from DEAE-Sepharose is too low to be visualised by SDS-PAGE followed by silver staining. This makes estimation of decorin purity and recovery after application to DEAE-Sepharose media difficult. In an attempt to increase the amount of decorin that could be loaded, 1ml of fibroblast-conditioned medium was concentrated by methanol/chloroform precipitation, separated by SDS-PAGE and analysed for decorin content by Western blotting. However, the relatively high amount of proteins present compared to decorin obscured the signal. After purification on Source Q15, samples represented extensively purified preparations of decorin, and were concentrated enough to facilitate analysis (Figure 2.4A).

A different approach was taken to estimate decorin purification efficiency on DEAE-Sepharose media. The total protein content at each step of purification was estimated by BCA assay (Figure 2.6). The amount of decorin at each step of purification was quantitatively estimated by Western blotting by comparison to known concentrations of purified decorin. To facilitate densitometric analysis, samples were subjected to exhaustive digestion of the glycosaminoglycan moiety with chondroitin ABC lyase prior to Western blotting (Figure 2.7). Decorin recovery and enrichment were then calculated for each stage of purification (DEAE-Sepharose, Source Q15 and ultrafiltration) and are shown in Figure 2.8. For analysis, samples were taken before and after application to DEAE-Sepharose matrix from each of three litres of media (L1-L3). Decorin eluted from these three litres was pooled together (D1-D3) and applied to Source Q15 matrix. A sample was taken after purification on Source Q15. Before ultrafiltration, purified decorin was additionally pooled with decorin separately purified from a fourth litre of conditioned media (L4), and a sample taken.

Stage of Purification	Volume (mL)	Conc (mg/mL)	Total Protein (mg)	SD
L1 Media	1000	0.65	655.00	292.45
L2 Media	1000	0.80	803.00	77.25
L3 Media	1000	0.69	690.00	7.23
Average Media	1000	0.72	**716.00**	**77.26**
L1 after DEAE-Sepharose (D1)	96	0.03	2.62	2.01
L2 after DEAE-Sepharose (D2)	82	0.02	1.47	0.98
L3 after DEAE-Sepharose (D3)	106	0.02	1.68	0.99
Average after DEAE-Sepharose	95	0.02	**1.92**	**0.61**
L1-L3 after Source Q15	8	0.04	**0.31**	**0.13**
L1-L4 before ultrafiltration	14	0.03	**0.42**	
L1-L4 after ultrafiltration	0.52	0.61	**0.32**	**0.01**

Figure 2.6: Analysis of total protein content by BCA assay at each stage during decorin purification. Samples were taken throughout purification and protein content analysed by BCA assay on 2 to 4 repeats (with the exception of L1-L4 before ultrafiltration, which was analysed only once due to restricted sample amount). The average protein concentration and standard deviations (SD) were calculated in mg/ml using BSA as a standard. Litres 1 to 4 are denoted L1, L2, L3 and L4. L1-L3 were combined after purification on DEAE-Sepharose and purified on Source Q15. Before ultrafiltration, decorin purified from Source Q15 was pooled with decorin from L4, which was purified separately. L1-L4 was sampled before and after ultrafiltration.

A



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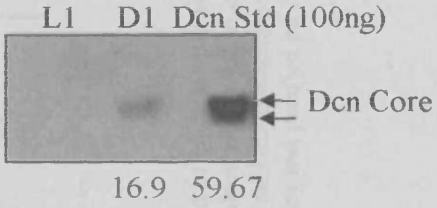

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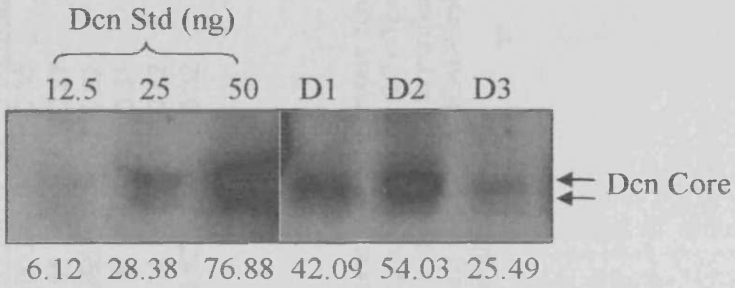


D

Stage of Purification	Dcn Concentration (mg/ml)	Volume (ml)	Total Dcn (µg)
L1	0.0003	1000	346
D1 (B)	0.0022	96	210
D1 (C)	0.0023	96	220
D2	0.0030	82	250
D3	0.0014	106	150

Figure 2.7: Densitrometric analysis of decorin content at each stage of purification. Samples from before (L1) and after (D1-D3) purification on DEAE-Sepharose were digested with chondroitin ABC lyase. Decorin standards from previous purifications (Dcn Std) were similarly treated. Samples were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with antibodies to decorin as described in materials and methods. In (A) Litre 1 of media was analysed before (L1, 12.8µL of 1000ml) and after (D1, 12.8µL of 96ml) purification on DEAE-Sepharose matrix. A second exposure of the blot is shown in part (B), to visualise decorin standard that was not over-exposed. Part (C) shows samples of Litres 1 (D1), 2 (D2) and 3 (D3) after purification on DEAE-Sepharose. All blots were analysed by densitometry and values are shown under each band. Values were compared to standards and used to calculate amounts of decorin contained in each sample, shown in (D).

Stage of Purification	Total Protein (mg)	Volume (mL)	Total Dcn (mg)	Decorin Purity (%)	Recovery (%)	Enrichment
Before DEAE-Sepharose (Average L1-L3)	716	1000	0.35	0.05		1x
After DEAE-Sepharose (Average D1-D3)	1.9	95	0.21	12	60	240x
Before Source Q15 (D1-D3)	5.8	285	0.62	12		
After Source Q15 (D1-D3)	0.31	8	0.31	100	50	2000x
Before Ultrafiltration (L1-L4)	0.42	14	0.42	100	76	
After Ultrafiltration (L1-L4)	0.32	0.52	0.32	100		

Figure 2.8: Summary of decorin purification efficiency and enrichment. The total protein contained in each sample was estimated by BCA assay (Figure 2.6), and total decorin estimated by quantitative analysis of Western blots (Figure 2.7). These values were used to calculate the purity, recovery and enrichment of decorin at each stage of purification. L1 denotes Litre 1 of media prior to purification on DEAE-Sepharose, D1 denotes this litre after purification on DEAE-Sepharose. D1 was pooled with D2 and D3 (similarly purified on DEAE-Sepharose) and purified on Source Q15. Purified decorin was pooled with a separately purified litre, L4 and sampled before and after ultrafiltration.

2.3.2 Decorin Characterisation

2.3.2.1 Decorin binding to Collagen type I

Decorin has been shown previously to have binding affinity for the matrix component collagen type I (Scott and Orford 1981). To check that purified decorin had retained binding affinity for collagen type I, an ELISA-based assay was used. Microtitre plates were coated with collagen type I or BSA (as a negative control) and non-specific binding sites blocked with BSA. Solutions of decorin at 2 and 10μg/ml were incubated with wells and bound decorin detected using antibodies to decorin as detailed in materials and methods. Under these conditions, decorin binding to collagen type I was greater than binding to the negative control, BSA, indicating that decorin had retained binding affinity for collagen type I (Figure 2.9).

2.3.2.2 Decorin Activation of Akt

Since this study will investigate signalling properties of decorin, the activity of purified decorin was tested in a relevant biological assay. It has been shown previously that decorin activates Akt maximally after 10 min in endothelial cells (Schönherr et al., 2001a). Purified decorin was similarly incubated with endothelial cells and Akt activation visualised by Western blotting of cell lysates, using antibodies directed against Akt phosphorylated at serine 473. As a loading control, the membrane was probed with antibodies to FAK. Figure 2.10 demonstrates that decorin activates Akt after 10 min incubation with endothelial cells. Densitometric analysis revealed that Akt was activated approximately 1.6x in comparison to 1.4 x as previously described (Schönherr et al., 2001), indicating that decorin had retained expected signalling properties.

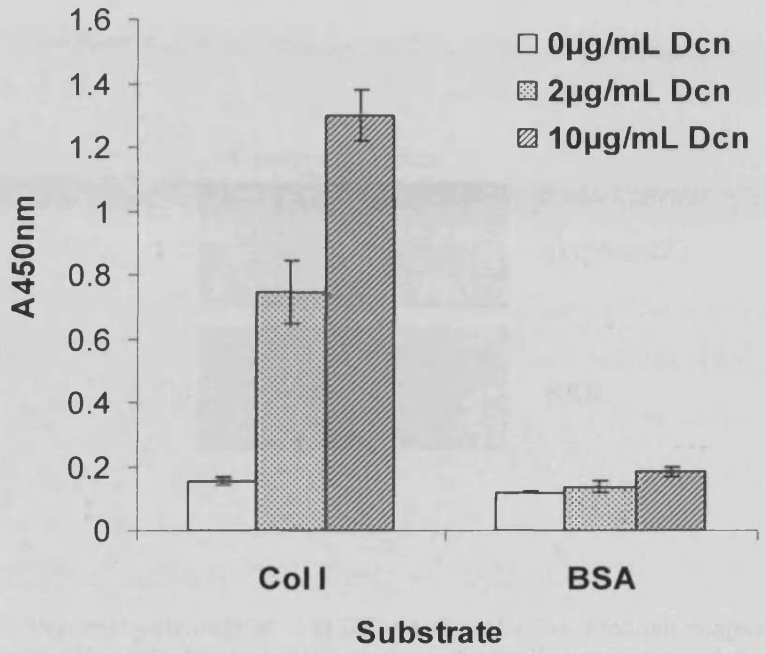


Figure 2.9: Decorin binding to immobilised collagen type I. Microtitre plates were coated with collagen type I or BSA and non-specific binding sites blocked with BSA. Solutions of decorin at 2 and 10 µg/ml were incubated with wells and bound decorin detected using antibodies to decorin as detailed in materials and methods.

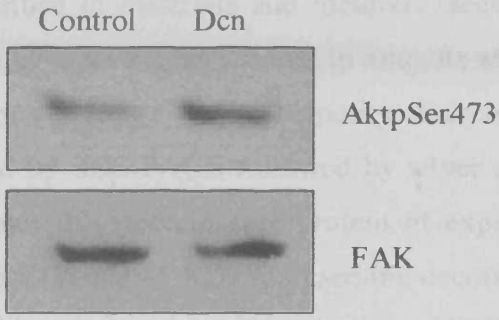


Figure 2.10: Decorin activation of Akt in endothelial cells. Medium supplemented with purified decorin (70μg/ml), Dcn, or medium alone (Control), was incubated for 10 minutes with serum-starved endothelial cells cultured for 48 hours on thin collagen gels. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with antibodies to Akt phosphorylated at serine 473, and to FAK as a loading control. Bound antibodies were both detected using HRP-conjugated antibodies to rabbit IgG, developed by chemiluminescence and visualised by exposure to photographic film.

2.3.3 Decorin Core Protein Production and Purification

Decorin core protein was obtained by exhaustive digestion of decorin glycosaminoglycan with chondroitin ABC lyase followed by purification on DEAE-Trisacryl as described in materials and methods (section 2.2.3.1 and 2.2.3.2). Purified decorin core protein was stored in aliquots at -20°C to avoid loss of activity due to repeated freeze-thaw. The purity of decorin core protein preparations was checked by SDS-PAGE followed by silver staining (Figure 2.11). Lane 1 demonstrates that decorin core protein of expected molecular weight, approximately 43 KDa and 45 KDa representing decorin linked to 2 or 3 N-linked oligosaccharides respectively (Glossl et al., 1984), was obtained after digestion of the GAG moiety with chondroitin ABC lyase. Lane 2 (enzyme alone) indicates that unwanted proteins are introduced into the decorin core protein preparation. Lane 3 shows that the majority of the contaminating proteins were removed after purification on DEAE-Trisacryl, and that decorin core protein had been isolated (Figure 2.11).

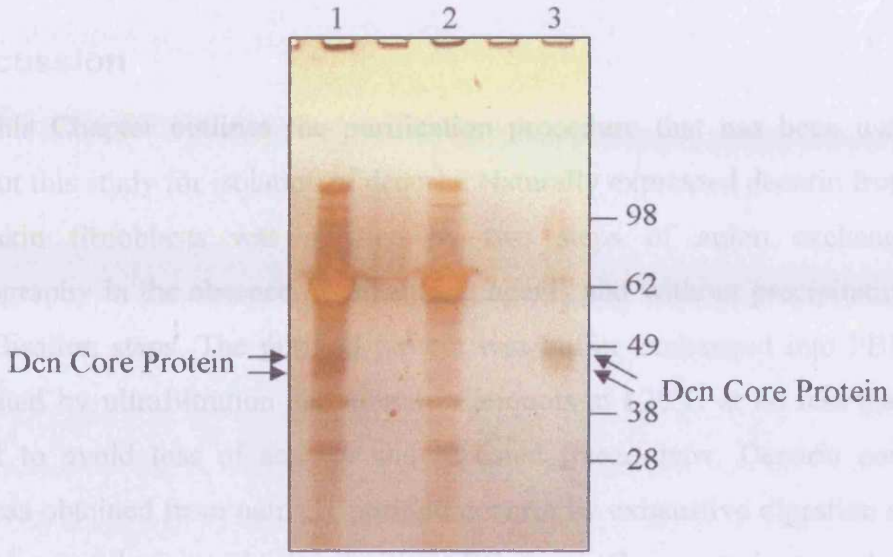


Figure 2.11: Purity of decorin core protein was visualised by silver staining of samples separated by SDS-PAGE. Purity of decorin core protein preparations was assessed by SDS-PAGE followed by silver staining. Samples were as follows. Lane 1: 4µg purified decorin digested with chondroitin ABC lyase Lane 2: Chondroitin ABC lyase, equivalent amount to lane 1. Lane 3: 4µg purified decorin core protein. Decorin core protein is indicated by arrows.

2.4 Discussion

This Chapter outlines the purification procedure that has been used throughout this study for isolation of decorin. Naturally expressed decorin from human skin fibroblasts was purified by two steps of anion exchange chromatography in the absence of denaturing agents and without precipitation or lyophilisation steps. The purified protein was buffer exchanged into PBS, concentrated by ultrafiltration and stored in aliquots at –20°C at no less than 0.5mg/ml to avoid loss of activity and repeated freeze-thaw. Decorin core protein was obtained from natively purified decorin by exhaustive digestion of the glycosaminoglycan with chondroitin ABC lyase. Core protein was then purified under native conditions by one step of anion-exchange chromatography and stored under similar conditions to intact proteoglycan. The molecular weight of both decorin and decorin core protein was similar to that of decorin purified from human skin fibroblasts in previous studies (Glossl et al., 1984; Schönherr et al., 2005).

An additional band (80-90kDa) was detected which was also immunoreactive to antibodies directed against decorin. This band was also observed in decorin preparations similarly purified by anion-exchange from conditioned medium of stably transfected, decorin-expressing HEK293 cells and from EA.hy926 endothelial cells transiently expressing decorin (induced by transfection with adenovirus containing human decorin cDNA). This band would therefore not appear to be exclusive to the fibroblast cell line used in the current study. Additionally, this band appears to represent some form of decorin, since it was detected using both monoclonal and polyclonal antibodies directed against decorin. Further, within our laboratories, this band has been excised and analysed by mass spectrometry. This analysis confirmed the presence of decorin, moreover, no other proteins could be detected (Gregoire Martin, Elke Schönherr, personal communication).

Contaminants in decorin preparations obtained by anion exchange have been previously described; biglycan (Pearson and Gibson, 1982) and the serum component α2HS glycoprotein (Sugars et al., 2002). Regarding the latter, it was previously shown that two anion-exchange steps were required for efficient separation of decorin and α2HS glycoprotein, and suggested that urea should be included during purification for efficient removal of α2HS glycoprotein (Sugars et al., 2002). In the current study, decorin and α2HS glycoprotein appear to be efficiently separated, since no contaminating proteins were visualised by silver staining of purified decorin preparations. Previously, α2HS glycoprotein was shown to elute from Source Q15 media below 0.35 M NaCl (Sugars et al., 2002), while in our protocol, decorin eluted above 0.7 M NaCl. It would appear likely that any complexes of decorin and α2HS glycoprotein had dissociated under these conditions and were therefore efficiently separated. Further, N-terminal sequencing and BLAST analysis previously detected the presence of α2HS glycoprotein (Sugars et al., 2002). Mass spectrometry of excised bands in the current study did not detect any significant matches with proteins other than decorin using MASCOT (Gregoire Martin, Elke Schönherr, personal communcation). It would therefore seem unlikely that our preparation was contaminated with α2HS glycoprotein or with biglycan. Co-purification of decorin with biglycan would also seem unlikely in the current study since we appeared to isolate proteins that only corresponded to the expected molecular weight of decorin (visualised by silver staining), and that were immunoreactive with both monoclonal and polyclonal antibodies to decorin. Further, the fibroblast cell line used in this study as a source of decorin does not efficiently synthesise biglycan; biglycan comprises less than 5% of the total proteoglycans synthesised by these cells (Schönherr et al., 1995).

The efficiency of decorin purification was also estimated in this Chapter. Previously, between 85% and 100% of decorin was recovered after purification on DEAE anion-exchange matrix under similar conditions (Schaefer et al., 2000) suggesting that losses during the first step of purification should not be extensive. Losses of decorin during the second anion-exchange step (Source Q15 matrix) may be explained by the exclusion of triton X-100 which has been shown to enhance recovery from anion-exchange media (Ramamurthy et al., 1996; Schaefer et al., 2000). Purification of decorin on this matrix indicated the presence of two populations of decorin, with about 10% eluting in peak I, and the remainder eluting in peak II. This contributed to overall losses since only the population eluting in peak II was retained and accounted for as purified decorin. Two decorin subpopulations have been previously partially resolved from preparations of recombinant decorin purified by nickel chelating chromatography (Ramamurthy et al., 1996). These populations were suggested to relate to different size and disaccharide compositions of the glycosaminoglycan moiety (Ramamurthy et al., 1996). This could explain our observation of two populations of decorin. However there is another explanation. Separation of decorin eluted in peak I on re-application to the Source Q15 matrix demonstrated that a second peak could be resolved, which eluted at identical conductivity to purified decorin. This indicates that decorin in peak I could be aggregated material with altered affinity for anion-exchange media, representing either self-aggregated decorin, or decorin complexed with another protein species. On re-purification, a small proportion of these complexes may dissociate, allowing decorin to interact more efficiently with the anion-exchange media and elute as a pure preparation.

Removal of the GAG moiety resolved decorin into a discrete doublet on separation by SDS-PAGE, corresponding to decorin covalently linked to 2 or 3 N-linked oligosaccharides as previously described (Glossl et al., 1984). However, in Figure 2.11, comparison of lanes 1 and 3 indicates that prior to core protein purification the 2- or 3-N linked oligosaccharide populations of decorin are approximately equal, while after purification, a major band becomes apparent representing decorin with 3 oligosaccharides. During core protein purification, a proportion of decorin with 2 oligosaccharides is lost, presumably by binding to the anion-exchange media (DEAE-Trisacryl). It is unlikely that intact decorin proteoglycan with 2 or 3 oligosaccharides interacts differentially with anion-exchange matrix, since this interaction is mediated primarily by interactions with the polyanionic glycosaminoglycan moiety, not *via* the oligosaccharides. In the case of the core protein however the absence of the glycosaminoglycan may allow the weaker interactions of the oligosaccharides to become apparent.

Various other observations were made throughout this study. The yield of decorin ranged from approximately 100μg to 500μg per litre of conditioned media. Variation in yield is commonly observed with this cell line for unknown reasons (Heinz Hausser, personal communication). However, one factor was determined to be the source of FCS used in maintenance of this cell line. In the first two thirds of the study, FCS was obtained from Biochrom AG, Berlin, Germany. During this time, the yield varied between 120μg/L and 483μg/L, where the average was 242μg per litre of fibroblast-conditioned medium. However, when FCS was obtained from Gibco, Invitrogen in the final third of the study, the yield varied between 78μg/L and 127μg/L, where the average was 99μg per litre of fibroblast-conditioned medium. The low expression of decorin in the latter third of the study could also have contributed to overall losses during purification of decorin. Previous studies have further characterised decorin purified from this cell line. The GAG moiety of decorin was shown to contain approximately 50% iduronic acid (Seidler et al., 2006). The activities of this source of decorin are therefore more relevant to non-

mineralised tissues, since mineralised tissue contains chondroitin sulphate-bearing decorin only (Franzen and Heinegard, 1984).

It has also been shown that biological activities of decorin are sensitive to purification under denaturing conditions (Gu et al., 1997; Hildebrand et al., 1994; Svensson et al., 1995; Truppe and Kresse, 1978), to lyophilisation (Goldoni et al., 2004) and to storage conditions (Grant et al., 2002; Winnemoller et al., 1991), as discussed in the introduction to this Chapter. In this study, we will investigate decorin effects on endothelial cell behaviour and signalling. It is therefore imperative that the biological activities of decorin preparations are not compromised. The biological activity of the decorin preparation was assessed in two ways. Decorin has been previously shown to interact with collagen type I (Scott and Orford, 1981). In an ELISA-based assay, we confirmed that decorin could interact with immobilised fibrillar collagen type I. It was also confirmed that this preparation of decorin activated Akt in endothelial cells in a similar manner as previously described (Schönherr et al., 2001). This demonstrates that the binding and signalling activities of decorin were retained. The source of decorin described in this Chapter does not facilitate purification of large amounts of decorin. However, since decorin is naturally expressed by this cell line, decorin expression would be unlikely to exceed the capacity of enzymes involved in synthesis of decorin. This product would therefore be expected to contain naturally occurring post-translational modifications, and can be considered to be a physiologically relevant preparation. In summary, we have demonstrated an efficient purification procedure for isolation of naturally expressed decorin that retains expected biological activities. These methods for decorin purification were used throughout this study to investigate the signalling properties of decorin in endothelial cells.

Chapter 3

The Influence of Decorin on Endothelial Cell Attachment and Migration

3.1 Introduction

Regulation of endothelial cell adhesion and migration is critical in control of angiogenesis. Throughout angiogenesis, endothelial cells rearrange their contacts with the extracellular matrix in order to form abluminal sprouts, to migrate and proliferate and to develop into mature vessels. Moreover, endothelial cell interactions with the remodelling microenvironment are crucial for regulated responses to growth factors and migratory signals. Factors such as decorin that modulate adhesion and migration thus have the potential to contribute to various aspects of angiogenesis. The role of decorin in angiogenesis however is controversial, and the effects of decorin on endothelial cell behaviour are not well characterised. Additionally the mechanisms by which decorin influences endothelial cell behaviour remain unknown. In this Chapter, the effects of decorin on endothelial cell behaviour will be characterised.

Throughout angiogenesis, endothelial cells may encounter different forms of decorin. Decorin can be presented to the cells either as a soluble effector, or as an immobilised substrate in association with collagen type I. Endothelial cells undergoing angiogenic morphogenesis *in vitro* initiate expression of decorin (Jarvelainen et al., 1992), while decorin expression is associated with nascent vessels *in vivo* (Nelimarkka et al., 2001). Decorin may present as a soluble form in this context. During sprouting angiogenesis, endothelial cells degrade the basement membrane and migrate through the interstitial matrix, of which decorin is a component. Endothelial sprouts therefore encounter collagen-bound decorin. Additionally, during matrix remodelling, MMP-mediated matrix degradation could liberate soluble decorin or decorin fragments (since decorin is also an MMP substrate (Imai et al., 1997)) from fibrillar collagen which may activate alternative signalling pathways. The role of decorin in various aspects of angiogenesis could therefore relate to presentation of decorin or decorin fragments as soluble or collagen-bound, immobilised forms.

The role of decorin in tube formation is controversial. Decorin expression has been shown to enhance tube formation in collagen lattices (Schönherr et al., 1999). However, as a substrate, decorin inhibited collagen-induced tube formation (Davies et al., 2001). In contrast, exogenous decorin was found to have no influence on tube formation on matrigel, or on microvessel sprouting from aortic explants (Grant et al., 2002) while in another study, exogenous decorin inhibited VEGF-induced tube formation on matrigel (Sulochana et al., 2005). Endothelial tube formation is itself a complex process governed by modulation of cell adhesion and migration, in turn controlled by interactions between integrins and their matrix ligands. Therefore, investigation of decorin effects on cell adhesion and migration when presented in different forms is crucial in understanding the role of decorin in angiogenesis.

The role of decorin in endothelial cell adhesion has not been thoroughly investigated. However, soluble decorin was recently shown to inhibit endothelial cell adhesion to fibronectin and to collagen type I (Sulochana et al., 2005). As a substrate, decorin supported endothelial cell adhesion but impaired actin stress fibre formation (Davies et al., 2001). This indicates that decorin alters the availability of receptor binding sites within classic adhesion-supporting matrix substrates such as fibronectin and collagen type I, or that decorin signalling negatively regulates focal adhesion and actin cytoskeleton assembly. In comparison to endothelial cells, much work has been conducted on decorin effects on fibroblast adhesion. Consideration of these studies could shed light on the mechanism of decorin effects on endothelial cells.

As a substrate, decorin is frequently incapable of supporting fibroblast adhesion (Lewandowska et al., 1987; Winnemoller et al., 1991; Noyori and Jasin, 1994; Huttenlocher et al., 1996) and where fibroblasts were able to adhere to decorin, actin stress fibre formation was impaired (Bidanset et al., 1992). Proper fibroblast attachment and spreading requires binding to both the heparin-binding and cell-binding domains (CBD) of fibronectin, although cells can adhere to some extent to either alone (Woods et al., 1986). In 1987, Lewandowska and colleagues (Lewandowska et al., 1987) demonstrated that a

mixture of cartilage-derived decorin and biglycan could inhibit fibroblast adhesion to both intact fibronectin and the cell binding domain (CBD) of fibronectin. Bidanset and colleagues (Bidanset et al., 1992) extended this study to investigate the individual role of decorin and biglycan. In contrast, neither proteoglycan could influence fibroblast adhesion to intact fibronectin, but either could inhibit adhesion to a CBD-containing fragment. A similar inability of decorin to influence fibroblast adhesion to intact fibronectin was also observed in later studies (Noyori and Jasin, 1994; Huttenlocher et al., 1996), although another study found that decorin inhibited adhesion to both intact and the cell binding domain (CBD) of fibronectin (Winnemoller et al., 1991). The study by Lewandowska and colleagues (Lewandowska et al., 1987) also demonstrated that while a mixture of decorin and biglycan could inhibit fibroblast adhesion to fibronectin, it could not interfere with adhesion to collagen type I, indicating that the effect of decorin could be substrate dependent. In support of this, it was later confirmed that decorin alone could not influence fibroblast adhesion to collagen type I (Bittner et al., 1996), or to vitronectin (Huttenlocher et al., 1996). More recently however, decorin was shown to inhibit fibroblast adhesion to collagen type I (Bhide et al., 2005).

As discussed, when cells were seeded onto adhesion-supporting matrix components, in particular, fibronectin, decorin could in some cases, interfere with attachment and spreading. The mechanism was suggested to be through masking of integrin-binding sites in the matrix by decorin. Indeed, steric inhibition of cell-surface receptor-matrix interactions by decorin has been demonstrated. Decorin and cell-surface CD44 both interact with a GAG-binding site on collagen XIV, thus decorin competitively inhibited fibroblast adhesion to this substrate (Ehnis et al., 1998). Similarly, decorin binding to a KKTR (lysine-lysine-threonine-arginine) motif in the cell-binding domain on thrombospondin competitively inhibited fibroblast adhesion to this substrate (Winnemoller et al., 1992; Merle et al., 1997). This does not however, explain why decorin inhibited fibroblast adhesion to fibronectin or collagen type I in some studies but not in others. Additionally, the steric hindrance model suggests that decorin does not directly interact with cell-surface receptors. However, immobilised decorin was

shown recently to support platelet adhesion *via* the collagen-binding α2β1 integrin suggesting that decorin can in fact interact directly with cell surface adhesion receptors (Guidetti et al., 2002). Additionally, it has been suggested that a binding site for chondroitin sulphate exists within the α4β1 integrin (Iida et al., 1998). Therefore, decorin effects cannot be presumed to depend entirely upon its interactions with matrix substrates.

The contrasting effects of decorin on cell adhesion may be explained at least partially by use of different purification procedures for decorin isolation, which can influence decorin activity (discussed in Chapter 2). Alternatively, differential interactions between decorin and matrix substrates, or cell-specific integrin affinity for matrix substrates (including decorin) could explain contrasting observations. For example, α2β1 integrin on fibroblasts and platelets mediates adhesion to collagen type I, but not to laminin. Human melanoma, bladder carcinoma and neuroblastoma cells however, use the same integrin to adhere to both substrates. Experiments with purified integrin from either cell type demonstrated that there were no differences in integrin specificity (Elices and Hemler, 1989) indicating that integrin-ligand interaction is controlled by cells, or may additionally require cell-specific co-receptors or co-factors. Ligand specificity has also been shown to be dependent on the degree of activation of the integrin (Kramer et al., 1989; Languino et al., 1989) which can be controlled by inside-out signalling (Van de Walle et al., 2005). These cell- and context-specific factors could result in differential responses to decorin.

Also pertinent to sprout migration and tube formation in angiogenesis, decorin has been shown to modulate endothelial cell migration. Decorin inhibited endothelial cell migration towards a chemotactic stimulus through gelatin- or fibrillar collagen type I-coated inserts (Kinsella et al., 2000; Sulochana et al., 2005). Endogenous or exogenous decorin also inhibited endothelial monolayer outgrowth on collagen type I (Kinsella et al., 2000). Under these conditions enhanced pericellular fibronectin fibrillogenesis was observed, and suggested to be a mechanism which could be responsible for inhibition of cell migration by decorin; through stabilisation of pericellular matrix (Kinsella et al., 2000). Investigations on fibroblastic cell types

demonstrated that matrix-bound decorin inhibited the migration-promoting effects of fibronectin and collagen type I on osteosarcoma cells (Merle et al., 1999). In contrast, exogenously added decorin enhanced lung fibroblast migration on plastic toward a chemotactic stimulus (Tufvesson and Westergren-Thorsson, 2003). In the first of these studies (Merle et al., 1999), the inhibitory effect required intact decorin, but was suggested to be mediated *via* the GAG moiety. Neither the isolated core protein nor GAGs could influence migration. However DS-bearing decorin was more potent than CS-bearing decorin. Interestingly, this study postulated that decorin could interact with substrates *via* the core protein, and simultaneously with cell-surface integrins *via* the GAG moiety (Merle et al., 1999). Enhanced fibroblast migration on plastic by decorin in solution was suggested to relate to decorin-mediated activation of small Rho GTPases through an unknown receptor (Tufvesson and Westergren-Thorsson, 2003). Both studies support a role for decorin in modulating migration through interactions with cell-surface receptors. Thus while decorin has been postulated to inhibit adhesion by masking receptor-substrate interactions, in contrast, decorin has been proposed to modulate migration through direct interaction with cell-surface receptors.

3.1.1 Aims

- To establish how decorin influences endothelial cell behaviour (attachment and migration), focusing particularly on collagen type I as a substrate
- To investigate whether decorin presentation as a soluble effector, bound to collagen type I or as a substrate, can differentially influence endothelial cell behaviour

3.2 Materials and Methods

3.2.1 Protein Purification and Reagents

Intact decorin and decorin core protein were purified from fibroblast-conditioned media as described in Chapter 2 and were used at concentrations between 10μg/ml and 70μg/ml (0.1μM to 0.7μM respectively). Dermatan sulphate glycosaminoglycan (DS) (stock concentration 100μM, from porcine skin, 90% L-iduronic acid, 10% C4S and C6S, catalogue number C-4259) was obtained from Sigma-Aldrich, UK and used at concentrations between 0.1μM and 0.5μM. Rhodocetin, a venom component of the Malayan pit viper (*Calloselasma rhodostoma*) was a kind gift of Dr. Johannes A. Eble (Muenster University, Germany, (Eble et al., 2001)). Rhodocetin (stock concentration ranging from 0.5mg/ml to 1mg/ml in 70mM NaCl, 20mM sodium phosphate, pH6.5) was used at concentrations between 5μg/ml and 10μg/ml (0.15μM to 0.3μM respectively). Fibronectin (F1141, 1mg/ml in 0.5M NaCl, 0.05M Tris, pH7.5) was obtained from Sigma-Aldrich, UK.

3.2.2 Cell Culture and Maintenance

Cells of the permanent human endothelial cell line EA.hy926 (Edgell et al., 1983) were maintained in growth medium; MCDB 131 medium supplemented with 10% foetal calf serum (both Invitrogen, UK), 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine (HAT) and 100 units/ml penicillin, 100μg/ml streptomycin (both Sigma-Aldrich, UK), at 37°C, 5% CO_2. Cells were passaged at least twice weekly on attaining 70% to 90% confluency and split to 1 in 5 to 1 in 10 for re-seeding. This cell line is described in detail in Chapter 2, section 2.2.1

3.2.3 Experimental Outline

In this Chapter, decorin or decorin moieties were presented to cells as a collagen-bound or a soluble form. In adhesion assays, for presentation of soluble decorin or decorin moieties, cell suspensions were mixed with decorin immediately prior to seeding. Alternatively, cells were seeded onto decorin incorporated into collagen gels, prepared by addition of decorin or decorin moieties prior to gelling. For migration assays, spheroids were plated and decorin immediately added to the medium. Alternatively, spheroids were plated onto decorin incorporated into collagen gels generated in the presence of decorin.

3.2.4 Preparation of Matrix Substrates for Adhesion and Migration Assays

Fibrillar collagen type I gels were prepared as described in Chapter 2, section 2.2.5.1. Briefly, hydrophilic tissue-culture 96-well plates (Greiner bio-one, Gloucestershire, UK) were coated with neutralised collagen solutions and incubated at 37°C for 30 min to allow gelling. For migration assays, thick gels were prepared by addition of 50μL of the neutralised collagen solution to each well of a 96-well plate (Greiner Bio-one). For adhesion assays, wells were thinly coated with collagen by addition of 100μL neutralised collagen solution to each well of a 96-well plate, followed by immediate removal of as much of the solution as possible. For experiments where collagen-bound decorin or decorin moieties were required, decorin, decorin core protein or DS glycosaminoglycan were added to a solution containing the appropriate volume of NaOH (for neutralisation) and proteins equally dispersed by repeated pipetting. Collagen type I was then added and proteins similarly dispersed. Under these conditions therefore, collagen fibrils formed in the presence of decorin or its moieties. Alternatively, decorin was added after addition of collagen type I, but prior to addition of NaOH, or was added after addition of NaOH and collagen type I (to the neutralised solution). Similar results were obtained with decorin under these conditions, and all subsequent experiments were carried out as described.

To coat wells with immobilised BSA, fibronectin or decorin, wells were coated overnight at 4°C with 100µL per well of a 96-well plate with 1mg/ml BSA, 10µg/ml fibronectin or 30µg/ml decorin, each diluted in PBS. Before use, wells were washed twice with PBS, and unoccupied binding sites blocked with 1% BSA in PBS for 30 min at 37°C. Wells were washed three times with PBS before use.

3.2.5 Endothelial Cell Attachment Assay

96-well plates were prepared by coating with matrix substrates or BSA as described (section 3.2.4). For preparation of cell suspensions, endothelial cells were detached by treatment with trypsin (Gibco, Invitrogen), and the trypsin inactivated by addition of growth medium. Cells were collected by centrifugation at 725 x g for 5 min and resuspended in serum-free Waymouth medium containing antibiotics (100 units/ml penicillin, 100µg/ml streptomycin). Cell number was determined using a haemocytometer, and cells were diluted to 1.5 x 10^5 per ml in serum-free Waymouth medium. 100µL of the solution (15,000 cells) was added to each well. For experiments where cell suspensions were mixed with decorin, decorin core protein, DS glycosaminoglycan or rhodocetin, cells were diluted to 3 x 10^5 per ml and immediately prior to seeding, decorin, decorin moieties, rhodocetin, or the equivalent volume of PBS was added to the cell suspension and the volume adjusted to 1.5 x 10^5 cells per ml using serum-free Waymouth medium. Alternatively, cells were seeded onto decorin, decorin core protein or DS glycosaminoglycan incorporated into collagen gels, or immobilised decorin or fibronectin as described in section 3.2.4. Cells were allowed to adhere for 10, 60 or 180 min at 37°C, 5% CO_2. Loose or non-adherent cells were removed by washing once with 100µL per well PBS pre-warmed to 37°C. Adherent cells were fixed by addition of 100µL per well 4% paraformaldehyde in PBS for 15 min at room temperature and stained with 50µL per well 0.1% (w/v) crystal violet in H_2O for 25 min. Wells were washed extensively with H_2O (200µL per well, five times), and crystal violet contained in stained cells was released by solubilisation with 100µL per well 2% (w/v) SDS in water. After 2 h, absorbance at 540nm was measured in a

microplate reader (EL311, BioTek Instruments, Winooski, VT, USA). Five wells were prepared for each variable and the average taken. The percentage of adherent cells was calculated relative to the positive control which was defined as adhesion to a collagen substrate for one hour in the absence of any additives, and which was set to 100%. As a negative control, matrix-coated wells to which cells had not been added were similarly treated, or cells were allowed to adhere to BSA-coated wells. In all cases, values were less than 25% of the control.

3.2.6 Endothelial Cell Migration Assay

A spheroid migration assay was developed based on the studies of Korff and Augustin (Korff and Augustin, 1998). Endothelial cell spheroids have been used successfully to investigate various aspects of angiogenesis (Korff and Augustin, 1998; Korff et al., 2001). 100% stock of methyl cellulose solution was prepared by adding 100ml Waymouth MAB 87/3 medium pre-warmed to 60°C, to 1.33g methyl cellulose (Sigma-Aldrich, UK). The solution was agitated at room temperature for 1 h using a magnetic stir bar, followed by agitation overnight at 4°C. The solution was cleared by centrifugation at 4,000 x g for 90 min and the supernatant retained as 100% stock solution.

Uniform endothelial cell spheroids were generated by incubating 1000 cells per well of a hydrophobic 96-well microtitre plate (Greiner bio-one) in 100μL Waymouth MAB 87/3 medium supplemented with 5% heat-inactivated FCS, antibiotics (100 units/ml penicillin, 100μg/ml streptomycin) and 30% methyl cellulose at 37°C, 5% CO_2 for 48 hours. In this hydrophobic environment, all the cells contained in each well aggregated to form a single spheroid. Spheroids were removed from individual wells using a 10ml stripette and collected in a 50ml Falcon tube and the volume adjusted to 50ml using serum-free Waymouth MAB 87/3 medium supplemented with antibiotics. After mixing by inverting the solution three times, spheroids were collected by centrifugation at 725 x g for 2 min. Spheroids were resuspended in Waymouth MAB 87/3 medium supplemented with 0.5% heat-inactivated FCS and antibiotics to a volume of approximately 100μL per spheroid. 100μL of the suspension was then transferred to each well of a 96-well hydrophilic microtitre

plate pre-coated with collagen gels, decorin-collagen gels or with fibronectin as described in section 3.2.4. A minimum of 10 wells containing a single spheroid located close to the centre of the well was selected for each variable by phase contrast microscopy (approximately 3x the required numbers of spheroids were plated to ensure that data from 10 replicas per variable could be collected).

The endothelial cells of each spheroid migrated radially outwards. Migration was recorded by capturing images at time of plating (t = 0) and approximately every 24 hours thereafter up to 5 days after plating, using an Olympus CK2 microscope and Nikon E4500 digital camera. The height and width of the furthest extensions of each spheroid were measured using the ruler tool on Adobe Photoshop. Occasionally, single cells that were no longer associated with the spheroid were observed and these were excluded from measurements. The actual measurements were calculated in μm using an image of a 1mm graticule captured under identical conditions as a standard. The average spheroid diameter (from 2 measurements) for each variable was then calculated (n=10).

In some experiments, spheroid migration was analysed in the presence of decorin or rhodocetin. For this purpose, single spheroids were plated in 50μL per well and 10 wells selected. 50μL of 2x concentrated decorin or rhodocetin was then added to the medium (to yield final concentrations of decorin 0.1μM to 0.7μM, or rhodocetin 0.1μM to 0.5μM).

3.2.7 Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Unless otherwise indicated, adhesion assays were performed with at least 5 replicates in 3 independent experiments, and migration assays with at least 10 replicates in 3 independent experiments. The mean, SEM and significance were calculated using In-STAT software (Reed Elsevier, Arizona, USA). Unless otherwise indicated, repeated measures ANOVA and Dunnett multiple comparison test were performed to test statistical significance between control and multiple variables (i.e. adhesion assays). Alternatively, Bonferroni was used to test significance between selected variable pairs (i.e. migration assays).

3.3 Results

3.3.1 Influence of Decorin on Endothelial Cell Adhesion

3.3.1.1 Adhesion to Immobilised Decorin or Collagen type I

We first investigated whether decorin as a substrate could support endothelial cell adhesion. Endothelial cells were seeded onto substrates of decorin, BSA or fibrillar collagen type I. A proportion of endothelial cells adhered to collagen type I within 10 min of seeding. Adhesion increased significantly by 60 min after seeding, and increased slightly between 60 and 180 min after seeding (Figure 3.1A). As a negative control, cells were seeded onto BSA for 60 min, and as expected, no adhesion was observed. Endothelial cell adhesion to immobilised decorin after 60 min was similar to adhesion on BSA, indicating that decorin cannot support endothelial cell adhesion. Additionally, endothelial cells could not adhere to immobilised decorin at any of the time-points tested, nor was any increase in adhesion observed over the time-course of the experiment (Figure 3.1A). Bright-field images of wells were captured 60 min and 180 min after seeding of cells onto decorin or collagen type I (Figure 3.1B). In accordance with the data shown in Figure 3.1A, few cells can be seen adhering to immobilised decorin, while at the same time-points endothelial cells have efficiently adhered and spread on collagen type I.

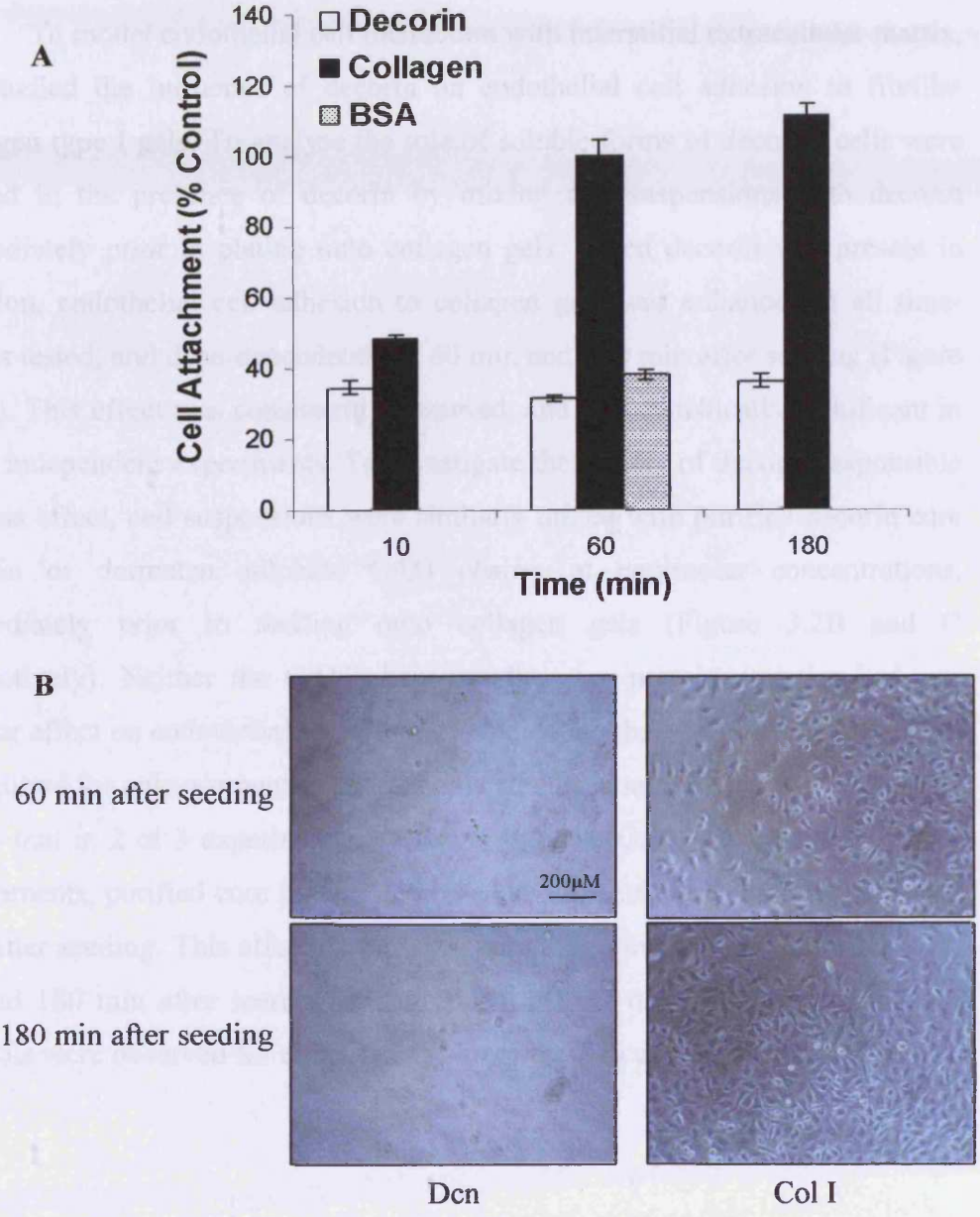


Figure 3.1: Endothelial cell adhesion to decorin, collagen type I or BSA. Microtitre plates were coated with 0.3µM decorin, BSA or fibrillar collagen type I, and blocked with 1% BSA. Endothelial cells ($1.5x10^4$) were seeded onto each substrate and adherent cells fixed and stained with crystal violet at the indicated times. Wells were coated in triplicate for each variable, and the average calculated. Standard errors are shown as error bars and data is representative of two independent experiments (A). Bright-field images were captured during fixation, 1h and 3h after seeding onto decorin or collagen type I (B).

3.3.1.2 Adhesion to Collagen type I in the Presence of Decorin

To model endothelial cell interaction with interstitial extracellular matrix, we studied the influence of decorin on endothelial cell adhesion to fibrillar collagen type I gels. To analyse the role of soluble forms of decorin, cells were seeded in the presence of decorin by mixing cell suspensions with decorin immediately prior to plating onto collagen gels. When decorin was present in solution, endothelial cell adhesion to collagen gels was enhanced at all time-points tested, and dose-dependently at 60 min and 180 min after seeding (Figure 3.2A). This effect was consistently observed, and was statistically significant in three independent experiments. To investigate the moiety of decorin responsible for this effect, cell suspensions were similarly mixed with purified decorin core protein or dermatan sulphate GAG chains, at equimolar concentrations, immediately prior to seeding onto collagen gels (Figure 3.2B and C respectively). Neither the GAG chain nor the core protein moieties had any similar effect on endothelial cell adhesion, indicating that the intact proteoglycan is required for enhancement of endothelial cell adhesion to collagen. It should be noted that in 2 of 3 experiments, dermatan sulphate GAG chains and in 1 of 3 experiments, purified core protein decreased endothelial adhesion to collagen 10 min after seeding. This effect of dermatan sulphate is evident in Figure 3.2C. At 60 and 180 min after seeding no significant effects on adhesion compared to controls were observed for either DS or core protein in any of three experiments.

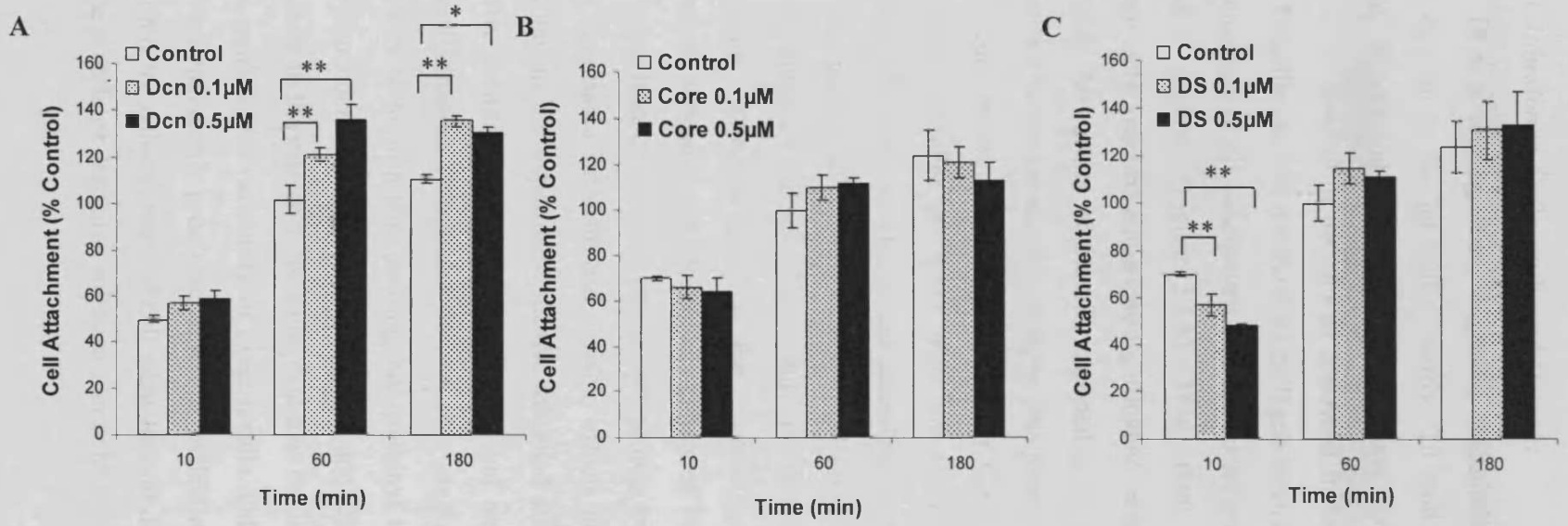


Figure 3.2: Endothelial cell adhesion to collagen type I in the presence of decorin, decorin core protein, or dermatan sulphate. Endothelial cell suspensions were mixed with decorin (A), purified core protein (B) or dermatan sulphate GAG chains (C) immediately prior to seeding on thin collagen gels at 15,000 cells per well. Adherent cells were fixed and stained with crystal violet at indicated times after plating. Five wells were seeded for each variable, and the average calculated. Standard errors are shown as error bars and data is representative of three independent experiments. Statistical significance is indicated, where $p < 0.05$ is represented as * and $p < 0.01$ as **.

3.3.1.3 Adhesion to Collagen-bound Decorin

During angiogenesis, migrating endothelial cells encounter collagen-bound decorin in the interstitial matrix. To study the role of collagen-bound decorin, endothelial cells were seeded onto collagen gels prepared in the presence or absence of decorin as described in materials and methods, section 3.2.4. Endothelial cell adhesion to collagen-bound decorin was greater than to collagen alone at all time-points tested, and adhesion was enhanced in a dose-dependent manner (Figure 3.3A). This effect was consistently observed, although only reached statistical significance in two of three experiments. To investigate the moiety of decorin responsible for enhancing cell adhesion, cell suspensions were seeded onto collagen gels prepared in the presence of purified decorin core protein or dermatan sulphate GAG chains (Figure 3.3B and C respectively). Neither the GAG chain nor the core protein moieties had any consistent effect on endothelial cell adhesion. In the experiment shown (Figure 3.3B) core protein enhanced adhesion of cells 60 min after seeding. Core protein similarly enhanced cell adhesion 60 min after seeding in two further independent experiments, although only at the highest concentration tested. In one experiment, adhesion was additionally enhanced 10 min after seeding. Hence, in 3 of 3 experiments, collagen-bound core protein promoted adhesion to a certain degree. Dermatan sulphate had variable effects on adhesion at all time-points tested, and in the experiment shown, inhibited adhesion dose-dependently 180 min after seeding (Figure 3.3C). In a second experiment, dermatan sulphate enhanced adhesion 10 min and 60 min after seeding, while in a third, adhesion was enhanced 60 min after seeding, but inhibited 180 min after seeding, both in a dose-dependent manner. The core protein and DS moieties of decorin may not be retained in the matrix to the same extent as the intact proteoglycan which may in part, explain the variability of these results. Only intact decorin consistently and dose-dependently promoted adhesion, indicating that the intact proteoglycan is required for enhancement of cell adhesion although contributions from either the core protein or dermatan sulphate cannot be excluded.

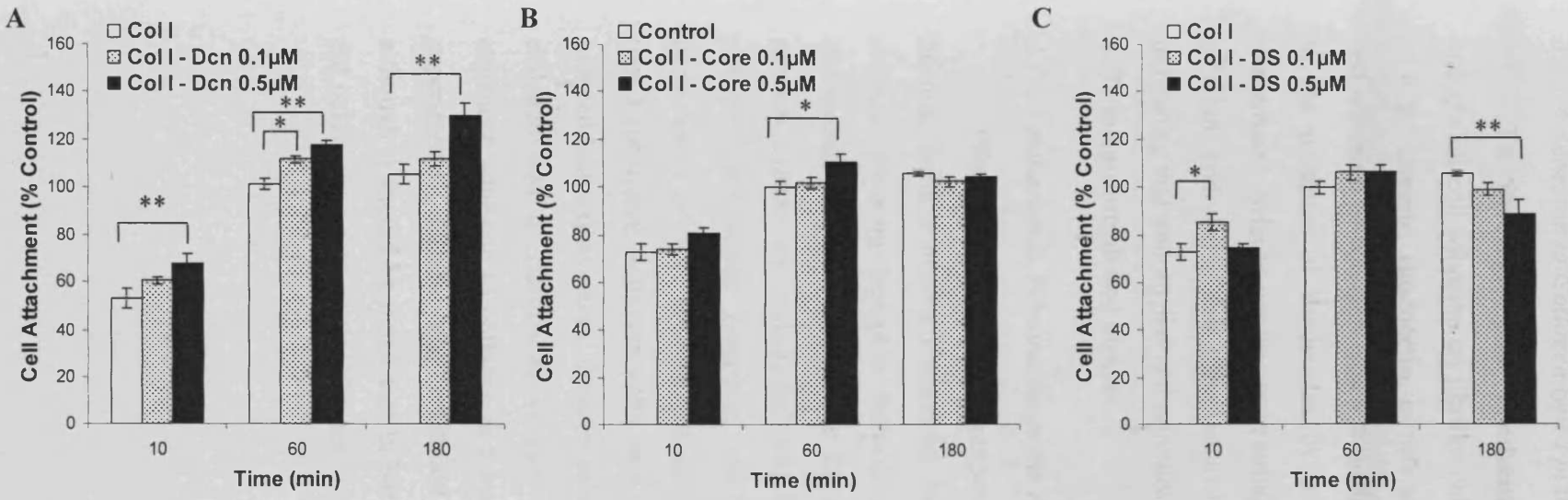


Figure 3.3: Endothelial cell adhesion to collagen type I, collagen bound-decorin, or to collagen-bound decorin moieties. Endothelial cells (15,000 per well) were seeded onto collagen gels prepared in the presence of decorin (A), purified core protein (B) or dermatan sulphate GAG chains (C). Adherent cells were fixed and stained with crystal violet at the indicated times after plating. Five wells were coated for each variable, and the average calculated. Standard errors are shown as error bars and data shown is 1 of 3 independent experiments. Statistical significance is shown where $p < 0.05$ is represented as * and $p < 0.01$ as **.

3.3.1.4 Adhesion to Collagen type I in the Presence of Rhodocetin

To investigate the involvement of collagen-binding integrins in endothelial cell adhesion on fibrillar collagen type I, we used a specific inhibitor of α2β1 integrin, rhodocetin, in this assay. To investigate whether endothelial cell adhesion to collagen type I is mediated by α2β1 integrin, cells were seeded in the presence of rhodocetin by mixing cell suspensions with rhodocetin immediately prior to seeding onto collagen gels. When rhodocetin was present in solution, endothelial cell adhesion to collagen gels was inhibited (Figure 3.4), indicating that endothelial cell adhesion to collagen type I is at least partially an α2β1 integrin-mediated process.

3.3.1.4 Adhesion to Fibronectin in the Presence of Decorin

Fibronectin is a major component of the provisional matrix in wound healing. Soluble plasma fibronectin from blood serum is deposited in vascular damage, forming insoluble matrices in the local environment. Immobilised fibronectin serves as a substrate for migrating endothelial cells. To analyse decorin effects on endothelial cell behaviour on substrates recognised by different cell-surface receptors, cell suspensions were mixed with decorin immediately prior to seeding onto immobilised fibronectin or fibrillar collagen type I substrates. Adherent cells were quantified 60 min after seeding. In three independent experiments, decorin enhanced endothelial cell adhesion to both collagen and to fibronectin (Figure 3.5). In all three experiments decorin enhanced adhesion to collagen in a statistically significant manner. In all three experiments, decorin also enhanced adhesion to fibronectin (Figure 3.5) although it should be noted that in one of these experiments, this enhancement did not reach statistical significance.

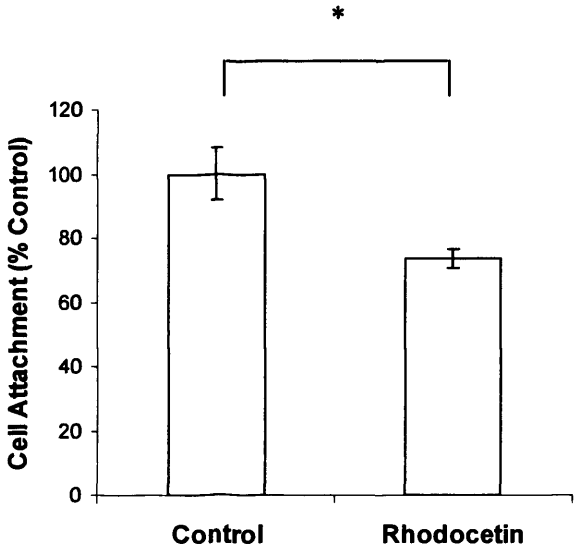


Figure 3.4: Endothelial cell adhesion to collagen type I in the presence of a specific α2β1 integrin inhibitor, rhodocetin. Endothelial cells (15,000 per well) were mixed with 0.15μM rhodocetin immediately prior to seeding onto collagen gels. Adherent cells were fixed and stained with crystal violet one hour after plating. Four wells were coated for each variable, and the average calculated. Standard errors are shown as error bars and data shown is representative of 1 of 2 independent experiments. Statistical significance was calculated using 2-tailed student's t-test, where * represents $p < 0.05$.

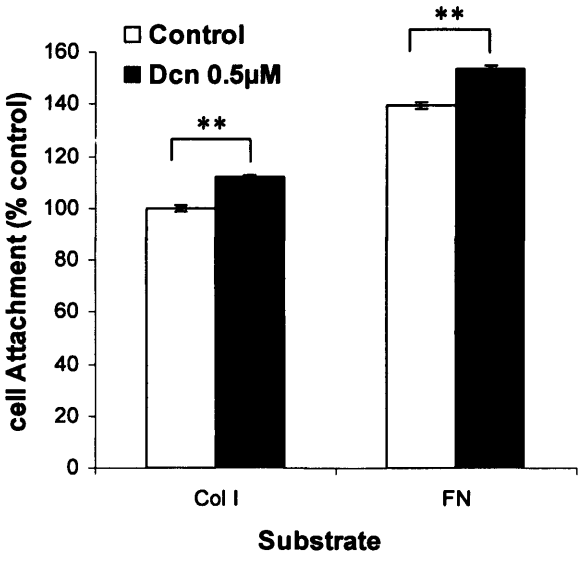


Figure 3.5: Endothelial cell adhesion to collagen type I or to fibronectin in the presence of decorin. Microtitre plates were coated with fibronectin at 10μg/ml and blocked with 1% BSA, or were coated with fibrillar collagen gels. Endothelial cell suspensions were mixed with 0.5μM decorin and seeded onto fibronectin or collagen for 60 min. Adherent cells were fixed and stained with crystal violet. Five wells were coated for each variable, and the average calculated. Standard errors are shown as error bars and data is representative of three independent experiments. Statistical significance is shown where $p < 0.01$ is represented as **.

3.3.2 Influence of Decorin on Endothelial Cell Migration

3.3.2.1 Migration on Collagen type I in the Presence of Decorin

A spheroid migration model was developed to investigate the effect of different forms of decorin on endothelial cell migration. To analyse decorin effects on endothelial cell migration, spheroids were plated onto collagen type I gels and decorin (at 0.1μM and 0.5μM) was immediately added to the medium. Endothelial cell migration was quantified by measuring the cross-section of the spheroid at different times as detailed in materials and methods and shown in Figure 3.6A (48 hours after plating spheroids). When endothelial cells migrated from spheroids in the presence of decorin in solution, endothelial cells formed elongated, polarised sprout-like extensions that extended over a larger area than control spheroids (Figure 3.6). Figure 3.7 illustrates quantitative analysis of the results and shows that spheroid migration in the presence of decorin was enhanced in a dose-dependent manner.

3.3.2.2 Migration on Collagen-bound Decorin

When incorporated into collagen gels, decorin also enhanced spheroid migration (Figure 3.8). It should be noted that the degree by which decorin enhanced migration varied between experiments. Figures 3.7 and 3.8 are not directly comparable since experiments were conducted on different occasions.

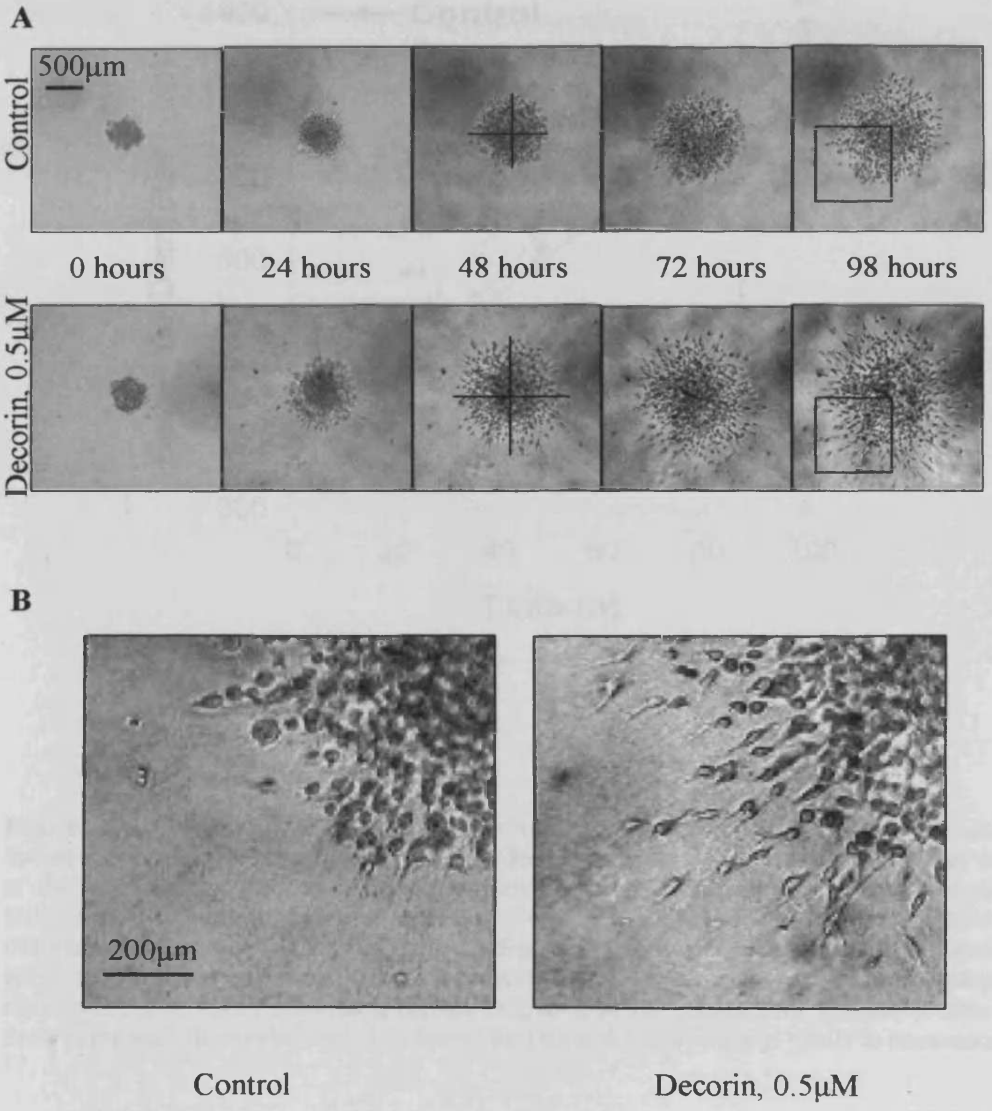


Figure 3.6: Morphology of endothelial cells migrating on collagen type I in the presence or absence of decorin. Spheroids were plated onto collagen gels and decorin at a final concentration of 0.5µM was immediately added to the medium where indicated. Migration was followed by capturing images at the indicated times after plating and quantified by measuring the height and width of each spheroid as indicated at 48 hours after plating (A). An enlarged section of images captured 98 hours after plating is shown in (B), indicated by the black box in (A), to highlight changes in cell morphology.

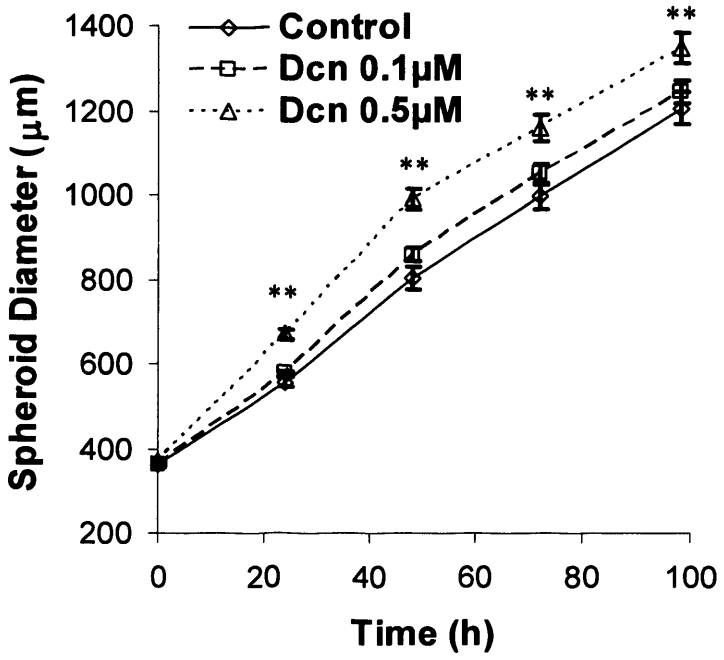


Figure 3.7: Endothelial cell migration on collagen type I in the presence of decorin. Spheroids were plated onto collagen gels and decorin at the indicated concentrations was added to the medium. For each variable, a minimum of 10 spheroids were plated, and migration followed by capturing images at indicated times. Migration was quantified as detailed in materials and methods. The average spheroid diameter is shown for each time-point and standard errors are shown as error bars. Data is representative of 3 independent experiments. Statistical significance was tested between spheroids migrating in the presence or absence (control) of decorin for each time-point, and is indicated for Dcn at 0.5μM, where $p < 0.01$ is represented as **.

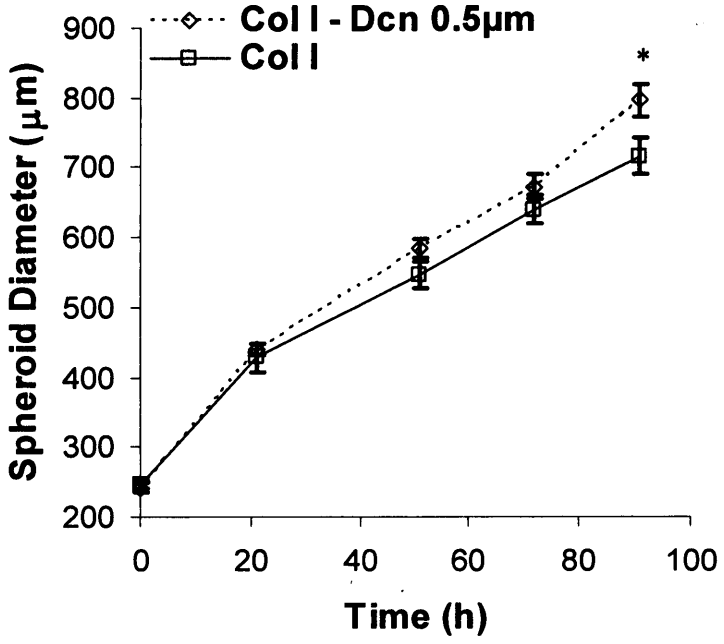


Figure 3.8: Endothelial cell migration on collagen-bound decorin. Spheroids were plated onto collagen gels or gels containing decorin (prepared by addition of decorin or the equivalent volume of PBS prior to gelling). For each condition, a minimum of 10 spheroids were plated, and migration followed by capturing images at indicated times. Migration was quantified as detailed in materials and methods. The average spheroid diameter is shown for each time-point and standard errors are shown as error bars. Data is representative of 2 independent experiments. Statistical significance was tested between spheroids migrating in the presence or absence (col I) of decorin for each time-point, and is indicated, where $p < 0.05$ is represented as *.

3.3.2.3 Migration on Collagen type I in the Presence of Rhodocetin

Migration is an integrin-mediated process. To investigate the involvement of collagen-binding integrins in endothelial cell migration on fibrillar collagen type I, we used a specific inhibitor of α2β1 integrin, rhodocetin, in this assay. Spheroids were plated onto collagen gels and rhodocetin immediately added to the medium at 0.15μM or 0.3μM. Endothelial cell migration on collagen was dose-dependently inhibited in the presence of rhodocetin (Figure 3.9). At 0.15μM rhodocetin, spheroid migration is reduced however this was not statistically significant. At 0.3μM rhodocetin, spheroid migration is reduced in a statistically significant manner. These data indicate that spheroid migration on collagen type I is at least partially an α2β1 integrin-mediated process. The data shown in Figure 3.7 and Figure 3.9 is directly comparable since these experiments were conducted in parallel.

3.3.2.4 Migration on Fibronectin in the Presence of Decorin

To analyse the role of decorin in endothelial cell migration on different matrix substrates, spheroids were allowed to migrate on a substrate of fibronectin. When soluble decorin was added to the medium, endothelial cell migration was slightly enhanced at all time-points tested, but only to a statistically significant degree at the last time-point (Figure 3.10A).

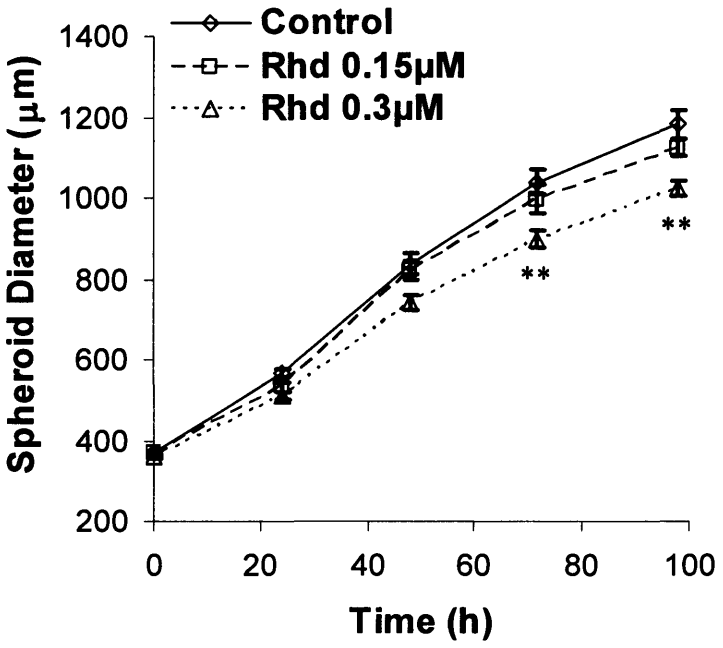


Figure 3.9: Endothelial cell migration on collagen type I in the presence of rhodocetin. Spheroids were plated onto collagen gels and rhodocetin was immediately added to the medium at the indicated concentrations. For each condition, a minimum of 10 spheroids was plated, and migration was followed by capturing images at indicated times. Migration was quantified as detailed in materials and methods. The average spheroid diameter is shown for each time-point and standard errors are shown as error bars. Data is representative of 3 independent experiments. Statistical significance was tested between spheroids migrating in the presence or absence (control) of rhodocetin for each time-point, and is indicated for Rhd at 0.3µM, where $p < 0.01$ is represented as **.

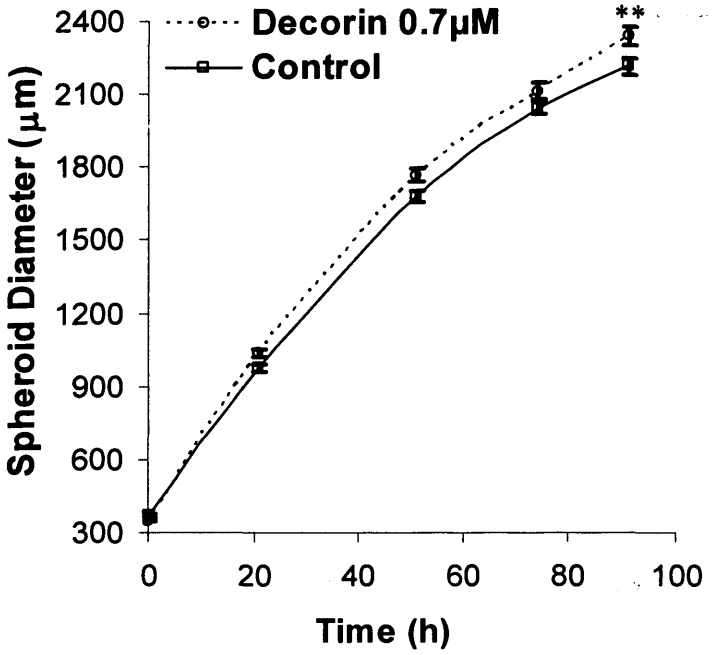


Figure 3.10: Endothelial cell migration on fibronectin in the presence of decorin. Wells were coated with fibronectin and blocked with 1% BSA. Spheroids were plated and decorin immediately added to the medium where indicated. For each condition, a minimum of 10 spheroids were plated, and migration was followed by capturing images at indicated times. Migration was quantified as detailed in materials and methods. The average spheroid diameter is shown for each time-point and standard errors are shown as error bars. Data is representative of 2 independent experiments. Statistical significance was tested between spheroids migrating in the presence or absence (control) of decorin for each time-point, and is indicated, where $p < 0.01$ is represented as **.

3.4 Discussion

In this Chapter, we characterised the role of decorin in endothelial cell attachment and migration. Decorin by itself as an immobilised substrate could not support endothelial cell attachment. In contrast, when decorin was presented in conjunction with collagen, endothelial cell attachment was enhanced compared to collagen alone. When added to cells in suspension immediately prior to seeding, decorin also enhanced attachment to both collagen type I and to fibronectin. Decorin effects on attachment (whether collagen-bound or soluble) required the intact proteoglycan, since neither the purified core protein or dermatan sulphate GAG chains alone could exert any consistent influence. Similarly, in a spheroid outgrowth model, decorin enhanced endothelial cell migration irrespective of presentation as a collagen-bound or soluble form. It should be noted that exogenous decorin would also interact with matrix substrates and thus represents both soluble and to some extent, collagen-bound decorin. However, under these conditions, collagen fibrils would have already formed thus decorin is unlikely to have a major effect on fibrillogenesis or to be incorporated extensively into collagen fibrils. Since soluble decorin (similarly to collagen-bound decorin) was able to enhance endothelial cell attachment and migration on collagen, decorin effects appear to be independent of its role in collagen fibrillogenesis.

In contrast to this study, immobilised decorin by itself was previously shown to support endothelial cell adhesion, although stress fibre formation was impaired indicating that decorin negatively regulates this processes compared to classic adhesion-supporting matrix substrates (Davies et al., 2001). Studies on fibroblasts also indicate variability in the role of decorin in supporting cell attachment. Immobilised decorin generally could not support fibroblast attachment (Huttenlocher et al., 1996; Lewandowska et al., 1996; Noyori and Jasin, 1994; Winnemoller et al., 1991), while one study found that decorin could support adhesion, but that stress fibre formation was impaired (Bidanset et al., 1992).

Unlike migration experiments, which may be significantly influenced by cellular adaptation during time in culture, cell attachment to matrix substrates in the short-term is mediated primarily by integrin ligation. In our study, decorin enhanced endothelial cell attachment to both collagen type I and fibronectin, although this effect was more pronounced in the former case. Cell attachment to these substrates involves ligation of RGD-independent and RGD-dependent integrins respectively, indicating that decorin may influence activity of a number of integrins. This data contrasts to a recent study, where decorin was shown to inhibit endothelial cell adhesion to both fibronectin and collagen type I (Sulochana et al., 2005). Differences in cell lines used, or in experimental methods could account for contradictory effects of decorin on endothelial cells. In our experiments, cell suspensions were mixed with decorin and seeded immediately. Previously however, cell suspensions were pre-incubated with decorin for 30 minutes prior to seeding (Sulochana et al., 2005), which may have altered the pattern or activation state of cell-surface receptors. Alternatively, natively purified decorin used in the current study could have revealed previously unseen effects of decorin on endothelial cell behaviour.

Indeed, it was previously shown that natively purified decorin interfered with fibroblast adhesion to intact fibronectin and to a CBD-containing fragment (Winnemoller et al., 1991) while decorin purified under denaturing conditions could inhibit fibroblast adhesion to the CBD-containing fragment of fibronectin only (Bidanset et al., 1992). Work from the laboratory where natively purified decorin was used (Winnemoller et al., 1991) demonstrated two binding sites for both intact decorin and isolated core protein on intact fibronectin and on a CBD-containing fragment (Schmidt et al., 1991). Decorin purified under denaturing conditions however, interacted with one binding site on intact fibronectin and on a CBD-containing fragment, while the isolated core interacted with two sites on each substrate (Bidanset et al., 1992). Differential decorin-substrate interactions may therefore occur depending on conditions of decorin purification.

There is no definitive mechanism by which decorin influences cell adhesion. Steric inhibition of cell-surface receptor interactions with matrix substrates by decorin has been demonstrated (Merle et al., 1997; Ehnis et al.,

1998). In support of this, decorin inhibits fibroblast adhesion to substrates that contain binding sites for decorin (Lewandowska et al., 1987; Winnemoller et al., 1991; Winnemoller et al., 1992; Bidanset et al., 1992; Gu and Wada, 1996; Merle et al., 1997; Bhide et al., 2005). However, this mechanism does not explain decorin-mediated enhancement of endothelial cell adhesion to both collagen type I and to fibronectin in our experiments. It has been suggested that decorin inhibits fibroblast adhesion to collagen independently of interactions with cell surface receptors, including α2β1 integrin (Bhide et al., 2005). However, the possibility of decorin interacting with cell-surface receptors under different conditions cannot be excluded, and may be the mechanism responsible in our system. As noted by the authors, an endocytosis receptor for decorin on fibroblasts has been established (Hausser et al., 1998). Further to this, decorin interacts with growth factor receptors on adherent cell types (Iozzo et al., 1999; Schönherr et al., 2005) and with α2β1 integrin on platelets (Guidetti et al., 2002). Interestingly, CHO cells do not express α2β1 integrin (Nykvist et al., 2001), but still adhere to collagen type I, evidently through alternative collagen-binding integrins. Decorin could not influence CHO cell adhesion to collagen (Bittner et al., 1996), indicating that of the collagen-binding integrins, decorin may more specifically influence α2β1 integrin-mediated adhesion.

Cell motility requires a fine balance of de-adhesion and re-adhesion at the trailing and leading edge of the cell respectively. Cells adhering too tightly would be unable to migrate. Conversely, insufficient cell-matrix contacts would prevent migration since matrix-integrin contacts exert tractional forces on the cytoskeleton. A recent study has highlighted a complex relationship between endothelial cell attachment, migration and tube formation (Sulochana et al., 2005). Decorin inhibited VEGF-induced tube formation on matrigel. Peptides derived from LRR5 of decorin core protein mimicked this effect, implicating this region of decorin in control of tube formation. Interestingly, inhibition of either adhesion or migration alone could not account for inhibition of tube formation. A LRR5-derived peptide that inhibited tube formation could not interfere with adhesion, while the most effective peptide in inhibiting migration could not inhibit tube formation (Sulochana et al., 2005). This indicates that

distinct activities of decorin could be involved in different aspects of angiogenesis. However, experiments conducted with decorin-derived peptides should be regarded with caution in considering the role of the intact proteoglycan in angiogenesis. Decorin-derived peptides exhibited some different activities to intact decorin in modulation of endothelial behaviour (Sulochana et al., 2005), therefore differential interactions of GAG and intact core protein could result in very different effects of the intact proteoglycan.

The spheroid outgrowth model used in our experiments could be considered to represent endothelial cell migration on interstitial matrix prior to tube formation. In contrast to previous studies on endothelial cells however (Kinsella et al., 2000; Sulochana et al., 2005), decorin enhanced migration. Previous investigation of alternative cell types such as osteosarcoma cells also indicated inhibitory effects of decorin on cell migration (Merle et al., 1999). However in agreement with our data, (Tufvesson and Westergren-Thorsson, 2003) reported that decorin enhances fibroblast migration. As highlighted in the earlier discussion of decorin effects on adhesion, natively purified decorin (used in this study) may have had different effects to decorin purified under denaturing conditions (Merle et al., 1999; Tufvesson and Westergren-Thorsson, 2003; Sulochana et al., 2005). Additionally, there may be cell-type and/or assay-specific effects. In classic chemotaxis assays, cell suspensions are seeded onto matrix-coated porous membranes and migration towards a chemotactic stimulus analysed (Merle et al., 1999; Kinsella et al., 2000; Tufvesson and Westergren-Thorsson, 2003; Sulochana et al., 2005). In the current study, cells were seeded as a three-dimensional spheroid where cell-cell contacts were pre-established prior to plating, and a migratory phenotype induced by matrix contact. In a different approach, decorin inhibited endothelial cell monolayer outgrowth possibly through enhancing pericellular organisation of fibronectin (Kinsella et al., 2000). Over the time-course of our assay, the cells could deposit fibronectin. However, if decorin were enhancing pericellular fibronectin network formation, it might be expected that migration would be inhibited rather than promoted. It should also be noted that in our system, migration was largely independent of proliferation. Under the conditions of the assay (conducted in 0.5% heat-

inactivated FCS), endothelial cell proliferation is unlikely to be supported since migration reached a plateau after 7 days in culture, indicating that there was a limited cell pool which could contribute to migration. Also, decorin has been shown not to influence endothelial cell proliferation (Kinsella et al., 2000; Sulochana et al., 2005).

While a definitive mechanism by which decorin influences migration does not exist, the involvement of direct signalling through unknown cell-surface receptors by decorin has been proposed (Merle et al., 1999; Tufvesson and Westergren-Thorsson, 2003). Decorin also enhanced pericellular fibronectin network organisation (Kinsella et al., 2000), a process dependent upon matrix interaction with fibronectin-binding integrins (Mao and Schwarzbauer, 2005). A candidate receptor for enhancement of migration by decorin is the IGF-I receptor (IGF-IR). Decorin signals through the IGF-IR in endothelial cells (Schönherr et al., 2005) and the classic IGF-IR ligand IGF-I promotes α2β1, α3β1 and α5β1-mediated migration (Lynch et al., 2005). This provides a potential mechanism for decorin enhancement of cell migration on collagen or fibronectin through IGF-IR-mediated signalling and 'inside-out' activation of integrins. Another possibility is direct interaction of decorin with integrins, since decorin interacts with α2β1 integrin on platelets (Guidetti et al., 2002). In support of either mechanism, use of the integrin inhibitor rhodocetin in this study demonstrates that endothelial cell adhesion and migration on collagen type I in our system is at least partially mediated by α2β1 integrin. This indicates that decorin could influence α2β1 integrin activity through direct and/or indirect mechanisms.

In summary, decorin has been suggested to influence adhesion by masking integrin binding sites, but to influence migration by interacting with cell-surface receptors. The former mechanism does not explain our observations, additionally, decorin promoted attachment independently of its influence on collagen fibrillogenesis. Thus, direct interactions of decorin with cell-surface receptors should be considered, and IGF-IR and α2β1 integrin represent candidate receptors. Further investigation of decorin interactions with and signalling through IGF-IR and/or α2β1 integrin is therefore required to elucidate the mechanism underlying decorin influences on endothelial cell behaviour.

Chapter 4

Investigation of Decorin Interactions With the Collagen-binding Integrin, $\alpha_2\beta_1$

4.1 Introduction

In chapter 3 it was demonstrated that decorin enhances endothelial cell adhesion and migration on collagen type I. In contrast, previous studies have shown that decorin inhibits endothelial cell adhesion (Sulochana et al., 2005) and migration (Kinsella et al., 2000; Sulochana et al., 2005) on collagen type I. These differences may reflect different methodologies and sources of decorin used in these studies. However, there is currently no clear mechanism for decorin influences on endothelial behaviour, making further explanation of these differences difficult. One possible mechanism for decorin effects could be through direct interactions with cell-surface integrins. Cell adhesion and migration are controlled by interactions between integrins and the extracellular matrix. Immobilised decorin has recently been shown to interact with α2β1 integrin on platelets and in solid-phase assays (Guidetti et al., 2002). Additionally, decorin is an important regulator of collagen fibrillogenesis (Danielson et al., 1997) and the α2β1 integrin has also been recently implicated in the cellular control of this process (Jokinen et al., 2004). Therefore, decorin could influence cell adhesion and migration by modulating integrin-collagen interactions. Indeed, it was demonstrated in Chapter 3 that decorin enhanced endothelial attachment and migration on collagen and that in our system this was at least partially, an α2β1 integrin mediated process.

Irrespective of whether decorin enhances or inhibits adhesion and migration on a collagen substrate, these events are under the control of the collagen type I binding integrins, α1β1, α2β1, α10β1 and α11β1. The latter two integrins have not been shown to be expressed by endothelial cells, and their distribution is reported to be restricted to cartilage (Camper et al., 1998) and embryonic mesenchymal cells, respectively (Tiger et al., 2001). Based on this restricted expression and as the molecular tools to investigate the α10 and α11 subunits are not yet widely available, these integrins have not been considered further in this study. In this chapter therefore, interactions of decorin with α2β1 integrin and the other major collagen type I-binding integrin α1β1 will be investigated.

Several studies support key roles for α2β1 and α1β1 integrins in angiogenesis. During angiogenesis, the matrix microenvironment is remodelled and integrin expression is altered to allow endothelial cell migration and proliferation. In accordance with this, vascular endothelial growth factor (VEGF) upregulates the α2 and α1 integrin subunits on endothelial cells *in vitro*, and blocking antibodies to these integrins inhibit VEGF-driven angiogenesis *in vivo* (Senger et al., 1997). Furthermore, inhibitors of both alpha subunits inhibit tumour angiogenesis (Senger et al., 2002), and both subunits have been shown to promote endothelial cell migration (Byzova et al., 2000; Senger et al., 2002). Additionally, the α2β1 integrin is suggested to play a role in vessel maturation. For example, ligation of α2β1 but not α1β1 with collagen type I is required for capillary morphogenesis (Sweeney et al., 2003). Similarly, vacuole formation and coalescence precedes lumen formation (Kamei et al., 2006) and is dependent on α2β1 but not α1β1 integrin (Davis and Camarillo, 1996). Recently, endorepellin, a fragment of perlecan, was also suggested to interact with α2β1 integrin (Bix et al., 2004) adding to the growing list of ligands for this integrin that are associated with angiogenesis.

Integrins switch between inactive (low affinity) and active (high affinity) states in response to extracellular and intracellular signals that act by eliciting conformational changes in the integrin. Indeed, conformational changes in the extracellular domain are required for integrin activation (Sims et al., 1991). Several integrin α subunits (the collagen-binding α1, α2, α10, α11 subunits and the leukocyte subunits αD, αE, αL, αM, αX) contain an additional domain, the A-domain (homologous to Von Willebrand factor A-domain, also referred to as the I- domain, for 'insertion'). The A-domain (pink domain, Figure 4.1) contains a conserved metal binding site or MIDAS (metal ion–dependent adhesion site) required for ligand binding and integrin activation (Kamata et al., 1999; Kamata and Takada, 1994; Tuckwell et al., 1995) (arrow, Figure 4.1), and the propeller of the α subunit head region (dark pink and pink regions, Figure 4.1) interacts with the β subunit (Xiong et al., 2001). The β subunit is not involved in ligand binding, and instead plays a regulatory role (Lu et al., 2001a). However, this

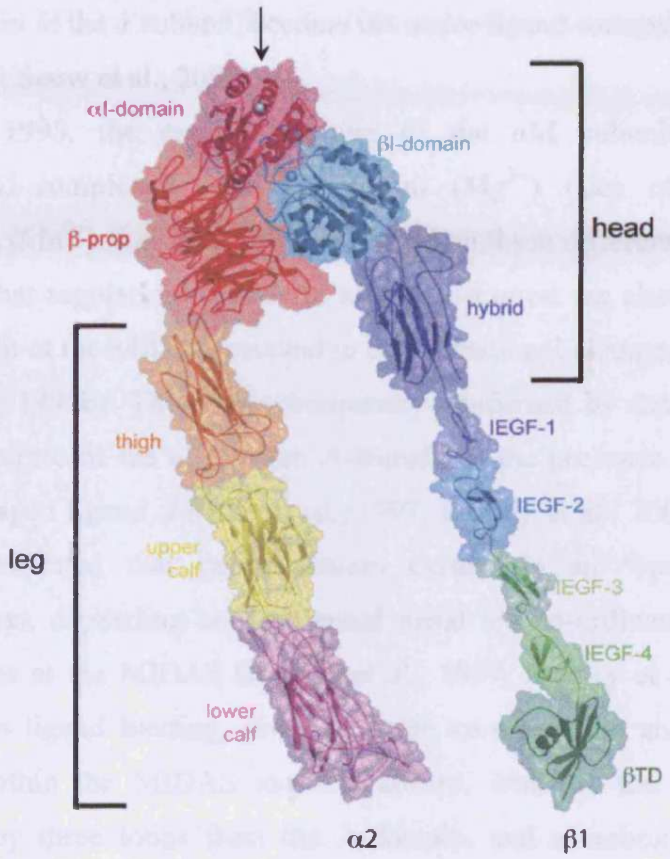


Figure 4.1: A model of α2β1 integrin in a fully open and active, high affinity conformation. The domains of the head and leg regions are shown, where the C-terminal helices in αA- and βA-domains (labelled as I domains) are in the active/open conformation, and the leg regions separated. On the left, the α2 subunit is shown in orange/yellow/pink. On the right, the β1 subunit is shown in purple/blue/green. The MIDAS site is indicated by the grey sphere (shown by an arrow) in the A-domain of the α domain (labelled αI, pink). Collagen binds at the αA-domain MIDAS. Reproduced from (White et al., 2004).

subunit also contains an A-domain and propeller region, which in the absence of an A-domain in the α subunit, become the major ligand recognition sites (Mould et al., 1997; Seow et al., 2002).

In 1995, the crystal structure of the αM subunit A-domain was determined, complexed with magnesium (Mg^{2+}) (Lee et al., 1995a) or manganese (Mn^{2+}) (Lee et al., 1995b). Based on these different structures, it was proposed that regulation of integrin affinity occurred *via* changes in metal ion coordination at the MIDAS coupled to conformational changes in the A-domain (Lee et al., 1995b). This was subsequently confirmed by determination of the crystal structure of the α2 subunit A-domain in the presence and absence of a model collagen ligand (Emsley et al., 1997; Emsley et al., 2000). These studies also demonstrated that the A-domain existed in an 'open' and 'closed' conformation, depending on differential metal ion co-ordination by the ligand and residues at the MIDAS (Emsley et al., 1997; Emsley et al., 2000), Figure 4.2B. Upon ligand binding, divalent cation co-ordination and the position of residues within the MIDAS motif is altered, whereby the metal ion is co-ordinated by three loops from the A-domain, and a carboxylate group from glutamic or aspartic acid of the ligand (Figure 4.2B). This rearrangement is coupled to a shift of the C-terminal helix, α7 (Figure 4.2B, yellow helix) which is propagated to the rest of the molecule (reviewed in Arnaout et al., 2005).

Ligand binding by integrins is modulated by the presence of the divalent cations Mg^{2+}, Mn^{2+} and calcium (Ca^{2+}). Mn^{2+} or Mg^{2+} co-ordination at the MIDAS is proposed to participate in regulation of ligand binding by stabilising a more active integrin conformation (Gotwals et al., 1999). While the MIDAS is essential for ligand binding, further metal ion binding sites also contribute (Dickeson et al., 1997), and these divalent cations each induce or stabilise different integrin conformations. For example, each cation altered the proteolytic susceptibility of α2β1 integrin, producing different patterns after tryptic digestion (Staatz et al., 1990). It has been shown that Mg^{2+}, but not Ca^{2+}, supports α2β1 integrin (Staatz et al., 1989) and isolated α2 A-domain interaction with collagen type I (Tuckwell et al., 1995). However, interaction of α2β1 integrin with collagen type I in the presence of Mg^{2+} was inhibited by Ca^{2+} in a

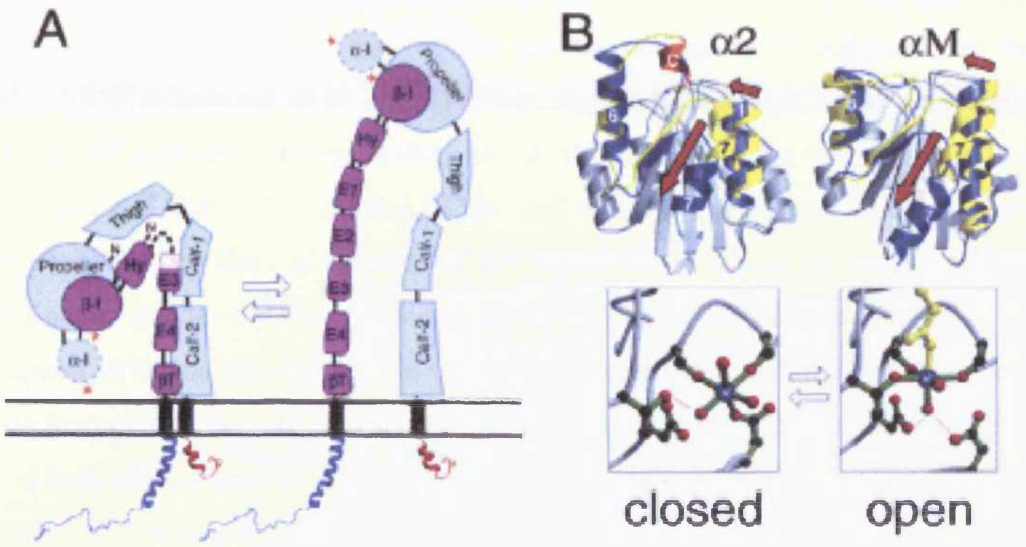


Figure 4.2: A model of active and inactive integrin conformations. In part (A), the left-hand side illustrates an integrin in a closed, bent conformation, with association between the cytoplasmic, transmembrane and leg domains of the integrin. On the right-hand side, an integrin in the active conformation is shown, where the cytoplasmic, leg and tail domain are separated. In part (B), different conformations of isolated α2 and αM A-domains are shown. The upper panels indicate open and closed forms of the α2 A-domain and αM A-domain respectively. The red arrows indicate major conformational changes. Regions that undergo large structural changes are shown in blue (open/liganded) and yellow (closed/unliganded). The lower panels detail the MIDAS motif in the closed and open (ligand-bound) conformations. Reproduced from (McCleverty and Liddington, 2003).

non-competitive manner (Staatz et al., 1989). In contrast, Ca^{2+} could not inhibit interaction of the isolated A-domain with collagen type I in the presence of Mg^{2+} (Dickeson et al., 1997). These studies (Staatz et al., 1989; Dickeson et al., 1997) indicate that separate binding sites exist for these cations. In contrast, other studies indicate that Ca^{2+} can support ligand binding by co-ordination at the MIDAS (Calderwood et al., 1997). This may be ligand or integrin dependent, since Ca^{2+} supported ligand binding to the α1 subunit but not the α2 subunit A-domain, and Ca^{2+} co-ordination at the MIDAS may only be supported when aspartate rather than glutamate of the ligand is involved in metal ion co-ordinaton at the MIDAS (Calderwood et al., 1997; Ajroud et al., 2004). In support of ligand involvement in Ca^{2+}-dependent effects, isolated αM A-domain has low affinity for Ca^{2+} irrespective of integrin conformation (open or closed), and high affinity for Mg^{2+} but only in the open, active state (Ajroud et al., 2004). However, binding of ligands to the A-domain of αM enhanced affinity for Ca^{2+} (Ajroud et al., 2004).

In the absence of ligand, interactions between the legs, tails and head regions are thought to hold the head in an 'inactive' or 'resting' conformation that has low affinity for ligand (Hughes et al., 1996; Takagi et al., 2001) and the integrin is thought to be in a bent conformation (Takagi et al., 2002), Figure 4.2A. Cytoplasmic interactions between integrin subunits controls 'inside-out' signalling, whereby interruption of these interactions results in separation of the leg domains and integrin activation (Hughes et al., 1996; Hughes et al., 1995; O'Toole et al., 1994), Figure 4.2A. In support of this, forced association of integrin subunits prevents activation (Lu et al., 2001b). Specifically, a cytoplasmic GFF*XX* sequence in the α2 subunit of α2β1 integrin acts as a hinge, maintaining the integrin in an inactive conformation (O'Toole et al., 1994), and cell-permeable peptides containing this sequence activated the integrin (Wang et al., 2003). There are likely multiple sites at which cytoplasmic tail interactions are regulated. For example, the platelet integrin αIIbβ3 has multiple electrostatic and hydrophobic contacts between the cytoplasmic tails collectively referred to as a 'handshake' or 'clasp'. These interactions are disrupted by point mutations leading to 'unclasping' and integrin activation (Vinogradova et al., 2002).

Multiple interactions of αIIbβ3 integrin contribute differentially to integrin activation, whereby the integrin may exist in a variety of states between fully active 'unclasped' and fully inactive 'clasped' conformations (Ma et al., 2006). During 'outside-in' signalling, the head region binds to extracellular matrix proteins, triggering a 'switchblade' opening of the integrin head that is propagated down the legs and through the plasma membrane. The cytoplasmic and trans-membrane domains separate, exposing binding sites for intracellular proteins (Takagi et al., 2002). During 'inside-out' signalling, cytosolic proteins interact with one or both of the cytoplasmic tails, triggering conformational changes in the head that lead to a high-affinity, active conformation of the integrin (Vinogradova et al., 2002: Garcia-Alvarez et al., 2003).

The existence of three conformational states has been demonstrated for α2β1 integrin: a resting, inactive state; an intermediate, low affinity state induced by outside stimulation; and an active high affinity state, induced by inside-out stimulation. Both the intermediate and fully active states have collagen binding affinity (Van de Walle et al., 2005). The presence of three conformational states of the isolated A-domains of the integrin subunits αL and αM has also been reported (Jin et al., 2004), and different conformational states of αL have different binding affinities (Shimaoka et al., 2003). This phenomena may be common to all integrins since it has also been observed in integrins that do not contain an A-domain in the α subunit. For example, αvβ3 integrin has been shown to exist in a bent (low affinity) conformation, in an extended conformation with a closed headpiece, and in an extended conformation with an open head-piece (Takagi et al., 2002), similar to the observed structures of α2β1 integrin (Van de Walle et al. 2005).

A high affinity binding site for α2β1 integrin was identified on collagen type I, which contained an essential triple helical GER (glycine-glutamic acid-arginine) sequence (Knight et al., 1998). It was later demonstrated that the α2 A-domain bound to three GER-containing sites per collagen molecule (Xu et al., 2000). Further, α2β1 integrin may interact with up to 11 potential GER-containing motifs, depending on the activation state of the integrin (Siljander et al., 2004). In accordance with these observations, angiogenesis within a collagen type I environment is dependent on ligation of α2β1 integrin with a GER-containing sequence (Sweeney et al., 2003). A triple helical structure however, is not always a requirement for ligand binding to α2β1 integrin; the snake venom component rhodocetin, binds to the A-domain of the α2 subunit at a site overlapping that of the collagen-binding site, but does not contain a triple helical structure (Eble and Tuckwell, 2003).

The α2β1 integrin interacts with collagen in a cation-dependent manner. Mg^{2+} or Mn^{2+} support this interaction, while Ca^{2+} does not (Staatz et al., 1989). Further, monoclonal antibodies have been described which can preferentially interact with, or stabilise inactive (Tuckwell et al., 1995) or active states of α2β1 integrin (Schoolmeester et al., 2004). Collagen type I is the classic ligand for α2β1 integrin. Collagen interacts with the A-domain of the α2 subunit, and more specifically, preferentially interacts with an active conformation of the integrin (Van de Walle et al. 2005). Rhodocetin is a component of the venom of the Malayan pit viper (*Calloslelasma rhodostoma*), that has been shown to be a potent inhibitor of α2β1 integrin (Eble et al., 2001; Eble et al., 2002). In contrast to collagen type I, rhodocetin preferentially interacts with, and possibly stabilises, an inactive conformation of the integrin (Eble and Tuckwell 2003). Like collagen, rhodocetin also interacts with the A-domain of the α2 subunit, moreover, the binding site overlaps that of collagen type I, thus rhodocetin may also act in a competitive manner (Eble and Tuckwell, 2003). These well-characterised ligands and modulators of α2β1 integrin activation can therefore be used to investigate interactions of decorin with this integrin.

4.1.1 Aims

- To investigate the mechanism and consequence of interactions between decorin and α2β1 integrin, using collagen type I (which interacts with an active integrin conformation) and rhodocetin (which interacts with an inactive conformation) as tools
- To compare interaction of α2β1 integrin with immobilised collagen or decorin under conditions where inactive or active conformations are supported
- To investigate the location of the binding site for decorin on α2β1 integrin, and the moiety of decorin responsible for this interaction
- To investigate whether decorin interacts with and/or stabilises an active or inactive conformation of α2β1 integrin
- To investigate whether decorin interacts with the related collagen type I-binding integrin, α1β1

4.2 Materials and Methods

4.2.1 Protein Purification and Glycosaminoglycan Preparation

Decorin and decorin core protein were obtained and purified as described in Chapter 2. Dermatan sulphate (DS) (porcine skin, 90% L-iduronic acid, 10% chondroitin-4 sulphate (C-4S) and chondroitin-6 sulphate (C-6S), catalogue number C-4259) and CS (bovine trachea, 70% C-4S, 30% C-6S, catalogue number C-852) were obtained from Sigma-Aldrich, UK, and dissolved at concentrations of 1mg/ml in TBS/2mM $MgCl_2$. Collagen type I was purified from rat-tail tendons by acid extraction as described in Chapter 2, section 2.2.5.

The monoclonal antibodies JA221 and 9EG7 raised in mouse and rat respectively, were generous gifts of Dr. Johannes A. Eble (Muenster University) and have been previously described (Eble and Tuckwell, 2003). JA221 recognises a site in the human α2 A-domain (C-terminus of helix α4). This antibody enhances α2β1 integrin binding to collagen type I and inhibits integrin interactions with rhodocetin (Eble and Tuckwell 2003). 9EG7 is directed against the integrin β1 subunit (Lenter et al., 1993) and enhances integrin binding to collagen type I (Eble et al. 2001). Both antibodies were used at 200nM.

Human soluble α2β1 and α1β1 integrins and GST-α2 A-domain fusion protein and Rhodocetin were also obtained from Dr. J. A. Eble. Human soluble α2β1 integrin and GST- α2 A-domain fusion protein, which were recombinantly expressed in Drosophila Schneider cells and E. coli, respectively, were isolated as described previously (Eble et al. 2001, Eble and Tuckwell 2003). The human soluble α2β1 integrin contains a dimerisation motif of the transcription factor Fos that replaces both the membrane-anchoring transmembrane domains and the cytoplasmic tails of both integrin subunits (Eble et al. 2001). Human soluble α1β1 integrin was similarly generated and isolated as described (Eble et al., 2006). Rhodocetin was purified from the venom of the Malayan pit viper (*Calloselasma rhodostoma*) according to a previously published protocol (Eble et al., 2001), and binds to the A-domain of the α2 subunit at a site overlapping the collagen-binding site (Eble and Tuckwell, 2003).

4.2.2 Binding of α2β1 or α1β1 Integrin to Immobilised Ligand

Microtitre plates were coated overnight at 4°C (100μL per well, in duplicate) with solutions of decorin at 30μg/ml in PBS or collagen type I at 10μg/ml in 0.1M acetic acid. After washing twice with TBS (50mM Tris/HCl, pH7.4, 150mM NaCl) containing 2mM $MgCl_2$ (TBS/Mg), non-specific binding sites were blocked with 1% BSA in the same buffer for 90 min at room temperature. The α2β1 or α1β1 integrins (at concentrations up to 400nM, otherwise at 100nM) were diluted in blocking buffer containing 1mM $MnCl_2$, unless otherwise indicated. Specified additives or competitors were added (2μM rhodocetin, up to 10μM DS, up to 3μM C-4S, up to 3μM decorin core protein, 200nM JA221 or 9EG7 antibodies, 10mM EDTA, 2mM $CaCl_2$) and 100μL of the integrin-containing solution immediately added to each well of the plate. For analysis of pH dependence, ampholines (pH4-10; Amersham Biosciences) at 40mg/ml were used as buffering agents and the pH adjusted to between pH4.5 and pH7.5. The integrin solution was incubated with immobilised substrates for 2 h at room temperature. Unbound integrin was removed by washing twice with 50mM Hepes, pH7.0, containing 150mM NaCl 2mM $MgCl_2$ and 1mM $MnCl_2$. Bound integrin was immobilized with 2.5% (v/v) glutaraldehyde in the same buffer for 10 min at room temperature, and quantified by ELISA using a rabbit antiserum directed against the β1 subunit and anti-rabbit IgG alkaline-phosphatase-conjugated antibodies (Sigma), diluted 1:600 and 1:1000 respectively, in blocking buffer. Each antibody step incubation step was carried out for 90 min and was followed by three washes with TBS/Mg buffer. The ELISA was developed with 50μL per well of *p*-nitrophenyl phosphate (Sigma-Aldrich, UK) as a phosphatase substrate, in 0.1M glycine (pH10.4), supplemented with 1mM $MgCl_2$ and zinc acetate. The dye reaction was stopped by addition of 50μL 1.5M NaOH and the relative absorbance at 405nm versus 595nm measured in an ELISA reader (Dynatech, Germany).

4.2.3 Binding of α2 Integrin Subunit GST-A-domain to Immobilised Ligand

The methodology for investigation of GST-alpha2A-domain binding to decorin was conducted in a similar manner to binding of α2β1 integrin. GST-alpha2A-domain was incubated with decorin or collagen coated microtitre plates for 2 h at room temperature in binding buffer (1% BSA/TBS supplemented with 2mM $MgCl_2$) containing 1mM $MnCl_2$. Binding was detected using polyclonal rabbit antibodies to the GST moiety (Molecular Probes, Leiden, The Netherlands) at 1:600, followed by incubation with anti-rabbit IgG alkaline-phosphatase-conjugated antibodies (Sigma-Aldrich, UK). The ELISA was developed thereafter following the same experimental protocol as to that used for binding of α2β1 integrin to immobilised substrates (section 4.2.2).

4.2.4 Binding of Rhodocetin to Decorin

Rhodocetin, an inhibitor of α2β1 integrin interactions with collagen type I, will be used in this study to investigate decorin interactions with this integrin. However, it is not known whether decorin and rhodocetin have binding affinity. To investigate this, decorin binding to immobilised rhodocetin was analysed by ELISA or by dot-blotting. Rhodocetin binding to immobilised decorin could not be investigated, since antibodies to rhodocetin were not available.

4.2.4.1 Solid-Phase: ELISA

Each well of a 96-well microtitre plate was coated overnight at 4°C by incubation with 100μL of collagen type I or rhodocetin each at 80μg/ml in PBS. All subsequent steps were carried out at room temperature. After washing extensively with PBS, remaining binding sites were blocked by incubation with 200μL per well 1% BSA/PBS for 1 h. Wells were washed twice with PBS and incubated with 100μL of 2 or 10 μg/ml decorin in blocking buffer for 2h. Unbound decorin was removed by washing three times with PBS, and bound decorin detected using polyclonal rabbit antibodies to human decorin (1:1000), followed by HRP-conjugated anti-rabbit antibodies (1:5000, Dako Cytomation).

Polyclonal rabbit antiserum to human decorin has been previously described (Hausser et al., 1992), Chapter 2. Antibody incubations were carried out for 1h, and were followed by 3 washes with PBS/0.05% Tween. The ELISA was developed by addition of 50μL SureBlue tetramethylbenzidine dihydrochloride (TMB) Microwell Substrate (520003) for approximately 2 min or until colour had sufficiently developed. The reaction was stopped with 50μL of (TMB) stop solution (508506, both KPL, Insight Biotechnology, Wembly, UK) and the absorbance at 450nm measured using a 96-well microplate reader (EL311, BioTek Instruments, Winooski, VT, USA).

4.2.4.2 Solid Phase: Dot-blot Assay

Nitrocellulose membrane was prepared by soaking in transfer buffer (20x SDS-PAGE NuPAGE transfer buffer, Invitrogen, UK containing 10% methanol and Antioxidants, Invitrogen) and placing onto two sheets of similarly soaked blotting paper (Whatman Number 4, Whatman International, UK) in turn placed onto several sheets of dry blotting paper. 10μg amounts each of rhodocetin, collagen type I, plasma fibronectin (solution 1mg/ml, F1141, Sigma-Aldrich, UK) or BSA were spotted onto the membrane in a volume of 10μL PBS. 10μL of PBS alone was spotted as a control. The liquid was drawn through the membrane by capillary action. Remaining binding sites were blocked by incubation with 5% non-fat dried milk (NFDM, Marvel) in TBS/0.1% Tween overnight at 4°C. A solution of 40μg/ml decorin in TBS containing 1% NFDM/0.1% Tween was incubated with the membrane for 2h at room temperature. The membrane was washed three times between antibody incubations and prior to development with TBS/0.1% Tween for 5 min. Bound decorin was detected by incubation with anti-decorin antibodies diluted in 1% BSA/PBS (1:1000) followed by HRP-conjugated anti-rabbit antibodies (1:2000) for 1h each at room temperature. The membrane was developed by chemiluminescence by incubation with SuperSignal (Pierce Biotechnology, Perbio Science, Northumberland, UK) following the manufacturers instructions and visualised by exposure to photographic film (Hyperfilm ECL, Amersham Biosciences, UK).

4.3 Results

4.3.1 Interaction of α2β1 Integrin with Immobilised Decorin

To investigate whether α2β1 integrin interacts with decorin, a well-established *in vitro* assay was used. A soluble form of the integrin consisting of the ectodomain only was incubated with immobilised decorin. The integrin was incubated under activating (in the presence of Mn^{2+} and the activating antibody 9EG7) or inactivating (in the presence of EDTA) conditions. Under activating conditions, dose-dependent and saturable binding was observed (Figure 4.3). Binding was abolished in the presence of EDTA, indicating that binding occurs in a divalent-cation dependent manner (Figure 4.3). Collectively, this indicates that a relevant interaction exists between decorin and α2β1 integrin.

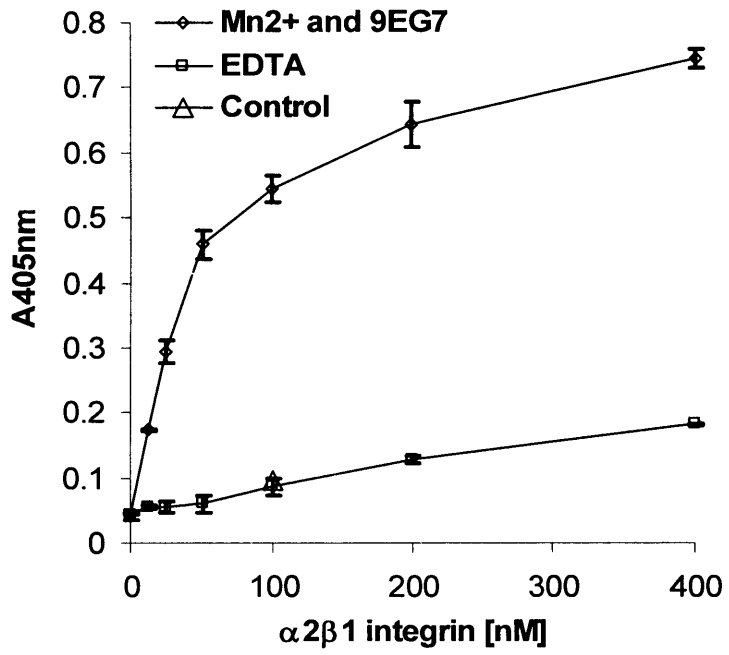


Figure 4.3: The α2β1 integrin interacts with immobilised decorin in a divalent cation dependent manner. Microtitre plates were coated with decorin and non-specific binding was blocked with BSA. Immobilised decorin was incubated with up to 400nM α2β1 integrin in the presence of an activating antibody 9EG7 and 1mM Mn^{2+}(Mn2+ and 9EG7) or 10mM EDTA (EDTA). To assess non-specific binding, wells were coated with BSA, and incubated with one concentration of α2β1 integrin, 100nM, in the presence of 9EG7 and 1mM Mn^{2+} (Control). Bound integrin was detected in duplicate wells using the ELISA-based method described in materials and methods. Data is representative of three independent experiments, and error bars indicate standard deviation.

4.3.2 Interaction of α1β1 Integrin with Immobilised Decorin

To investigate whether decorin also interacts with another collagen-binding integrin, α1β1, immobilised decorin was incubated with 100nM soluble α1β1 integrin ectodomain under various conditions. This concentration gave near saturation levels of binding for soluble α2β1 integrin (Figure 4.3). The integrin was incubated with immobilised decorin in binding buffer alone; in the presence of 1mM of the integrin activator Mn^{2+}; in the presence of the activating antibody 9EG7 and Mn^{2+} simultaneously; or with 10mM EDTA. No specific binding of this integrin with immobilised decorin was observed under any of the conditions tested, since the binding signal did not exceed that observed using immobilised BSA alone for coating (Figure 4.4). As a positive control, collagen type I, a known ligand for α1β1 integrin was tested under similar conditions. Under conditions where the integrin is activated, significant binding to collagen type I was observed. Binding was abolished in the presence of the cation chelator, EDTA. This demonstrates that the assay was working as expected, and that under conditions allowing collagen binding, no specific binding of α1β1 integrin to immobilised decorin could be detected.

4.3.3 Interaction of Integrin α2 Subunit A-domain with Immobilised Decorin

To investigate whether decorin interacts with the A-domain of the α2 subunit, immobilized decorin or collagen type I was titrated with a recombinant GST-fused integrin α2 A-domain (Figure 4.5). GST-tagged A-domain bound dose-dependently to immobilised collagen type I. However, even at concentrations where the α2 subunit A-domain-collagen interaction was saturated, no binding of the A-domain to decorin could be detected (Figure 4.5). This suggests that the interaction of α2β1 integrin with decorin is not mediated by the A-domain, or that the A-domain alone is not sufficient for binding.

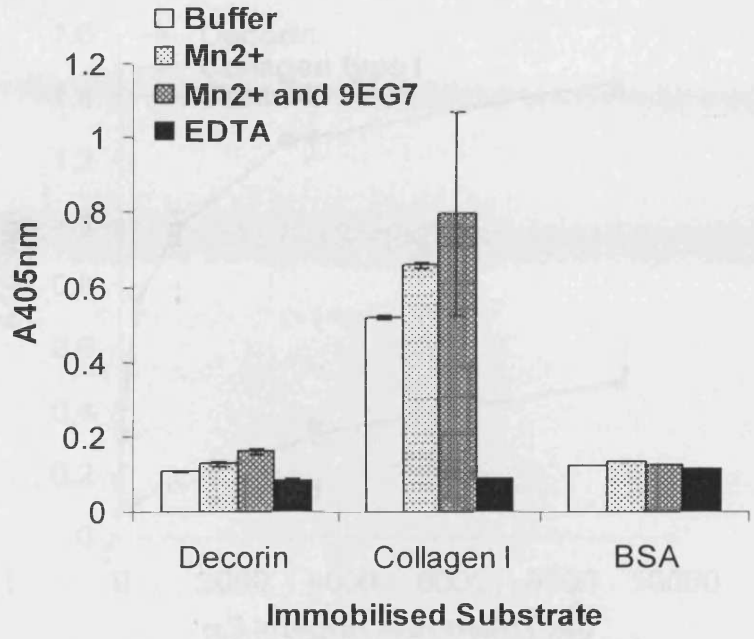


Figure 4.4: The α1β1 integrin does not interact with immobilised decorin. Microtitre plates were coated with decorin, collagen type I or BSA and non-specific binding was blocked with BSA. Immobilised substrates were incubated with 100nM α1β1 integrin in binding buffer alone, in the presence of 1mM Mn^{2+}, 9EG7 and 1mM Mn^{2+} simultaneously or 10mM EDTA. Bound integrin was detected in duplicate wells using the ELISA-based method described in materials and methods. Data is representative of two independent experiments, and error bars indicate standard deviation.

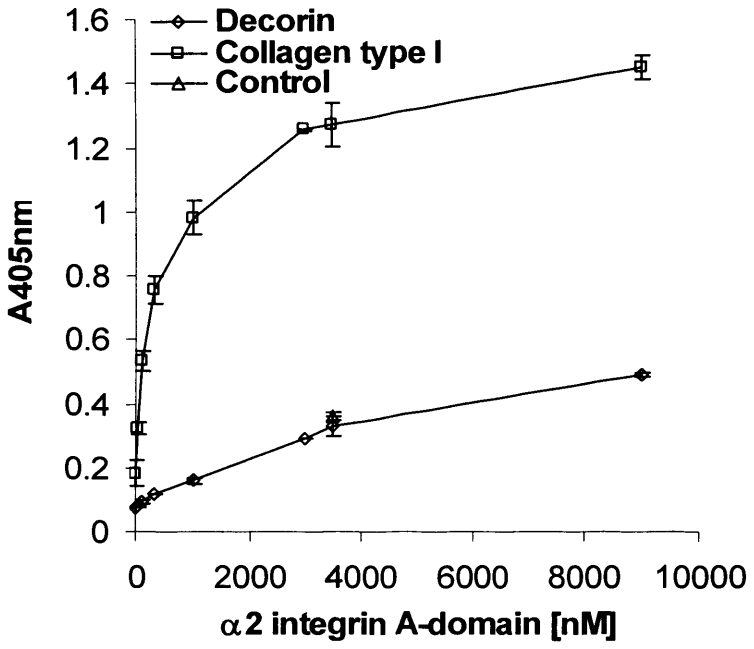


Figure 4.5: The α2 subunit A-domain does not interact with immobilised decorin. Mictotitre plates were coated with decorin, collagen type I or BSA and non-specific binding was blocked with BSA. Immobilised decorin or collagen type I were incubated with a titration of GST-tagged A-domain of the α2 subunit in binding buffer containing 1mM Mn^{2+}. BSA was incubated with A-domain at a single concentration, 4000nM. Bound protein was detected in duplicate wells using the ELISA-based method described in materials and methods. Data is representative of two independent experiments, and error bars indicate standard deviation.

4.3.4 Mechanistic Studies on α2β1 Integrin-Decorin Interaction

The decorin interaction with α2β1 integrin was further investigated by examining binding under various conditions that modulate the integrin activation state, in comparison to the classic ligand, collagen type I. Some important differences between binding of the integrin to immobilised decorin or collagen type I were observed. For the following calculations, BSA values were first subtracted as non-specific binding. Both Ca^{2+} and rhodocetin induce and/or stabilise an inactive conformation of the integrin. Binding of α2β1 integrin to immobilised collagen was slightly inhibited in the presence of Ca^{2+} and more strongly inhibited (by 0.8-fold) in the presence of rhodocetin (Figure 4.6, compared to buffer alone in both cases). In contrast, binding to immobilised decorin was not influenced by Ca^{2+}, while rhodocetin enhanced binding by approximately 2-fold (Figure 4.6, compared to buffer alone in both cases). The divalent cation Mn^{2+}, which stabilises an active conformation of the integrin, enhanced binding to both decorin and collagen, by 1.6-fold and 0.8-fold respectively (compared to buffer alone in each case). The antibody 9EG7, which also stabilises an active conformation of the integrin by interactions with the β1 subunit, slightly enhanced binding of the integrin to both decorin and collagen type I in combination with Mn^{2+}, over Mn^{2+} alone. Another activating antibody, JA221, which interacts with the A-domain, enhanced binding of the integrin to collagen by 0.9-fold compared to buffer alone, but only slightly enhanced integrin binding to decorin compared to buffer alone (Figure 4.6).

Decorin can interact with both active and inactive conformations of the integrin, indicating that different binding sites for decorin may be exposed in different integrin conformations. Additionally, rhodocetin is known to interact with the A-domain proximal to, or overlapping the collagen binding site, and may thereby competitively interfere with collagen type I-A-domain interactions. The lack of inhibition of decorin-integrin interaction by rhodocetin is consistent with the absence of an interaction of decorin with the recombinant A-domain (Figure 4.5), further supporting a mechanism of integrin-decorin interaction distinct from both the collagen and rhodocetin binding sites on the A-domain.

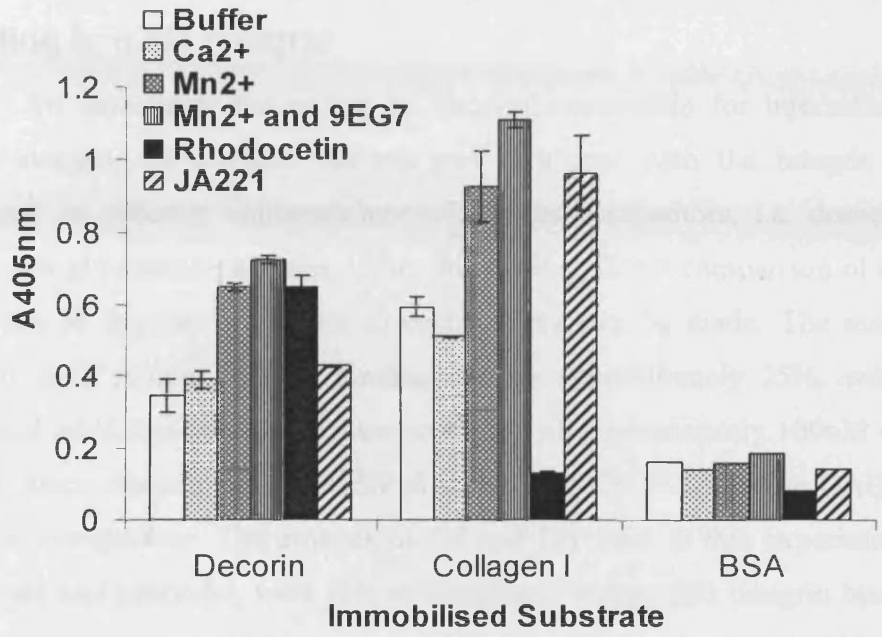


Figure 4.6: α2β1 integrin interactions with immobilised decorin or collagen type I under various conditions. Microtitre plates were coated with decorin, collagen type I or BSA and non-specific binding was blocked with BSA. Immobilised substrates were incubated with 100nM α2β1 integrin in binding buffer alone, in the presence of 2mM Ca^{2+}, 1mM Mn^{2+}, 9EG7 and 1mM Mn^{2+} simultaneously, 2μM rhodocetin, or in the presence of another activating antibody, JA221. Bound integrin was detected in duplicate wells using the ELISA-based method described in materials and methods. Data is representative of two independent experiments, and error bars indicate standard deviation.

4.3.5 Identification of the Moiety of Decorin Responsible for Binding to α2β1 Integrin

To investigate the moiety of decorin responsible for interaction with α2β1 integrin, immobilised decorin was incubated with the integrin in the presence of different concentrations of various competitors, i.e. decorin core protein or glycosaminoglycans. Using this method, direct comparison of binding affinities of decorin in relation to each moiety can be made. The maximum decorin core protein reduced binding was by approximately 25%, with half-maximal inhibition reached at a concentration of approximately 100nM (Figure 4.7A). Since decorin can be modified with either CS or DS, these GAGs were used as competitors. The sources of CS and DS used in this experiment (see materials and methods), were able to completely block α2β1 integrin binding to immobilised decorin. Half-maximal inhibition was obtained with 400nM and 100nM of DS and CS respectively (Figure 4.7B and C). The concentration of DS and CS required to achieve half-maximal inhibition in repeat experiments was variable (400nM, 1000nM, 1800nM for DS and 100nM, 150nM for CS). These results indicate that the glycosaminoglycan moiety plays an essential role in the interaction of decorin with α2β1 integrin. Unfortunately, intact decorin could not be used as a soluble competitor due to limited reagent availability.

Interaction of α2β1 with immobilised decorin could be competed for by both DS and CS. However, this competition reduced binding of the integrin to decorin to levels below that of binding of the integrin to the negative control, BSA (Figure 4.7B and C). This could be explained by some non-specific binding of the integrin to BSA, which might have been reduced in the presence of competitor. However, analysis of integrin binding to BSA was conducted only in the absence of competitor due to limited reagent availability. In experiments where controls of primary and secondary antibodies or secondary antibodies only were included (both in the absence of the integrin), the level of binding was minimal to either BSA or decorin substrates, and was approximately equal.

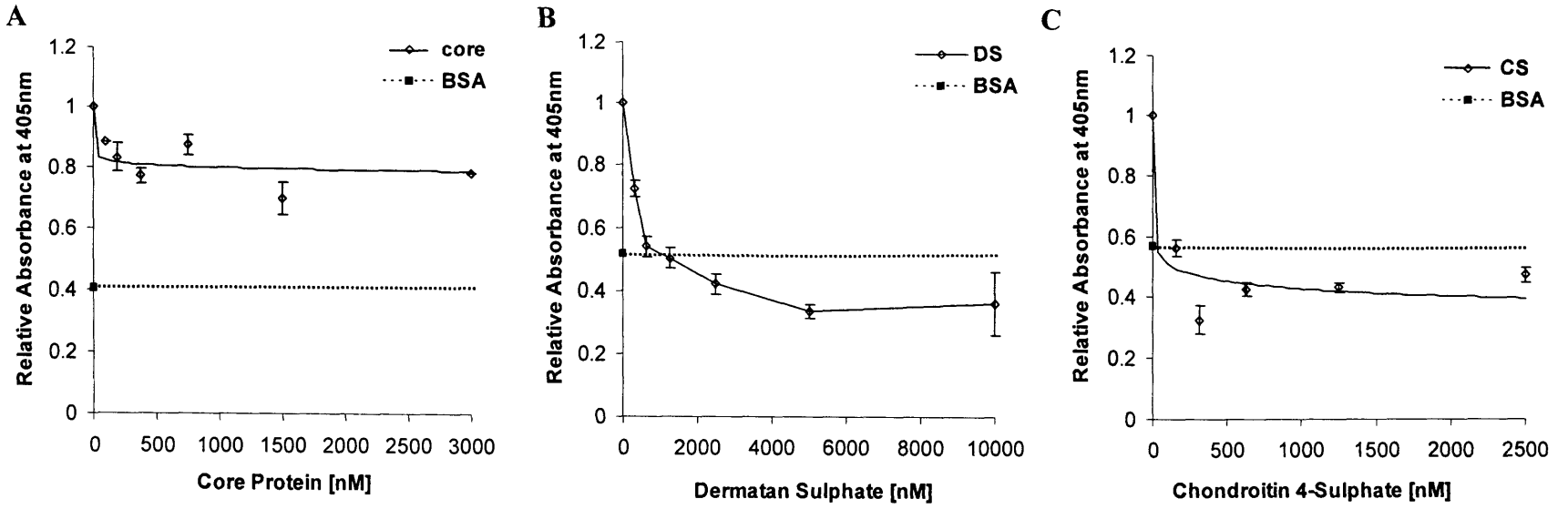


Figure 4.7: Decorin core protein does not compete for binding of α2β1 integrin to immobilised decorin, while the GAG chains DS and C-4S can compete for binding of α2β1 integrin to immobilised decorin. Microtitre plates were coated with decorin and blocked with BSA. Wells were incubated with 100nM α2β1 integrin in the presence of activating antibody 9EG7 and 1mM $Mn^{2+}$and a titration of either decorin core protein (A), dermatan sulphate (B) or chondroitin-4-sulphate glycosaminoglycans (C) as competitors. Bound integrin was detected in duplicate wells using an ELISA-based method as described in materials and methods. Data is representative of a minimum of two independent experiments, and error bars indicate standard deviation. The dotted line in each figure indicates the level of α2β1 integrin binding in the negative control, BSA, in the absence of competitor.

4.3.6 Interaction of Inactive α2β1 Integrin with Decorin

4.3.6.1 Effects of Rhodocetin on Integrin-Decorin Interaction

In Figure 4.6, it was demonstrated that under conditions where α2β1 integrin is maintained in an inactive, low affinity conformation for ligand binding (in the presence of Ca^{2+} or rhodocetin), the integrin could efficiently interact with immobilised decorin. The fact that rhodocetin substantially enhanced binding could indicate that decorin preferentially interacts with an inactive conformation of the integrin, or that a high affinity binding site for decorin is exposed in an inactive integrin conformation. To investigate this hypothesis, binding was analysed under conditions where the integrin adopts an inactive conformation. Immobilised decorin or collagen type I was incubated with α2β1 integrin in the presence of different concentrations of rhodocetin (Figure 4.8A). As previously demonstrated (Eble et al., 2001), rhodocetin almost completely abolished integrin binding to immobilised collagen type I. In contrast, rhodocetin did not inhibit the interaction of α2β1 integrin with immobilised decorin at concentrations at which it blocked integrin binding to collagen. However, above 400nM rhodocetin (where integrin binding to collagen was abolished), binding of α2β1 integrin to decorin was sharply increased 4-fold (for discussion see section 4.3.8).

Since rhodocetin enhanced binding of α2β1 integrin to decorin at high concentrations, we also tested whether decorin could bind the A-domain of α2 in the presence of rhodocetin. Under these conditions, no binding to decorin was observed, while binding to collagen type I was inhibited (Figure 4.8B). This further supports an interaction of decorin with a site on the α2 subunit distinct from the classical ligand-binding A-domain.

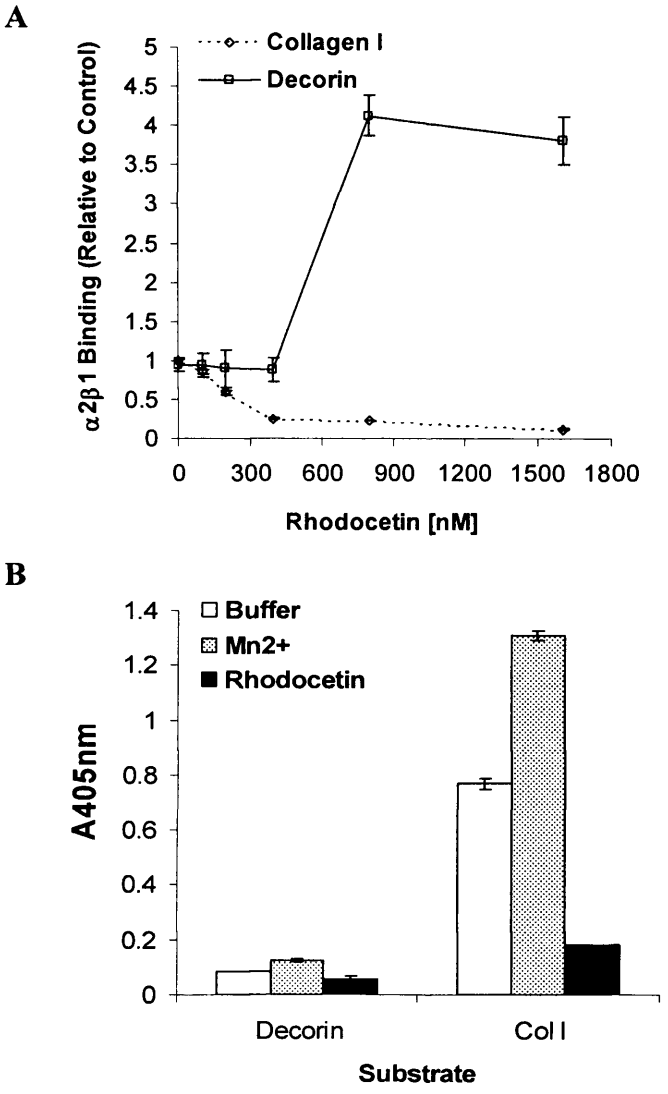


Figure 4.8: α2β1 integrin binding to immobilised decorin is enhanced under conditions where interactions with collagen are inhibited (A). Under these conditions of enhanced binding, the α2 subunit A-domain cannot interact with immobilised decorin (B). Microtitre plates were coated with decorin or collagen type I and non-specific binding was blocked with BSA. Immobilised substrates were incubated with 100nM α2β1 integrin in the presence of rhodocetin at the indicated concentrations (A). Alternatively, immobilised substrates were incubated with 1.8μM GST-tagged A-domain in buffer alone (TBS/Mg), or supplemented with 1mM Mn^{2+} or 2μM rhodocetin (B). Bound integrin or GST-tagged A-domain was detected in duplicate wells using the ELISA-based method described in materials and methods. Data is representative of two independent experiments (A) or shows a single experiment (B) and error bars indicate standard deviation.

4.3.6.2 Effect of pH on Decorin-α2β1 Integrin Interaction

At slightly acidic pH, it has been previously demonstrated that an inactive conformation of α2β1 integrin is induced. This process is reversible and is not due to protein denaturation, while conformational changes become irreversible below pH4.5. Binding of α2β1 integrin to collagen I is reduced below pH7 (Eble and Tuckwell, 2003) and Figure 4.9, indicative of a low affinity (for collagen type I) conformation of α2β1 integrin. We therefore tested binding of the integrin to immobilised decorin or collagen type I at a range of pH values between pH 4.5 and pH 7.5 (Figure 4.9), where binding of the integrin to decorin or collagen type I at pH 7.5 was set to 1 respectively. The integrin bound optimally to immobilised decorin at pH 5 and to collagen above pH 6.5. Decreasing the pH from 7.5 to 5 progressively enhanced α2β1 integrin binding to decorin, an effect observed previously with immobilised rhodocetin, which interacts preferentially with an inactive conformation of the integrin (Eble and Tuckwell, 2003). Below pH5, integrin binding to decorin was abolished presumably due to denaturation of α2β1 integrin under strongly acidic conditions.

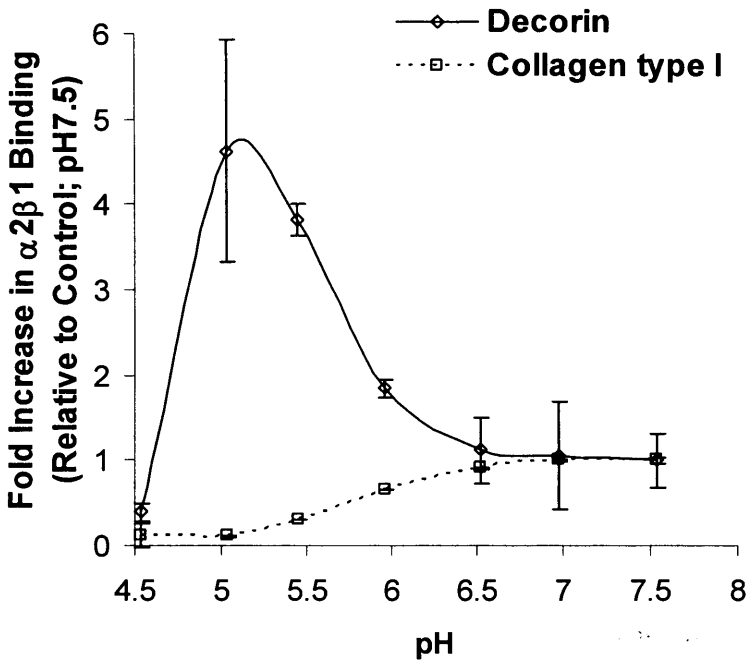


Figure 4.9: α2β1 integrin binding to immobilised decorin is enhanced under conditions where interactions with collagen are inhibited. Microtitre plates were coated with decorin or collagen type I and non-specific binding blocked with BSA. Immobilised substrates were incubated with α2β1 integrin in binding buffer supplemented with 1mM Mn^{2+} and in ampholine buffered solutions, adjusted to the indicated pH. Data is represented as relative binding of α2β1 integrin, where absorbance at pH7.5 was set to 1 for each of collagen type I and decorin. Bound integrin was detected in duplicate wells using the ELISA-based method described in materials and methods. Data is representative of two independent experiments and error bars indicate standard deviation.

4.3.7 Influence of Decorin on α2β1 Integrin-Collagen type I Interaction

4.3.7.1 Interaction of Decorin with a Collagen-derived Peptide, FC3

To investigate whether decorin could influence α2β1 integrin interaction with immobilised collagen type I, a synthetic collagen peptide, FC3, was used, and will be described in a forthcoming publication (Stephan Niland and Johannes A. Eble). Since decorin is known to interact with both collagen type I and α2β1 integrin, it would be difficult to interpret data in which collagen type I, decorin and α2β1 integrin were all present. In order to investigate whether decorin could directly influence integrin affinity, a different approach was taken. Firstly, to investigate whether decorin could interact with FC3, the peptide was immobilised and incubated with decorin. Decorin did not have binding affinity for FC3, however, decorin also did not interact with the positive control, collagen type I (Figure 4.10A, data supplied by Johannes A. Eble), presumably due to non-fibrillar structures adopted under the coating conditions used, since decorin-collagen I interaction could be demonstrated in an identical assay where fibrillogenesis was supported (Figure 4.11A). It is apparent however, that under these conditions, decorin does not interact with FC3 (Figure 4.10A), and thus could not influence integrin-collagen interaction through masking of integrin binding sites on collagen. This substrate was therefore used to investigate whether decorin directly influences integrin affinity for collagen substrates through interaction with the integrin.

4.3.7.2 Decorin Influence on α2β1 Integrin Interaction with a Collagen-derived Peptide, FC3

The FC3 peptide FC3 was immobilised under similar conditions to Figure 10A, and incubated with α2β1 integrin in the presence of 20nM decorin. Decorin inhibited integrin binding to the collagen peptide (Figure 10B, data supplied by Johannes A. Eble) in a statistically significant manner at all integrin concentrations tested above 1.25nM.

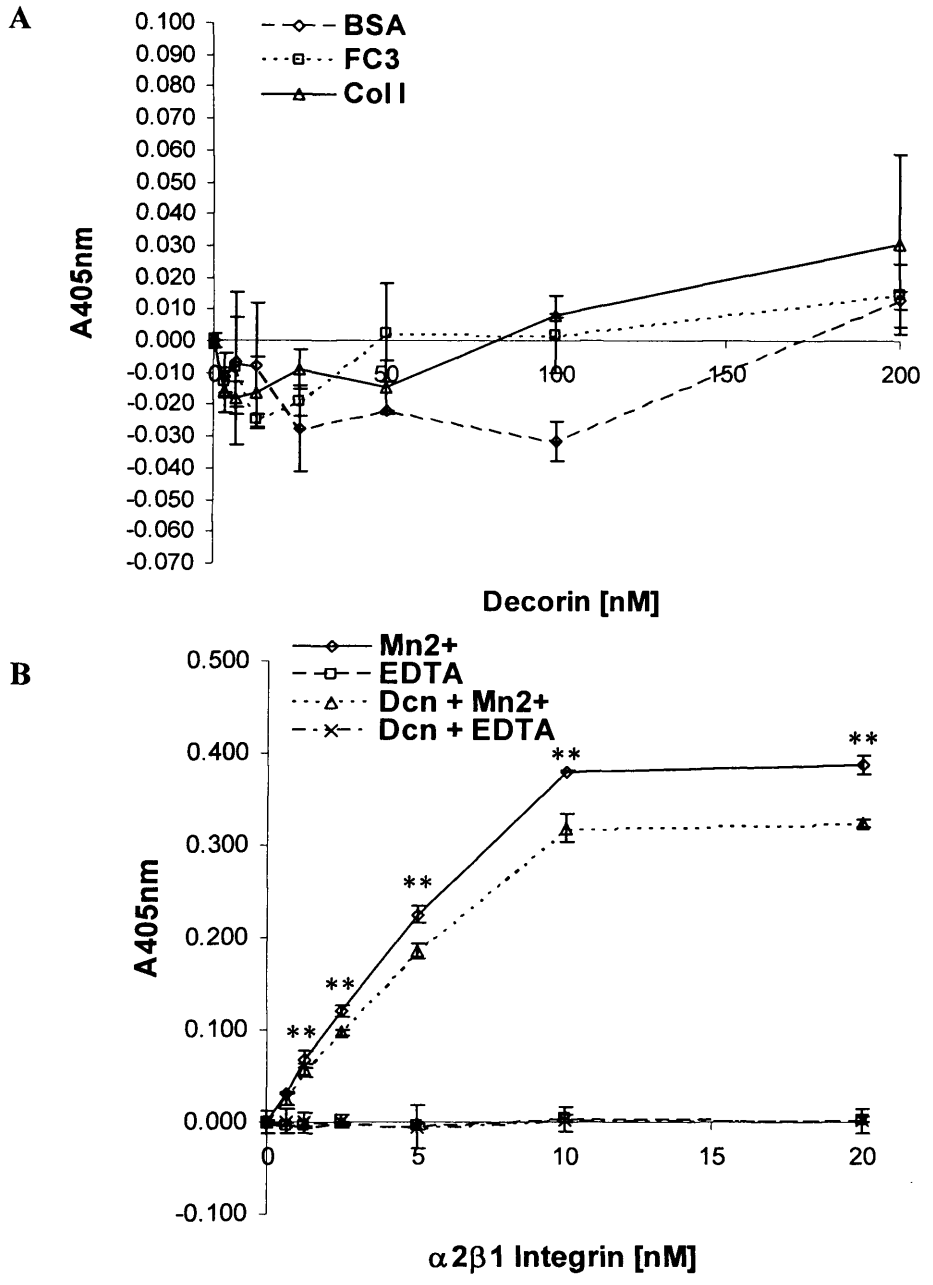


Figure 4.10: Decorin Influence on α2β1 Integrin Interaction with a synthetic collagen peptide, FC3. BSA, collagen type I (10μg/ml in acetic acid), or FC3 peptide (2.5mg/ml) were immobilised and non-specific binding sites blocked as described in materials and methods, section 4.2.2. Substrates were incubated with a solution of 20μg/ml decorin, and bound decorin detected by ELISA as described in materials and methods, section 4.2.4.1 with the exception that decorin antibodies were detected using anti-rabbit IgG alkaline-phosphatase-conjugated antibodies(A). FC3 peptide (2.5mg/ml) was immobilised and incubated with a titration of α2β1 integrin in the presence of 20μg/ml decorin, in 2mM $MgCl_2$, 1mM $MnCl_2$ in 1% BSA/TBS, pH7.4, or in the presence of EDTA. Bound integrin was detected by ELISA, as described in section 4.2.2 (B). Statisitcal significance was tested using ANOVA and Bonferroni, where ** indicates $p < 0.01$.

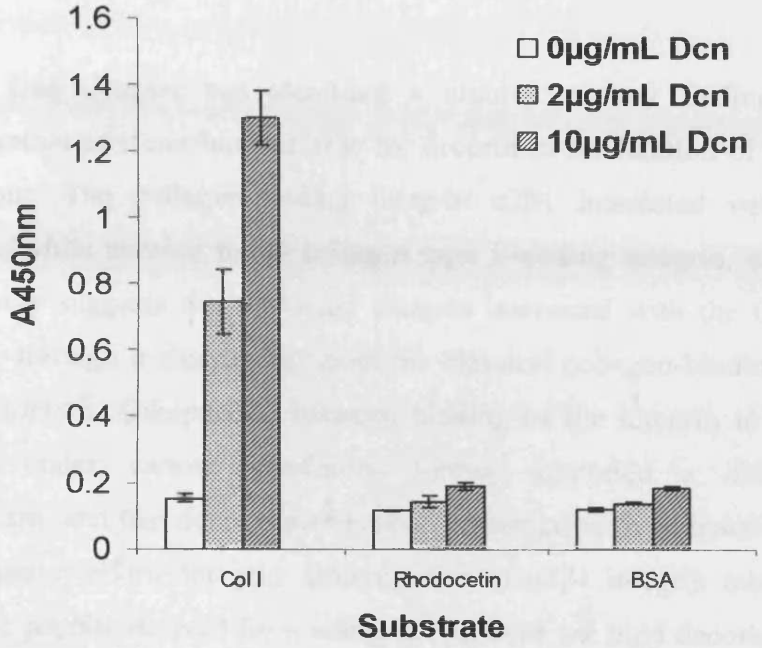
4.3.8 Interaction of Decorin and Rhodocetin

4.3.8.1 Interaction of Decorin with Immobilised Rhodocetin

At concentrations below 400nM, rhodocetin had no effect on α2β1 integrin interaction with decorin, while at concentrations above 400nM, rhodocetin enhanced α2β1 interactions with decorin (Figure 4.8A). A possible explanation is that rhodocetin binds to decorin with high affinity and that complexed rhodocetin is unable to interact with the integrin to induce an inactive conformation. At higher concentrations (above 400nM rhodocetin), free rhodocetin could interact with the integrin to influence decorin-integrin interactions through stabilisation of an inactive state of the integrin. Alternatively, rhodocetin could bind to decorin with low affinity. High concentrations of rhodocetin could affect decorin in a way that further enhances decorin interactions with the integrin.

To ascertain whether a decorin-rhodocetin interaction exists, immobilised rhodocetin was incubated with a solution of decorin, and bound decorin was detected by ELISA, using antibodies to decorin. Under these conditions, no interaction of decorin with rhodocetin could be detected over that of the control (immobilised BSA), while decorin bound effectively to the positive control; immobilised collagen (Figure 4.11A). As an additional method, rhodocetin was immobilised on nitrocellulose membrane and incubated with a solution of decorin. Bound decorin was detected using specific antibodies, and visualised by chemiluminescence and exposure to photographic film. Again, no interaction of decorin with rhodocetin could be detected, while decorin bound effectively to immobilised collagen and fibronectin (Figure 4.11B).

A



B

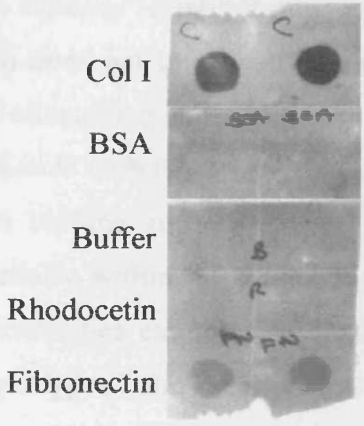


Figure 4.11: Decorin in solution does not interact with immobilised rhodocetin. Microtitre plates were coated with collagen type I (positive control), BSA (negative control) or rhodocetin. Non-specific binding sites were blocked with BSA and wells were incubated with a solution of decorin diluted in PBS to the indicated concentrations. Binding was detected using antibodies to decorin and developed by ELISA. Data is representative of 2 independent experiments, and standard deviations are shown as error bars (A). In (B), nitrocellulose membrane was spotted in duplicate with 10µg each of Collagen type I [Col I], BSA, PBS [Buffer], Rhodocetin or fibronectin. Non-specific binding sites were blocked with 5% non-fat dried milk, and the membrane incubated with a solution of 40µg/ml decorin in PBS. Binding was detected using antibodies to decorin, and visualised by chemiluminescence and exposure to photographic film (B).

4.4 Discussion

This Chapter has identified a number of key findings towards the investigation of a mechanistic role for decorin in modulation of endothelial cell behaviour. The collagen-binding integrin α2β1 interacted with immobilised decorin, while another major collagen type I-binding integrin, α1β1, could not. This study suggests that the α2β1 integrin interacted with the GAG moiety of decorin, through a site distinct from the classical collagen-binding A-domain of the α2 subunit. Comparison between binding of the integrin to collagen or to decorin under various conditions further supported a different binding mechanism, and that decorin preferentially interacts with an inactive, low affinity conformation of the integrin. Investigation of α2β1 integrin interaction with a synthetic peptide derived from collagen that does not bind decorin indicated that decorin could reduce the affinity of the integrin for collagen. Of further note, decorin does not appear to mask the binding site of the integrin on collagen since decorin retains the capacity to inhibit α2β1 interactions with a collagen peptide with which decorin does not interact. Decorin may therefore influence integrin conformation in an allosteric manner.

Decorin interacts with α2β1 integrin but not the related collagen-binding integrin α1β1. The binding site for decorin within this integrin is therefore located at least partially within the α2 subunit. This may be explained by the different ligand specificities exhibited by these integrins. While both integrins interact with collagen types I and IV, α1β1 binds preferentially to type IV, while α2β1 binds preferentially to type I (Calderwood et al., 1997). Surface-exposed side chains surrounding the MIDAS motif of the α-subunit A-domain are variable, and provide additional ligand contact points that contribute to ligand specificity (Dickeson et al., 1999; Kamata et al., 1999; Kamata and Takada, 1994; Kern et al., 1993).

Our data confirms that of a previous study (Guidetti et al., 2002), that the collagen-binding integrin α2β1 interacts with immobilised decorin. However, there are important differences between this and the current study. In the former

(Guidetti et al., 2002), the core protein of decorin was suggested to interact with the collagen-binding A-domain of the α2 subunit. In contrast, we could not detect direct binding of decorin to isolated A-domain and decorin appeared to interact with the integrin primarily *via* the GAG moiety. It should be noted that the DS and CS used as competitors in this study are likely very different to the GAG contained within our decorin, since the extent and pattern of modifications such as epimerisation and sulphation varies considerably between GAGs from different sources. However, we could not demonstrate consistent inhibition using isolated core protein, while DS and CS from different sources could both completely block binding of the integrin to decorin. These data together suggest that it is the GAG moiety of decorin rather than the core protein which is primarily involved in interaction with the integrin.

The core protein was formerly implicated (Guidetti et al., 2002) since the antibody P1E6, which binds to the A-domain, interfered with platelet interactions with decorin. Further, in the same study, platelets were found to adhere to a substrate of decorin core protein (Guidetti et al., 2002). In the current study, attempts were made to also investigate the antibody P1E6, however, formation of a precipitate upon mixing the antibody with the integrin in binding buffer prevented interpretation of results. Using the methodology shown in this thesis, a direct comparison of the ability of either GAG or core protein to interact with α2β1 integrin could be made. Using this method, the core protein could not significantly compete with integrin-decorin interaction, while the glycosaminoglycans investigated completely inhibited this interaction in a dose-dependent manner. In the former study a contribution of the GAG moiety was not investigated (Guidetti et al., 2002).

Both DS and CS could compete for binding of the integrin to immobilised DS-bearing decorin, indicating that the integrin did not interact with a motif specific to dermatan sulphate presented by our source of decorin. More variable results were observed when using DS than with CS as a competitor, which could be explained by DS self- association in solution interfering with its interaction with the integrin. Indeed, DS has a greater propensity to self-associate in solution than CS (Fransson, 1976; Scott, 1992;

Scott, 1995). It should also be noted that while our data suggests that decorin interacts with α2β1 integrin primarily *via* the GAG moiety, we could not rule out minor interactions of the core protein with α2β1 integrin. The core protein moiety may also play a role in stabilising GAG-integrin interactions. Interestingly, the integrin did not have binding affinity for immobilised biglycan, while in the same assay, the integrin bound effectively to decorin and collagen type I (Johannes Eble, personal communication). This data indicates either a contribution of the core protein of decorin in interacting with the integrin, or that the source of biglycan used may not contain the correct pattern of GAG modification required for interaction with α2β1 integrin.

Interestingly, the relevance of GAG-mediated interactions is supported *in vivo* by the phenotype of patients with a progeroid variant of Ehlers-Danlos Syndrome (EDS). These patients exhibit skeletal and craniofacial abnormalities (Faiyaz-Ul-Haque et al., 2004; Kresse et al., 1987) and have loose skin and deficiencies in wound healing (Kresse et al., 1987). These defects are directly linked to mutations in β4 galactosyltransferase I (Almeida et al., 1999; Gotte and Kresse, 2005; Seidler et al., 2006) which results in abnormal decorin and biglycan glycosylation, where approximately half the population of decorin is secreted as the core protein only (Kresse et al., 1987; Gotte and Kresse, 2005; Seidler et al., 2006). Interestingly, biosynthesis of large CS/DS-containing proteoglycans was unaffected (Kresse et al., 1987). Of note, the defect in loose skin and in wound healing is similar to the phenotype of the decorin knockout mouse (Danielson et al., 1997; Jarvelainen et al., 2006), indicating an essential role for properly glycosylated decorin *in vivo*. Further, decorin inhibited osteosarcoma cell migration *in vitro* in a GAG-dependent manner, where dermatan sulphate exerted a greater inhibitory effect than chondroitin sulphate (Merle et al., 1999).

Interactions with the A-domain classically convey ligand occupancy to intracellular signalling pathways to support cell adhesion (Hynes, 2002; Lee et al., 1995b). In contrast to data on platelets (Guidetti et al., 2002), decorin by itself did not support endothelial cell adhesion (Chapter 3), indicating that decorin is not capable of inducing A-domain-mediated productive alterations in integrin conformation in endothelial cells. This result is consistent with solid-

phase studies which failed to show an interaction between decorin and the isolated α2 integrin subunit A-domain. In contrast to platelets, endothelial cells may require activation of additional receptors in order to adhere and spread. Alternatively, natively purified decorin used in the current study could have different binding activities to denatured decorin used in the previous study (Guidetti et al., 2002).

Important differences between binding of α2β1 integrin to decorin and collagen type I were observed. Under conditions where a high affinity conformation of the integrin was stabilised (with Mn^{2+} or the antibodies 9EG7 or JA221), binding of α2β1 integrin to collagen was enhanced, while only Mn^{2+} and 9EG7 could enhance binding to decorin. Where a low affinity conformation of the integrin was stabilised (with Ca^{2+}, rhodocetin or low pH) binding of α2β1 integrin to collagen was inhibited or abolished in all cases, while binding to decorin was either not influenced (Ca^{2+}) or was enhanced (rhodocetin or low pH). The enhancement of α2β1 integrin interaction with decorin under these conditions could be explained by decorin preferentially interacting with an inactive conformation of α2β1 integrin, although binding sites for decorin clearly also exist on the active integrin conformation.

The results presented herein demonstrate that below 400nM rhodocetin, α2β1 integrin binding to decorin was unaffected, while above 400nM rhodocetin, integrin binding to decorin was enhanced. This could be explained by decorin-rhodocetin interactions preventing rhodocetin from stabilising an inactive conformation of the integrin. Once binding sites for rhodocetin on decorin reach saturation (above 400nM), the integrin-decorin interaction could be influenced by rhodocetin-integrin interactions, whereby rhodocetin stabilises an inactive conformation. This would be consistent with decorin preferentially interacting with an inactive conformation of the integrin. However, we have not been able to demonstrate an interaction between rhodocetin and decorin, at least under solid-phase conditions. Another explanation could be that simultaneous binding of decorin and rhodocetin to the integrin creates additional, normally unavailable, ligand binding sites on the integrin.

It has been previously shown that in a low affinity conformation, α2β1 integrin cannot interact with collagen, while both the intermediate and fully active conformations have binding affinity for this ligand (Van de Walle et al., 2005). Investigation of decorin influence on integrin interaction with the FC3 peptide suggests that decorin reduces integrin affinity for collagen. This could indicate that decorin allosterically induces an intermediate affinity conformation of the integrin; a low affinity conformation would not interact with collagen, while a high affinity conformation would not be expected to exhibit reduced interactions with collagen. It should also be noted that conflicting results were obtained during analysis of decorin interaction with immobilised collagen type I. This may be explained by different coating conditions of collagen used in the respective experiments. Decorin interacts with a triple helical conformation of collagen type I (Hedbom et al., 1993; Tenni et al., 2002). Where binding of decorin to collagen type I could not be detected (Figure 4.10A), a monomeric form of collagen type I was likely to have been supported (collagen type I was coated at 10 μg/ml in acetic acid). At collagen concentrations below 20 μg/ml, fibrillogenesis is not supported (Williams et al., 1978), while neutral pH is required for fibrillogenesis to occur. Decorin binding to immobilised collagen type I could be demonstrated (using the same antibodies and similarly purified decorin), when 80 μg/ml collagen type I was coated in PBS, pH7.4 (Figure 4.11A). Collagen fibrillogenesis is likely to have been supported under these conditions, facilitating decorin interaction.

Of note, α2β1-expressing CHO cells have been reported to spread significantly more on immobilised collagen in the presence of Mn^{2+} (which induced an intermediate affinity conformation) than when α2β1 integrin was activated *via* inside-out signalling (high affinity) (Van de Walle et al., 2005). Certain conformational states of the integrin may therefore be more relevant for transient adhesion involved in cell spreading and migration, whereas high affinity conformations may support more stable adhesion (Van de Walle et al., 2005). Indeed, as noted by the authors, distinct conformational states of another A-domain containing integrin, αLβ2 support differential states of adhesion (Salas et al., 2004). Transient adhesion of leukocytes (rolling) was supported by

intermediate affinity integrin conformations, while firm adhesion was supported by high affinity integrins (Salas et al., 2004). The intermediate affinity structure of αLβ2 is suggested to have an open headpiece but closed A-domain, while in the high affinity conformation both the headpiece and A-domain are open. Blocking conversion of the A-domain from a closed to open, high-affinity conformation was also suggested to block firm adhesion (Salas et al., 2004). The recombinant α2 subunit A-domain exists in at least two distinct conformations; open (high affinity) and closed (low affinity) (Emsley et al., 1997; Emsley et al., 2000). On ligand binding, a conformational change occurs in the A-domain at the C-terminal helix, α7 (Arnaout et al., 2005). Similar conformational changes occur in other A-domain containing integrins, αL and αM upon ligand binding (Lee et al., 1995a; Lee et al., 1995b), therefore studies on these integrins are relevant to consideration of α2β1 integrin. It is possible therefore, that decorin could similarly block conversion of the A-domain of the α2 subunit to an open, high-affinity state. By altering the affinity of the α2β1 integrin for collagen type I, decorin could support more intermediate states of adhesion through supporting an intermediate affinity conformation of the integrin. In summary, this Chapter demonstrates that decorin could directly modulate α2β1 integrin-collagen interactions. This could provide a mechanism for decorin effects on endothelial cell adhesion and migration on collagen type I. Whether decorin can activate signalling pathways through interacting with α2β1 integrin, and whether these pathways are required for decorin-mediated modulation of endothelial cell behaviour however, requires further investigation.

Chapter 5

Investigation of Decorin Signalling Through IGF-IR and/or α2β1 Integrin

5.1 Introduction

In Chapter 3 it was demonstrated that decorin enhances endothelial cell adhesion and migration on collagen type I. However, the mechanisms for these effects are unknown. Decorin has been previously shown to directly signal through α2β1 integrin in platelets (Guidetti et al., 2002) and through IGF-IR in endothelial cells (Schönherr et al., 2005), providing candidate receptors with which decorin could interact to influence endothelial cell behaviour. In Chapter 4, we confirmed and further characterised the interaction of decorin with α2β1 integrin ectodomain. In this Chapter, we will investigate whether decorin acts directly on classical integrin-mediated signalling pathways in endothelial cells adhering to collagen type I. Further, we will investigate the involvement of decorin signalling through IGF-IR in modulation of endothelial cell behaviour.

Interactions between α2β1 integrin and extracellular ligands are classically mediated by the A-domain of the α2 subunit (Kamata et al., 1999; Kamata and Takada, 1994; Tuckwell et al., 1995). A conformational change occurs in the A-domain upon collagen binding, which confers structural alterations to intracellular domains of the integrin heterodimer (Hynes, 2002; Lee et al., 1995). Thus signals of ligand occupancy are transduced to intracellularly associated protein complexes. These complexes include adaptor proteins and tyrosine kinases that create docking sites for, and activate proteins involved in diverse signalling pathways. A classic example is focal adhesion kinase (FAK).

Ligand occupancy in the appropriate signalling context results in FAK autophosphorylation at tyrosine 397 (Burridge et al., 1992). Phosphorylation of this residue can therefore be used to assess if a productive conformational change is induced in the integrin ectodomain. After FAK autophosphorylation, Src kinases are recruited (Schaller et al., 1994), which phosphorylate tyrosines 576 and 577 of FAK, both of which are required for maximal catalytic activity (Calalb et al., 1995). Tyrosines 861 (Calalb et al., 1996) and 925 are also sites for phosphorylation by Src, the latter of which creates a docking site for the adaptor protein Grb2, leading to activation of the MAPK pathway (Schlaepfer et

al., 1994). FAK localises to focal adhesions through interactions of the C-terminal focal adhesion targeting (FAT) domain with integrin-associated proteins such as paxillin (Turner and Miller, 1994). FAK can also associate with growth factor receptors through an N-terminal domain (FERM), thus can directly link integrin and growth factor signalling (Sieg et al., 2000). Indeed, FAK autophosphorylation occurs in response to growth factor signalling (Abu-Ghazaleh et al., 2001).

Ligand occupancy leads to intracellular association of focal adhesion components at sites of adhesion, for example vinculin, which in turn is linked to the actin cytoskeleton (Goldmann and Ingber, 2002). These points of adhesion link actin filaments to the ECM, to generate the traction and force required for migration. A number of focal adhesion components are implicated in focal adhesion turnover, which is important in cell motility; FAK deficiency results in reduced motility and formation of large focal adhesions (Ilic et al., 1995), while over-expression enhances migration (Cary et al., 1996). As an activator of FAK, Src activity is also implicated in control of focal adhesion turnover (Cary et al., 1996; Fincham and Frame, 1998). Other key focal adhesion kinases implicated in focal adhesion formation include PI3-K (discussed in more detail later in this introduction), and PKC (Woods and Couchman, 1992). The protease calpain-2 also promotes focal adhesion turnover by cleaving focal adhesion components. Subsequent focal complex disassembly reduces cell-matrix interactions (Dourdin et al., 2001), enhancing migration in response to matrix components (Carragher et al., 1999) and growth factors (Glading et al., 2000). Correspondingly, calpain inhibitors impair cell migration (Huttenlocher et al., 1997).

Other focal adhesion proteins such as talin, vinculin, α-actinin, paxillin and tensin provide a structural role in linking integrins to the cytoskeleton, or in mediating integrin activation. Talin for example, is activated by calpain-mediated proteolysis (Yan et al., 2001) or by binding of locally generated phosphoinositides to the head domain of talin, inducing a conformational change that exposes an integrin binding site (Martel et al., 2001). Talin interaction with β1 integrin results in conformational changes in the integrin leading to activation (Calderwood et al., 2002; Calderwood et al., 1999; Garcia-Alvarez et al., 2003)

by 'unclasping' of integrin cytoplasmic domains (Vinogradova et al., 2002). (inside-out signalling). These changes result in exposure of the extracellular ligand-binding site. Binding of extracellular ligands in turn alters metal ion coordination in the MIDAS site, resulting in conformational changes within the A-domain which are propagated to the intracellular cytoplasmic domains (Arnaout et al., 2005; Hynes, 2002). These conformational changes expose further binding sites for focal adhesion components in the cytoplasmic regions to elicit outside-in signalling (Takagi et al., 2002). Vinculin plays an essential structural role in linking integrins with the cytoskeleton (Goldmann and Ingber 2002) by interacting simultaneously with talin (which is integrin-associated) *via* the head domain and with F-actin *via* the tail domain. In an inactive state, these binding sites are masked through self-association of the head and tail domains. Co-operative binding of both talin and F-actin relieves autoinhibition (Chen et al., 2006). Indeed, the importance of associations between integrins and the cytoskeleton is illustrated by a study in which integrins with mutations in the cytoplasmic regions retained ligand affinities, but modulated adhesion (Peter and O'Toole, 1995). Further, this demonstrates that cytoskeleton-integrin interactions can modulate adhesion independently of alterations in integrin affinity.

Focal adhesion formation and actin polymerisation are controlled by the activity of the small Rho GTPases, Rac, Cdc42 and Rho. These GTPases regulate actin polymerization and focal adhesion assembly in actin-driven structures; stress fibers, lamellipodia and filopodia respectively (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992). A sequential activation of these GTPases can occur whereby Cdc42 activates Rac, leading to association of filopodia and lamellipodia, while Rac in turn activates Rho (Nobes and Hall, 1995). Rac activity is implicated in forward movement, while Cdc42 maintains cell polarity, including localisation of lamellipodia to the leading edge (Nobes and Hall, 1999). Rho stimulates contraction and force generation by regulating actin-myosin filament formation to generate stress fibers (Ridley and Hall, 1992) although Rho-induced stress fibres and focal adhesions can also inhibit migration (Nobes and Hall, 1999). Indeed FAK can stimulate migration by inhibiting Rho activity to enhance focal adhesion turnover (Ren et al., 2000).

Growth factors stimulate motility by signalling through their receptors to activate Rho GTPases (Nobes et al., 1995; Ridley and Hall, 1992; Ridley et al., 1992). The signalling mechanisms by which Rho GTPases are activated and by which they control motility are not completely understood, however PI3-K appears to play a key role. The growth factor EGF activates Rac and Cdc42 in a PI3-K dependent manner (Menard and Mattingly, 2003). Similarly, PDGF and insulin activate Rac through PI3-K activation (Nobes et al., 1995; Ridley and Hall, 1992; Ridley et al., 1992), and IGF-I stimulates motility by activating a pathway involving PI3-K, small GTPases and FAK (Cheng et al., 2000). Conversely, Rho (Zhang et al., 1993), and Cdc42 (Zheng et al., 1994) can activate PI3-K, and epithelial cell migration required activation of PI3-K by Cdc42 and Rac (Keely et al., 1997). However, in fibroblasts, constitutively active Rac did not require PI3K activity to induce lamellipodia (Nobes et al., 1995). Small GTPases can also be activated by integrin associated kinases. FAK can activate Rac (Hsia et al., 2003), while another integrin-linked kinase, ILK, activates Rac in a PI3-K dependent manner (Qian et al., 2005). Interestingly, initial stages of cell attachment (resulting in FAK autophosphorylation) are independent of Rho GTPase activity, while subsequent spreading and further FAK autophosphorylation is dependent on Rho GTPase activity (Clark et al., 1998), indicating that complexity also exists in integrin-mediated activation of Rho GTPases.

Platelet adhesion *via* α2β1 integrin to substrates such as collagen type I or von Willebrand factor leads to platelet activation through tyrosine phosphorylation of a number of proteins. One such protein is Syk, which activates PLCγ2 (Keely and V. Parise, 1996). Platelets can also adhere to decorin, similarly, a number of proteins were tyrosine phosphorylated, two of which were identified as Syk and PLCγ2. It was suggested that these proteins were likely to be activated by decorin interactions with α2β1 since platelets interacted with decorin through this receptor (Guidetti et al., 2002).

Considering these findings in platelets, decorin could similarly modulate endothelial cell behaviour by directly signalling through, or by modulating collagen type I signalling through α2β1 integrin. Conformational changes

induced by decorin could therefore be transduced to intracellularly associated protein complexes involved in control of endothelial cell behaviour. However, the classic IGF-IR ligand IGF-I is also implicated in modulation of cell adhesion and migration. This provides an alternative mechanism by which decorin could influence endothelial cell behaviour, since decorin has been shown to signal through the IGF-I receptor in endothelial cells (Schönherr et al., 2005). In other cell types, IGF-I promotes cell migration through its receptor in a β1 integrin (Kiely et al., 2005; Tai et al., 2003) or an αVβ3 integrin dependent manner (Jones et al., 1996; Marelli et al., 2006). IGF-I signalling through IGF-IR has also been shown to enhance α2β1, α3β1 and α5β1-mediated migration (Lynch et al., 2005). The mechanism(s) by which IGF-I promotes migration may be cell-type specific. In myeloma cells (Tai et al., 2003), IGF-I promoted direct association of IGF-IR and β1 integrin in lipid rafts, resulting in phosphorylation of FAK and paxillin and enhancement of β1 integrin interactions with these proteins. These effects were dependent on IGF-IR autophosphorylation induced by IGF-I ligation, and on subsequent activation of PI3-K and Akt (Tai et al., 2003). A number of studies also demonstrated that IGF-I-mediated stimulation of migration required activation of PI3-K (Cheng et al., 2000; Lynch et al., 2005; Marelli et al., 2006; Wenli et al., 2001) and Akt (Marelli et al., 2006). While in some systems IGF-I also activated MAPK, this activity was not required for motility (Wenli et al., 2001; Meyer et al., 2001). In other systems MAPK was not activated (Tai et al., 2003; Lynch et al., 2005), while in another, activation of both MAPK and PI3-K contributed to IGF-I mediated stimulation of migration (Meyer et al., 2001).

Of relevance, IGF-I has been shown to promote endothelial cell migration (Grant et al., 1987; Ikeo et al., 2001; Liu et al., 2001; Nakao-Hayashi et al., 1992) and tube formation (Nakao-Hayashi et al., 1992). IGF-I activated both MAPK and PI3-K/Akt pathways in endothelial cells, however only the latter pathway was involved in IGF-I stimulated migration (Liu et al., 2001). Signalling by decorin through the IGF-IR/PI3-K/Akt pathway in endothelial cells has been demonstrated in our laboratory, and resulted in upregulation of the cell-cycle inhibitor, p21. The related protein p27 was also upregulated but this

was independent of Akt activation. Protracted signalling by decorin through IGF-IR led to receptor downregulation (Schönherr et al., 2005). Decorin has also been shown to signal through the ErbB family of growth factor receptors. Similar to decorin signalling through IGF-IR, signalling through ErbB receptors also results in down-regulation of the receptor and upregulation of p21, but through activation of the MAPK pathway rather than Akt (De Luca et al., 1996; Iozzo et al., 1999; Moscatello et al., 1998; Santra et al., 2000; Santra et al., 1997). However, ErbB-mediated signalling by decorin appears to be restricted to neoplastic cells; decorin does not activate ErbB receptors in endothelial cells (Schönherr et al., 2005), macrophages (Xaus et al., 2001), or neuronal progenitor cells (Santra et al., 2006). Further, the signalling pathways activated by decorin through ErbB receptors (MAPK and intracellular calcium release) were not activated in endothelial cells (Schönherr et al., 2001).

5.1.1 Aims

- To investigate the mechanisms by which decorin influences endothelial cell behaviour
- To investigate whether putative signalling pathways activated by decorin are involved in regulation of endothelial cell behaviour
- To determine whether decorin signalling through IGF-IR is involved in decorin influences on endothelial cell behaviour
- To determine whether decorin can directly influence classical integrin-mediated signalling in endothelial cells

5.2 Materials and Methods

5.2.1 Cell Culture and Maintenance

For production of adenovirus, HEK-293 (human embryonic kidney) cells were maintained in MEM containing glutamine (Gibco, Invitrogen) supplemented with 10% FCS, 100 units/ml penicillin, 100μg/ml streptomycin. Cells were passaged at least twice weekly on reaching 60% to 90% confluency, and split 1 in 6 to 1 in 12 for re-seeding.

For investigation of decorin signalling in endothelial cells, the human endothelial cell line EA.hy926 (Edgell et al., 1983) was maintained in MCDB 131 medium (Gibco, Invitrogen) supplemented with 10% FCS, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), 100 units/ml penicillin, 100 μg/ml streptomycin and 2mM glutamine (all Gibco, Invitrogen). Cells were passaged at least twice weekly on attaining 70% to 90% confluency and split to 1 in 5 to 1 in 10 for re-seeding. This cell line is described in detail in Chapter 2, section 2.2.1.

5.2.2 Preparation of Matrix Substrates

Tissue-culture plates or dishes were coated with collagen or decorin incorporated into collagen as described in Chapter 3, section 3.2.4. Briefly, wells were thinly coated by covering the surface with neutralised collagen mixtures and immediately removing as much of the solution as possible. Gels were allowed to set by incubating plates or dishes at 37°C, 5% CO_2 for 30 min.

5.2.3 Investigation of Decorin Signalling Through IGF-IR

To investigate the involvement of decorin signalling through the IGF-I receptor in control of endothelial cell behaviour, IGF-IR signalling was specifically blocked using tyrphostin AG1024. This chemical inhibitor prevents IGF-I receptor and to some extent, insulin receptor, autophosphorylation and tyrosine kinase activity (Parrizas et al., 1997) by binding to the active site of the receptor and distort the conformation to prevent substrate and ATP binding

(Posner et al., 1994). Thus activation of signalling pathways activated by IGF-IR is prevented. Tyrphostin AG1024 has been used previously by us in the EA.hy926 endothelial cell line to demonstrate decorin mediated signalling through the IGF-I receptor, and a suitable inhibitor concentration established (Schönherr et al., 2005). In some experiments an inhibitor of the EGFR, tyrphostin AG1478, was used with AG1024 to simultaneously inhibit decorin signalling through both EGFR and IGF-IR.

5.2.3.1 Endothelial Cell Attachment

Endothelial cell adhesion to collagen or mixed collagen-decorin gels was investigated under conditions where growth factor signalling was blocked. Adhesion assays were carried out exactly as described in Chapter 3, section 3.2.5. To block growth factor signalling, cell suspensions were incubated with 10μM tyrphostin AG1024 or AG1478 (inhibitors of IGF-IR or EGFR signalling respectively, Alexis Biochemcals, Axxora, Nottingham, UK, 10mM in DMSO) for 1h, 37°C, 5% CO_2. Equal volumes of the vehicle, DMSO, were added to cells as a control. Cells were then mixed with decorin in solution (final concentration 0.4μM decorin) immediately prior to seeding onto collagen gels in the continued presence of tyrphostins. Alternatively, cells were seeded onto mixed decorin-collagen gels (0.4μM decorin) also in the continued presence of tyrphostins. Adherent cells were quantified 1h after seeding by staining with crystal violet as previously described (Chapter 3, section 3.2.5). The percentage of adherent cells was calculated relative to the control, defined as adhesion to a collagen substrate for one hour in the presence of the DMSO vehicle. This control was set to 100% adhesion for each experiment. Five wells were prepared for each variable and the average taken.

5.2.3.2 Endothelial Cell Migration

Spheroid migration on collagen gels, mixed decorin-collagen gels, or on collagen in the presence of decorin in solution was analysed as described in Chapter 3, section 3.2.6. To quantify decorin-induced enhancement of migration under conditions where IGF-IR signalling was blocked, spheroids were allowed

to migrate in the presence of 10μM AG1024 in a final volume of 100μL Waymouth medium containing 0.5% heat-inactivated serum and antibiotics (penicillin/streptomycin). To block IGF-IR signalling in the presence of soluble decorin, spheroids were plated onto 40μL collagen gels in a volume of 50μL and 10 wells selected. 2x tyrphostin AG1024 followed by decorin were sequentially added each in a volume of 25μL (final concentration 10μM and 0.4μM respectively). As controls, the equivalent volume of DMSO (vehicle) or PBS were added respectively. Alternatively, spheroids were plated onto 40μL collagen-bound decorin (0.6μM) gels in a volume of 50μL medium and 10 wells selected. 50μL of 20μM (2x) AG1024 or the equivalent volume of DMSO was added to each well.

5.2.4 Analysis of Migration-Conditioned Medium by Western Blotting

To investigate whether decorin was released into the media from mixed decorin-collagen gels, and whether decorin was degraded over the time-course of the migration experiments, medium was collected at the conclusion of migration assays (5 days after plating) and stored at -20°C. Medium was collected from wells in which spheroids had migrated in the presence of soluble decorin, or on mixed decorin-collagen gels. To ascertain whether decorin could be passively released from mixed decorin-collagen gels in the absence of spheroids or serum, media was also collected from wells containing collagen or mixed decorin-collagen gels in the absence of spheroids. Media was also collected from spheroid-free wells which had been incubated in medium containing 0.5% heat-inactivated serum (normal conditions) or in serum-free medium.

Decorin in conditioned media was analysed by Western blotting. 20μL of each sample was incubated with 0.01 units Chondroitin ABC lyase (Seikagaku, Tokyo, Japan) in 0.1M Tris pH8.0, 0.03M sodium acetate at 37°C for 2h in a total volume of 30μL. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane as described in Chapter 2, section 2.2.4.4. Membranes

were blocked, incubated with anti-decorin antibodies, and bound antibodies detected as described (Chapter 2, section 2.2.4.4).

5.2.5 Analysis of Integrin Signalling by Western Blotting Analysis

Focal adhesion kinase (FAK) is a classic mediator of integrin signalling and the activation state of this protein can be measured by analysis of tyrosine phosphorylation at 397 (autophosphorylation) (Burridge et al., 1992) and at other key residues including 576 (Calalb et al., 1995) and 925 (Schlaepfer and Hunter, 1996). This property of FAK was used as a measure of classic integrin signalling. The experimental procedures detailed in this section have been previously described for analysis of FAK phosphorylation (Stephens et al., 2004).

5.2.5.1 Integrin Signalling in Adherent Cells

Adherent cells were prepared by seeding 500,000 cells per 35mm collagen gel-coated dish in Waymouth medium supplemented with 0.5% FCS and antibiotics (penicillin/streptomycin). Cells were cultured at 37°C, 5% CO_2 for 48 h prior to incubation with either rhodocetin (0.15μM or 0.3μM) or decorin (0.5μM or 0.7μM) for 10, 30, 60 and 120 min in serum-free medium containing antibiotics (penicillin/streptomycin) at 37°C, 5% CO_2. Alternatively, cells were incubated with rhodocetin and decorin simultaneously for 1 h, or incubated with decorin for 1 h prior to stimulation with rhodocetin for 1 h.

Medium was removed and cell lysates were harvested into 150μl ice-cold extraction buffer per dish, and protein content estimated by BCA assay as previously described (Chapter 2, section 2.2.6.2). Equal amounts (10μg to 15μg) of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane as described (Chapter 2, section 2.2.4). The membrane was blocked with 5% NFDM (non-fat dried milk) in TBS/0.1% Tween-20, and antibodies were incubated in 1% NFDM in TBS/0.1% Tween-20. Bound antibodies were detected as described (Chapter 2, section 2.2.4.4). To allow for normalisation, membranes probed with antibodies to phosphorylated proteins were stripped for

30 minutes at 50°C, in stripping buffer (2% SDS, 60mM Tris-HCl, pH7.4, 0.7% 2-mercaptoethanol) and re-probed. Antibodies were used at the following dilutions or concentrations: β-tubulin (Clone TUB2.1, 1:500, Sigma) detected with HRP-conjugated antibodies to mouse IgG (1:2000, Dako Cytomation, UK), FAK (1:100, PC314, Oncogene), FAKpY397 (0.5 μg/ml, Calbiochem), FAKpY576 and FAKpY925 (Santa-Cruz Biotechnology, Santa Cruz, CA, USA both 1:200). All antibodies to FAK were raised in rabbits and were detected with HRP-conjugated anti-rabbit IgG antibodies (1:2000, Dako Cytomation).

5.2.5.2 Integrin Signalling During Cell Attachment

100,000 cells were seeded per well of a collagen-gel coated 24-well plate in serum-free Waymouth medium containing antibiotics (100 units/ml penicillin, 100μg/ml streptomycin). Cell suspensions were mixed with decorin immediately prior to seeding onto collagen gels, or were seeded onto mixed decorin-collagen gels. Media was removed 1 h after seeding and attached cells were extracted into cold lysis buffer and lysates analysed by Western blotting as described (section 5.2.5.1).

5.2.6 Electromobility Shift Assay (EMSA)

EMSA was used to investigate whether decorin could activate the group O subfamily of forkhead transcription factors. These factors, FoxO1, FoxO3 and FoxO4, also known as FKHR, FKHR-L1, AFX respectively (Burgering and Kops, 2002), recognize a common DNA-binding element (Furuyama et al., 2000).

5.2.6.1 Preparation of Cell Extracts

Endothelial cells (2 x 10^6 per 60mm diameter dish) were cultured on thin collagen gels for 48h in Waymouth medium containing 0.5% FCS and antibiotics. Medium was replaced with serum-free medium containing antibiotics and decorin at 0, 1, 10, 50 or 100μg/ml and cells were cultured for 1h or 24h. After incubation with decorin, dishes were placed on ice and the medium discarded. 150μl of ice-cold low salt buffer (10mM HEPES pH7.9, 0.1% NP40,

1.5mM $MgCl_2$, 10mM KCl, 1mM EDTA, 1mM EGTA, 5mM NaF, 0.5mM DTT, 0.5mM PMSF, 10μg/ml each of leupeptin and pepstatin) was added to each dish and cells were extracted by scraping. Lysates were vortexed and incubated on ice for 5min. This step was repeated twice. Lysates were then centrifuged for 10min at 10,000 x g and at 4°C, and the supernatant collected as cytoplasmic extract. The pellet was washed twice with 1ml ice-cold low salt buffer by repeated resuspension (by flicking and vortexing) and centrifugation for 10 min, at 10,000 x g and at 4°C. The nuclei were resuspended in 50μl ice-cold high salt buffer (20mM HEPES pH7.9, 25% glycerol, 1mM $MgCl_2$, 420mM NaCl, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF, 10μg/ml each of leupeptin and pepstatin) and incubated at 4°C for 4 to 6 h while shaking. The resulting nuclear extract was cleared by centrifugation for 30min at 10,000 x g and at 4°C and stored at -80°C. The protein concentration of each extract was estimated using the BCA assay as described (Chapter 2, section 2.2.4.1).

5.2.6.2 Radioactive Labelling of Probes

Probes specific to FoxO transcription factors were designed by Gregoire Martin, based on a previous study (Furuyama et al., 2000), which demonstrated that the consensus sequence for binding of the FoxO family of transcription factors is TTGTTTA (shown in bold). Probes were obtained from Life Technologies, Invitrogen, UK. The sequences were:

FoxO + 5' GATCTCATC**TTGTTTAC**GGCCTGGATC 3'

FoxO - 5' GATCCAGGCC**GTAAACAA**GATGAGATC 3'

Probes were resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1mM EDTA) at 1μg/ml. To generate double stranded probes, 100μg each of corresponding single probes were combined in a total volume of 1ml TE buffer containing 100mM NaCl. The solution was heated to 90-100°C by placing in a heated beaker of water. Annealing was induced by allowing water to cool to room temperature overnight. Double stranded DNA was diluted to 2pmol/μL in a total volume of 1ml H_2O and stored at -20°C.

Double stranded probes were radioactively 5' end-labelled with $\gamma^{32}P$ using T4 Polynucleotide Kinase (M4101, Promega, UK). For efficient labelling,

the DNA was first dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (M1821, Promega, UK). 8pmol DNA (4μL) was twice treated with 0.1 units CIAP at 37°C for 30 min. DNA was purified by phenol-chloroform extraction and ethanol precipitation. Briefly, one volume (50μL) of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The solution was mixed, and after centrifugation, the upper phase collected and 5M NaCl added to a final concentration of 0.2M, followed by 150μL ethanol. The precipitated DNA was collected by centrifugation for 15 min at 12,000 x g and the pellet was air-dried at room temperature for 1 h, before resuspension in 30μL H_2O. For radioactive end-labelling, the 30μL FoxO dephosphorylated double-stranded oligonucleotides were mixed with 5μL 10x T4 polynucleotide kinase buffer (Promega), 5μL $^{32}\gamma$P-ATP (10μCi/μL), 1μL T4 polynucleotide kinase (10 units), and the final volume was made up to 50μL with H_2O. After 2 h at 37°C, the reaction was stopped by addition of 2μL 0.5M EDTA and radiolabelled probes were purified by phenol-chloroform extraction and ethanol precipitation as described. The pellet was finally resuspended in 200μL H_2O to yield a solution of approximately 20 fmol/μL.

5.2.6.3 EMSA

For EMSA, 5μg nuclear extract was incubated with FoxO binding buffer (40mM HEPES pH7.9, 20% glycerol, 4mM $MgCl_2$, 1mM EDTA, 4mM DTT, 100mM KCl, 0.2mg/ml BSA), 20 fmol radiolabelled probes (1μL), 1μg poly-deoxyinosinic-deoxycytidylic (dI:dC) (Sigma-Aldrich, UK) and the volume adjusted to 20μl (for accuracy, master mixes were used which contained binding buffer, poly dIdC and radioactively labelled probes). The solution was incubated at room temperature for a maximum of 15 min and 19μL of each sample was loaded in each well of a 6% polyacrylamide gel.

For competition of binding of labelled probes to nuclear proteins, cold probes were added at 10-fold, 50-fold and 100-fold excess of labelled probes. For these experiments, samples of nuclear extract for EMSA analysis were prepared exactly as described but without radioactively labelled probes. Cold probes were then added and the mixture incubated for 15 min at room

temperature. Radioactively labelled probes were then added and the solution incubated for a further 15 min at room temperature.

Samples were separated in 6% self-made polyacrylamide gels (25.5ml H_2O, 3.5ml 5x Tris-Borate-EDTA (TBE), 8ml acrylamide/Bis-acrylamide [30% solution, 37.5:1, A3699, Sigma-Aldrich, UK], 100µL 10% ammonium persulphate [A3678, Sigma-Aldrich, UK], 100µL N,N,N',N'-Tetramethylethylenediamine (TEMED) [T9281, Sigma-Aldrich, UK]). 5x TBE contained 0.445M Tris, 0.445M boric acid and 0.01M EDTA. Electrophoresis was carried out in 0.5x TBE at a constant voltage (125V, limit 35mA) for 90 min. Gels were sandwiched between two sheets of wetted (with water) cellophane (grade 325-PV-38, Bitish Cellophane Ltd., Somerset, UK) and placed onto filter paper (Whatman filter paper Number 4, Whatman International, Maidstone, UK). Gels were dried using a gel dryer (Model 543, Bio-Rad, UK) and vacuum pump (Edwards RV5, BOC Ltd., Sussex, UK). Dried gels were exposed to photographic film (Hyperfilm ECL, Amersham Biosciences, UK) for 1h to 48h in a film cassette with an intensifying screen (BioMAX TranScreen HE, product code Z37,431-8, Kodak, Sigma-Aldrich, UK).

5.2.7 Adenovirus Characterisation and Transfection

5.2.7.1 Adenovirus Stock Preparation

Adenoviruses were amplified by large-scale infection of a packaging cell line, HEK-293. The following adenoviruses were amplified: AdvControl (insert-free adenovirus), AdvDcn (adenovirus containing human decorin cDNA), AdvdnAkt (dominant negative Akt mutant), AdvdpAkt (dominant positive Akt mutant). The control and decorin adenovirus have been previously described (Schönherr et al., 1999). AdvdnAkt (T308A,S473A) protein cannot be activated by phosphorylation (Alessi et al., 1996) and functions in a dominant-negative manner (Kitamura et al., 1998). Constitutively active Akt consists of wild-type Akt fused to the c-src myristoylation sequence (Fujio and Walsh, 1999). For amplification, 5 x 10^5 HEK-293 cells were seeded in 2ml growth medium per well of a 6-well plate (Greiner Bio-one) and incubated at 37°C, 5% CO_2 for 20-

24 h (cells were approximately 90% confluent at this time). For infection, growth medium was discarded. 20µl of adenovirus was added to 500µL infection medium (MEM containing 2% heat-inactivated FCS, 100 units/ml penicillin, 100µg/ml streptomycin) and added to one well (500µL infection medium without adenovirus was added to a control well). After 90-120 min incubation at 37°C, 5% CO_2, a further 1ml infection medium was added to each well. The plate was incubated at 37°C, 5% CO_2 and checked every 20-24 h. Once the cytopathic effect of the adenovirus infection was visible (cells become rounded and start to detach compared to control well which had no visible cytopathic effect, between 1 and 5 days), cells were removed by repeated pipetting and collected by centrifugation for 5 min at 725 x g. Cells were resuspended in 250µL infection medium and stored at -80°C. When required, adenovirus-containing cells were freeze-thawed four times by thawing in a 37°C water-bath followed by vortexing for 1 min and freezing in liquid nitrogen. The cell lysate was cleared from cell debris by centrifugation for 10 min, at 12,000 x g, 4°C and the supernatant collected as primary adenovirus stock.

For the next step of amplification, which followed the same procedure, 3 x 10^6 HEK-293 cells were seeded in 10ml growth medium per T-75 flask (11 flasks per adenovirus, one flask was used as a control). Flasks were incubated at 37°C, 5% CO_2 for 20-24 h and were between 70-90% confluent at this time. For infection, 200µl of each adenovirus stock was added to 21ml of infection medium and 2ml was added to each of 10 T-75 flasks. After 90-120 min incubation, 10ml growth medium was added to each flask. During infection, care was taken to keep flasks level, ensuring contact between cells and adenovirus. Flasks were checked every 20-24 h. Once the cytopathic effect was visible (1 to 5 days), cells were detached by sharply tapping the flask and collected by centrifugation as described above. Cells were resuspended and pooled in a total of 5ml growth medium and immediately placed at -80°C for storage. Adenovirus-containing lysates were generated by freeze-thaw as described above, and aliquoted for long-term storage at -80°C.

5.2.7.2 Adenovirus Analysis: PCR

To confirm identity of adenovirus constructs, DNA constructs were extracted from adenovirus and analysed by PCR, using primers to the decorin sequence or control primers to the adenovirus construct. PCR products were separated by agarose gel electrophoresis and visualised by inclusion of ethidium bromide in the gel and exposure to ultraviolet light.

For extraction of adenovirus DNA, 50 µl TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and 50 µl Pronase E (20 mg/ml, Sigma-Aldrich, UK) were added to 100 µl adenovirus preparation. SDS was added to a final concentration 0.5%, and the solution incubated for 30 min at 37°C. DNA was extracted using a QIAprep® Miniprep kit (Qiagen, Sussex, UK) and all buffers and columns used were those supplied with the kit. 2ml buffer PB was added to the adenovirus solution and applied in three aliquots to QIAprep® Miniprep-columns. The column was washed with 750 µl PE buffer and eluted with 50 µl EB buffer pre-heated to 60°C. Adenovirus-derived DNA was amplified by PCR using the following primers:

Adenovirus specific: sense: 5'-GTAGAGTCATAATCGTGCATCAGG-3',
antisense: 5'-TTTATATGGTACCGGGAGGTGGTG-3'.

Decorin-specific: sense 5'-CGACTTCGGAGCCTCCCT-3',
antisense: 5'-GAATTACTTATAGTTTCCGAG-3'.

For PCR, each tube contained 50µL comprised of 10µL purified adenovirus DNA, 5pM of each primer, 5µL poly 10x buffer (Promega, Southampton, UK), 2mM $MgCl_2$, 200µM dNTPs, 2.5 units Taq polymerase (Promega), made up to 50µL with RNAse free H_2O. PCR was carried out with a hotstart, under the following conditions: For adenovirus-specific primers 35 cycles were performed (denaturation: 94°C, 1min, annealing: 50°C, 1min; elongation: 72°C, 1min). For decorin-specific primers 35 cycles were performed (denaturation: 94°C, 1min, annealing: 56°C, 1min; elongation: 72°C, 1min). PCR

products were then examined by agarose-gel electrophoresis. 10μL of PCR products were added to 2μL of 6x sample buffer (0.25% bromophenol blue, 30% glycerol, 0.06M EDTA pH8.0) and loaded onto 1 % agarose gels in TAE buffer (40 mM Tris acetate, 2 mM Na_2EDTA, pH 8.5) supplemented with 0.1 μg/ml ethidium bromide (Sigma-Aldrich, UK). 300ng of a 1 Kb DNA ladder molecular weight marker (15615-016, Invitrogen, UK) was loaded to calibrate the gel. Products were separated by electrophoresis at constant 50V. PCR products had been previously checked by sequencing (Schönherr et al., 1999).

5.2.7.3 Calculation of Adenovirus Plaque Forming Units (pfu)

The plaque forming units (amount of active adenovirus) of adenovirus preparations were calculated by incubating a dilution series of adenovirus with HEK-293 cells. Infected cells were visualised by staining with antibodies that recognise hexon proteins of the adenovirus, using diaminobenzidine (DAB) as a substrate. Plaque forming units are visualised as orange-stained cells proximal to weakly stained cells. For each adenovirus, one 6-well plate (Greiner Bio-one) was seeded with HEK-293 cells at 1 x 10^6 cells per well in 2ml growth medium. Plates were incubated at 37°C, 5% CO_2 for 20-24 hours. At this time, the cells were confluent. Adenovirus stocks were diluted in infection medium to between 1 x 10^5 and 1 x 10^9. 500μL of adenovirus dilution was transferred to 5 wells of the 6-well plate (500μL infection medium without adenovirus was added to the sixth well as a control). After incubation at 37°C, 5% CO_2 for 90 min, a further 1ml infection medium was added to each well and care was taken at all stages not to disturb the cell layer. After 48 hours, cells were fixed with 500μL 4% paraformaldehyde/PBS per well for 10 min at room temperature and permeabilised with 70% EtOH (pre-cooled to –20°C). 500μL was added to each well and the plate was incubated for 10 min at –20°C. Wells were blocked by washing twice with 1% BSA/PBS (BSA fraction V, 160069, ICN Biomedicals Inc, Ohio) and incubated with 500μL anti-hexon antibody (goat anti-adenovirus polyclonal antibody (AB1056) Chemicon, Hampshire, UK) diluted 1:500 in 1% BSA/PBS. After incubation for 90 min at 37°C, wells were washed three times with PBS. Bound primary antibody was detected by incubation with 500μL per

well rabbit anti goat-HRP (sc-2768, Santa Cruz Biotechnology, California, USA) at 1:1000 in 1% BSA/PBS for 90 min at 37°C. Wells were washed five times with 1ml PBS and developed with 500μL diaminobenzidine (DAB) solution per well containing 1.5mM DAB, 15mM sodium phosphate pH 6.4, 0.1% hydrogen peroxide. Development (1h) was stopped by washing five times with 1ml H_2O per well. Plaques were identified as a strongly stained cell or cells proximal to weakly coloured cells (orange-brown colour, using light microscopy). Plaques were counted in control wells (this did not exceed 2 plaques) and in a minimum of two test wells and the control subtracted from test well values. The average plaque forming units per ml (pfu) were calculated, where pfu = number of plaques x dilution in well x 2 (since 500μL was added to each well).

5.2.7.4 Adenovirus Transfection of EA.hy926 Endothelial Cells

For adenoviral transfection (to transiently express either decorin or dominant negative Akt), 3 x 10^6 EA.hy 926 cells were seeded per T-75 flask in growth medium and transfected to the next day (cells were approximately 80% confluent). 2ml infection medium was added to each flask, containing required amount of adenovirus (for over-expression of dominant negative Akt, this and the control adenovirus were used at 100 pfu per cell; where high levels of over-expression were not required, for example for expression of decorin, this and the control adenovirus was used at 1 pfu per cell). Flasks were incubated with adenovirus at 37°C, 5% CO_2 for 90-120 min. 10ml EA.hy926 growth medium was then added and flasks incubated for a further 20-24 h. Cells were washed with PBS, trypsinised and collected by centrifugation for 5 min, 725 x g. Cells were washed three times in growth medium by repeated resuspension and centrifugation and finally resuspended in medium necessary for experiment and seeded as required.

5.2.8 Analysis of Transfected Cells

5.2.8.1 Analysis of Decorin Transfected Cells by Western Blotting

Endothelial cells were transfected with 1 pfu per cell of decorin or control adenovirus as described in section 5.3.5.4. Cells were seeded at 500,000 cells per 35mm collagen gel-coated dish in Waymouth medium supplemented with 0.5% FCS and antibiotics (penicillin/streptomycin). After culturing at 37°C, 5% CO_2 for up to 48h, lysates were prepared and analysed by Western blotting as described (section 5.2.6.1) Antibodies to FAK and β-tubulin were used as described (section 5.2.6.1). Alternative antibodies were used at the following dilutions or concentrations: β-chain of the IGF-I receptor or β1 integrin subunit (sc-713 and sc-8978 respectively, both at 1:200, Santa Cruz Biotechnologies, Santa Cruz, USA), both raised in rabbits and detected with HRP-conjugated anti-rabbit IgG antibodies (Dako Cytomation, 1:2000). Alternatively, transfected cells were similarly cultured for 48 hours prior to incubation with rhodocetin at 0.15μM or 0.3μM for 1h in serum-free medium containing antibiotics. Lysates were analysed using antibodies to FAK or FAKpY397 as described (section 5.2.6.1).

5.2.8.2 Confirmation of Decorin Expression

To confirm that decorin expression was induced by transfection with decorin adenovirus, medium was collected from decorin-expressing and control transfected cells at different time-points up to 48 h after seeding. Proteins were precipitated from 600μL medium by addition of 600μL each of methanol and chloroform and collected by centrifugation at 12,000 x g, 2 min. The top layer was discarded, and 600μL methanol added. After vortexing, the mixture was centrifuged at 12,000 x g for 10 min. The supernatant was discarded and the pellet retained as precipitated protein. The pellet was air-dried prior to digestion with 0.01 units of chondroitin ABC lyase in 0.1M Tris pH8.0, 0.03M sodium acetate buffer at 37°C for 2h. Digested material was separated by SDS-PAGE and transferred to nitrocellulose membrane as described in Chapter 2, section

2.2.4.4. Membranes were blocked, incubated with anti-decorin antibodies, and bound antibodies detected as described (Chapter 2, section 2.2.4.4).

5.2.8.3 Migration of Dominant Negative Akt Transfected Cells

To investigate whether Akt activation by decorin (Schönherr et al., 2001) was involved in stimulation of migration by decorin (Chapter 3), endothelial cells were transfected with adenovirus harbouring dominant negative Akt. Spheroids were generated from transfected cells. Endothelial cells were transfected with 100 pfu AdvdnAkt per cell as described (see section 5.2.8.4), and previously demonstrated to inhibit Akt-mediated signalling in the EA.hy926 cell line (Schönherr et al., 2001). After transfection, spheroids were generated by incubation in a hydrophobic environment for 48h in a similar manner as for generation of untransfected spheroids (Chapter 3, section 3.2.6). Spheroids were plated onto 50μL collagen gels and stimulated with 0.7μM decorin in solution as previously described (Chapter 3, section 3.2.6).

5.2.9 Statistical Analysis

Statistical analysis was carried out where appropriate exactly as described in Chapter 3, section 3.2.7.

5.3 Results

5.3.1 Decorin Signalling Through IGF-IR

5.3.1.1 Decorin Signalling through IGF-IR during Adhesion to Collagen type I or Collagen-bound Decorin

To investigate involvement of decorin signalling through the IGF-I receptor in decorin-mediated enhancement of cell attachment to collagen type I, endothelial cells in suspension were incubated with 10μM tryphostin AG1024 or the vehicle, DMSO, for one hour. Cells were then mixed with decorin in solution immediately prior to seeding onto collagen gels. It should be noted however, that under these conditions overall levels of adhesion were reduced by approximately half in the presence of DMSO, compared to experiments carried out in Chapter 3 (by comparing absorbance values at 540nm; previously, endothelial cells were trypsinised and the suspensions immediately treated with decorin and seeded). Additionally, under the current conditions, AG1024 further reduced adhesion over DMSO by 2% to 14%, while a mixture of both tyrphostin AG1024 and AG1478 further reduced adhesion (over DMSO) by 13% to 30%. It was not tested whether incubating cells in suspension for one hour in the absence of DMSO also resulted in reduced adhesion.

However, under these conditions, soluble decorin was consistently able (in all 3 experiments conducted) to significantly enhance cell adhesion to collagen type I both in the presence and absence of AG1024 (Figure 5.1A). In contrast, when IGF-IR signalling was blocked using AG1024 prior to seeding onto collagen-bound decorin, although decorin slightly enhanced adhesion in two of three experiments (one of which is shown in Figure 5.1B), none of these experiments reached statistical significance. In all three experiments, collagen-bound decorin significantly enhanced adhesion in the presence of the vehicle, DMSO (Figure 5.1B).

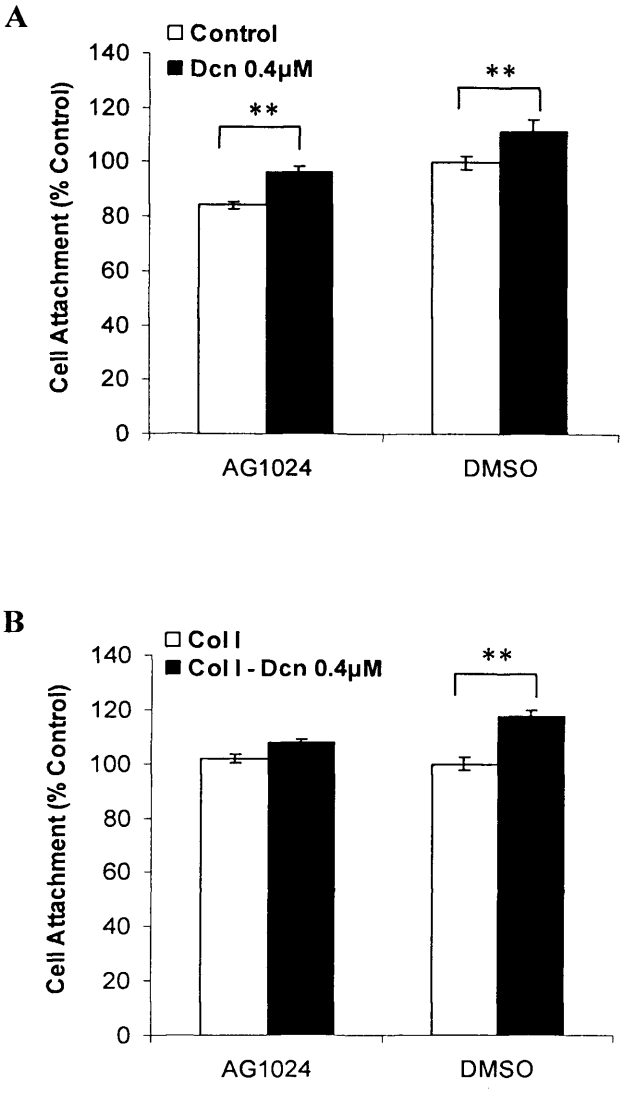
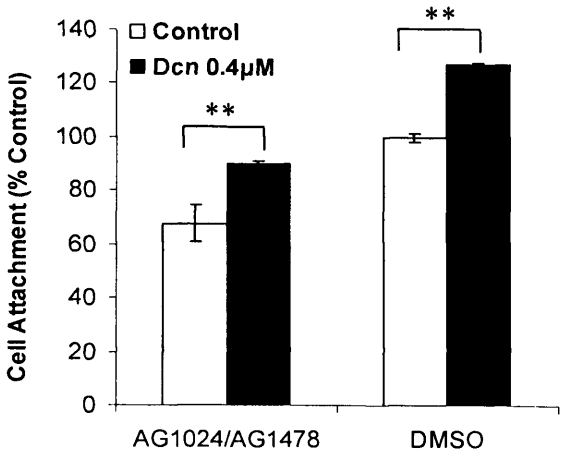


Figure 5.1: Soluble decorin enhances adhesion independently of signalling through the IGF-I receptor, while collagen-bound decorin may partially signal through the IGF-I receptor to enhance adhesion. Endothelial cells were trypsinised and trypsin activity stopped by addition of growth medium. Cells were exchanged into serum-free medium and incubated with tyrphostin AG1024 at 10μM for one hour, 37°C, 5% CO_2. Cells were mixed with 0.4μM decorin in solution prior to seeding onto collagen gels (A). Alternatively, cells were plated onto mixed decorin 0.4μM-collagen gels (B). Adherent cells were stained with crystal violet one hour after plating. Data shown is one experiment, representative of three independent experiments. Standard errors are shown as error bars. Statistical significance is shown, where ** denotes $p < 0.01$.

5.3.1.2 Decorin Signalling through IGF-IR or EGFR during Adhesion to Collagen type I or Collagen-bound Decorin

To investigate compensation from decorin signalling through the EGFR when IGF-I receptor signalling was blocked, signalling through both IGF-IR and EGFR was simultaneously blocked using tyrphostins AG1024 and AG1478 respectively. Under these conditions, soluble decorin was able to enhance adhesion in both experiments carried out (Figure 5.2A) although in one experiment this did not reach statistical significance. In contrast, collagen-bound decorin consistently (in 3 of 3 independent experiments) failed to enhance adhesion in the presence of the inhibitors (Figure 5.2B). In all three experiments, collagen-bound decorin consistently enhanced adhesion in the absence of the inhibitors, although in one experiment this did not reach statistical significance.

A



B

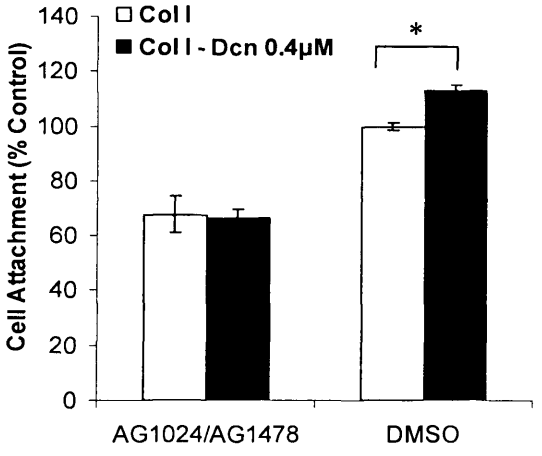


Figure 5.2: Soluble decorin enhances adhesion independently of growth factor receptor signalling, while collagen-bound decorin signals through growth factor receptors to enhance adhesion. Endothelial cells were trypsinised and trypsin activity stopped by addition of growth medium. Cells were exchanged into serum-free medium and incubated with tyrphostin AG1024 and tyrphostin AG1478 simultaneously at 10μM each for one hour. Cells were mixed with 0.4μM decorin in solution prior to seeding onto collagen gels (A). Alternatively, cells were plated onto mixed decorin 0.4μM-collagen gels (B). Adherent cells were stained with crystal violet one hour after plating. Data is representative of two independent experiments. Standard errors are shown as error bars. Statistical significance is shown where * denotes $p < 0.05$, and ** denotes $p < 0.01$.

5.3.1.3 Decorin Signalling through IGF-IR During Endothelial Cell Migration on Collagen type I or Collagen-bound Decorin

To investigate involvement of decorin signalling through the IGF-I receptor in decorin-mediated enhancement of cell migration, signalling through the receptor was blocked using tyrphostin AG1024. Endothelial cell spheroids were plated onto collagen gels and incubated with 10μM tryphostin AG1024 or DMSO vehicle for one hour prior to addition of 0.4μM decorin. Decorin failed to enhance endothelial cell migration on collagen, while in the presence of the DMSO vehicle (control), decorin enhanced endothelial cell migration (Figure 5.3A). Alternatively, spheroids were plated onto mixed 0.6μM decorin-collagen gels and tyrphostin AG1024 added to the medium. Similarly, decorin failed to enhance endothelial cell migration, while in the control (DMSO vehicle), endothelial cells migrated further on mixed decorin-collagen gels than on collagen gels (Figure 5.3B). This suggests that decorin could enhance endothelial cell migration through IGF-IR mediated signalling however, since treatment with AG1024 alone already reduced endothelial cell migration (over DMSO vehicle) it could be argued that the cells could not respond to decorin.

Since it had been previously shown that decorin could activate Akt dependent and independent signalling pathways in endothelial cells, spheroids were generated from endothelial cells expressing a dominant negative mutant of Akt (using adenovirus mediated gene transfer), and plated onto collagen. When decorin was added to the media, migration was enhanced, (Figure 5.3C), indicating that decorin enhances migration through Akt independent pathways. The inset of Figure 5.3C confirms that Akt was over-expressed.

It should also be noted that data presented in Figures 5.3A and 5.3B were performed in parallel and are therefore directly comparable. These figures demonstrate that collagen-bound decorin (5.3B) enhanced migration more than soluble decorin. Further, at 0.4μM soluble decorin, 4μg decorin was present in each well (in 100μL media), while at 0.6μM collagen-bound decorin, 2.4μg decorin was present in each well (in a 40μL collagen-decorin gel), thus less decorin was present when collagen-bound.

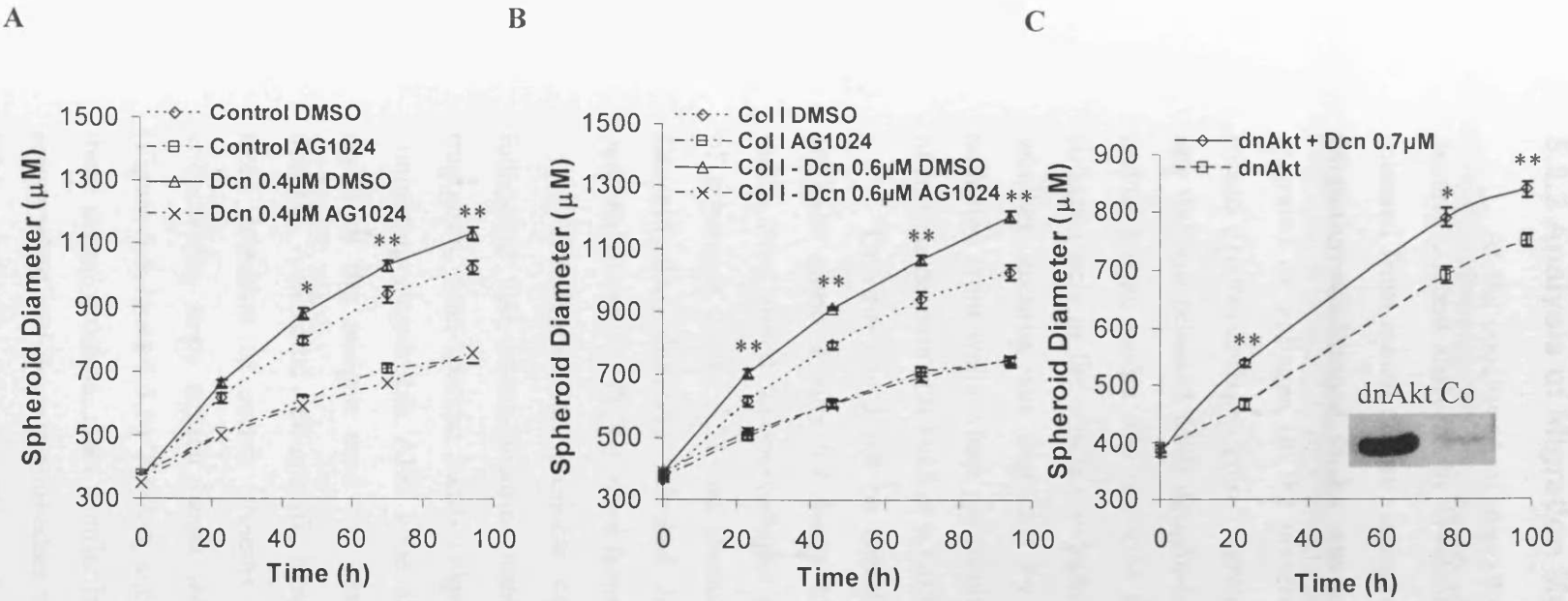


Figure 5.3: Decorin enhances endothelial cell migration on collagen or on mixed decorin-collagen gels by signalling through the IGF-I receptor, but independently of Akt activation. Spheroids were plated onto 40μL collagen gels and incubated with AG1024 at 10μM (or the equivalent volume of DMSO) for 60 minutes before addition of decorin at 0.4μM (or the equivalent volume of PBS) and migration analysed (A). Spheroids were plated onto mixed decorin 0.6μM-collagen gels (40μL) and AG1024 at 10μM (or the equivalent volume of DMSO) added, and migration analysed (B). Alternatively, spheroids were generated from endothelial cells over-expressing dominant negative Akt. Spheroids were plated onto collagen gels and decorin at 0.7μM (or the equivalent volume of PBS) added and migration analysed (C). Data is representative of two independent experiments. Standard errors are shown as error bars. In (C), an inset of a Western blot using antibodies directed against Akt (detailed in Chapter 2, section 2.3.2.2) is shown, from cell lysates of endothelial cells transfected with dominant negative Akt (dnAkt) or control adenovirus (Co). Statistical significance is shown compared to the control (control DMSO (A), Col I DMSO (B)) at each time-point, where * denotes $p < 0.05$, and ** denotes $p < 0.01$.

5.3.2 Analysis of Migration Medium

At the conclusion of migration experiments, medium was collected and decorin content analysed by Western blotting to examine whether decorin was released from mixed decorin-collagen gels, and whether decorin was degraded. Migration-conditioned media was collected from wells where spheroids had migrated on collagen (in the presence and absence of soluble decorin) or on mixed decorin-collagen gels (migration data, Figure 5.3). To examine whether any decorin released from decorin-collagen gels required the presence of cells and/or serum, media was collected from wells containing collagen or decorin-collagen gels in the absence of spheroids and/or serum. To further investigate whether decorin was degraded by the end of the experiment, media was collected from wells where spheroids had migrated on collagen in the presence of soluble decorin at 0.1μM or 0.5μM (migration data, Figure 3.7, Chapter 3).

Decorin could not be detected in conditioned medium from cells on collagen alone (Figure 5.4, lanes 4,5,10,11), but was detected in conditioned media from mixed decorin-collagen gels in the absence (lanes 2,3) and presence of spheroids (lanes 6,7) and absence of both spheroids and serum (lane 3). Decorin was therefore released independently of the presence of either endothelial cells or FCS derived factors, indicating a passive process.

No degradation products could be detected (Figures 5.4 and 5.5), indicating that intact decorin, rather than degradation products, stimulated migration. Non-specific bands (Figures 5.4 and 5.5) were apparent in wells containing chondroitin ABC lyase alone, and those labelled NS were likely a result of the antigen used for immunisation being chondroitinase digested decorin. Additional non-specific bands seen only in Figure 5.4 likely originate from detection of serum proteins by biotinylated anti-rabbit and/or HRP-streptavidin, since similar bands were not detected in the absence of serum (Figure 5.4, lanes 1,3,5). Further, antibodies to decorin are unlikely to recognise these serum proteins, since similar bands were not apparent in the presence of serum when anti-decorin antibodies were detected using one antibody step, with HRP-anti-rabbit antibodies (Figure 5.5).

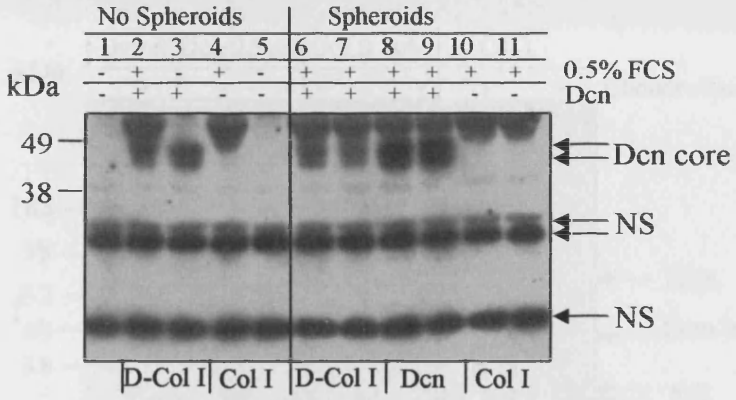


Figure 5.4: Decorin is released from mixed decorin-collagen gels, and is not degraded during migration experiments. Medium was collected at the conclusion of migration experiments shown in Figure 5.3 (A). Media from migration experiments contained 0.5% heat-inactivated FCS (+). In addition, medium containing 0.5% heat-inactivated FCS (+) or no FCS (-) was collected from wells containing collagen or mixed decorin-collagen gels but no cells (denoted no spheroids). Lanes 6 to 11 show media collected from wells containing spheroids. Decorin (Dcn) was used at 0.6μM for mixed gels (Dcn-Col I) or at 0.4μM soluble decorin (Dcn). All media was digested with Chondroitin ABC lyase, separated by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with polyclonal antibodies to decorin followed by biotin-conjugated anti-rabbit and HRP-conjugated steptavidin. Lanes are as follows. **1** Chondroitin ABC lyase;.wells 2 to 11 contain medium collected from wells containing: **2,3** Dcn-col I gel; **4,5** Col I gel; **6** Dcn-col I gel + AG1024; **7** Dcn-col I gel + DMSO; **8** Col I gel + AG1024 + Dcn; **9** Col I gel + DMSO + Dcn; **10** Col I gel + AG1024; **11** Col I gel + DMSO. Non-specific bands originating from chondroitin ABC lyase are indicated (NS).

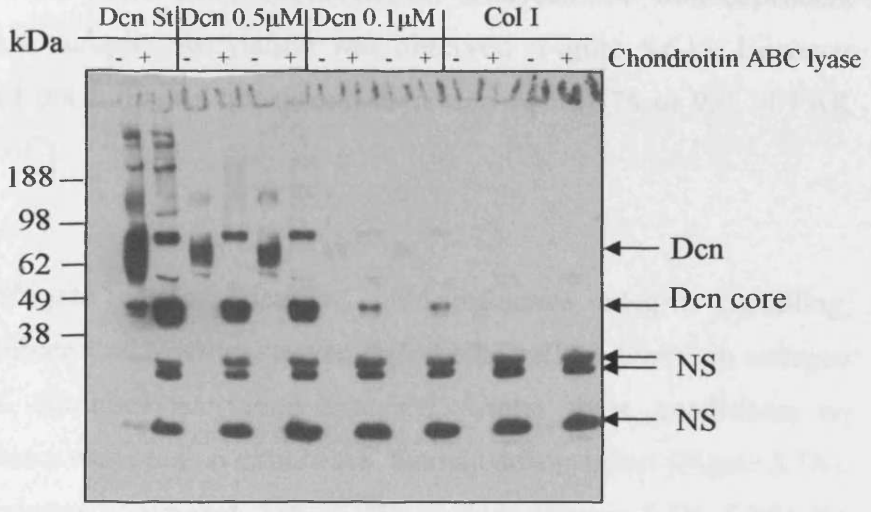


Figure 5.5: Decorin is released from mixed decorin-collagen gels, and is not degraded during migration experiments. Medium was collected at the conclusion of a previous experiment from duplicate wells (Figure 3.7, Chapter 3), where spheroids migrated on collagen gels in the presence of soluble decorin at 0.1μM or 0.5μM, in media containing 0.5% FCS. Media were digested with Chondroitin ABC lyase and, along with undigested media, was separated by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with polyclonal antibodies to decorin and HRP-conjugated anti-rabbit IgG antibodies (Dcn St – 2.5μg purified decorin standard). Non-specific bands originating from chondroitin ABC lyase are indicated (NS).

5.3.3 Decorin Signalling Through Integrins

5.3.3.1 Rhodocetin

To investigate whether rhodocetin could influence integrin signalling, rhodocetin was incubated with serum-starved endothelial cells cultured on collagen gels, and FAK autophosphorylation analysed. A time-dependent decrease in FAK autophosphorylation was observed (Figure 5.6A). However rhodocetin could not influence phosphorylation at tyrosine 576 or 925 of FAK (Figure 5.6B, 5.6C).

5.3.3.2 Decorin

To investigate whether decorin could influence integrin signalling, decorin was incubated with serum-starved endothelial cells cultured on collagen gels, and FAK autophosphorylation analysed. Under these conditions, no influence of decorin was seen on either FAK autophosphorylation (Figure 5.7A), or on phosphorylation at tyrosine 576 or 925 of FAK (Figure 5.7B, 5.7C). To investigate whether decorin can influence FAK autophosphorylation under conditions where cells are attaching to matrix substrates (identical to adhesion experiments conducted in Chapter 3), endothelial cells were mixed with decorin in solution immediately prior to seeding on collagen gels. Alternatively, cells were seeded onto mixed decorin-collagen gels. At 1h and 3h after seeding, adherent cell lysates were analysed by Western blotting. Under these conditions, decorin also could not influence FAK autophosphorylation (Figure 5.7D).

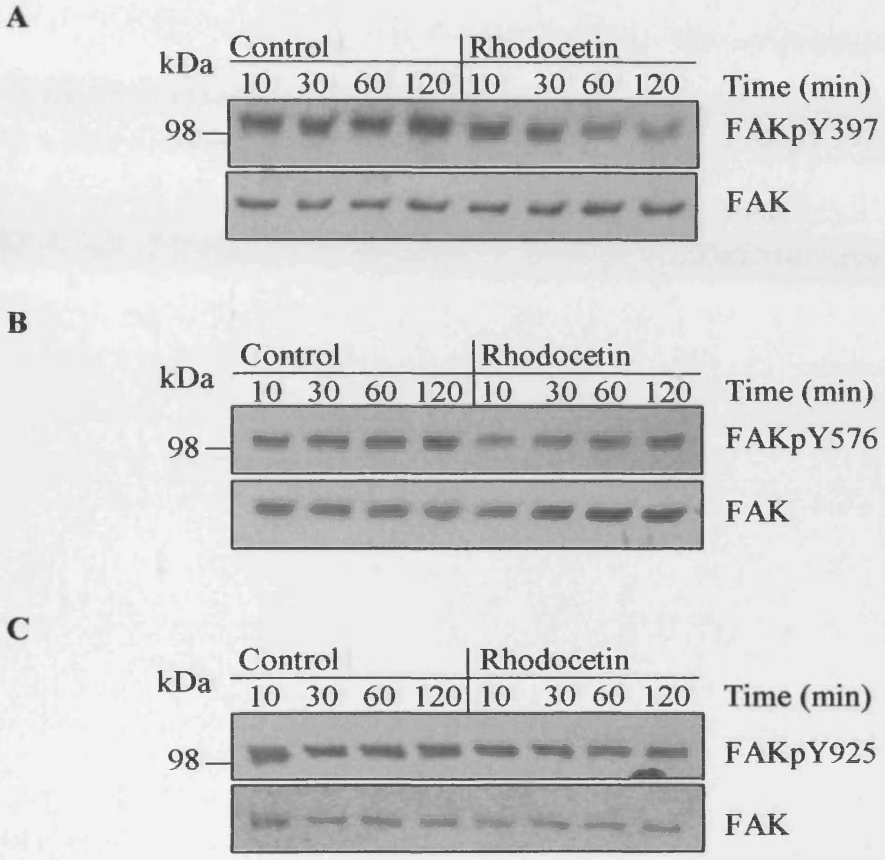


Figure 5.6: Rhodocetin inhibits FAK autophosphorylation. Endothelial cells were cultured on collagen gels in medium containing 0.5% FCS for 48 hours prior to incubation with rhodocetin at 5μg/ml for the indicated times. Lysates were separated by SDS-PAGE and analysed by Western blotting using antibodies to phosphorylated tyrosine residues of FAK, tyrosines 397 (A), 576 (B) or 925 (C). As a loading control, membranes were stripped and probed with antibodies to non-phosphorylated FAK. All experiments shown are representative of 3 independent experiments.

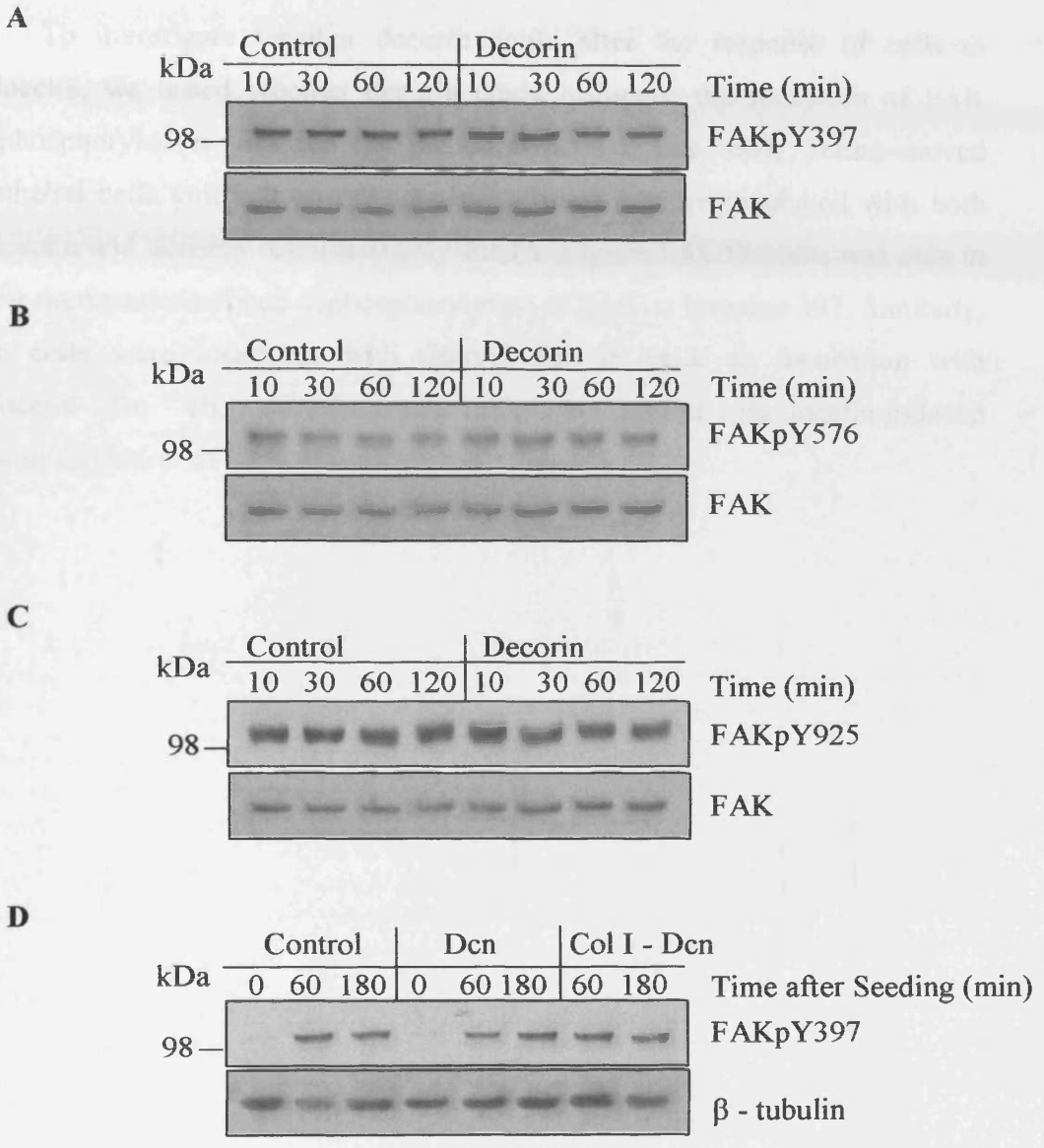


Figure 5.7: Exogenous decorin does not influence FAK autophosphorylation. Endothelial cells were cultured on collagen gels in medium containing 0.5% FCS for 48 hours prior to incubation with decorin at 70μg/ml for the indicated times. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and analysed by Western blotting using antibodies to phosphorylated tyrosine residues of FAK, tyrosines 397 (A), 576 (B) or 925 (C). As a loading control, membranes were stripped and probed with antibodies to non-phosphorylated FAK. Alternatively, endothelial cells were seeded onto collagen (Control), in the absence or presence of 0.5μM decorin (Dcn), or were seeded onto mixed decorin 0.5μM-collagen gels (Col I – Dcn). Cells were allowed to adhere for 60 or 180 min. Proteins were extracted, separated by SDS-PAGE, transferred to nitrocellulose membrane and analysed by Western blotting using antibodies to FAK phosphorylated at tyrosine 397. As a loading control, unstripped membranes were probed with antibodies to β-tubulin (D). All experiments shown are representative of 3 independent experiments.

5.3.3.3 Decorin and Rhodocetin

To investigate whether decorin could alter the response of cells to rhodocetin, we tested whether decorin could influence the reduction of FAK autophosphorylation induced by rhodocetin. To this end, serum-starved endothelial cells cultured on collagen gels for 48 h were incubated with both rhodocetin and decorin simultaneously for 1h (Figure 5.8). Decorin was able to inhibit rhodocetin-induced dephosphorylation of FAK at tyrosine 397. Similarly, when cells were incubated with decorin for 1h prior to incubation with rhodocetin for 1h, decorin was able to inhibit rhodocetin-induced dephosphorylation of FAK at tyrosine 397 (Figure 5.8).

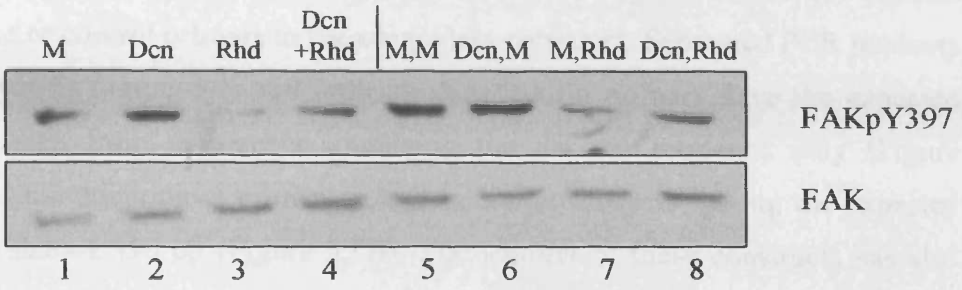


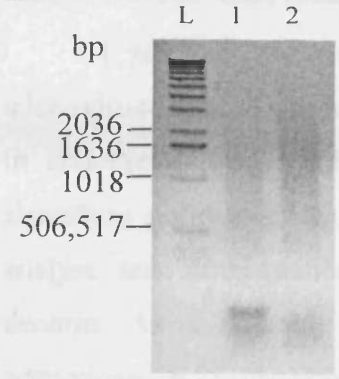
Figure 5.8: Decorin inhibits rhodocetin-induced reduction in FAK autophosphorylation. Endothelial cells were cultured on collagen gels in medium containing 0.5% FCS for 48 hours prior to stimulation with 0.5μM decorin, 0.15μM rhodocetin or both simultaneously for 1h (lanes 1-4). Alternatively, endothelial cells were stimulated with 0.5μM decorin for 1h, followed by incubation with 0.15μM rhodocetin for a further hour (lanes 5-8). Lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and analysed by Western blotting using antibodies to phosphorylated tyrosine 397 of FAK. As a loading control, membranes were stripped and probed with antibodies to non-phosphorylated FAK. (M denotes medium, Rhd, rhodocetin, Dcn, decorin. For example, M,Rhd denotes 1h incubation in medium followed by 1h incubation with rhodocetin). Data is representative of 3 independent experiments.

5.3.4 Adenovirus Characterisation

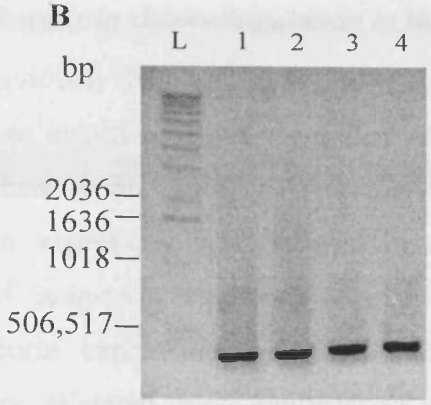
To confirm identity of adenovirus constructs, DNA constructs were extracted from adenovirus and analysed by PCR using primers to the decorin sequence or control primers to the adenovirus construct. Separated PCR products are shown in Figure 5.9, and indicate that decorin primers gave the expected product size from constructs containing the decorin sequence only (Figure 5.9A), while adenovirus primers recognised all constructs, giving the expected product size of 350 bp (Figure 5.9B). The identity of these constructs has also been previously confirmed by sequencing (Schönherr et al., 1999).

The plaque forming units (pfu) of adenovirus preparations were calculated by incubating a dilution series of adenovirus with HEK-293 cells. Plaques of infected cells were visualised by staining with antibodies to hexon proteins of the adenovirus. The pfu obtained for adenovirus preparations are shown in Figure 5.9C. The pfu are in the expected range, with the control adenovirus Adl312 having the highest pfu as commonly found (Elke Schönherr, personal communication).

A



B



C

	pfu/ml virus stock			
Dilution	**AdvDcn**	**AdvControl**	**AdvdnAkt**	**AdvdpAkt**
1.00E+08	7E+09	1.95E+10	3.05E+09	1.55E+09
1.00E+09	4E+09	1.95E+10	n/a	2.5E+09
Average	5.5E+09	1.95E+10	3.05E+09	2.02E+09

Figure 5.9: Adenovirus characterisation and plaque forming units. The adenovirus constructs AdvControl, dnAkt, dpAkt and AdvDcn were analysed by PCR, using primers recognising decorin (A) or adenovirus constructs (B). PCR products were resolved by separation on agaraose gels and visualised using ethidium bromide. Images were captured under UV light. Lanes are as follows. L – Molecular Weight markers, bp denotes base-pairs, 1 - AdvDcn, 2 - AdvAdl312, 3 - AdvdpAkt, 4 – AdvdnAkt. Adenovirus pfu was calculated by infection of HEK-293 cells with dilutions of adenovirus preparations. Plaques were visualised by staining with antibodies to adenovirus hexon proteins. Plaques were counted in two wells and the number of pfu per ml calculated (C).

5.3.5 Decorin Expression and Integrin Signalling

5.3.5.1 Decorin Expression Results in Down-Regulation of the IGF-IR

It has been shown previously that long-term exposure to decorin through adenovirus-induced expression results in down-regulation of the IGF-I receptor in endothelial cells (Schönherr et al., 2005). To further investigate decorin signalling pathways, decorin expression was induced in endothelial cells to analyse the consequence of long-term exposure to endogenously produced decorin. As a control, decorin expression in endothelial cells induced by adenovirus was checked by Western blot analysis of the medium using antibodies to decorin (Figure 5.10A). This demonstrates that decorin expression was induced in endothelial cells by adenovirus containing decorin cDNA but not by control adenovirus. Cell lysates of transfected cells were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies to IGF-IR (Figure 5.10B). Cell lysates were analysed between 18h and 66h after transfection to encompass maximum decorin expression at 48h after transfection as previously described (Schönherr et al., 2001). Figure 5.10B demonstrates that decorin expression results in down-regulation of the IGF-I receptor. Densitometric analysis was carried out by normalisation to β-tubulin, however levels of β-tubulin also decreased in the presence of decorin. Western blotting of the same cell lysates in a further experiment (Figure 5.11A) demonstrated that β-tubulin levels were not altered by expression of decorin. It is possible therefore that transfer of proteins to nitrocellulose membrane was not equal for the data shown in Figure 5.10B. However, IGF-IR levels appeared to be generally decreased when decorin was expressed.

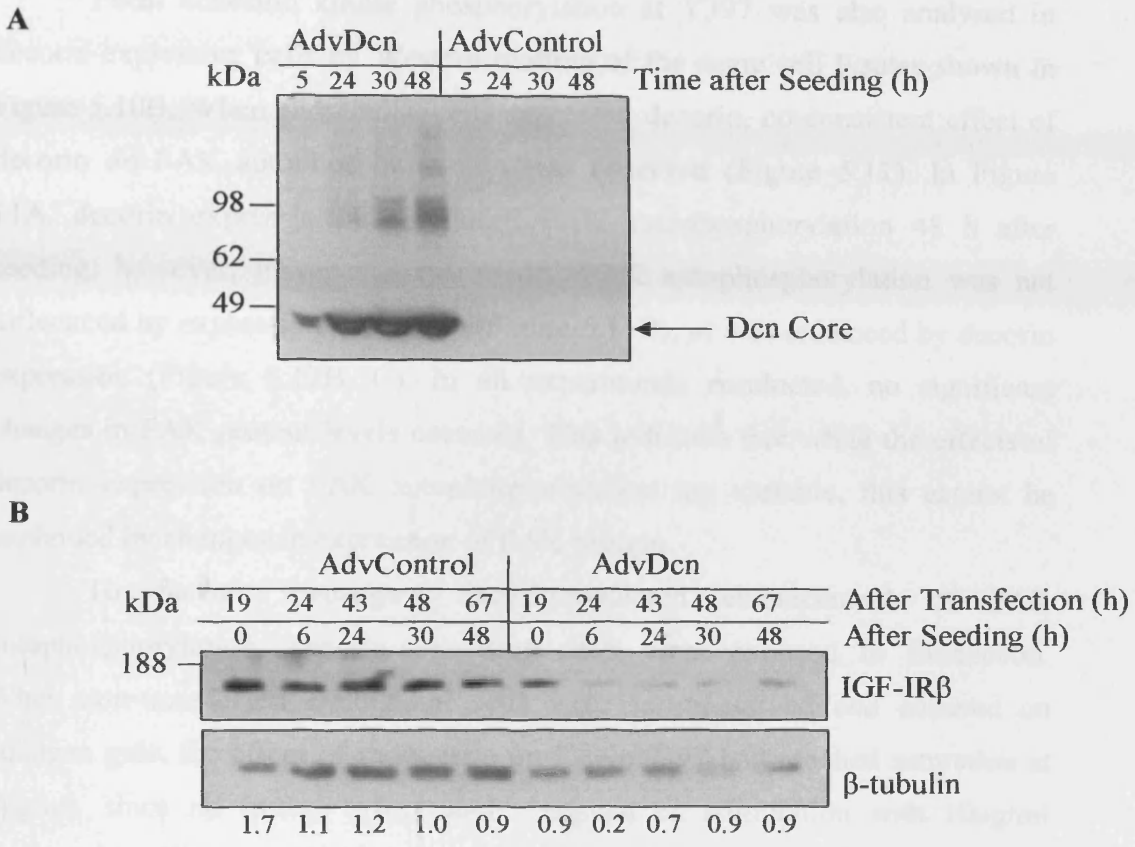
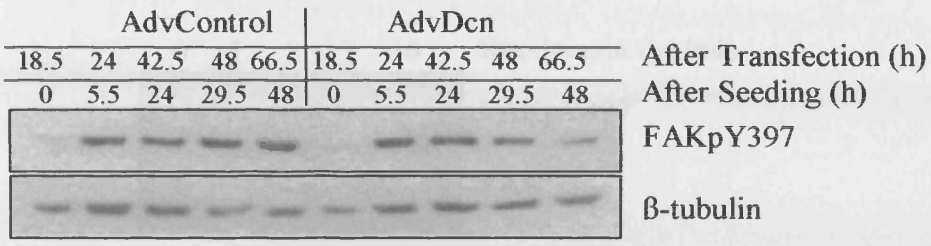


Figure 5.10: Decorin expression in endothelial cells results in down-regulation of the IGF-I receptor. Decorin expression was induced in endothelial cells using an adenovirus containing the cDNA of human decorin (AdvDcn). Decorin or control adenovirus transfected cells were cultured on fibrillar collagen type I in medium containing 0.5% FCS for the indicated times. At this time, the medium was collected, precipitated using methanol/chloroform, digested using chondroitin ABC lyase and analysed by Western blotting using antibodies to decorin (A). Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies to IGF-IR. As a loading control, the membrane was stripped and re-probed with antibodies to β-tubulin. Densitrometric analysis of IGF-IR normalised to β-tubulin is shown (B).

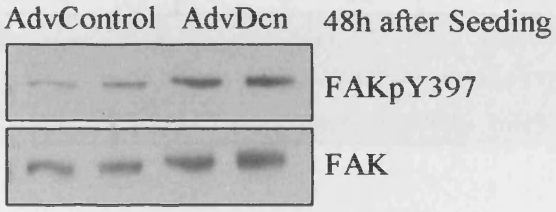
Focal adhesion kinase phosphorylation at Y397 was also analysed in decorin-expressing cells by Western blotting of the same cell lysates shown in Figure 5.10B. When endothelial cells expressed decorin, no consistent effect of decorin on FAK autophosphorylation was observed (Figure 5.11). In Figure 11A, decorin expression had reduced FAK autophosphorylation 48 h after seeding, however, in repeat experiments, FAK autophosphorylation was not influenced by expression of decorin (Figure 5.11B), or was enhanced by decorin expression (Figure 5.12B, C). In all experiments conducted, no significant changes in FAK protein levels occurred. This indicates that while the effects of decorin expression on FAK autophosphorylation are variable, this cannot be explained by changes in expression of FAK protein.

To further investigate decorin-mediated enhancement of FAK autophosphorylation, decorin-expressing cells were exposed to rhodocetin. When non-transfected endothelial cells were serum-starved and cultured on collagen gels, the effect of rhodocetin on FAKpY397 had reached saturation at 5μg/ml, since no further effect could be seen on stimulation with 10μg/ml rhodocetin (Figure 5.12A). Similarly, in cells transfected with control adenovirus, 5μg/ml rhodocetin was sufficient to produce maximal effects (Fgiure 5.12B, C). However, in decorin-expressing cells, dose-dependent effects up to 10μg/ml rhodocetin on FAK autophosphorylation were observed (Figure 5.12B, C), suggesting that FAK autophosphorylation was slightly enhanced in endothelial cells expressing decorin. In the experiment shown in Figure 5.12B, a clear enhancement of FAK phosphorylation at tyrosine 397 can be seen in decorin expressing cells compared to control transfected cells, which is not explained by changes in FAK protein levels. However, when FAKpY397 levels in decorin-expressing or control transfected cells were averaged from three independent experiments, statistical significance was not reached (Figure 5.12C). This is likely due to the variability in decorin effects on FAK autophosphorylation between different experiments (Figure 5.11, 5.12).

A



B



C

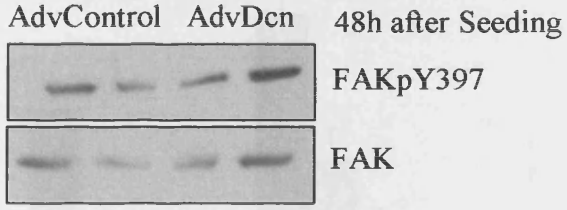


Figure 5.11: Decorin expression in endothelial cells variably enhances FAK activity. Decorin expression was induced in endothelial cells the adenovirus containing the cDNA of human decorin (AdvDcn). Decorin or control (AdvControl) transfected cells were cultured on fibrillar collagen type I in medium containing 0.5% FCS. At the indicated times, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies to FAKpY397. As a loading control, the membrane was re-probed with antibodies to FAK. Part (A) shows analysis of identical cell lysates shown in Figure 5.9B. In (B and C) another experiment is shown where lysates were analysed at one time-point; 48 hours after plating. As a loading control, the membranes were stripped and re-probed with antibodies to FAK. Two separate experiments were performed (both shown in B and C), and within each experiment, each condition was tested in duplicate.

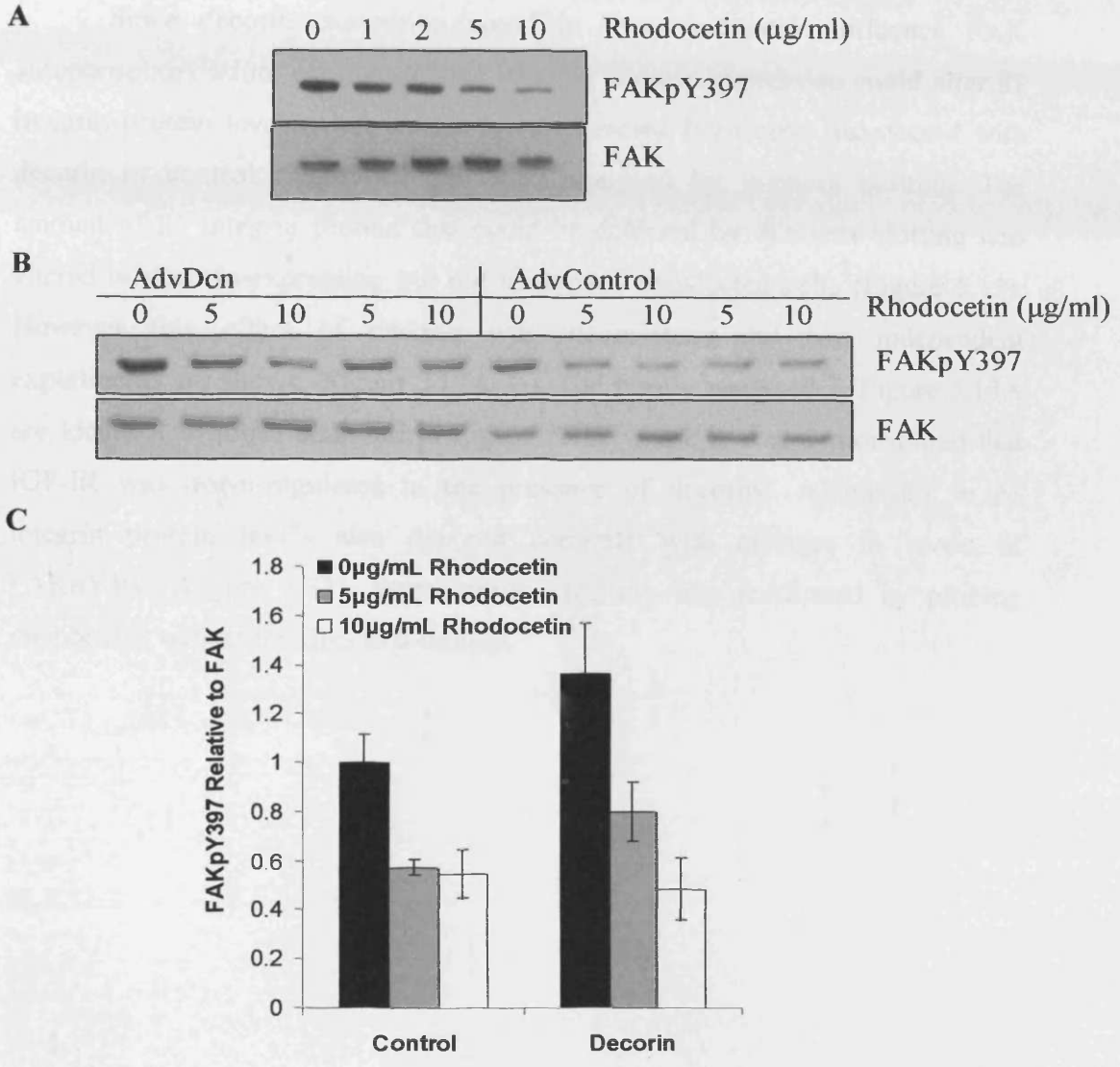


Figure 5.12: The influence of decorin expression on FAK activity in endothelial cells. Endothelial cells were cultured on collagen gels in medium containing 0.5% FCS for 48 hours prior to incubation with rhodocetin at 1, 2, 5, or 10µg/ml for 1h in serum-free medium. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and analysed by Western blotting using antibodies to phosphorylated tyrosine residues of FAK. The membrane was stripped and re-probed with antibodies to FAK (A). Decorin expression was induced in endothelial cells using adenovirus containing cDNA of decorin (AdvDcn). As a control, cells were transfected with insert-free control adenovirus (AdvControl). Adenovirus transfected cells were cultured on fibrillar collagen type I in medium containing 0.5% FCS for 48 hours. At this time, cells were exposed to 5 or 10µg/ml rhodocetin in duplicate. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies to FAKpY397. The membrane was stripped and re-probed with an antibody to FAK (B). Western blots were analysed by densitometry from 3 independent experiments carried out as described in (B), and FAK autophosphorylation calculated relative to FAK. The average was calculated and the standard error of the mean is indicated as error bars (C).

5.3.5.2 Decorin Influence on β1 Integrin Expression

Since decorin expression could in some instances, influence FAK autophosphorylation, we investigated whether decorin expression could alter β1 integrin protein levels. Cell lysates were extracted from cells transfected with decorin or control adenovirus and were analysed by Western blotting. The amount of β1 integrin protein that could be detected by Western blotting was altered in decorin-expressing but not in control transfected cells (Figure 5.13). However this effect of decorin was inconsistent and two independent experiments are shown (Figure 5.13A, B). The lysates analysed in Figure 5.13A are identical to those analysed in Figure 5.10 (where it was demonstrated that IGF-IR was down-regulated in the presence of decorin). Alterations in β1 integrin protein levels also did not correlate with changes in levels of FAKpY397 (Figure 5.13). Equal protein loading was confirmed by probing membranes with antibodies to β-tubulin.

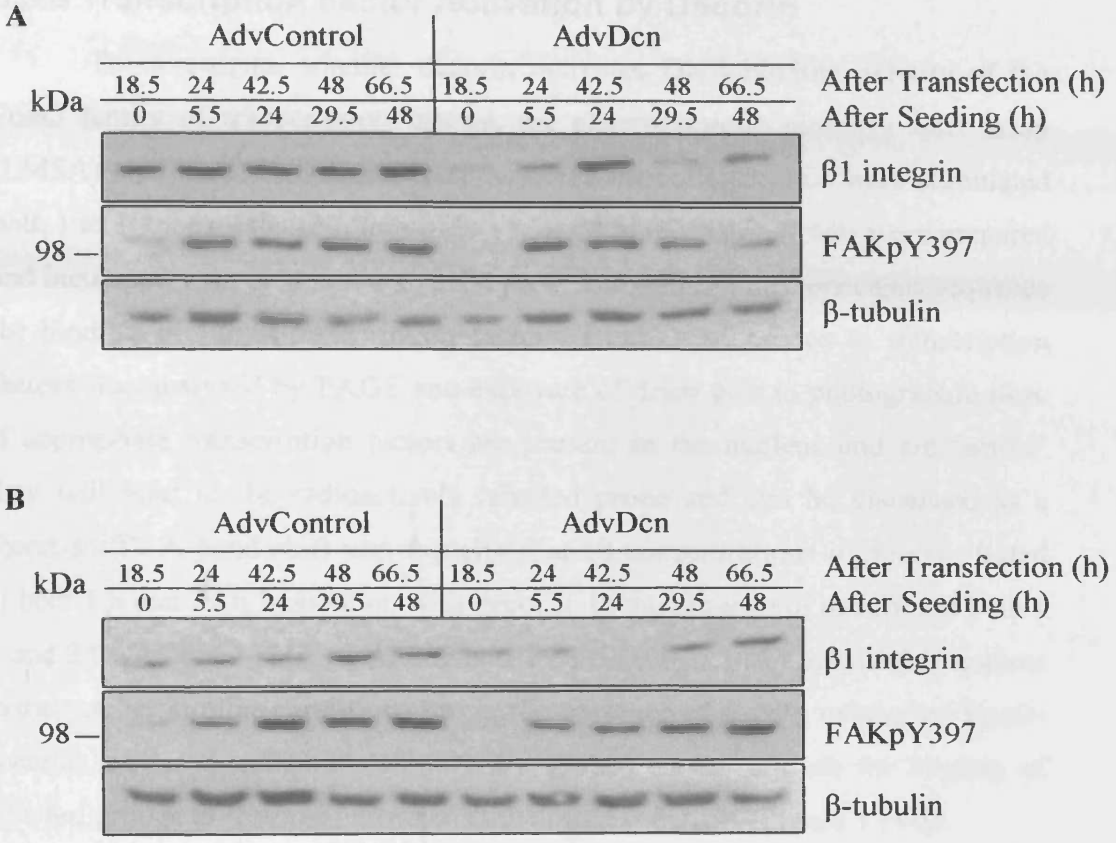


Figure 5.13: Decorin expression in endothelial cells influences extraction of the β1 integrin subunit. Decorin expression was induced in endothelial cells using adenovirus containing the cDNA of human decorin (AdvDcn). Adenovirus transfected cells were cultured on fibrillar collagen type I under serum free conditions for the indicated times. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies to β-integrin and FAKpY397. As a loading control, the membrane was stripped and re-probed with antibodies to β-tubulin. Two identical experiments are shown in (A) and (B). Cell lysates analysed in (A) are identical to those shown in Figure 5.9 and Figure 5.10A.

5.3.6 Transcription Factor Activation by Decorin

To investigate whether decorin activates DNA-binding activity of the FoxO family of transcription factors, the electrophoretic mobility shift assay (EMSA) was used. Endothelial cells cultured on collagen gels were stimulated with 1 to 100μg/ml decorin for either 1 h or 24 h. Nuclear extracts were prepared and incubated with $\gamma^{32}P$ labelled DNA probes containing the consensus sequence for binding of FoxO transcription factors. Binding of probes to transcription factors was analysed by PAGE and exposure of dried gels to photographic film. If appropriate transcription factors are present in the nucleus and are 'active' they will bind to the radioactively labelled probe and can be visualised as a 'band-shift'. A band-shift was visualised at all concentrations of decorin tested at both 1 h and 24 h incubation with decorin. In the absence of decorin, at both 1 h and 24 h no band-shift could be visualised (Figure 5.14A). Incubating nuclear extract under similar conditions but in the presence of excess unlabelled FoxO-specific probes demonstrated that 'cold' probes could compete for binding of labelled probes to forkhead proteins in the nuclear extract (Figure 5.14B).

To further investigate activation of FoxO transcription factors, cytoplasmic and nuclear extracts were separated by SDS-PAGE and membranes probed with antibodies directed against phosphorylated FoxO1 (Ser256) and FoxO4 (Ser193) (1:1000, raised in rabbit, code 9461, Cell signalling Technology, Danvers, MA, USA). However, no signal could be obtained even after prolonged exposure to photographic film.

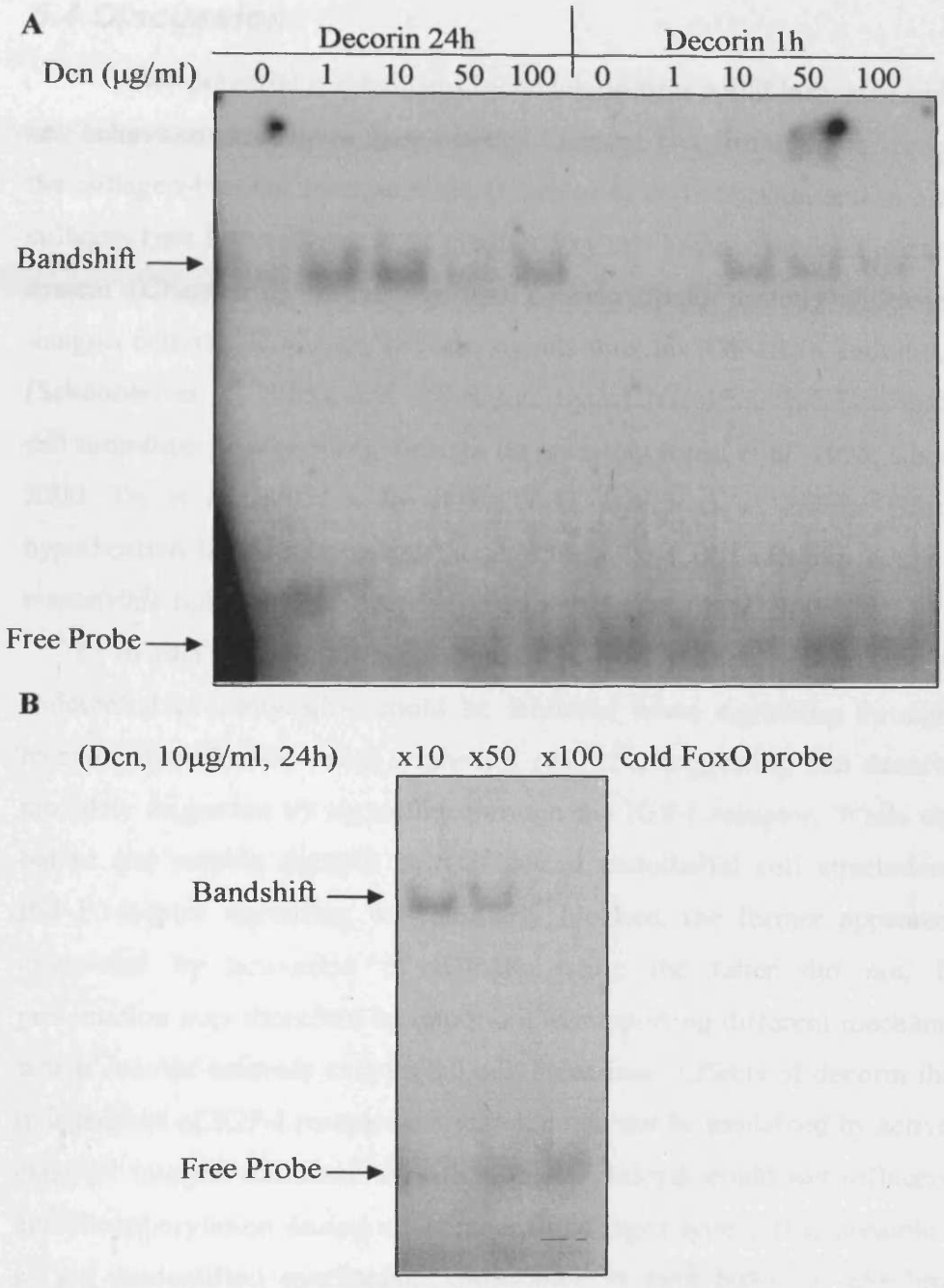


Figure 5.14: Decorin activates FoxO transcription factors. An electromobility shift assay was carried out incubating serum-starving endothelial cells cultured on collagen gels with 1, 10, 50 or 100μg/ml decorin. After incubation for 1h or 24h, cells were lysed and the nuclear extracts obtained. Nuclear extracts were incubated with ^{32}P labelled DNA probes containing consensus sequence for binding of FoxO transcription factors. Protein-DNA complexes were separated by PAGE and binding of probes to transcription factors was analysed by exposure of dried gels to photographic film (A). Alternatively, one sample of nuclear extract (Inucbation with 10μg/ml decorin for 24h) was incubated under identical conditions as for (A), but additionally with 10-fold, 50-fold or 100-fold excess unlabelled FoxO specific probe (B).

5.4 Discussion

Two potential mechanisms by which decorin could influence endothelial cell behaviour were investigated in this Chapter. Decorin interacts directly with the collagen-binding integrin α2β1 (Chapter 4) and adhesion and migration on collagen type I was shown to be mediated by this integrin, at least in part, in our system (Chapter 3), indicating that decorin could directly influence α2β1 integrin activity. However, decorin signals through IGF-IR in endothelial cells (Schönherr et al. 2005), and the classic ligand IGF-I can promote endothelial cell migration by signalling through its receptor (Jones et al. 1996, Cheng et al. 2000, Tai et al. 2003, Kiely et al. 2005, Lynch et al. 2005). Thus it was hypothesised that decorin signalling through the IGF-I receptor could also be responsible for decorin influences on endothelial cell behaviour.

In this Chapter, it was shown that decorin-mediated enhancement of endothelial cell migration could be inhibited when signalling through IGF-I receptor was blocked using tyrphostin AG1024, suggesting that decorin could modulate migration by signalling through the IGF-I receptor. While collagen-bound and soluble decorin both enhanced endothelial cell attachment when IGF-I receptor signalling was similarly blocked, the former appeared to be modulated by activation of IGF-IR, while the latter did not. Decorin presentation may therefore be important in supporting different mechanisms by which decorin controls endothelial cell behaviour. Effects of decorin that were independent of IGF-I receptor activation could not be explained by activation of classical integrin-mediated signalling, since decorin could not influence FAK autophosphorylation during attachment to collagen type I. It is possible that an as yet unidentified mechanism contributes to modulation of attachment by decorin.

When IGF-I receptor activation was blocked, soluble decorin could still enhance cell attachment, while collagen-bound decorin effects were inhibited to some extent. Presentation of decorin as an immobilised substrate with collagen type I, or as a soluble form may therefore act differentially in control of endothelial cell behaviour. However, it should be noted that soluble decorin

would also bind to the surface of the collagen gels, while analysis of migration-conditioned media demonstrated that decorin is passively released from decorin-collagen gels (after 5 days), indicating that decorin presentation as a soluble or a collagen-bound form could in either case represent both conditions. During cell attachment however (1h), it is not known whether collagen-bound decorin was released. These observations also would not explain differential requirements of collagen-bound or soluble decorin for IGF-I receptor activation in modulating attachment. Further, a more potent effect of collagen-bound decorin could also be suggested; less is required to achieve similar stimulation. 5 to 10-fold less decorin was present when bound to collagen than when added to the media, but the degree by which attachment was stimulated is similar (Figures 5.2 and 5.3). For instance, at 0.4μM, in each well, cells were seeded in the presence of 4μg soluble decorin, but onto only 0.4μg to 0.8μg decorin bound to collagen (gels of approximately 10μL to 20μL volume). These observations cannot be explained by saturation of decorin effects, since attachment was dose-dependent up to at least 0.5μM decorin, and the degree by which decorin stimulated attachment was also similar at 0.1μM, irrespective of presentation (Figures 3.2 and 3.3, 60 mins, Chapter 3).

Decorin has not been previously found to signal through EGFR in endothelial cells (Schönherr et al. 2005). However, to investigate compensation from this receptor when IGF-IR signalling was blocked, activation of both receptors was inhibited simultaneously. Under these conditions, soluble decorin could still enhance attachment while collagen-bound decorin could not. This is likely to be an effect of blocking IGF-IR signalling since blocking this receptor alone already inhibited collagen-bound decorin effects.

Decorin activates Akt dependent and independent signalling pathways in endothelial cells (Schönherr et al. 2001), and activation of the IGF-I receptor is responsible for activation of Akt (Schönherr et al. 2005). Since decorin did not require Akt activation to stimulate endothelial cell migration, activation of Akt independent signalling pathways by decorin appears to be involved in stimulating migration. In Chapter 3, it was demonstrated that endothelial cell migration on collagen was mediated at least partly, by α2β1 integrin. In support

of the involvement of decorin signalling through the IGF-IR in stimulating motility, the classic ligand IGF-I was shown to stimulate α2β1-mediated migration (Lynch et al. 2005). In another study, IGF-I promoted co-localisation of IGF-IR and β1 integrin in lipid rafts, resulting in enhanced FAK autophosphorylation in an Akt dependent manner (Tai et al. 2003). In our system however, migration was independent of Akt activity and decorin did not stimulate FAK autophosphorylation, therefore this mechanism appears unlikely. A requirement for MAPK in IGF-I mediated motility has also been demonstrated in another system (Meyer et al. 2001). Since decorin does not activate MAPK signalling in the endothelial cell line used in this study (Schönherr et al. 2001), this mechanism would also appear unlikely in our system. Other studies have demonstrated that IGF-I stimulation of migration required activation of PI3-K (Tai et al. 2003, Lynch et al. 2005) and occurred independently of Src, ROCK or MAPK activity (Lynch et al. 2005), although the same group found that IGF-I-induced motility induced FAK dephosphorylation (Guvakova and Surmacz, 1999). Activation of PI3-K by decorin through IGF-IR could not be previously confirmed in the cell line used in the current study since various inhibitors did not completely inhibit PI3-K activity (Elke Schönherr, personal communication). Activation of PI3-K could be involved in decorin-mediated enhancement of endothelial cell migration. Activation of Rho GTPases by decorin could also be involved. Indeed, growth factor signalling stimulates motility by activating Rho GTPases (Nobes et al., 1995; Ridley and Hall, 1992; Ridley et al., 1992) in a PI3-K dependent manner (Cheng et al., 2000; Menard and Mattingly, 2003; Nobes et al., 1995) and Rho GTPases are implicated in control of integrin clustering and affinity (for further discussion see Chapter 6).

The tyrphostin AG1024 by itself inhibited endothelial cell attachment and migration. It could be argued therefore that under these conditions the cell response to decorin was altered. However our data indirectly supports decorin signalling through IGF-IR in modulation of cell migration. Decorin down-regulates the IGF-IR (Schönherr et al. 2005), therefore could only transiently enhance migration through this mechanism. Indeed, the rate of migration in the

presence of soluble decorin increases within the first 48 hours (Figure 5.4A and Chapter 3, Figure 3.7), after which the rate of migration appears similar to the control. This observation would be in accordance with a down-regulation of the IGF-IR by decorin. Additionally, when tyrphostin AG1024 was used to block IGF-IR signalling during adhesion, decorin could still enhance adhesion, suggesting that the cells were still able to respond to stimulation by decorin. It should also be noted that analysis of migration-conditioned medium confirmed that intact decorin was still present. This would exclude that the cell response was inhibited by decorin degradation or removal by endocytosis. Interestingly, decorin core protein could not modulate adhesion to collagen type I, mediated at least in part, by α2β1 integrin (Chapter 3). In Chapter 4 it was found that decorin likely interacted with this integrin *via* the GAG moiety. This could support a mechanism by which decorin GAG interaction with α2β1 integrin mediates adhesion, while decorin core protein interaction with IGF-I receptor (or another unknown receptor) mediates migration.

Since soluble and collagen-bound decorin in our migration system in fact represents both forms of decorin, it is difficult to ascertain conclusively whether decorin presentation is important in modulation of endothelial cell migration. Despite this, subtle differences can still be observed. Decorin added to the medium increased the rate of migration only within the first 48 hours, while collagen-bound decorin enhanced the rate of migration throughout the experiment (Figure 5.4 and Chapter 3, Figure 3.8). Less decorin was present bound to collagen than as an additive in the medium (2.4μg and 4μg per well respectively), but collagen-bound decorin stimulated migration to a greater degree (Figure 5.4). This indicates that presentation of decorin as a soluble effector or immobilised in the interstitial matrix may be a factor in differential control of endothelial cell behaviour.

Decorin alone could not influence FAK autophosphorylation, indicating that productive conformational changes in the integrin ectodomain were not induced. This is consistent with the observations that decorin could not interact with the A-domain of the α2 subunit (Chapter 4), nor could decorin as a substrate alone support endothelial cell adhesion (Chapter 3). Rhodocetin is a

potent inhibitor of α2β1 integrin (Eble et al., 2001; Eble et al., 2002), and reduced FAK autophosphorylation. Decorin inhibited rhodocetin-mediated reduction of FAK autophosphorylation. This could be explained by a number of mechanisms. Firstly however, it is unlikely that decorin competed with rhodocetin for interaction with α2β1 integrin since decorin does not interact with the rhodocetin (and collagen) binding A-domain. By interacting with an alternative site on the α2 subunit (Chapter 4), decorin could allosterically stabilise a more active conformation. While this is not consistent with decorin inhibiting α2β1 integrin interaction with the collagen peptide FC3 (Chapter 4), it should be considered that the conditions of these assays may support different integrin responses. On the cell-surface, unlike the ELISA experiments carried out in Chapter 4, integrin activity is additionally under the control of inside-out activation and alterations in integrin clustering. Further, on the cell-surface, unligated integrins are suggested to be maintained in a preferentially inactive conformation, while in Chapter 4, decorin effects on integrin activity were examined under conditions where a preferentially active conformation was supported. Thus on the cell-surface, it cannot be excluded that decorin might support an active integrin conformation. Alternatively, decorin-rhodocetin interaction could prevent binding of rhodocetin to α2β1 integrin however we could not demonstrate such an interaction (Chapter 4). Another explanation could be that by activating the IGF-I receptor, decorin activates α2β1 integrin by inside-out signalling. A more active conformation may be unable to interact with rhodocetin.

In transfected decorin-expressing cells, rhodocetin-mediated dephosphorylation of FAK at Y397 was also reduced. Under these conditions, it appears that inhibition of rhodocetin activity may reflect enhanced levels of basal FAK autophosphorylation by decorin. This could indicate that decorin expression alters focal adhesion dynamics. Further, in decorin-expressing cells, the levels of β1 integrin protein that could be detected by Western blotting varied, while levels were consistently similar in control-transfected cells. It is not clear whether this was a result of changes in β1 integrin protein expression, or whether the localisation of β1-containing integrins was altered. Signalling by

IGF-I through IGF-IR can induce translocation of β1 integrin into lipid rafts (Tai et al. 2003), which could alter protein solubility in cell lysis buffer. Decorin could induce a similar translocation into detergent resistant membranes (lipid rafts) by signalling through the IGF-I receptor. It should be noted however that this modulation occurred under conditions where the IGF-I receptor was down-regulated by decorin although some receptor could still be detected. Alternatively, down-regulation of IGF-IR could have altered the activity or levels of other cell-surface receptors.

The data presented also revealed that decorin activated FoxO transcription factors, indicating that decorin modulates gene expression in endothelial cells. These factors can upregulate p27 (Medema et al., 2000), and are associated with long-term cell survival of non-haematopoietic cells (Birkencamp and Coffer, 2003; Burgering and Medema, 2003). Decorin upregulates p27 in endothelial cells (Schönherr et al. 2001) and enhances endothelial cell survival (Schönherr et al., 1999). These effects of decorin could therefore be explained by activation of FoxO transcription factors. However, in endothelial cells, decorin transiently activates Akt (Schönherr et al. 2001) and Akt is an inhibitor of FoxO activity (Birkencamp and Coffer, 2003; Burgering and Medema, 2003). These activities of decorin would therefore seem contradictory. However, in colon carcimoma cells, IGF-I signalling through the IGF-IR also transiently activated Akt, but upregulated p27 through activation of FoxO proteins (Ewton et al., 2002). Decorin could therefore activate FoxO transcription factors by signalling through the IGF-I receptor.

As discussed, since decorin does not interact with the A-domain of α2β1, it is not unsurprising that decorin alone could not modulate FAK activity. However it may be surprising that decorin does not influence FAK activity through IGF-IR activation. IGF-I commonly promotes cell migration through dephosphorylation of FAK (Guvakova and Surmacz, 1999; Konstantopoulos and Clark, 1996). Further, peptides derived from LRR5 of decorin modulated VEGF-induced FAK autophosphorylation, although it was not shown whether the peptides alone could modulate FAK autophosphorylation (Sulochana et al., 2005). Decorin-derived peptides also exhibited some different activities to intact

decorin in modulation of endothelial behaviour (Sulochana et al., 2005), therefore simultaneous interactions of GAG and intact core protein moieties of decorin could result in very different activities to decorin-derived peptides. Interestingly, FAK expression, but not its kinase activity, was required for PDGF and EGF mediated cell migration, indicating that FAK may act as a scaffold protein in growth-factor induced migration (Sieg et al., 2000). Thus modulation of FAK activity may not be critical in modulation of migration in our system. In the current study, phosphorylation at Y576 of FAK was also unaffected, indicating that decorin did not activate Src kinases, nor was the catalytic activity of FAK likely to be altered. Another key protein involved in cell migration is the protease calpain-2 (Huttenlocher et al., 1997; Dourdin et al., 2001). However this protein is also unlikely to be activated by decorin since no cleavage of FAK protein was observed by Western blotting. Thus decorin appears to modulate endothelial cell behaviour independent of alterations in FAK activity or cleavage.

Decorin activated Syk and PLCγ in platelets, most likely by signalling through α2β1 integrin (Guidetti et al., 2002). Binding of platelets to decorin through α2β1 integrin also activates the small GTPase Rap1b through PLC activation and calcium ion mobilisation (Bernardi et al., 2006). On binding of platelets to collagen through α2β1 integrin, Src and FAK are activated (in addition to PLCγ, Syk and calcium mobilisation) (Inoue et al., 2003). However Src and FAK were not activated in endothelial cells by decorin (shown in the current study) and decorin does not mobilise calcium in endothelial cells (Schönherr et al., 2001a). The reagents were not available to specifically investigate Syk, PLCγ or Rap1b activity within this study. Further it is not known whether these proteins together represent major signalling pathways in endothelial cells, while PLCγ is unlikely to be involved since this would lead to calcium mobilisation. These differences may be cell-type specific; in platelets, signals may involve cross talk with receptors not present on endothelial cells or *vice versa*. These differences could also be substrate dependent. In the current study, endothelial cells were cultured on collagen and decorin added

exogenously, while platelets were added to immobilised decorin (Bernardi et al., 2006; Guidetti et al., 2002) or collagen type I (Inoue et al., 2003).

In summary, activation of IGF-I receptor by decorin may be one mechanism by which decorin controls endothelial cell behaviour, and could provide a mechanism for activation of α2β1 integrin through inside-out signalling. Presentation of decorin as a collagen-bound substrate or as a soluble effector may also be important in control of endothelial cell behaviour. However, alternative mechanisms may also exist by which decorin can modulate endothelial cell behaviour which may involve direct interaction with α2β1 integrin or alternative, unknown receptors.

Chapter 6

The Influence of Decorin on Endothelial Cell Morphology

6.1 Introduction

To date, this thesis has indicated that activation of the IGF-I receptor by decorin could be responsible for decorin influencing endothelial migration, while direct interaction of decorin with α2β1 integrin could be responsible for decorin influencing endothelial cell adhesion. However additional mechanisms may also exist for decorin-mediated modulation of endothelial cell behaviour. Questions also remain as to the signalling pathways activated by decorin downstream of the IGF-I receptor which could modulate endothelial cell behaviour. To investigate further the mechanisms by which decorin influences endothelial cell behaviour, the influence of decorin on cell morphology will be investigated in this Chapter. The small Rho GTPases are critical in control of morphological changes associated with cell motility. Whether decorin influences small Rho GTPase activity in endothelial cells will therefore also be investigated.

Decorin has been previously shown to induce morphological changes in endothelial cells (Davies et al., 2001; Sulochana et al., 2005). In an investigation on the role of decorin in tube formation, endothelial cells were seeded onto decorin before overlaying with collagen type I. Decorin reduced tube formation, and visualisation of cells prior to addition of collagen demonstrated that decorin impaired focal adhesion and actin-stress fibre formation (Davies et al., 2001). Exogenous decorin also inhibited formation of VEGF-induced focal adhesions in endothelial cells cultured on glass, although any effect of decorin alone was not investigated (Sulochana et al., 2005).

The influence of decorin on cell morphology in general has not been thoroughly investigated; however decorin effects on fibroblast morphology have been more extensively studied than on endothelial cells. Decorin appears to be an important regulator of fibroblast morphology. Decorin-deficient fibroblasts from a patient with carbohydrate-deficient glycoprotein syndrome type-I had a more spread phenotype than normal fibroblasts, and exogenous decorin restored a normal morphology after 48 hours (Gu and Wada, 1996). Similarly, in patients with a mutation in galactosyltransferase I, which alters decorin and biglycan

glycosylation, isolated fibroblasts exhibited an abnormal, more highly spread phenotype (Seidler et al., 2006), particularly implicating the importance of proper decorin glycosylation as a mechanism by which decorin influences morphology. After incubation with human lung fibroblasts for 24 hours, exogenous decorin induced an elongated phenotype as a result of actin stress fibre formation and up-regulation and reorganisation of α-smooth muscle actin (Tufvesson and Westergren-Thorsson, 2003). Decorin has been suggested to modulate morphology indirectly by interacting with matrix substrates and sterically inhibiting integrin-matrix interactions (Bidanset et al., 1992; Davies et al., 2001; Bhide et al., 2005). Fewer focal adhesions formed in fibroblasts adhering to fibronectin coated with decorin than on fibronectin alone, although cell adhesion and spreading was unaffected (Bidanset et al., 1992). On a substrate of collagen coated with decorin, fibroblast adhesion was reduced and correspondingly, a less organised actin cytoskeleton was observed under these conditions (Bhide et al., 2005). Inhibition of cell-matrix interactions by decorin could account for inhibition of focal adhesion and stress fibre formation. In support of this, decorin could not influence pre-formed focal adhesions (Bidanset et al., 1992). However, as previously discussed, decorin can interact directly with α2β1 integrin (Guidetti et al., 2002; Chapter 4) and IGF-IR, and therefore decorin effects cannot be presumed to depend entirely upon its interactions with matrix substrates.

In this Chapter I will consider how integrin-ligand interactions are linked to formation of adhesions and thereby to morphological changes. Integrin-mediated events are under the control of the small Rho GTPases through modulation of focal adhesion turnover and actin polymerisation. Rho controls formation of focal adhesions and stress fibres (Ridley and Hall, 1992), while focal complexes and actin polymerisation in filopodia and lamellipodia are controlled by Cdc42 (Nobes and Hall, 1995) and Rac (Nobes and Hall, 1995; Ridley et al., 1992) activity respectively. Decorin was shown to activate RhoA and Rac1 in fibroblasts (Tufvesson and Westergren-Thorsson, 2003), indicating that decorin directly activates signalling pathways associated with morphological changes. Some of the mechanisms by which the Rho GTPases, particularly Rac, can be

activated by growth factors or focal adhesion components were discussed in Chapter 5. The mechanisms by which Rho GTPase activity controls cell adhesion and motility are similarly complex. However, Rho GTPase effectors are implicated directly in modulation of myosin and actin-based structures. Active RhoA binds to and activates the serine-threonine kinase Rho-kinase/ROCK-II/ROK-α, which phosphorylates and inhibits myosin phosphatase, resulting in elevated myosin light chain (MLC) phosphorylation (Kimura et al., 1996). MLC phosphorylation promotes myosin filament assembly and actin-activated myosin ATPase activity (Bresnick, 1999). Thus, RhoA activity generates bundles of actin filaments. The generated tension results in clustering of integrins associated with actin (Chrzanowska-Wodnicka and Burridge, 1996). A down-stream target of Rac and Cdc42, is p21-activated kinase (PAK), a serine-threonine kinase that modulates formation of lamellipodia, filopodia and vinculin-positive adhesions (Sells et al., 1997). PAK phosphorylates and inhibits MLCK (myosin light chain kinase), thereby inhibiting myosin filament assembly (Sanders et al., 1999). Conversely, another downstream target of PAK, MRCK, phosphorylates MLC thereby promoting myosin filament assembly (Leung et al., 1998). Another target of Cdc42 and Rac, PI3-K, is implicated in control of integrin avidity (clustering) (Keely et al., 1997; Kovacsovics et al., 1995) and affinity (Constantin et al., 2000). Thus another mechanism by which the small Rho GTPases control cell adhesion and motility is through modulation of integrin avidity and/or affinity (through inside-out signalling). However, these events are differentially regulated, adding to the level of complexity of the mechanisms by which small GTPases modulate integrin-mediated events. Indeed, Rho was shown to regulate integrin affinity and avidity through different effectors (Giagulli et al., 2004).

Unligated integrins are generally diffuse over the plasma membrane and do not appear to be linked to the actin cytoskeleton. Integrin association with actin is induced on binding to extracellular matrix components (Felsenfeld et al., 1996; Miyamoto et al., 1995), and integrins are maintained in a less diffusible state by associations with actin (Kucik et al., 1996). Integrin clustering is critical in linking integrins to the cytoskeleton and for integrin-mediated signalling. However, artificially-induced clustering of the platelet integrin αIIbβ3 in the

absence of ligand binding was sufficient to induce FAK and Syk activation in the absence of extracellular ligands (Hato et al., 1998) and integrin recruitment into focal adhesions can occur independently of ligand binding (Takada et al., 1992). Further, integrin clustering alone recruited different focal adhesion components than a combination of integrin clustering and ligation (Miyamoto et al., 1995). Integrin ligation must be coupled to the cytoskeleton (through recruitment of integrins and focal adhesion components) for control of adhesion and motility. Integrin clustering is not critical in modulation of integrin affinity; changes in avidity are not necessarily accompanied by changes in affinity. In leukocytes, β1 and β2 integrins clustered spontaneously when the small GTPase Rap-1 was constitutively activated, but integrin affinity was not influenced (Sebzda et al., 2002). Similarly, PKCζ activity in leukocytes modulated integrin mobility, but did not influence affinity (Constantin et al., 2000; Giagulli et al., 2004), while blocking of Src activity or actin polymerisation also prevented β2 integrin clustering without altering integrin affinity (Piccardoni et al., 2004). However, Src activity, which is regulated by growth factor activity, was suggested to couple integrin clustering and integrin affinity by inducing clustering of high affinity integrins (Piccardoni et al., 2004). By influencing both integrin avidity and affinity (Giagulli et al., 2004), Rac may also play a role in coupling ligand occupancy of high affinity integrins to cytoskeletal attachments. Indeed, Rac activity recruits high-affinity αvβ3 integrin to lamellipodia during endothelial cell migration (Kiosses et al., 2001).

The focal adhesion component vinculin plays an essential structural role in linking integrins with the cytoskeleton, by simultaneous interaction with integrin-associated proteins (talin) and the actin cytoskeleton (Chen et al., 2006; Goldmann and Ingber, 2002). In the absence of vinculin, cell spreading and lamellipodia formation was impaired, and interaction of vinculin with both talin and actin was required to restore a normal phenotype (Goldmann and Ingber, 2002). These studies also indicate a central role for vinculin in lamellipodia formation, a process which is under the control of the small GTPase, Rac (Nobes and Hall, 1995; Ridley et al., 1992). Rac regulates complex formation between the focal adhesion components vinculin, FAK and Src (Guo et al., 2006), and it

was shown that constitutively active Rac could only induce lamellipodia formation when vinculin was present (Goldmann and Ingber 2002). While many questions remain as to the mechanisms by which small GTPases influence integrin affinity and/or avidity in different systems, it is clear that GTPase activity is essential in control of integrin activation.

Integrins are maintained in an inactive conformation by interactions between the transmembrane domains and the membrane-proximal segments of the cytoplasmic regions. Inside-out activation occurs by disruption of these interactions, resulting in separation of the cytoplasmic tails (discussed in detail in Chapter 4). The focal adhesion protein talin has been implicated in this process. In response to extracellular signals (Martel et al., 2001; Yan et al., 2001), a phosphotyrosine binding (PTB)-like region in talin is exposed, which interacts with a highly conserved NPX(Y/F) motif found in β integrin subunits. This interaction results in conformational changes in the integrin leading to activation (Calderwood et al., 2002; Calderwood et al., 1999; Garcia-Alvarez et al., 2003; Vinogradova et al., 2002). Therefore extracellular signals can result in binding of PTB-containing proteins to integrins, promoting separation of the cytoplasmic tails and triggering long-range conformational changes (inside-out signalling). These changes result in exposure of the integrin ligand-binding site. Binding of extracellular ligands in turn alters metal ion coordination in the MIDAS site, resulting in conformational changes within the A-domain which are propagated to the intracellular cytoplasmic domains (Arnaout et al., 2005; Hynes, 2002). These conformational changes expose further binding sites for focal adhesion components in the cytoplasmic regions to elicit outside-in signalling (Takagi et al., 2002).

In summary, extracellular signals such as growth factors facilitate interactions with matrix ligands through inside-out activation of integrins. Ligand binding in turn transduces signals of ligand occupancy to intracellularly associated focal adhesion components (outside-in signalling). Modulation of integrin activity involves co-ordination between differential control of integrin affinity and avidity, and in associations with the actin cytoskeleton. Focal adhesion components and activity of the small Rho GTPases play an essential and

complex role in control of these processes. These proteins regulate and couple integrin activity with the actin cytoskeleton to elicit morphological changes associated with modulation of cell adhesion and motility.

6.1.1 Aims

- To investigate the effect of decorin on endothelial cell morphology to better understand how decorin influences endothelial cell attachment and motility
- To investigate whether decorin can influence pre-formed focal adhesions and induce cytoskeletal rearrangements in adherent cells.
- To investigate whether decorin influences endothelial cell morphology during adhesion to collagen
- To determine whether decorin influences the activity of small Rho GTPases

6.2 Materials and Methods

6.2.1 Cell Culture and Maintenance

The human endothelial cell line EA.hy926 (Edgell et al., 1983) was maintained as described in Chapter 2, section 2.2.1.

6.2.2 Preparation of Matrix Substrates

For fluorescence microscopy experiments, endothelial cells were seeded onto 13mm glass cover-slips (13mm diameter coverglasses, thickness number 1, Raymond Lamb, Sussex, UK) placed into each well of a 24-well plate (Greiner Bio-one, UK). Cover-slips were sterilised by first placing in 100% ethanol solution, followed by dipping in 70% ethanol. Cover-slips were air-dried under sterile conditions and placed in wells. Wells containing cover-slips were washed twice with 1ml PBS before coating with matrix substrates.

Collagen gels or mixed decorin-collagen gels (0.5μM or 0.7μM decorin) were prepared as previously described in Chapter 3, section 3.2.4. Briefly, wells were coated with neutralised collagen type I and incubated at 37°C for 30 min to allow gelling. Wells were thinly coated by addition of excess collagen solution and immediate removal of as much as possible. For experiments where collagen-bound decorin was required, decorin was added to the neutralised solution and protein equally dispersed by repeated pipetting prior to addition of collagen. To coat wells with immobilised fibronectin or decorin, 24-well plates were coated overnight at 4°C with 100μL per well of 10μg/ml fibronectin, or 40μg/ml decorin both in PBS. Before use, wells were washed twice with PBS, blocked with 1% BSA/PBS for 30 minutes at 37°C and washed three times with PBS.

6.2.3 Fluorescent Staining of Actin Stress Fibres and Focal Adhesions

Morphological changes induced by decorin in the actin cytoskeleton of endothelial cells were investigated by staining F-actin with FITC-conjugated phalloidin. Changes in focal adhesions were investigated by staining with antibodies directed against vinculin. Unless otherwise indicated, 100,000 endothelial cells (approximately 80-90% confluent) were seeded per well of a 24-well plate coated with matrix substrates on cover-slips as described (section 6.2.2). Cells were seeded in Waymouth medium supplemented with 0.5% heat-inactivated FCS and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). For fluorescent staining, cells were placed on ice and fixed in 200μL ice-cold 4% paraformaldehyde in PBS per well. On addition of paraformaldehyde, plates were placed at room temperature for 10 min. All subsequent incubations were carried out at room temperature. Wells were washed once with PBS before permeabilisation with 200μL per well of 0.2% Triton-X100 in PBS for 10 min. Wells were washed twice with PBS and non-specific binding sites were blocked by incubation with 200μL per well 1% BSA/PBS for 1 h. F-actin was visualised by staining with 1μg/ml FITC-conjugated phalloidin (Sigma-Aldrich, UK) for 40 min. Wells were washed three times for 5 min with PBS. At this point, cover-slips were removed and mounted on Superfrost Plus glass slides (Fisher Scientific, UK) with fluorescent mounting fluid (product code S3023, DakoCytomation, UK). Alternatively, for staining of focal adhesions, antibodies against vinculin (6 μg/ml mAb VIN-11-5, Sigma-Aldrich, UK) were incubated with cells for 1 h. Wells were washed three times for 5 min with PBS and bound antibodies detected with rhodamine-conjugated anti-mouse antibodies (1:50, ICN/Cappel) for 1 h. Wells were washed three times for 5 min with PBS before removing cover-slips and mounting on Superfrost Plus glass slides (Fisher Scientific, UK) with fluorescent mounting fluid (product code S3023, DakoCytomation, UK).

6.2.3.1 Analysis of Endothelial Cell Morphology on Matrix Substrates

For analysis of endothelial cell morphology on different matrix substrates, endothelial cells were seeded onto fibronectin, decorin, collagen gels or mixed decorin-collagen gels in Waymouth medium (supplemented with 0.5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin) and cultured for 24h at 37°C, 5% CO_2 prior to staining of F-actin as described (section 6.2.3).

6.2.3.2 Analysis of Endothelial Cell Morphology in the Presence of Decorin and/or Rhodocetin

For analysis of effects of soluble decorin on endothelial cell morphology, endothelial cells were seeded onto collagen gels, fibronectin or uncoated glass cover-slips, and cultured in Waymouth medium (supplemented with 0.5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin) for 48 h at 37°C, 5% CO_2, prior to stimulation with 0.7μM decorin. Decorin was added in serum-free Waymouth medium (containing antibiotics) for 10, 30, 60 or 120 min at 37°C, 5% CO_2 prior to staining of F-actin as described (section 6.2.3).

For analysis of effects of soluble decorin and/or rhodocetin on endothelial cell morphology, cells were similarly cultured on collagen gels for 48h prior to incubation with 0.5μM decorin, 0.15μM rhodocetin or both simultaneously in Waymouth medium (supplemented with 0.5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin) for 1h. Alternatively, cells were incubated with decorin for 30 min prior to incubation with rhodocetin for a further hour. As controls for this experiment, cells were incubated with medium alone for 30 min, followed by incubation with rhodocetin for 1h; or were incubated with decorin for 30 min followed by incubation with medium alone for 1h. As a further control, cells were incubated with medium alone for 30 min, followed by addition of more medium for 1h. For both experiments, staining of F-actin was subsequently carried out as described (section 6.2.3).

6.2.3.3 Analysis of Endothelial Cell Morphology and Focal Adhesions during Adhesion in the Presence of Decorin

To analyse decorin effects on endothelial cell morphology and focal adhesions during adhesion, 100,000 cells were seeded per well of a collagen-gel coated 24-well plate in Waymouth medium (supplemented with 0.5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin). Cell suspensions were mixed with 0.5μM decorin immediately prior to seeding onto collagen gels, or were seeded onto mixed decorin-collagen gels (0.5μM decorin). One hour after seeding, wells were washed once with PBS and adherent cells were fixed and stained as described. These conditions were identical to cell adhesion assays carried out in Chapter 3 and to FAK phosphorylation analysis of cells during adhesion carried out in Chapter 5.

6.2.4 Cell Attachment Assay

To further analyse decorin and rhodocetin effects on pre-adherent cells, 15,000 endothelial cells were seeded per well of a collagen-gel coated 96-well plate in Waymouth medium (supplemented with 0.5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin). After 48h incubation at 37°C, 5% CO_2, cells were incubated with 0.5μM decorin, 0.15μM rhodocetin or both simultaneously. After 1h incubation at 37°C, 5% CO_2, wells were washed once with PBS. Adherent cells were quantified by staining with crystal violet as previously described (Chapter 3, section 3.2.5). Briefly, cells stained with crystal violet were solubilised with 2% sodium dodecylsulfate. The absorbance at 540nm was measured, and the percentage of adherent cells was calculated relative to the control, defined as adhesion to a collagen substrate for one hour in the absence of any additives. This control was set to 100% adhesion for each experiment. Background (staining of matrix coated wells in the absence of cells) was less than 20% of the control.

6.2.5 GTPase Activity

For investigation of decorin effects on small GTPase activity in endothelial cells, Rac-1, 2 and 3 activation was assessed using a commercially available kit (Rac activation kit, Cytoskeleton Inc. Denver, CO, USA). For investigation of the involvement of decorin signalling through the IGF-I receptor in activation of Rac, a commercially available ELISA kit was used (Rac-1, 2 and 3 activation G-LISA^{TM} kit BK125 Cytoskeleton Inc. Denver, CO, USA). Less cell lysate can be used for this method, further, the data is quantitative.

6.2.5.1 Active Rac Precipitation

Endothelial cells ($3x10^6$ per 10 cm diameter dish) were seeded onto collagen gels in Waymouth medium (supplemented with 0.5% FCS, antibiotics) and cultured for 24 h prior to incubation with 70μg/ml decorin in serum-free medium supplemented with antibiotics. For each variable, lysates were pooled from two dishes. After incubation for 0, 2 or 15 min, cells were washed once with PBS and extracts were analysed for active Rac-1 as specified by the manufacturer. Briefly, cells were lysed with 500μL lysis buffer (supplied with the kit) and diluted to 700μg/ml. 1ml cell lysate was incubated with glutathione S-transferase-tagged p21- activated kinase binding domain (PAK-PBD) protein beads to capture the active form of Rac. As a positive control, total Rac from unstimulated cell lysates was captured by incubating lysates with non-hydrolysable GTPγS prior to precipitation with PAK-PBD beads. Proteins were recovered by boiling the beads in loading buffer, and Rac was detected by Western blotting as described (Chapter 3, section 2.2.4.4) using antibodies to Rac-1,2 and 3 (ARC01, Cytoskeleton Inc.), detected with HRP-conjugated antibodies to rabbit IgG (1:1000, Dako Cytomation, UK).

6.2.5.2 Active Rac Capture using an ELISA Assay

Endothelial cells (350,000 per 35mm diameter dish) were seeded onto collagen gels in Waymouth medium (supplemented with 0.5% FCS, antibiotics) and cultured for 24 h. Cells were cultured for a further 24 h in serum-free

medium (supplemented with antibiotics). IGF-IR signalling was blocked by incubating cells with 10μM AG1024 (10mM stock) or equal volumes of the vehicle, DMSO as a control, for 1 h at 37°C, 5% CO_2. Cells were incubated with 0.6μM decorin or with the equivalent volume of PBS (control) for 15 min and extracted into 100μL lysis buffer (supplied with kit). Protein concentration was determined by BCA assay, equalised, and 25μL of lysate was incubated per well of the supplied microtitre plate (in duplicate). Plates were pre-coated by the manufacturer with p21- activated kinase binding domain (PAK-PBD) to capture active Rac. A positive control of constitutively active Rac was also included (supplied with the kit). Bound Rac was detected with specific antibodies to Rac-1,2 and 3 followed by HRP-conjugated secondary antibodies which were both supplied with the kit. As a negative control, which was subtracted from the final values, 25μL lysis buffer alone was similarly incubated with duplicate wells.

6.3 Results

6.3.1 Morphology of Endothelial Cells Cultured on Matrix Substrates

To visualise the morphology of endothelial cells cultured on various matrix substrates, cells were seeded onto fibronectin, decorin, collagen gels or mixed decorin-collagen gels. After 24h, cells were fixed and actin stress fibres visualised by staining of F-actin with FITC-conjugated phalloidin (Figure 6.1).

In accordance with previous observations (Chapter 3), endothelial cells did not adhere or spread on decorin as a substrate alone, even 24 h after seeding. On all other substrates, endothelial cells could efficiently adhere and spread. On fibronectin, cell morphology was considerably different to that of cells seeded onto collagen-containing substrates (Figure 6.1). Cells were spread over a larger surface area, and actin stress fibres also appeared more pronounced. Distinct lamellipodia were also observed (white arrows, Figure 6.1). On collagen gels, cells appeared more spindle-like, with fewer cell adhesion points. Lamellopodia could also be visualised (white arrows, Figure 6.1). On mixed decorin-collagen gels cells appeared to have longer, thin processes than on collagen gels, and lamellipodia were not clearly visible.

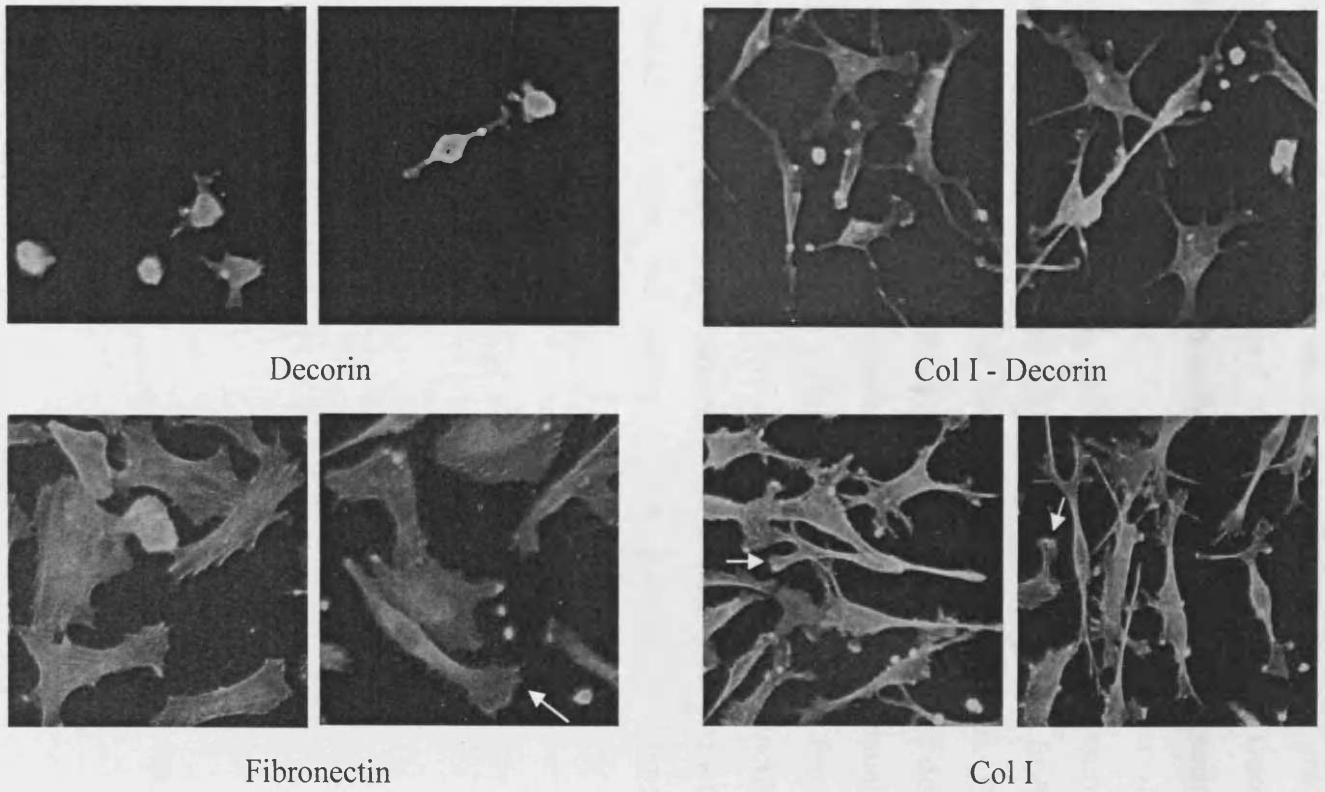


Figure 6.1: Morphology of endothelial cells cultured on different matrix substrates for 24 hours. Endothelial cells were seeded onto substrates of fibronectin (10μg/ml), decorin (0.4μM), collagen type I gels (Col I) or decorin incorporated into collagen gels (Col I – Decorin; decorin 0.7μM). Cells were seeded in media containing 0.5% FCS and cultured for 24 hours. Cells were fixed and permeabilised and actin stress fibres were stained with FITC conjugated phalloidin. Duplicate images are shown, and are representative of two independent experiments (40x magnification). Lamellipodia are indicated with white arrows.

6.3.2 Decorin Effects on the Morphology of Pre-Adherent Cells

6.3.2.1 Effects of Soluble Decorin on Morphology of Adherent Cells on Collagen

To assess whether decorin could influence pre-formed cytoskeletal structures in adherent cells, endothelial cells were seeded onto collagen gels and cultured under serum-reduced conditions (0.5% FCS). Decorin was added in solution and incubated with cells for up to 2 h, and the actin cytoskeleton was visualised by staining with FITC-phalloidin. Detailed observations of actin stress fibres could not be made under these conditions. Extensive scattering of fluorescence emission by the collagen gels and emission from above and below the plane of focus resulted in low resolution images. Despite this, some morphological changes were apparent in the presence of decorin (Figure 6.2). An induction of large actin-positive adhesions could be visualised within 30 min of incubation with decorin (white arrows, Figure 6.2). Pronounced long, thin cytoplasmic extensions by cells could also be seen after incubation with decorin for 60 to 120 min (white arrows, Figure 6.2). This phenotype may relate to formation of new cell-matrix contact without detachment of previously established contact points.

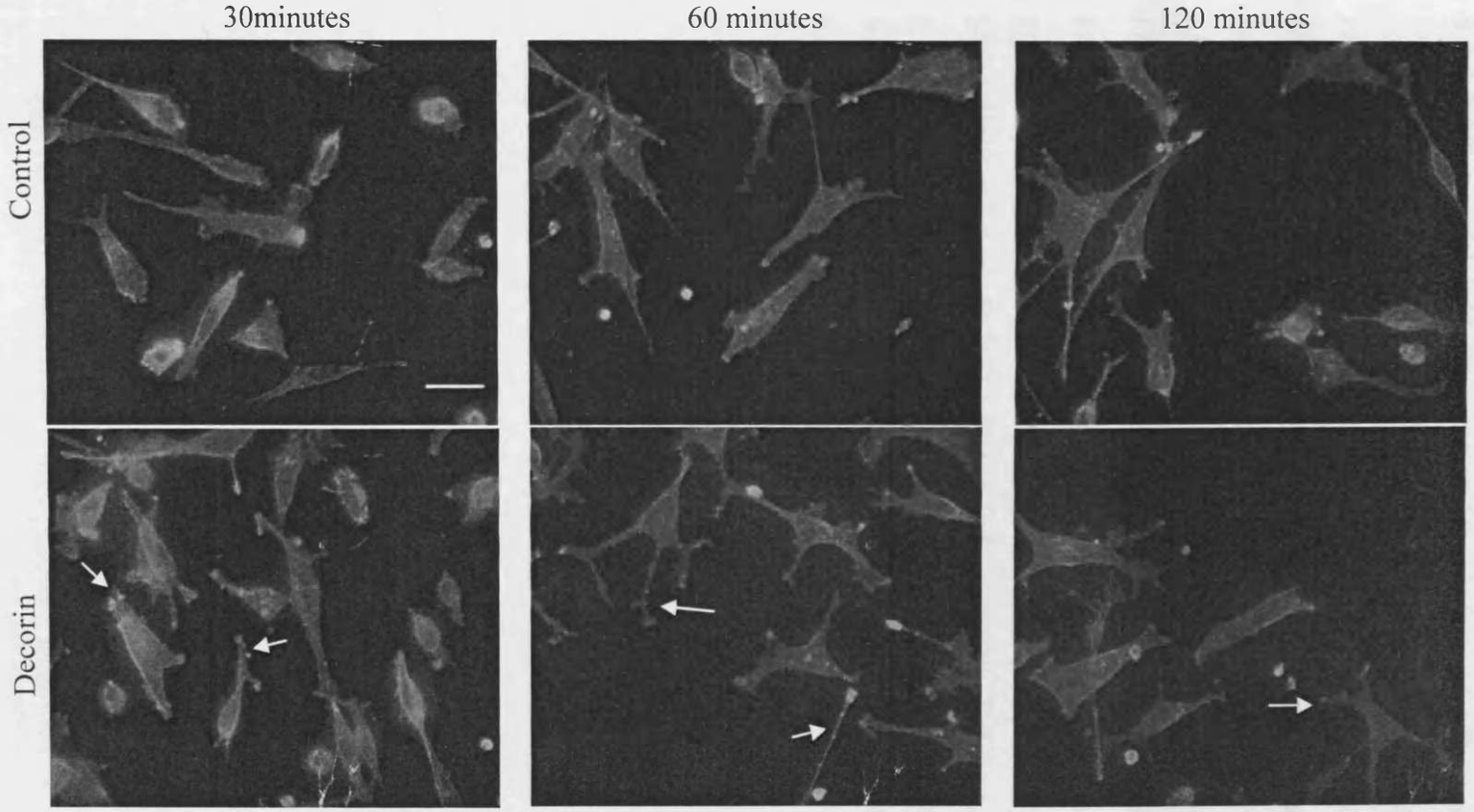


Figure 6.2: Morphology of endothelial cells cultured on collagen type I after stimulation with decorin for up to 2 hours. Endothelial cells were seeded onto collagen gels and cultured in media containing 0.5% FCS for 48h prior to stimulation with 0.7μM decorin in solution, in serum-free media. After 30, 60 or 120 min cells were fixed and permeabilised and actin stress fibres were stained with FITC conjugated phalloidin. Images are representative of two independent experiments. At 30 min incubation with decorin, large actin-containing adhesions are indicated by white arrows, and 60 and 120 min after incubation with decorin, long extensions are indicated by white arrows. Size-bar = 40μm.

6.3.2.2 Effects of Soluble Decorin on Morphology of Adherent Cells on Glass

To investigate the effects of decorin on the morphology of endothelial cells cultured on alternative substrates, endothelial cells were seeded onto glass cover-slips and were treated with decorin for 120 min. The actin cytoskeleton was visualised by staining with FITC-phalloidin. Under these conditions, decorin induced actin depolymerisation and membrane ruffling (white arrows, Figure 6.3A). Some extended cytoplasmic processes were also induced in the presence of decorin (white arrow-heads, Figure 6.3A). Additionally, in the presence of decorin, filopodia-like structures were frequently observed, particularly at intercellular junctions (white arrows, Figure 6.3B). These may be a consequence of loss of actin cytoskeleton integrity in the absence of focal adhesion turnover. In control cells, actin appeared more evenly distributed than in the presence of decorin (Figure 6.3A).

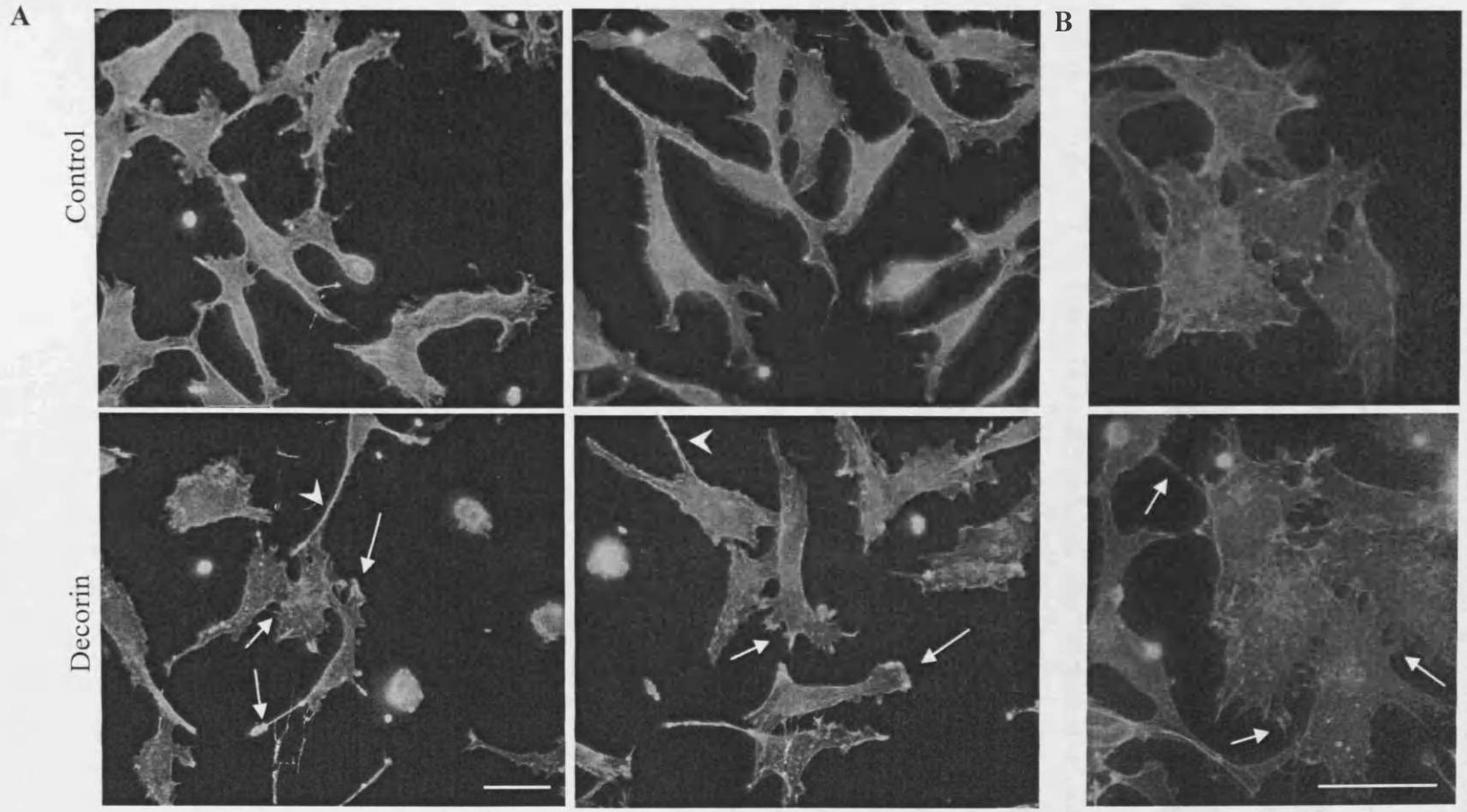


Figure 6.3: Morphology of endothelial cells cultured on glass cover-slips and after incubation with decorin for 2 hours. Endothelial cells (100,000 per well of a 24-well plate) were seeded onto glass cover-slips and cultured in media containing 0.5% FCS for 48h prior to stimulation with 0.7μM decorin in solution, in serum-free media. After 2 h, cells were fixed and permeabilised and actin stress fibres were stained with FITC conjugated phalloidin. Duplicate images are shown in (A). In (B) a further example is shown at higher magnification. Images are representative of two independent experiments. Long processes are indicated by white arrow-heads. Membrane ruffling or filopodia-like structures are indicated by white arrows. Size bar = 40μm.

6.3.2.3 Effects of Soluble Decorin on Morphology of Adherent Cells on Collagen in the Presence of Rhodocetin

Endothelial cells were cultured on collagen gels and serum-starved prior to incubation with rhodocetin, decorin or with both simultaneously for 2 h. Actin stress fibres were visualised by staining with FITC-conjugated phalloidin (Figure 6.5). After incubation with rhodocetin, endothelial cells had lost contacts with collagen and adopted a more rounded morphology than control cells. Some cells appeared to have lifted off of collagen, since cell number also appeared reduced (Figure 6.5). Decorin alone induced the formation of some large actin-positive adhesions. Simultaneous incubation with decorin and rhodocetin also resulted in a rounded morphology. However this effect was not as potent as that of rhodocetin alone (Figure 6.5). The same observations were made when endothelial cells were pre-incubated with decorin for 30 min prior to incubation with rhodocetin for a further 90 min. Decorin alone induced the formation of cell processes and enhanced cell spreading to a degree, while rhodocetin alone led to rounding up of the cells (Figure 6.6). When decorin was pre-incubated with cells for 30 min prior to incubation with rhodocetin for 90 min, decorin was able to reduce the induction of a rounded morphology by rhodocetin, although normal cell morphology could not be restored by decorin (Figure 6.6).

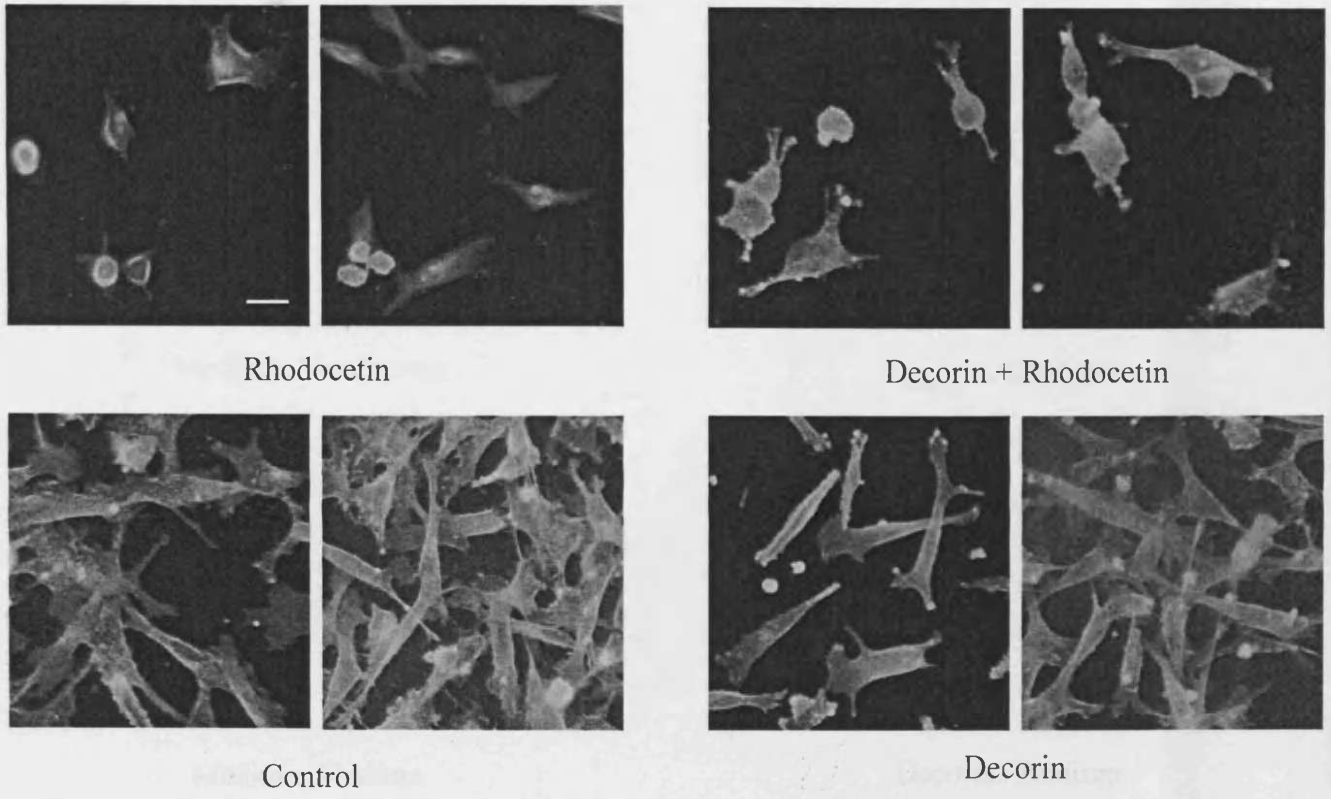


Figure 6.5: Morphology of endothelial cells cultured on collagen type I after incubation with decorin, rhodocetin or with both simultaneously. Endothelial cells were seeded onto collagen gels and cultured in media containing 0.5% FCS for 48h prior to stimulation with 0.5μM decorin, 0.15μM rhodocetin, or both simultaneously, in serum-free media. After 2h, cells were fixed and permeabilised and actin stress fibres were stained with FITC conjugated phalloidin. Two images are shown for each condition, from the same well, and are representative of two independent experiments. Size bar = 40μm.

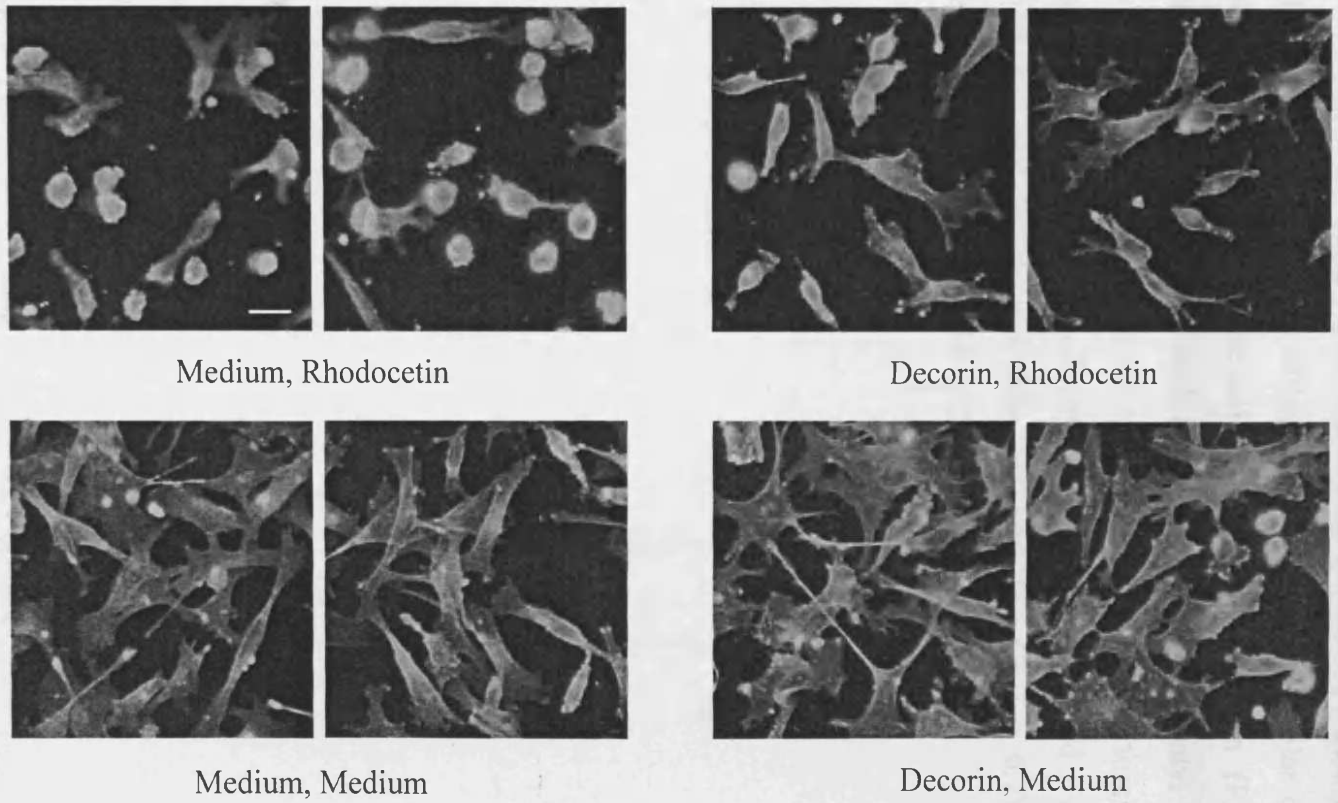


Figure 6.6: Morphology of endothelial cells cultured on collagen type I after incubation with decorin, rhodocetin, or pre-incubated with decorin prior to incubation with rhodocetin. Endothelial cells were seeded onto collagen gels and cultured in media containing 0.5% FCS for 48h prior to stimulation with 0.5μM decorin or medium alone, without serum. After 30 min, cells were stimulated with 0.15μM rhodocetin or medium for a further 90 min as follows: medium 30min, followed by rhodocetin 90min (medium, rhodocetin); decorin 30min, followed by rhodocetin 90min (decorin, rhodocetin); medium 30min, followed by medium 90min (medium, medium); decorin 30min, followed by medium 90min (decorin, medium). Cells were then fixed and permeabilised and actin stress fibres were stained with FITC conjugated phalloidin. Two images are shown for each condition, from the same well, and are representative of two independent experiments. Size bar = 40μm.

To investigate whether rhodocetin induced detachment of cells adherent to collagen type I, adherent cells were incubated with rhodocetin, decorin or both simultaneously for 2 h. Non-adherent or loosely attached cells were removed by washing with warm PBS (37°C) and adherent cells were quantified by staining with crystal violet. Rhodocetin induced cell detachment, since fewer cells remained attached to collagen whereas under similar conditions, decorin increased the proportion of cells attached to collagen (Figure 6.7). In the presence of both decorin and rhodocetin simultaneously, the proportion of adherent cells was similar to the control. Therefore when incubated simultaneously with rhodocetin, decorin inhibited rhodocetin-induced endothelial cell detachment from collagen gels (Figure 6.7). This observation correlates with that of Figures 6.5 and 6.6.

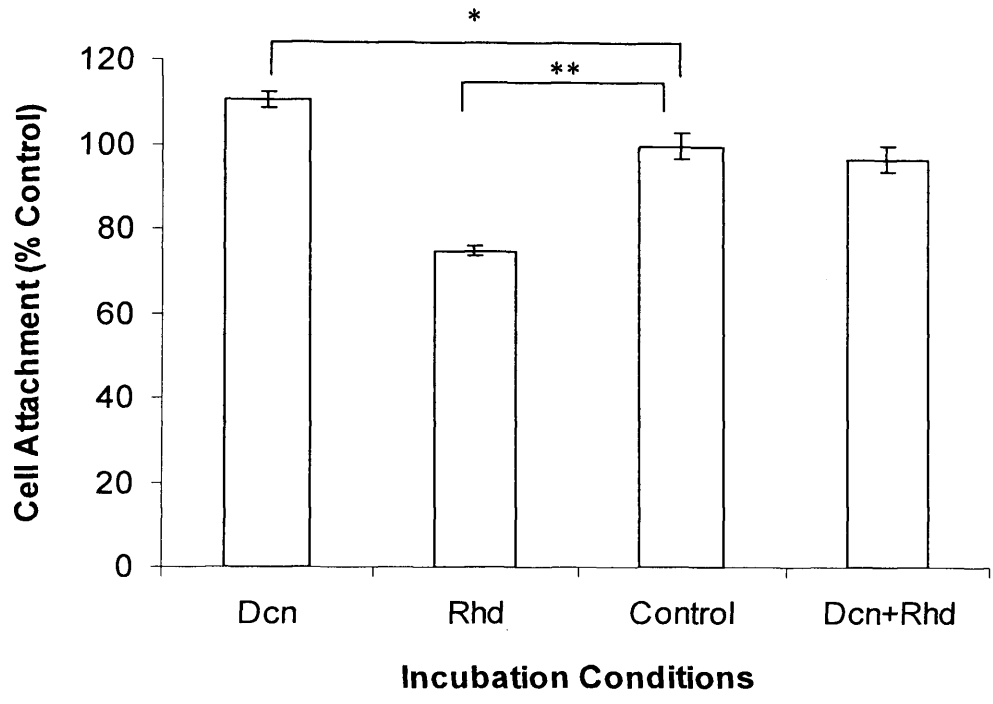


Figure 6.7: Influence of incubation with decorin (Dcn), rhodocetin (Rhd), or with both simultaneously (Dcn+Rhd) on the adhesion of endothelial cells to collagen type I. Endothelial cells were seeded onto collagen gels (15,000 per well of a 96-well plate) and cultured in media containing 0.5% FCS for 48h prior to stimulation with 0.5μM decorin, 0.15μM rhodocetin or both simultaneously in serum-free media. As a control, media alone was added. After 2h, wells were washed with PBS and adherent cells stained with crystal violet. Cell attachment represents optical density at 540nm relative to control (untreated cells). Data is representative of three independent experiments, and error bars represent the standard error of the mean. Statistical significance was tested using repeated measures ANOVA and Dunnett, and is shown relative to the control (media alone), where $p < 0.05$ is represented as * and $p < 0.001$ as **.

6.3.3 Decorin Effects on Morphology during Cell Attachment to Collagen

To investigate whether decorin could influence focal adhesion and stress fibre formation during cell attachment and spreading on collagen, endothelial cells were mixed with decorin and immediately seeded onto collagen gels, onto collagen gels alone, or onto mixed decorin-collagen gels (Figure 6.8, close-up images are shown in Figure 6.9). After one hour, adherent cells were fixed and stained to visualise the actin cytoskeleton (using FITC-phalloidin) and focal adhesions (using antibodies to vinculin). All conditions were carried out in serum-free media.

When cells were seeded in the presence of soluble decorin or onto mixed decorin-collagen gels, large vinculin-positive adhesion complexes formed at the periphery of a large proportion of the cells (white arrows, Figures 6.8, 6.9). In cells seeded onto collagen, smaller vinculin-positive structures were observed which were not constrained to the periphery of cells (white arrows, Figures 6.8, 6.9). These are characteristic of mature focal adhesions associated with stress fibres. In agreement with this, stress fibres were predominant in cells seeded onto collagen, but not when cells were seeded onto collagen in the presence of decorin, or onto mixed decorin-collagen gels (white arrow-heads, Figure 6.9).

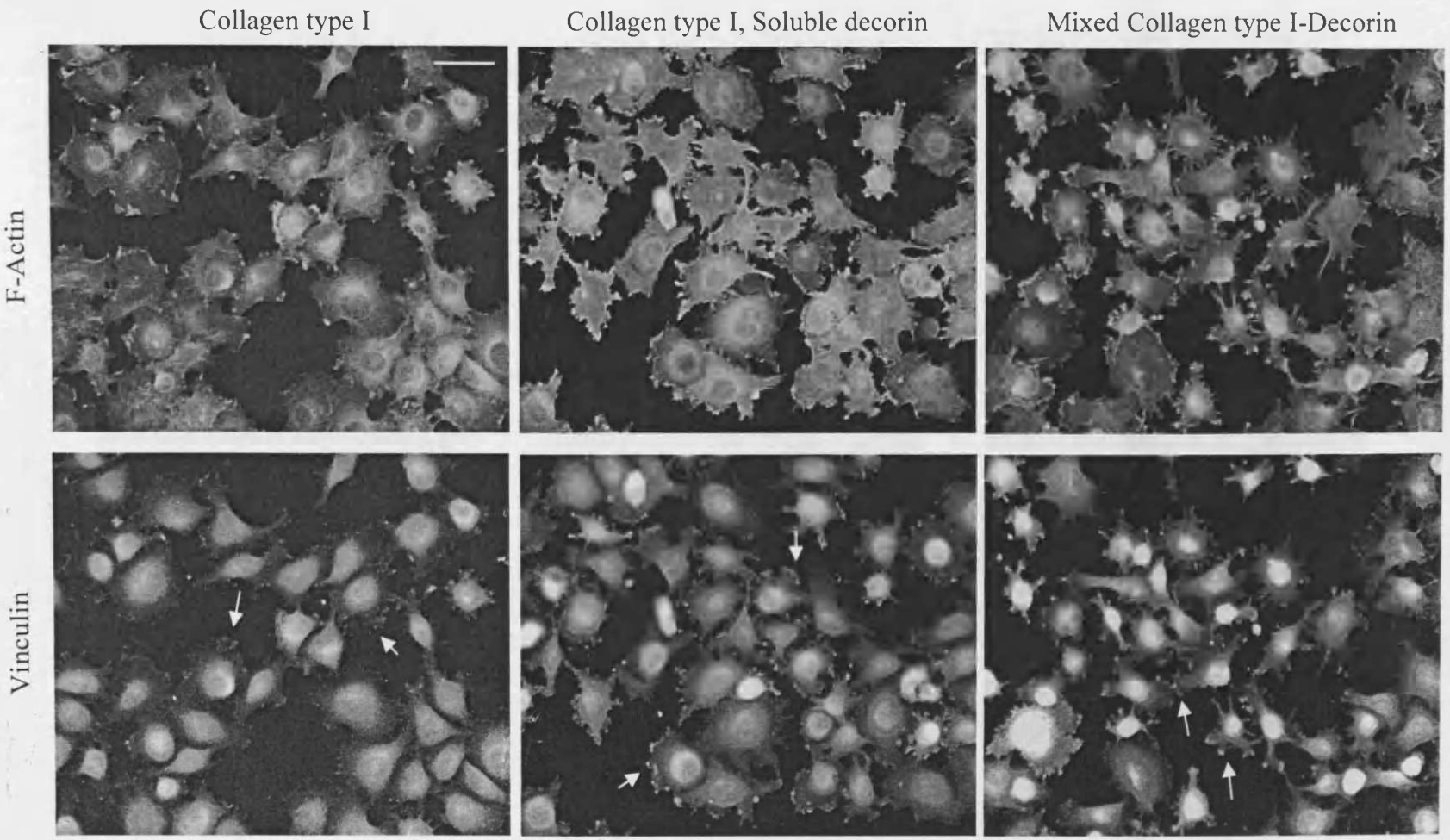


Figure 6.8: Influence of decorin on endothelial cell morphology during adhesion to collagen type I. Endothelial cells were seeded onto collagen type I, mixed decorin (0.5μM) - collagen, or were mixed with 0.5μM decorin prior to seeding onto collagen (100,000 cells per well of a 24-well plate). After 1h, cells were fixed and permeabilised. Actin stress fibres were stained with FITC conjugated phalloidin (upper panel), and focal adhesions with antibodies to vinculin (lower panel, corresponding images). Data is representative of two independent experiments. Focal adhesion structures are indicated with white arrows, and size bar = 40μm.

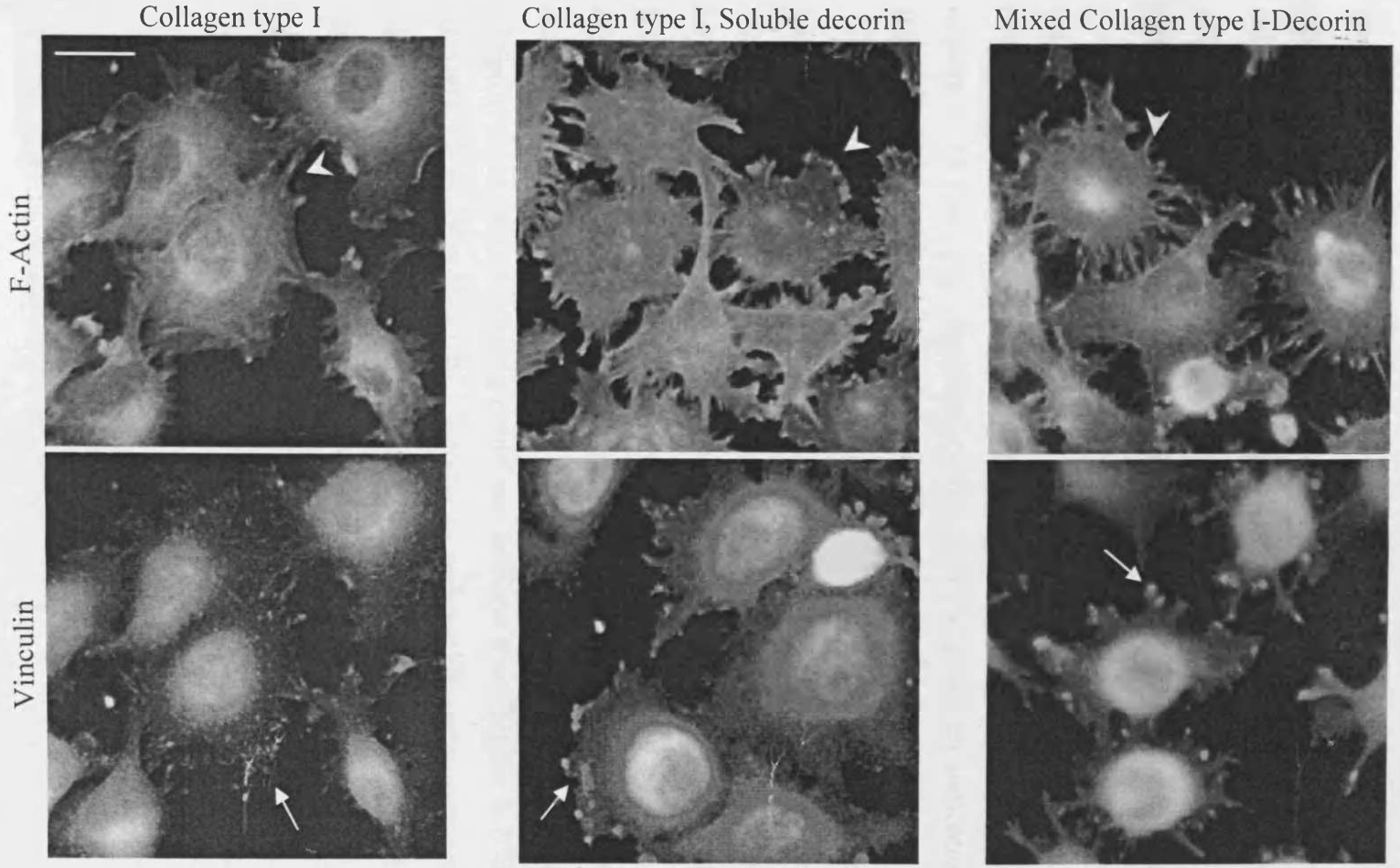


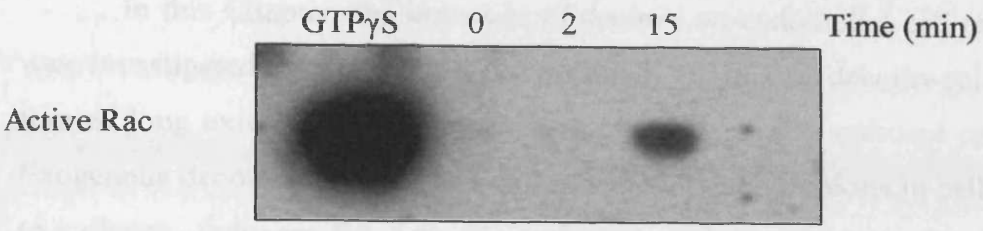
Figure 6.9: Influence of decorin on endothelial cell morphology during adhesion to collagen type I – Magnified Images. Endothelial cells were seeded onto collagen type I gels, mixed decorin (0.5μM) - collagen gels, or were mixed with 0.5μM decorin prior to seeding onto collagen gels, as indicated in Figure 6.8, from which these images are taken. Actin stress fibres were stained with FITC conjugated phalloidin (F-actin, upper panel), and focal adhesions with antibodies to vinculin (lower panel). Some focal adhesion structures are indicated with white arrows, and actin-containing structures with open arrow-heads. Size bar = 20μm.

6.3.4 Decorin-mediated Activation of Rac

To investigate whether decorin activates the small GTPase Rac, endothelial cells were cultured on collagen gels and serum-starved for 24 hours before incubation with 0.7μM decorin for 2 or 15 min. As a positive control, cells were incubated with non-hydrolysable GTPγS. Cells were extracted and activated Rac affinity precipitated using beads coupled to the Rac-GTP binding domain of the Rac effector, PAK (PAK-PBD). Bound activated Rac was visualised by Western blotting using specific antibodies to Rac. In unstimulated cells, or after incubation with decorin for 2 minutes, no active Rac could be detected. However, incubating cells with decorin for 15 minutes induced activation of Rac. The positive control, GTPγS, gives an indication of overall Rac levels in the cells (Figure 6.10A).

To investigate whether activation of Rac by decorin involved signalling through the IGF-I receptor by decorin, IGF-IR signalling in serum-starved endothelial cells cultured on collagen was blocked using tyrphostin AG1024. Cells were incubated with 0.6μM decorin or with the equivalent volume of PBS (control) for 15 min and Rac activation was analysed by ELISA, using PAK-PBD to capture active Rac from cell lysates. In the presence of DMSO (vehicle control) decorin activated Rac 1.4x. However, when signalling through the IGF-I receptor was blocked decorin could not activate Rac (Figure 6.10B). However, AG1024 by itself reduced Rac activity in endothelial cells compared to vehicle control. The data was within the range of 0.1 to 0.5 OD490 which is specified by the manufacturer as the quantitative range of this assay.

A



B

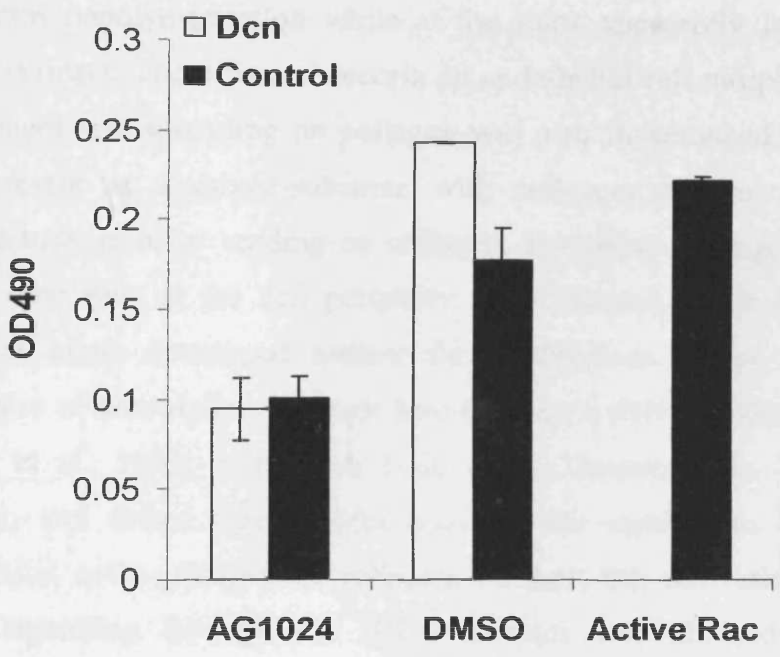


Figure 6.10: Influence of decorin on Rac activity and involvement of signalling through the IGF-I receptor by decorin. Endothelial cells were cultured on collagen gels and serum-starved for 24 h. Cells were incubated with decorin for 0, 2, 15 minutes and active Rac specifically precipitated using PAK-PBD beads. GTPγS cannot be hydrolysed to GDP, and therefore constitutively activates Rac (A). In (B), an ELISA assay was used to specifically detect active Rac. Endothelial cells were cultured on collagen gels and serum-starved for 48 h. IGF-I receptor signalling was blocked using 10μM tyrphostin AG1024 (DMSO represents the vehicle control). Endothelial cells were then incubated with decorin (Dcn) or the equivalent volume of PBS (Control) for 15 min. A positive control of constitutively active Rac was included (active Rac). Data is representative of two independent experiments.

6.4 Discussion

In this Chapter, the influence of decorin on endothelial cell morphology was investigated. Endothelial cells cultured on mixed decorin-collagen gels formed long extensions which were not apparent in cells cultured on collagen. Exogenous decorin also influenced pre-formed focal adhesions in cells adhering to collagen, inducing the formation of large actin-containing adhesions at the cell periphery. Further, a proportion of cells developed long processes, while others appeared to have retracted extended structures. Similarly, in endothelial cells cultured on glass cover-slips, exogenous decorin induced membrane ruffles and actin depolymerisation while at the same apparently leaving cell-matrix contacts intact. The effect of decorin on endothelial cell morphology during cell attachment and spreading on collagen was also investigated. Whether decorin was present as a mixed-substrate with collagen, or was mixed with cells immediately prior to seeding on collagen, formation of large vinculin-positive focal complexes at the cell periphery were induced, while cells seeded onto collagen alone developed mature focal adhesions. These observations are indicative of modulation of small Rho GTPase activity (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). Consequently, we analysed Rac activity, and found that decorin activates the small Rho GTPase, Rac, in endothelial cells cultured on collagen. Further, this activation was abrogated when signalling through the IGF-I receptor was blocked with tyrphostin AG1024, suggesting that decorin activates Rac by signalling through the IGF-I receptor. However, since AG1024 by itself reduced Rac activity in endothelial cells, further experiments using different approaches would be required to confirm this observation (discussed further in Chapter 7).

Decorin has been previously shown to induce morphological changes in endothelial cells. Endothelial cells adhering to a decorin substrate exhibited impaired focal adhesion and actin-stress fibre formation (Davies et al., 2001) while exogenous decorin inhibited formation of VEGF-induced focal adhesions (Sulochana et al., 2005). In the current study, decorin could not support endothelial cell adhesion therefore the influence of a decorin substrate on

endothelial cell morphology could not be investigated. Further, the effect of exogenous decorin on endothelial cell morphology was investigated rather than the influence of decorin on VEGF-mediated events. In the current study, decorin induced formation of large, immature focal adhesions, suggesting that focal complex turnover was inhibited. The former study therefore (Sulochana et al., 2005) is in agreement with our observation that decorin prevents formation of mature focal adhesions and stress fibres.

Previous studies on fibroblasts have also indicated that decorin impairs focal adhesion and stress fibre formation. Fewer focal adhesions were observed in cells seeded on fibronectin coated with decorin, although in contrast to the current study, decorin could not influence pre-formed focal adhesions or cell adhesion (Bidanset et al., 1992). In another study, fibroblast adhesion to collagen coated with decorin was reduced and correspondingly, a less organised actin cytoskeleton was observed (Bhide et al., 2005). These studies suggested that decorin interacts with matrix substrates and sterically inhibits integrin-matrix interactions, thus reducing focal adhesion and stress fibre formation (Bidanset et al., 1992; Bhide et al., 2005). However, our data indicates that decorin enhances cell-matrix interaction (decorin inhibits rhodocetin activities). Decorin also enhances cell attachment while at the same time inhibiting formation of mature focal adhesions and stress fibres. Decorin induced actin depolymerisation in adherent cells, and prevented formation of mature stress fibres during cell adhesion. This is likely due to stimulation of cytoskeletal reorganisation rather than through reduced cell-matrix interaction. A number of observations in the current study suggest that decorin did not mask integrin binding sites in the matrix. Masking of integrin binding sites in the matrix by decorin would not account for induction of large peripheral complexes by decorin (indicative of extensive integrin ligation and recruitment to adhesions). Additionally, decorin influenced pre-formed focal adhesions and induced cytoskeletal rearrangements in endothelial cells which were already adherent to various substrates, while induction of multiple cell processes by decorin would not be explained by a reduction in cell-matrix interaction.

As previously discussed, decorin can interact directly with α2β1 integrin (Guidetti et al., 2002 and Chapter 4). In contrast, a previous study (Bhide et al., 2005) suggested that decorin inhibited fibroblast adhesion to collagen type I by competing with α2β1 integrin for binding to the same site on collagen. A peptide derived from LRR3 of decorin bound to a triple helical peptide containing the essential α2β1 integrin-binding sequence glycine-glutamate-arginine (GER) (Bhide et al., 2005). However, it is important to consider that the intact binding partners might have very different binding activities. The α2β1 integrin for example, interacts with at least three GER-containing sequences on collagen type I, at the C- and N-termini and in the centre (Xu et al., 2000; Siljander et al., 2004), and may interact with up to 11 potential GER-containing motifs depending on the activation state of the integrin (Siljander et al., 2004). In contrast, decorin was proposed to bind to only one site containing the sequence GAKGDRGET (Scott et al., 1997), which notably does not contain the essential GER sequence for α2β1 integrin binding to collagen (Knight et al., 1998). Both GDR (glycine-aspartate-arginine) and GER containing sequences are abundant in collagen (Zhang et al., 2003), however α2β1 integrin cannot interact with the former (Knight et al., 2000). An alternative binding site for decorin near the C-terminus of collagen has also been proposed (Keene et al., 2000), however the histidine-tagged decorin used in this study may have influenced decorin-collagen interaction (Tenni et al., 2002). In addition, decorin has been suggested to interact with two N-terminal sites on collagen, in the vicinity of the d-band (Scott, 1993). Indeed, in numerous studies, decorin has been visualised bound to a region of the D-period of collagen, at the d-band (Scott and Haigh, 1988; Pringle and Dodd, 1990; Hedbom and Heinegard, 1993). In support of these studies, decorin also interacted with collagen-derived peptides containing sequences from the N-terminal and d-band regions (Tenni et al., 2002). Further, the DS moiety of decorin may also interact with collagen type I (Hedbom and Heinegard, 1993). Therefore, while studies utilizing isolated regions of collagen type I and/or decorin certainly suggest that the decorin binding site or sites on collagen could overlap that of α2β1 integrin, it is likely that alternative, independent binding sites exist for intact decorin and α2β1 integrin on fibrillar

collagen. Indeed, Bhide et al., note that the LRR3 peptide of decorin interacted with a collagen sequence not known to support integrin interaction physiologically (Bhide et al., 2005). As discussed in Chapter 4, decorin inhibited integrin interactions with a collagen peptide. Since decorin did not interact with the collagen peptide (thus did not competitively inhibit interaction), this would further support the observation that some separate binding sites appear to exist for decorin and α2β1 integrin on collagen.

In pre-adherent cells, rhodocetin induced a substantial loss of cell contacts with collagen (visualised by rounding up of cells in morphological studies). Rhodocetin also induced de-adhesion of a proportion of adherent cells, as indicated by cell attachment assays. These data indicate that endothelial cells adhere to collagen fundamentally through α2β1 integrin, although under the conditions of the assays some cells remained anchored to the matrix despite extensive disassembly of focal adhesions. This could indicate that EA.hy926 endothelial cells require α2β1 integrin activity in order to spread on collagen type I. It was shown in Chapter 5 that rhodocetin reduced FAK autophosphorylation, which would support the loss of integrin-matrix contacts visualised in the presence of rhodocetin. It was also shown in Chapter 5, that decorin inhibited the reduction in FAK autophosphorylation induced by rhodocetin. In accordance with this, in the present Chapter, decorin counteracted rhodocetin-mediated rounding up and detachment of endothelial cells. Interestingly, decorin alone also induced an apparent increase in endothelial cell adhesion when cells were already adhering to collagen. This could be explained by decorin stabilising or enhancing existing cell-matrix adhesions or by induction of large focal complexes by decorin under these conditions.

In stationary cells, larger adhesions exert a greater force per unit area than smaller adhesions (Balaban et al., 2001). This effect of decorin could therefore protect pre-adherent endothelial cells from low force detachment; our adhesion assay effectively measured the proportion of cells that resist low force detachment (applied during the wash steps). Interestingly, large adhesions are associated with both enhanced adhesion and enhanced motility. In motile cells, large adhesions exert less force per unit area than smaller adhesions (Beningo et

al., 2001), while the opposite was observed in stationary cells (Balaban et al., 2001). This would correlate with our finding that cells adhering in the presence of decorin (stationary) exhibit large adhesions and adhere more strongly, while decorin also stimulated migration. However, cytoskeletal dynamics also play a critical role in migration, and are clearly influenced by decorin. Indeed, morphological alterations induced by decorin are reminiscent of a migratory cell phenotype. Focal complexes at the cell periphery (observed in the presence of decorin) are associated with filopodia and lamellipodia, controlled by activity of the small GTPases Cdc42 and Rac respectively (Ridley et al., 1992; Nobes and Hall, 1995). Focal contacts in lamellipodia can fuse to form larger adhesions (Laukaitis et al., 2001) similar to those observed in the presence of decorin.

In contrast to the current study, exogenous decorin stimulated actin stress fibre formation in pre-adherent fibroblasts (Tufvesson and Westergren-Thorsson, 2003). The longer incubation period (24 hours) (Tufvesson and Westergren-Thorsson, 2003) may have resulted in decorin-mediated induction of actin stress fibres which were not seen in the current study. Alternatively, this may reflect differential activation of Rho GTPases by decorin in different cell types. Rho activity is associated with stress fibre formation (Ridley and Hall, 1992), indeed, Rho was activated by decorin where stress fibres were observed (Tufvesson and Westergren-Thorsson, 2003). While investigation of decorin effects on the activity of either Rho or Cdc42 could not be carried out in the current study (due to limited time and reagent availability), Rho activity may not be required for control of endothelial cell behaviour. Rac and Cdc42 modulated VEGF-induced motility and angiogenesis (Bayless and Davis, 2002; Connolly et al., 2002), but Rho was not involved in VEGF-induced chemotaxis (Soga et al., 2001b). Further, Rac activation alone was sufficient to stimulate endothelial cell motility and lumen formation (Soga et al., 2001a; Soga et al., 2001b) indicating an essential requirement for activity of this GTPase, but not Rho, in control of endothelial cell behaviour.

In this Chapter, it was demonstrated that decorin activates Rac in endothelial cells cultured on collagen type I, and that signalling through the IGF-I receptor by decorin could be responsible for this activation. However,

interpretation of the latter observation was difficult given that the IGF-I receptor inhibitor tyrphostin AG1024 by itself greatly reduced Rac activity in endothelial cells. This observation may also relate to reduced endothelial cell migration observed in the presence of this inhibitor, compared to the control (Chapter 5).

In Chapter 5, it was demonstrated that decorin could enhance adhesion to collagen type I, and that this was independent of decorin signalling through the IGF-I receptor. Visualisation and analysis of cell morphology in the present Chapter indicate that substantial morphological changes consistent with enhanced activation of small Rho GTPases were induced by decorin under identical conditions. These data indicate that activation of small GTPases by decorin can occur independently of IGF-I receptor activation. One possibility is that decorin could modulate collagen type I signalling through $\alpha 2\beta 1$ integrin, although we have not tested whether decorin alters FAK autophosphorylation when IGF-IR activity is blocked. In the case of collagen-bound decorin, enhancement of adhesion was at least partially dependent on IGF-IR activation (Chapter 5), while the morphological changes observed under identical conditions also support activation of Rho GTPases. Thus while both collagen-bound and soluble decorin enhance adhesion and induce morphological changes consistent with small GTPase activation, alternative mechanisms may exist, dependent on decorin presentation.

Decorin is likely to activate PI3-K through the IGF-I receptor, since decorin activates the PI3-K dependent kinase Akt (Schönherr et al., 2001) and PI3-K activation by growth factors has been shown to activate Rac (Nobes et al., 1995; Cheng et al., 2000; Menard and Mattingly, 2003). Both Rac and PI3-K activity is also implicated in control of integrin activity (Kovacsovics et al., 1995; Keely et al., 1997; Constantin et al., 2000, Kiosses et al., 2001; Maile and Clemmons et al., 2002), presenting a mechanism by which decorin could induce formation of large vinculin-positive focal complexes. Further, Rac requires vinculin to induce lamellipodia (Goldmann et al., 2002). Decorin-induced recruitment of vinculin into focal adhesions and activation of Rac could therefore together have contributed to formation of focal structures under the control of Rac.

Again, given the morphological changes induced by decorin, and that FAK is generally associated with focal adhesion assembly and turnover, it is surprising that decorin did not by itself influence FAK autophosphorylation, or indeed phosphorylation at other sites (Chapter 5). However in some systems FAK is re-distributed rather than activated. For example, IGF-I did not activate FAK, but caused a re-distribution of FAK away from focal adhesions, and enhanced cell migration (Zhang et al., 2005). IGF-I also sequentially activated RhoA, ROCK and p38 MAPK, and Erks 1 and 2 and PI3-K were also required for IGF-I stimulated cell motility. However, only PI3-K appeared to be directly activated by IGF-I (Zhang et al., 2005). Similarly, our laboratory has shown that decorin does not influence p38 MAPK or Erk1 and 2 activities in endothelial cells (Schönherr et al., 2001), consistent with activation of IGF-I receptor by decorin in control of cell migration. In another study, IGF-I stimulated migration of human proximal tubule cells without stimulating FAK autophosphorylation. Further, cell migration was unaffected by the absence of FAK protein (Cao et al., 2005). These studies demonstrate that cell motility induced by signalling through the IGF-I receptor can occur independently of FAK activity or indeed, the presence of FAK. In another study, IGF-I induced formation of actin microspikes at regions of cell-cell contact, inhibiting cell-cell interactions in a PI3-K dependent manner, and inducing redistribution of α-actinin to stimulate motility (Guvakova et al., 2002). In the current study, a redistribution of α-actinin was unfortunately not investigated. However the morphological changes observed in the presence of decorin could be consistent with this finding.

In summary, activation of Rac by decorin is likely to be an important mechanism by which decorin controls endothelial cell behaviour. Decorin may activate Rac by signalling through the IGF-I receptor, however, alternative mechanisms may exist by which decorin can influence Rho GTPase activity. Decorin also induced a reduction in actin stress fibres and appeared to impair focal adhesion turnover, resulting in formation of large peripheral adhesion complexes and membrane ruffling, all of which are associated with a migratory cell phenotype.

Chapter 7

General Discussion

7.1 Introduction and Summary

Angiogenesis is a complex process regulated by continuous feedback and co-ordination between signals from the extracellular matrix *via* integrins, and from growth factors to their receptors. Dysregulated angiogenesis contributes to the pathology of a wide range of diseases, while successful tissue engineering and reconstruction requires an adequate blood supply. Thus a better understanding of the basic mechanisms by which the extracellular matrix controls angiogenesis would contribute to development of successful therapeutic strategies. Decorin is a multifunctional component of the extracellular matrix involved in regulation of angiogenesis. In the absence of decorin, angiogenesis is dysregulated (Schönherr et al., 2004; Jarvelainen et al., 2006), while decorin expression is associated with neovessels formed under inflammatory conditions (Nelimarkka et al., 2001; Schönherr et al., 1999).

There are multiple levels at which decorin could act to regulate angiogenesis. Decorin regulates matrix structure and organisation (Danielson et al., 1997; Kinsella et al., 2000, Schönherr et al., 2001b) and growth factor (TGF-β) activity (Schönherr and Hausser, 2000). However, more recently, direct activities of decorin have been shown that could also be important in regulating angiogenesis. Decorin activates the IGF-I receptor in endothelial cells (Schönherr et al., 2005), and α2β1 integrin interacts with immobilised decorin (Guidetti et al., 2002). In this thesis, it was demonstrated that decorin activates the small GTPase Rac, consistent with morphological changes induced by decorin in endothelial cells. Decorin signalling through the IGF-I receptor may be a mechanism by which decorin activates Rac and stimulates endothelial cell migration. It was also demonstrated that decorin influences α2β1 integrin activity and interaction with collagen type I. These observations support a direct role for decorin in angiogenesis and contribute to a better understanding of the role of decorin in this complex process.

A summary of previously known signalling mechanisms by which decorin could influence endothelial cell behaviour is shown in Figure 7.1. It had been previously demonstrated that decorin enhances endothelial cell survival and tube formation in a collagen type I environment (Schönherr et al., 1999). A likely mechanism by which decorin promotes endothelial cell survival and differentiation is by upregulation of the cell cycle inhibitory proteins, p21 and p27 (Schönherr et al., 2005). Decorin upregulated p21 through activation of the IGF-I receptor, followed by activation of Akt, and could compete with IGF-I for receptor interaction. Upregulation of p27 was mediated by decorin through an Akt independent pathway, however the cell-surface receptor responsible is unknown (Schönherr et al., 2001; Schönherr et al., 2005).

In this thesis, some additional mechanisms by which decorin influences endothelial cell behaviour were identified and are summarised in Figure 7.2. Decorin activated the small GTPase Rac; activation of this pathway may involve activation of the IGF-I receptor, which would likely also involve activation of PI3-K. This pathway may relate to stimulation of migration by decorin. Additionally, while Akt was previously shown to be activated by decorin through the IGF-I receptor (Schönherr et al., 2005), decorin did not require Akt activity to stimulate migration. While decorin as a substrate alone could not support endothelial cell attachment, when presented to endothelial cells adhering to collagen type I, decorin enhanced attachment. This likely also requires activation of Rac by decorin. However, decorin could also enhance adhesion independently of IGF-I receptor activation, indicating that decorin could influence small Rho GTPase activity through an alternative cell-surface receptor, the identity of which could be $\alpha 2\beta 1$ integrin. Modulation of endothelial cell attachment and motility contributes differentially to tube formation (Sulochana et al., 2005), further implicating decorin in control of this process at multiple levels.

Decorin was also demonstrated in this thesis to activate the FoxO family of transcription factors, which are associated with long-term cell survival of quiescent cells, and with upregulation of p27. This pathway may therefore contribute to the earlier observation that decorin enhances cell survival and tube

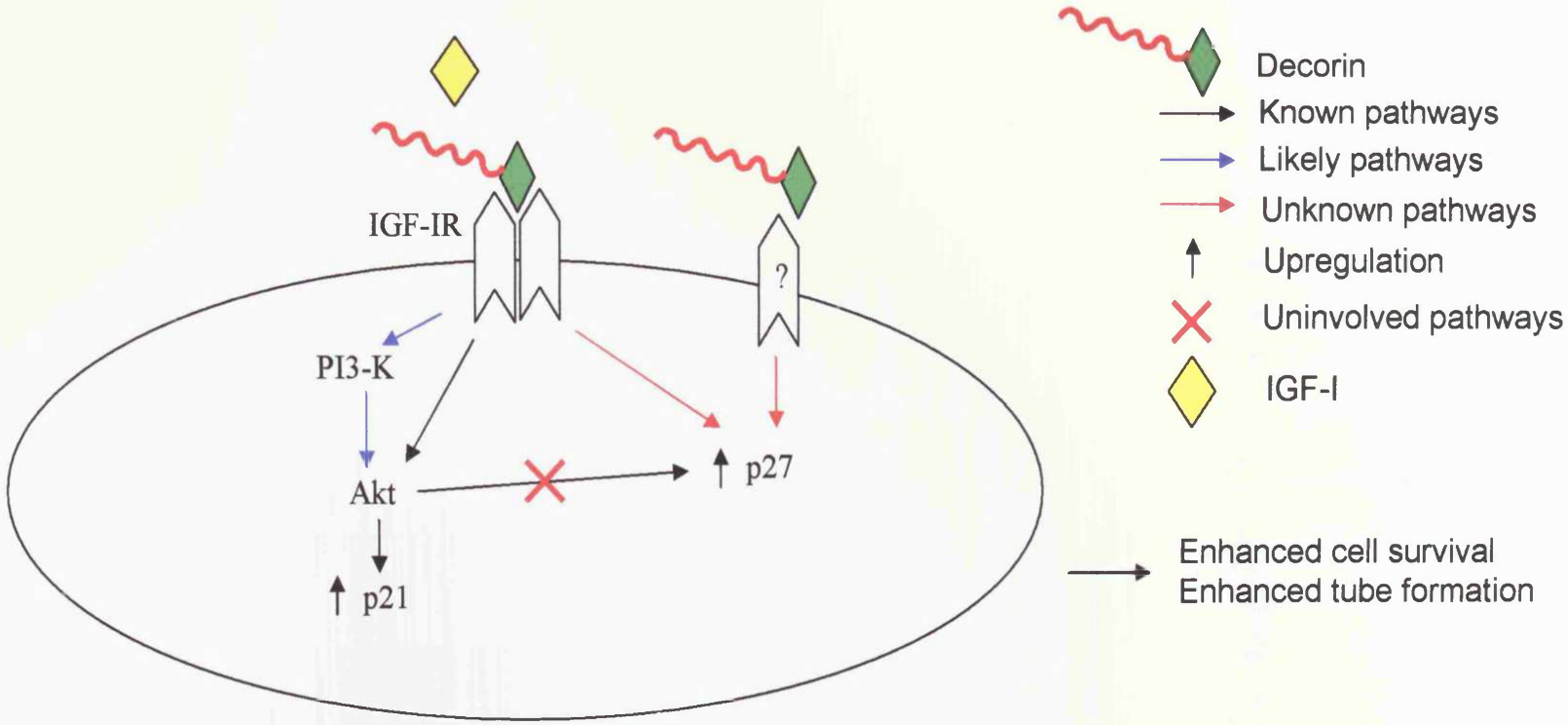


Figure 7.1: Summary of previously known roles of decorin in endothelial cell behaviour and the signalling mechanisms responsible. Decorin activates the IGF-I receptor, resulting in activation of Akt, and upregulation of the cell cycle inhibitor, p21. Decorin activates the related cell cycle inhibitor, p27 through unknown signalling pathways, which are independent of Akt activity. These pathways likely contribute to decorin-mediated enhancement of endothelial cell survival and tube formation in a collagen type I environment.

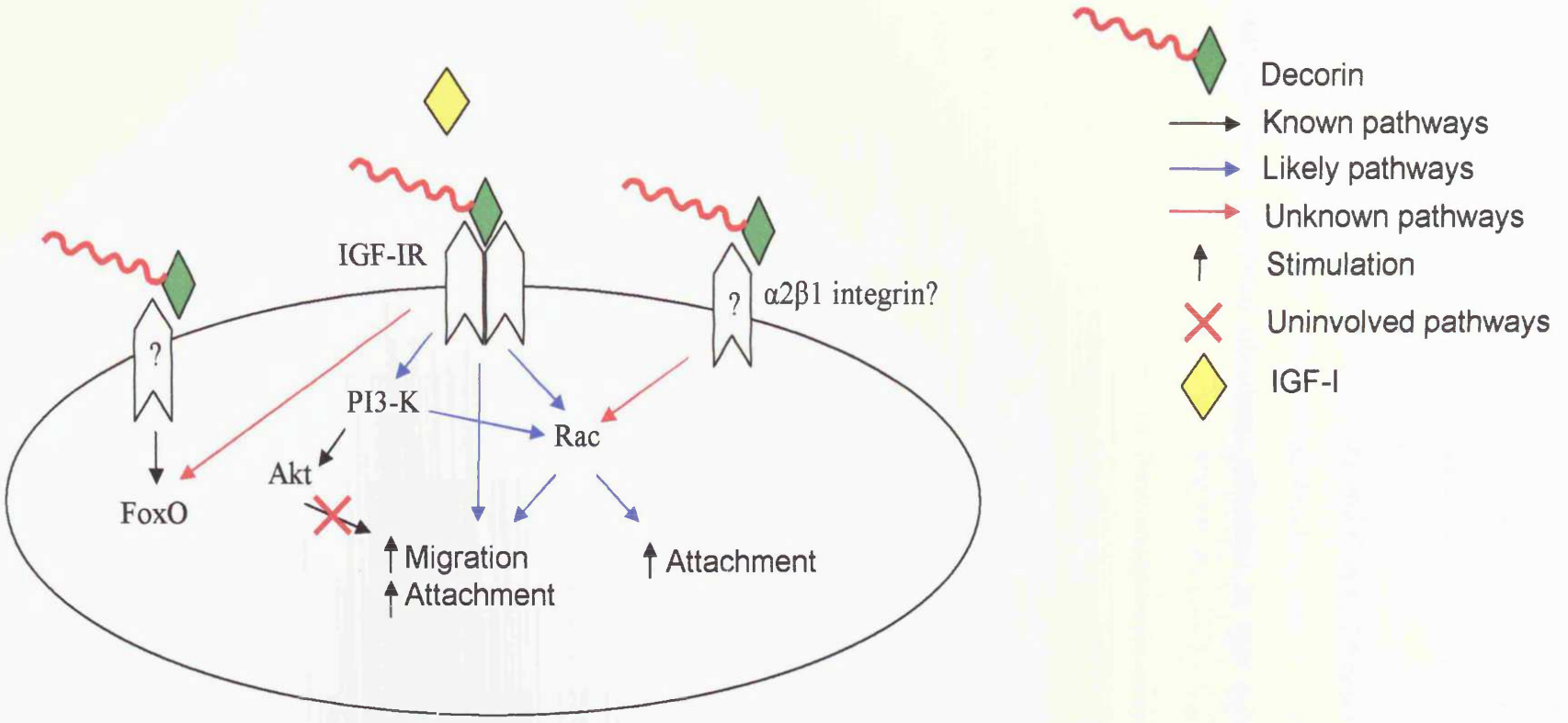


Figure 7.2: Summary of the current role of decorin in endothelial cell behaviour and the signalling mechanisms responsible. Decorin enhances endothelial attachment and migration on collagen type I. Decorin stimulates migration by activating the IGF-I receptor, but does not require Akt activation to do so. Decorin activates the small Rho GTPase, Rac, by activating the IGF-I receptor. Decorin may also activate small Rho GTPases through an alternative, unknown receptor. Decorin activates the FoxO family of transcription factors through an unknown receptor.

formation in a collagen type I lattice (Schönherr et al., 1999).

While this thesis has focussed on decorin interaction with collagen type I, IGF-I receptor and α2β1 integrin, decorin also interacts with other matrix components with which endothelial cells would come into contact during angiogenesis. Such components include fibronectin, collagen type XIV, and thrombospondin. As discussed in the introduction to Chapter 3, decorin and cell-surface CD44 both interact with a GAG-binding site on collagen XIV; decorin thus competitively inhibited fibroblast adhesion to this substrate (Ehnis et al., 1998). Similarly, decorin binding to a KKTR (lysine-lysine-threonine-arginine) motif in the cell-binding domain on thrombospondin competitively inhibited fibroblast adhesion to this substrate (Merle et al., 1997; Winnemoller et al., 1992). Whether a similar effect would be seen for endothelial cells adhering to fibronectin or collagen type XIV is not known, although it has been shown that combined substrates of decorin and thrombospondin inhibit collagen I-induced tube formation over thrombospondin alone (Davies et al., 2001). Decorin certainly has the potential to modulate endothelial motility and tube formation by competitively inhibiting such cell-matrix interactions. Further, DS-decorin has been suggested to compete with heparan sulphate proteoglycans for binding to the heparin-binding domains of fibronectin (Schmidt et al., 1987). Decorin could thus compete with endothelial cell-surface heparan sulphate proteoglycans for interaction with this substrate during angiogenesis, although it should be noted that decorin slightly enhanced endothelial cell attachment to fibronectin in our system (Chapter 3), indicating that alternative mechanisms may have been responsible for decorin modulating endothelial cell behaviour in this instance. The large complexity of potential interactions of decorin with multiple matrix components and cell-surface receptors, and therefore the possibility of numerous direct and indirect activities of decorin make it difficult to clearly ascertain the mechanisms responsible for decorin influencing endothelial cell behaviour. However, direct activation of signalling pathways by decorin rather than steric interference of cell-matrix interactions has been highlighted in this thesis as likely to play an important role.

7.2 The Relevance of Decorin Interaction with Endothelial Cell-Surface Receptors

One mechanism by which decorin influences endothelial cell behaviour is through modulation of α2β1 integrin activity (summarised in Figure 7.3). Endothelial cell attachment and migration on collagen type I in our system were mediated by α2β1 integrin, and direct interaction between decorin and this integrin was demonstrated in Chapter 4. This study (Chapter 4) also indicated that decorin preferentially interacted with an inactive integrin conformation, and reduced integrin affinity for collagen type I in an allosteric manner. This contrasts to the observation that decorin appears to enhance interaction between cell-surface integrins and matrix substrates (since endothelial cell attachment was enhanced by decorin), but may simply reflect the different conditions in which the integrin was presented to decorin. In the studies carried out in Chapter 4, isolated integrin-decorin interactions were investigated, where integrin affinity was manipulated by outside stimulation. In studies where decorin-integrin interactions would occur on the cell surface, integrin affinity is additionally under the control of inside-out signalling. Additionally, integrin clustering (avidity) contributes to integrin activity. Further, other cell-surface receptors are also present with which decorin interacts (IGF-IR). Indeed, activation of Rac by decorin signalling through the IGF-I receptor could represent a pathway by which decorin influences integrin activity through inside-out signalling. Large vinculin-positive adhesions visualised in cells adhering to collagen in the presence of decorin could support enhancement of integrin clustering by decorin. These studies indicate that decorin could influence integrin activity through multiple mechanisms.

The data presented in this thesis also indicates that activation of the IGF-I receptor by decorin could be one of the mechanisms by which decorin controls endothelial cell behaviour. In support of the relevance of this pathway in modulation of angiogenesis, this receptor is present on endothelium *in vivo* (Bar et al., 1988), while the classic IGF-IR ligand IGF-I, is released from wounded endothelial cells, and promoted proliferation (Taylor and Alexander, 1993).

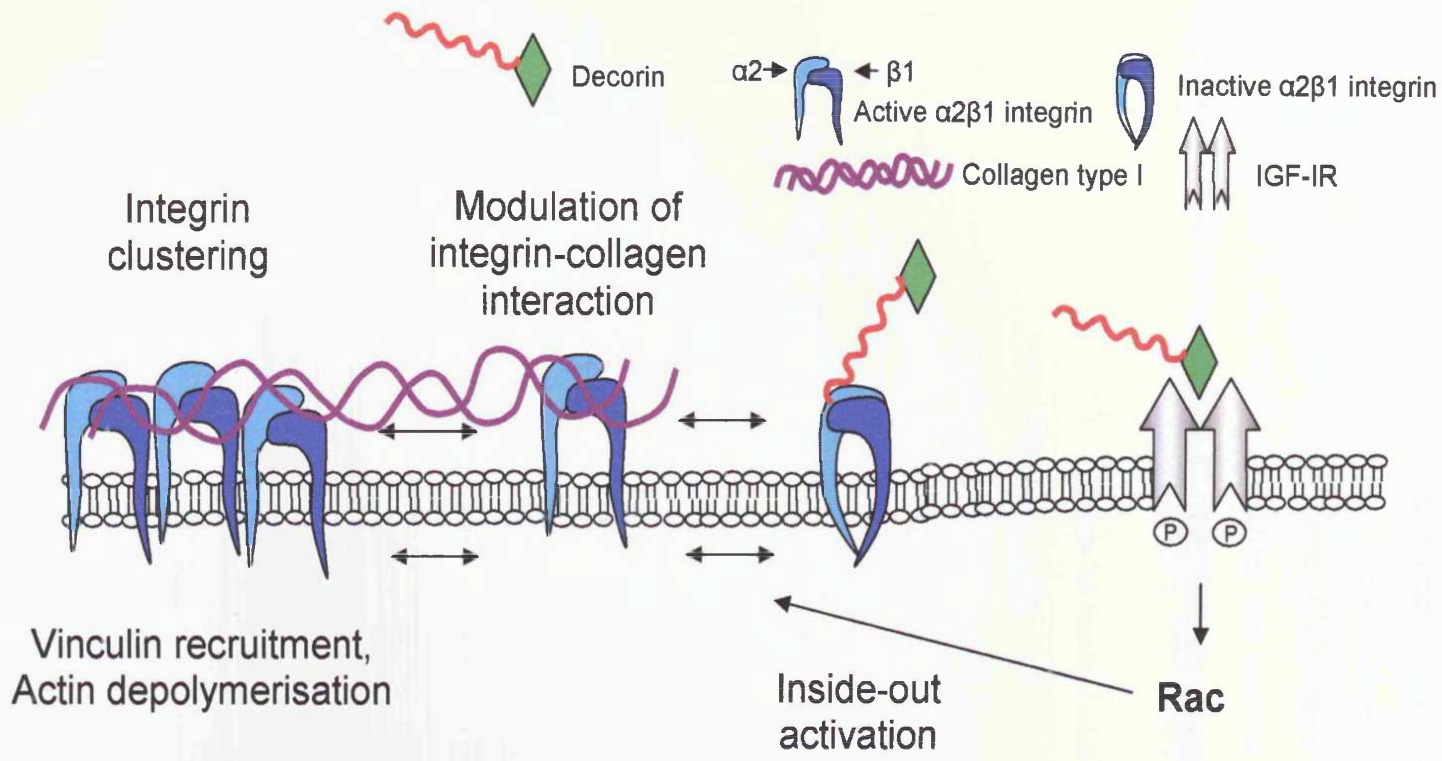


Figure 7.3: Summary of the current role of decorin in influencing α2β1 integrin-mediated events. Decorin activates Rac by signalling through the IGF-I receptor. This pathway could influence integrin affinity and/or clustering by inside-out signalling, to contribute to enhancement of cell attachment, migration and induction of large focal complexes by decorin. Decorin alone does not induce outside-in activation of integrins, but through interaction with α2β1 integrin, could allosterically influence its activity and/or clustering. The α2β1 integrin on the cell-surface exists in equilibrium between an inactive, active and clustered state.

Endothelium is thus responsive to signalling through IGF-IR during angiogenesis. Further, signalling by decorin through the IGF-IR is relevant *in vivo*, indicating that decorin is biologically available to interact with this receptor. Decorin down-regulates the IGF-I receptor *in vitro* (Schönherr et al., 2005), and increased IGF-I receptor expression was observed in native and neo-vessels in the decorin knockout mouse cornea (Schönherr et al., 2005), and in the kidney (Schönherr et al., 2005), indicating that control of IGF-IR levels on endothelial cells could potentially be an important mechanism by which decorin regulates angiogenesis *in vivo*.

In further support of a role for decorin in control of angiogenesis, decorin activated Rac (Chapter 6), and influences α2β1 activity. Both α2β1 integrin (Davis and Camarillo, 1996; Sweeney et al., 2003) and Rac are suggested to play a key role in lumen formation and vessel maturation (Bayless and Davis, 2002; Soga et al., 2001a; Soga et al., 2001b). Further, Rac activity is implicated in control of endothelium permeability and integrity through regulation of cell-cell junctions (Soga et al., 2001a; Soga et al., 2001b; Wojciak-Stothard et al., 2001), suggesting a role for decorin in endothelium homeostasis. Interestingly, modulation of both α2β1 integrin and Rac activity by decorin also has implications for regulation of collagen fibrillogenesis. Both α2β1 integrin and small GTPase activity is implicated in cellular control of pericellular collagen type I fibrillogenesis and in induction of long processes on collagen (Jokinen et al., 2004; Li et al., 2003; Sato et al., 2003). Some long cellular processes were observed in the presence of decorin although this may be in part due to actin depolymerisation induced by decorin in the absence of focal adhesion disassembly. While decorin is known to regulate collagen fibrillogenesis extracellularly, in the absence of cells (Vogel et al., 1984), these data indicate that decorin could additionally control cell-mediated regulation of collagen fibrillogenesis.

7.3 Presentation of Decorin as a Substrate, as a Collagen-bound form, or as a Soluble Effector

Several observations presented both in this thesis and in previous studies, indicate that the manner in which decorin is presented to endothelial cells may be important to mediate various functions of decorin. Decorin influences endothelial tube formation *in vitro*; however no clear pattern has emerged. Decorin enhanced tube formation when expressed endogenously by endothelial cells in a collagen type I lattice (Schönherr et al., 1999), but inhibited tube formation when presented as a substrate prior to addition of collagen type I (Davies et al., 2001). When added exogenously, decorin inhibited VEGF-induced tube formation on matrigel (Sulochana et al., 2005), but alone, decorin had no influence on tube formation on matrigel, or on microvessel sprouting from aortic explants (Grant et al., 2002). Although it cannot be excluded that the different methods used in these studies for isolation of decorin could be responsible for different effects, the importance of decorin presentation as either a soluble entity, an immobilised substrate or as a matrix-associated component is also supported in the current study.

As an immobilised substrate alone, decorin could not support endothelial cell attachment, but enhanced attachment when presented as an immobilised substrate with collagen type I, or added exogenously to endothelial cells both during attachment or already adherent to collagen type I. This could also suggest that endothelial cell-collagen type I interaction (mediated through $\alpha 2\beta 1$ integrin) is required in order for endothelial cells to respond to decorin signalling. Indeed, this is the case for growth factor signalling; integrin ligation is required for cell response to occur. Irrespective of presentation, decorin enhanced endothelial cell attachment and migration on collagen type I, however subtle differences were observed. As discussed in Chapter 5, exogenous decorin-mediated enhancement of endothelial cell migration was consistent with a mechanism dependent on activation of the IGF-I receptor. However, the observation that collagen-bound decorin continued to stimulate the rate of migration where soluble decorin could not, could indicate contributions of additional mechanisms by which collagen-

bound decorin could control motility. Thus while presentation of decorin does not appear to be critical for decorin to influence endothelial cell behaviour, the subtle differences observed in the current study could become more relevant in different contexts, such as during matrix remodelling.

While activation of the IGF-I receptor by decorin may have been involved in stimulation of migration, this does not exclude contributions of additional mechanisms. Further, due to the limitations of the inhibitor used to block IGF-I receptor signalling (tyrphostin AG1024), involvement of IGF-I receptor-mediated signalling pathways in control of endothelial cell behaviour by decorin would require further corroboration. Additionally, extracellular decorin interactions with α2β1 integrin cannot be excluded. Unfortunately, since we could not identify any antibodies that could block decorin interactions with the integrin, any involvement of this interaction could not be easily investigated.

Adding to the complexity of the role of decorin in angiogenesis, in an interstitial environment, simultaneous interactions could exist between decorin, collagen type I and α2β1 integrin, which may be differentially supported through various stages of angiogenesis. Many questions remain as to whether soluble and/or collagen-bound decorin can mediate these interactions and whether these interactions are relevant to angiogenesis. The availability of binding sites on decorin (which could be dependent on decorin presentation) could also contribute to different interactions. Within decorin, LRRs 3-6 are responsible for interaction with collagen (Bhide et al., 2005; Kresse et al., 1997; Schönherr et al., 1995; Svensson et al., 1995), while LRRs 1-5 of decorin contain the binding site for the IGF-I receptor (Schönherr et al., 2005). These binding sites could therefore either overlap or be in close proximity. An important question is whether the IGF-IR binding site is accessible on collagen-bound decorin. A mutant with reduced collagen-binding activity could still interact with IGF-IR, indicating that binding of decorin to collagen is not a requirement for this interaction (Schönherr et al., 2005). This does not however, rule out simultaneous interactions of decorin with collagen and the IGF-I receptor. Indeed, collagen-bound decorin appeared to enhance endothelial cell attachment by signalling through the IGF-I receptor (Chapter 5). Interestingly, decorin

interacts with IGF-IR *via* the core protein (Schönherr et al., 2005), and with α2β1 integrin *via* the GAG moiety (Chapter 4) suggesting that decorin could interact simultaneously with both cell-surface receptors. Such an interaction would have implications for direct regulation of integrin-growth factor cross-talk through influencing stability of receptor complexes at the cell-surface. In this model, the IGF-I receptor would interact with the convex, GAG-containing surface of decorin. Collagen-bound decorin could indeed present this surface to cell-surface receptors. As discussed in Chapter 2, it is a matter of some debate whether decorin exists predominantly as a monomer or as a dimer in a physiologically relevant environment. However, it has been recently proposed that decorin dimers would support convex surface interactions of decorin with binding partners (McEwan et al., 2006), as portrayed in Figure 7.4. Various binding interactions of decorin however may additionally depend on factors such as receptor availability and/or relative binding affinities. Indeed, it might seem unlikely that α2β1 integrin might interact with the GAG moiety of decorin rather than with collagen type I. While it was demonstrated that α2β1 integrin interacted with decorin at neutral pH (Chapter 4), the interaction was strongly enhanced where interaction with collagen was abolished (at acidic pH). In the context of tissue remodelling, hypoxic tumour tissue is acidic (Engin et al., 1995), while in wound healing, tissues also reach acidic pH, which contributes to the wound healing process (Greener et al., 2005). Under these conditions, interaction of α2β1 integrin with decorin could therefore become more relevant.

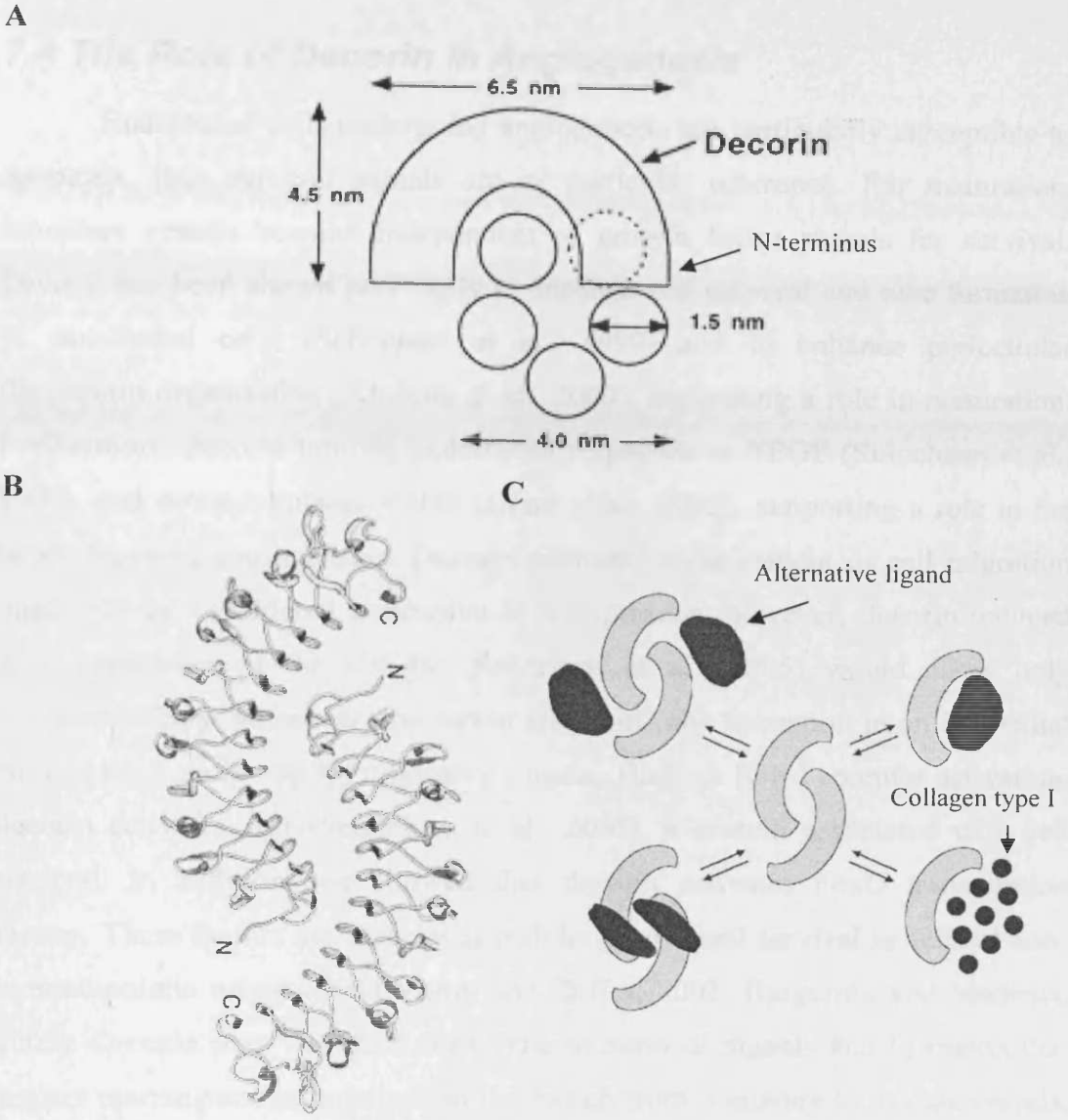


Figure 7.4: Interaction of decorin monomer or dimer with collagen type I and/or cell-surface receptors. As a monomer, decorin is proposed to interact with collagen type I through the concave surface, with the GAG and N-linked oligosaccharides located on the same side of the molecule. An additional, secondary collagen type I binding site was also proposed (at the N-terminus) (A), (Weber et al., 1996). As a dimer, decorin is proposed to interact through the concave surfaces of the core protein (B). In this model, the GAG and N-linked oligosaccharides are also proposed to be located on the same side of the molecule. Decorin dimer is proposed to interact with collagen,●, or alternative ligands such as EGFR, IGF-IR and TGF-β (filled black shapes) by dissociation into monomers, or through the surface or convex face of the dimer (C), (McEwan et al., 2006).

7.4 The Role of Decorin in Angiogenesis

Endothelial cells undergoing angiogenesis are particularly susceptible to apoptosis, thus survival signals are of particular relevance. For maturation, immature vessels become independent of growth factor signals for survival. Decorin has been shown previously to enhance cell survival and tube formation in endothelial cells (Schönherr et al., 1999) and to enhance pericellular fibronectin organisation (Kinsella et al., 2000), suggesting a role in maturation. Furthermore, decorin inhibits endothelial responses to VEGF (Sulochana et al., 2005), and down-regulates VEGF (Grant et al., 2002), supporting a role in the latter stages of angiogenesis. Decorin-mediated enhancement of cell migration might not be considered conducive to this process; however, decorin-induced down-regulation of the IGF-IR (Schönherr et al., 2005) would allow only transient effects. Moreover, the earlier stages of tube formation in an interstitial environment could require migratory signals. Through IGF-I receptor activation, decorin activates Akt (Schönherr et al., 2005), a protein associated with cell survival. In addition, we showed that decorin activates FoxO transcription factors. These factors are associated with long-term cell survival in cells of non-haematopoietic origin (Birkencamp and Coffer, 2003; Burgering and Medema, 2003). Decorin may therefore contribute to survival signals and to matrix-cell contact rearrangements involved in the switch from immature to mature vessels, and in vessel persistence after withdrawal of pro-angiogenic survival factors such as VEGF. Further, both α2β1 integrin (Davis and Camarillo 1996; Sweeney et al., 2003) and Rac (Connolly et al., 2002; Bayless and Davis, 2002; Soga et al., 2001a; Soga et al., 2001b) are critically involved in tube formation. Decorin interacts with α2β1 integrin, and activates the small Rho GTPase, Rac, providing potential mechanisms for decorin in modulation of tube formation. This would also further support a role for decorin in control of vessel maturation. Thus decorin could regulate angiogenesis by influencing matrix structure and cell behaviour through multiple mechanisms.

7.5 Future Perspectives

The effects of decorin on endothelial cell behaviour, demonstrated both within this study and in the literature, support a role for decorin in regulation of angiogenesis, particularly in vessel maturation. Some of the mechanisms by which decorin may control endothelial cell behaviour were presented in this thesis, however an important question is whether these mechanisms relate to enhancement of endothelial cell survival and tube formation by decorin. Questions also remain as to the involvement of IGF-IR in activation of FoxO transcription factors, and whether this pathway is responsible for upregulation of p27, and/or decorin-mediated enhancement of cell survival.

Other pertinent questions are whether the interaction between α2β1 integrin and decorin is biologically relevant, and whether decorin influences α2β1 integrin activity on the cell-surface directly, and/or by inside-out signalling through the IGF-I receptor. A number of approaches could be taken to further explore these questions. A requirement for decorin signalling through the IGF-I receptor in modulating endothelial cell attachment, migration and tube formation could be investigated using, for example, chemical inhibitors (tyrphostin AG1024), by over-expressing dominant negative IGF-IR mutants (using adenovirus-mediated gene transfer) or using siRNA to IGF-IR.

Further investigation of the location of the binding site for decorin on α2β1 integrin would facilitate analysis of whether decorin directly influences α2β1 integrin activity on the cell-surface, and/or whether IGF-I receptor activation is involved in inside-out activation of this integrin. Once the binding site is known, the effects of inhibition of decorin-integrin interaction on the cell-surface could then be investigated using mutated integrin receptors or by identifying or generating blocking antibodies. Alternatively, it was observed that decorin inhibited the effects of the specific α2β1 integrin inhibitor, rhodocetin, on endothelial cells. Whether decorin signalling through the IGF-IR results in inside-out activation of the integrin to block rhodocetin effects could be tested by blocking IGF-IR signalling in the presence of rhodocetin (using the approaches suggested above). Whether IGF-IR activation is required to mediate

the morphological changes induced by decorin during attachment to collagen type I (mediated by α2β1 integrin) could also be tested using similar methods. Further, the consequence of decorin interaction with other matrix components not considered in this study (such as collagen types VI, XII, XIV, fibronectin, thrombospondin, tenascin-X) in modulating endothelial cell behaviour would also contribute to an understanding of the role of decorin in control of various stages of angiogenesis.

In conclusion, a better understanding of the mechanisms by which the ECM regulates vessel formation, maturation and persistence is essential in manipulation of angiogenesis. Further investigation, in conjunction with this thesis, of the involvement and consequences of signalling pathways activated by decorin in endothelial cells would contribute to understanding how angiogenesis is dysregulated in a clinical setting, and how rational therapeutic strategies can be developed to restore tissue function and homeostasis.

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