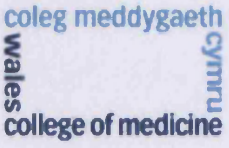
Host-microbial interactions and cellular responses associated with '*Streptococcus anginosus*' group infection.

Janine Landrygan-Bakri

Submitted in accordance with the requirements of the degree of Doctorate of Philosophy



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Summary

Interactions of the *Streptococcus anginosus* group (SAG) with the extracellular matrix (ECM) and subsequent effects on host connective tissue and bacterial cells is proposed to be important in the initial establishment of dentoalveolar infections. The aim of this thesis was to investigate the interactions of the SAG with small leucine-rich proteoglycans (SLRPs), decorin and biglycan, from periodontal tissues and recombinant decorin and biglycan. Additional aims were to investigate the subsequent effects of the SAG on cellular components within host tissues and the influence of ECM components on bacterial phenotype. Using surface plasmon resonance, this study indicated that both commensal and pathogenic strains of the SAG interact with SLRPs but there was preferential binding toward the dermatan sulphate-substituted decorin and biglycan present in gingival tissues. In addition, commensal and pathogenic SAG isolates were shown to influence periodontal ligament (PDL) and endothelial ECM responses, including cell growth and PG synthesis. The effects on the different cell types illustrates the complexity of disease caused by the SAG, and helps to highlight the complicated roles decorin and biglycan play within the ECM. This study has also shown that potential virulence factors of the SAG, including degradative enzymes, are up-regulated following exposure to ECM components derived from PDL cells, potentially causing destruction of the host ECM and possibly inhibiting remodelling of the ECM. Overall, this thesis provides valuable information on host-SAG interactions, highlighting complex roles for SAG and SLRPs in the establishment of periapical abscesses.

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Abbreviations used in this thesis

Abbreviation	Meaning
16S rRNA	Bacterial small subunit ribosomal RNA
BGN	Biglycan
BIA	Biomolecular interaction analysis
BHI	Brain heart infusion
bp	Base pairs
cDNA	Complementary Deoxyribonucleic acid
CO_2	Carbon dioxide
CSase	Chondroitinase
CS	Chondroitin sulphate
C4S	Chondroitin 4-sulphate
C6S	Chondroitin 6-sulphate
ddH_2O	Double distilled water
DCN	Decorin
DNA	Deoxyribonucleic acid
DS	Dermatan sulphate
ECM	Extracellular matrix
EGF(R)	Epidermal growth factor (receptor)
ER	Endoplasmic reticulum
FAA	Fastidious anaerobic agar
FBS	Foetal bovine serum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate
$GuCl_2$	Guanidinium chloride
HA	Hyaluronan
HAase	Hyaluronidase

HCl	Hydrochloric acid
HS	Heparan sulphate
ILY	Intermedilysin
KS	Keratan sulphate
mRNA	Messenger ribonucleic acid
N_2	Nitrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
O_2	Oxygen
PDL	Periodontal ligament
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Proteoglycan
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RU	Resonance unit
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLRPs	Small leucine-rich proteoglycans
SMG	*Streptococcus milleri* group
SPR	Surface plasmon resonance
TBS	Tris buffered saline
TGF-ß	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha

CHAPTER ONE

Chapter 1

Host-microbial interactions and cellular responses associated with *Streptococcus anginosus* infection.

1.0 General introduction

Acute infections associated with the teeth and surrounding supporting structures often progress to dentoalveolar abscesses (Rice and Crean, 2006). Causative agents are endogenous oral commensal bacteria, usually from the apex of a necrotic tooth or from the periodontal pockets, due to caries, exposure during cavity preparation or traumatic fractures. The outcome of such dentoalveolar abscesses depends upon the virulence of the causative microorganism, the local and systemic defence mechanisms of the host and the anatomical features of the region. Bacterial species frequently isolated from dentoalveolar abscesses are *Prevotella* spp., *Porphyromonas* spp., and facultative streptococci, such as the *Streptococcus anginosus* group (SAG) (Samaranayake, 2001).

Microbes that colonise or infect their mammalian hosts often exhibit both host and tissue tropism (Virji, 1996). Host-microbial interactions are two-way processes. Factors from eukaryotic cells are able to engage with and change the activity of prokaryocytic cells and vice-versa, this ability is a crucial first step in the development of infection. The extracellular matrix (ECM) is a major component of healthy tissues covered by epithelial or endothelial cells. Therefore, under normal circumstances, the ECM is unavailable for binding. However, any trauma that damages host tissues may expose the ECM and allow microbial colonisation and invasion (Nallapareddy *et al.*, 2000). Part of the defence mechanism against foreign

material and damaged tissue is to coat them with a film of various ECM components. However, these components can become involved in host-microbial interactions as receptors for colonising organisms so that the site of injury can be accessible for establishment of infection (Ljungh and Wadstrom, 1995). Gaining an insight into the interactions between matrix proteins and bacteria is important for understanding the mechanism of bacterial colonisation, a pre-requisite to infection, with a view to preventing or controlling infection (Holmes *et al.*, 1997).

1.1 Structure of the periodontium

The periodontium is a complex structure that provides the support required to maintain teeth in adequate function. The periodontium is a unique organ consisting of two soft connective tissues (gingiva and periodontal ligament) and two calcified tissues (alveolar bone and cementum) (see figure 1.1a). Together, these tissues retain the tooth within its socket and protect both the tooth root and adjacent structures from the external environment (Avery, 2000).

1.1.1 *Gingiva*

The connective tissue of the gingiva functions to support the tissue and to supply nutrients and innervation to the surrounding structures (Bartold, 2000).

The gingival unit comprises a number of distinct regions (see figure 1.1b), each with unique histological and clinical features. The free gingiva extends coronal to the attached gingiva and forms the walls of the gingival sulcus. In health, this is a narrow and shallow crevice of 1-2 mm

depth surrounding the tooth and often is not clinically obvious. The sulcus is lined by sulcular epithelium, which is continuous apically with the junctional epithelium and, although the demarcation between these two epithelia appears gradual clinically, it is distinct in histological sections. The sulcular epithelium is continuous coronally with the epithelium lining the oral cavity and is usually clinically demarcated from the attached gingiva on the oral-facing surfaces by the free gingival groove (Bartold *et al.* 2000; Clark and Loe, 2001).

The attached gingiva extends from the free gingiva and apically becomes continuous with the alveolar mucosa. This junction is clearly demarcated in health as the mucogingival junction or line. Palatal to the upper teeth, however, masticatory mucosa extends from the gingivae to cover the hard palate such that there is no alveolar mucosa and thus no mucogingival junction. The attached gingiva has a stratified squamous orthokeratinized epithelium and in health appears coral pink, firm and stippled, like orange peel, in contrast to the dark red and loosely bound alveolar mucosa to which it abuts (Clark and Loe, 2001).

Interdentally, the gingival papillary shape is determined by tooth contact. It is pyramidal in shape between the anterior teeth, but becomes more flattened in the premolar and molar regions, where approximal contact areas rather than contact points exist. In this region, *a col* (concavity) is formed, covered by a thin non-keratinized epithelium. This represents a structural weakness that may predispose to breakdown as a consequence of plaque-induced disease.

1.1.2 *Periodontal ligament*

The periodontal ligament (PDL) is a complex, vascular, and highly cellular soft tissue that is physically small but functionally important tissue in tooth support, proprioception and regulation of alveolar bone volume. It has a rapid turnover and an ability to adapt to alterations of mechanical loading, thus making it a model system to study connective tissue homeostasis and remodelling (McCulloch *et al.*, 2000). The main cell type found within the PDL is the fibroblast. Most PDL fibroblasts are highly active cells, exhibiting an elongated well-polarized cytoplasm with extensive areas of contact to collagen fibres. These fibroblasts contain a large number of rough endoplasmic reticulum and well-developed golgi complexes, indicating a high rate of protein synthesis (Beertsen *et al.*, 1997). Fibroblasts attach to the substratum of the ECM via surface receptors for structural matrix components such as, collagen and fibronectin. This attachment is essential for cell migration and organisation of the extracellular fibrillar matrix (Cho and Garant, 2000). Fibroblastic cells of the PDL are a suggested source of osteoblast-like and cementoblast-like cells, and are therefore suggested to be multipotent cells, or composed of heterogeneous cell populations that have the capacity to differentiate depending on the microenvironment (Roberts *et al.*, 1982; McCulloch and Bordin, 1991).

1.1.3 *Alveolar bone*

Alveolar bone forms during root development and is synthesised by osteoblasts derived from the denticle follicle. It is a specialised area of the mandibular and maxilla bones that lines the tooth sockets, providing

primary support for teeth (Avery, 2000). Alveolar bone can be described as a specialised form of connective tissue, whose mineral phase contributes to around two thirds of its weight with the remaining third attributable to the ECM (Sodek and McKee, 2000). Conversely, unlike other bone tissue within the body, alveolar bone is subject to continual and rapid remodelling associated with tooth eruption, adaptation and the functional demand of mastication. Considering the importance of this tissue, there is relatively little detailed information on its extracellular matrix, even though the type of ECM constituents known are thought to be similar for all bone types (Sodek and McKee, 2000).

1.1.4 *Cementum*

Cementum is a hard, calcified connective tissue covering the root dentine and providing the site for anchorage of PDL fibres. However, unlike bone, it lacks blood and lymph vessels and is not innervated or remodelled. Its thickness does, however, increase with age, with the rate of formation depending on the function of the tooth, and it contributes to repair if the root surface is damaged. In the cervical two-thirds of the root, it exists as a thin acellular tissue *(primary cementum)* laid down by an advancing front of cementoblasts. In the apical onethird of the root, cementum is cellular *(secondary cementum)* and is thus more comparable to bone. Sharpey's fibres are also evident (Hoag and Pawlak, 1990; Avery, 2000).

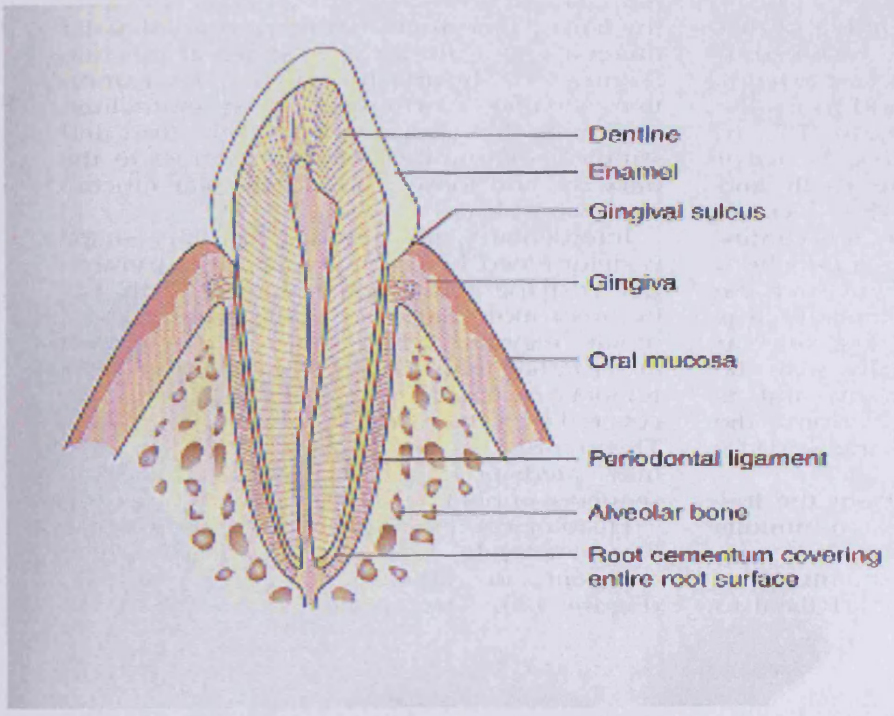


Figure 1.1a Periodontium, showing the periodontal ligament attaching the cementum of the tooth root to the alveolar bone of the socket

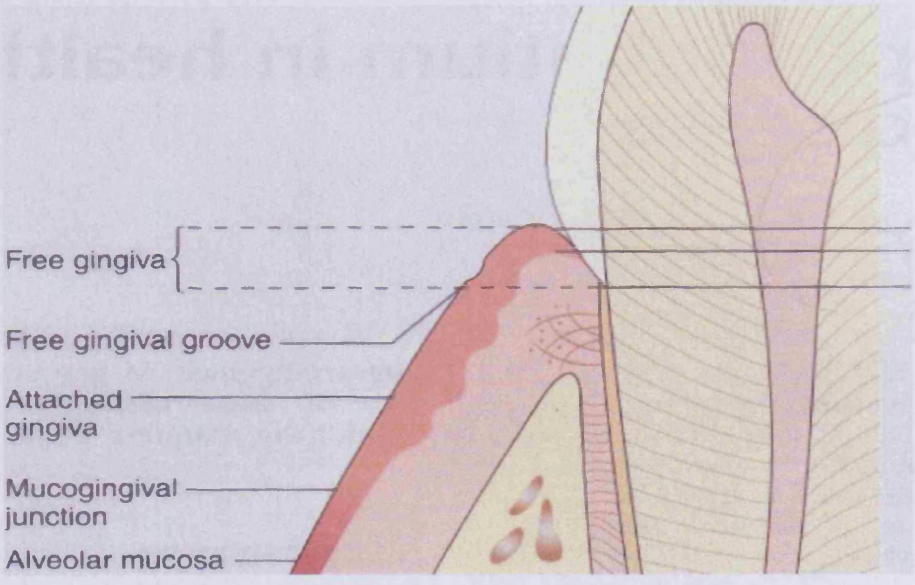


Figure 1.1b Periodontal tissue anatomy with gingival fibers

1.2 Extracellular matrix

Connective tissue serves a "connecting" function by supporting and binding other tissues. Unlike epithelial tissue, connective tissue typically has cells scattered throughout an ECM. The ECM is a complex array of secreted molecules including collagens, proteoglycans (PGs), glycosaminoglycans (GAGs) and a number of glycoproteins (Iozzo, 1998). This mixture of structural and functional proteins underlies all epithelia and endothelia and surrounds all connective tissue cells. The ECM is arranged into a unique, tissue-specific three-dimensional ultra-structure, providing a natural scaffold for tissue and organ morphogenesis, maintenance, and regeneration following injury (Embery *et al*, 2000; Waddington and Embery, 2001).

It has become clear that the pattern of non-collagenous and collagenous materials in the periodontium is similar to other soft and mineralised connective tissues present throughout the body (Waddington and Embery, 2001), albeit in differing compositions. However, the ECM of the periodontium has a much higher turnover and responds to stimuli more sensitively, such as mechanical stress and pathology (McCulloch *et al.*, 2000; Keles *et al.*, 2005).

1.2.1 Collagen

Collagens are the main organic constituents of the connective tissue matrix providing the tensile strength required by these tissues. They are involved in the general structural organisation of the ECM due to their

ability to interact with other ECM molecules and cells (Uitto and Larjava, 1991).

Collagen contains a triple helical conformation of tropocollagen which form fibrils in a tightly regulated manner, with a rod-like structure, 300nm in length and 1.5nm in diameter. Subsequently, multiple fibrils form into collagen fibres (Rushikof *et al.* 2004).

A collagen trimer may consist of two or three different α chains or three identical chains. There are at least 27 collagen types identified, with about 42 different α chains, and the number of members of this family is increasing continuously. Most collagens form supra-molecular assemblies, which are divided into nine distinct families: (1) fibril-forming collagens (types I, II, III, V, XI, XXIV, XXVII); (2) fibril-associated collagens with interrupted triple helices (FACITs) located on the surface of the fibrils, and structurally related collagens (types IX, XII, XIV, XVI, XIX, XX, XXI, XXII, XXVI); (3) the family of collagens VIII and X forming hexagonal networks; (4) the type IV collagens, located in basement membranes; (5) type VI collagen, forming beaded filaments; (6) type VII collagen, forming anchoring fibrils for basement membranes; (7) collagens containing trans-membrane domains (types XIII, XVII, XXIII, XXV); and (8) the family of type XV and XVIII collagens. The modes of supra-molecular assembly of families 7 and 8 are so far unknown. (Myllyharju & Kivirikko, 2001).

Collagens are the major ECM components of periodontal connective tissues, particularly type I collagen, providing mechanical attachment of the tooth to the bone and gingiva and mediate signals that regulate cell functions, including remodelling of the PDL and bone. Type I collagen is also present in bone, the sclera of the eyes, the cornea, lungs and skin (Glimcher, 1989; Hernandez *et al.,* 1991; Gentle *et al.*, 2003). Collagen type I may represent some 60% of the organic components within the soft connective tissues of PDL and gingiva, and 90% of the total organic matrix of mineralised tissues, such as alveolar bone (Waddington and Embery, 2001). Interaction with non-collagenous proteins, most notably PGs, helps in the assembly of the collagen matrix, in addition to a variety of other functions, including regulation of mineral deposition within the collagen fibrils.

1.2.2 Glycosaminoglycans

GAGs provide tissue hydration for fluid flow and molecular transport, charge repulsions and attractions for intermolecular spacing, and poly-anionic domains for growth factor or integrin signalling, cell adhesion, and migration or proliferation. Due to these functions, studies of ECM metabolism in genetic development and tissue engineering experiments include analysis of GAGs (Iozzo and Murdoch, 1996; Plaas *et al*, 2001).

GAG chains are the hallmark of PGs. They are highly anionic, linear polysaccharides that entail a backbone of repeating disaccharide units, hexuronic acid and an *n*-acetyl hexosamine. Seven different GAG species have been generally identified; chondroitin 4- and 6- sulphate (CS),

dermatan sulphate (DS), keratan sulphate (KS) I and II, heparan sulphate (HS), heparin and hyaluronan (HA) (see table 1). All GAGs are sulphated except hyaluronan, which is also the only GAG found non-covalently attached to a protein core (Hascall *et al*, 1995; Rostand & Esko, 1997; Waddington & Embery, 2001). Selective epimerisation of the hexuronic acid residue, within DS and HS, and selective sulphation of the hexosamine residue, within CS, DS, HS, heparin and KS, has permitted for a larger range of GAGs. This ultimately results in the production of specific properties (Hardingham & Fosang, 1992). Through biochemical characterisations and immunohistochemical studies, it has become apparent that the nature of GAGs varies in different tissue types (Fisher *et al.*, 1983; Heinegard *et al.*, 1985; Waddington *et al.*, 2003).

1.2.2.1 Biosynthesis of glycosaminoglycans

Biosynthesis of GAGs is initiated by transferring the reducing end of xylose from UDP-xylose to the hydroxyl serine residue of the core protein (threonine may also accept the xylose). The reaction is catalysed by xylosyl transferase I (XT-I) and II (XT-II) (Sugahara *et al.*, 1988; Cuellar *et al.*, 2006; Ponighaus *et al.*, 2006). Xylosylation is the rate-limiting factor in GAG biosynthesis, XT-I initiates the biosynthesis of the GAG linkage tetraaccharide in PGs whereas XT-II initiates the biosynthesis of both HS and CS GAGs (Cuellar *et al.*, 2006; Ponighaus *et al.*, 2006).

Subsequently, there is an addition of two galactose residues (from UDP-galactose), to the existing xylose, through galactosyl transferases. The carbohydrate backbone then forms by alternate transfer of N-

acetylhexosamine and hexuronic acid residues from the corresponding UDP-glycosyl donors onto the non-reducing end of the chain, linked by α and β chains (Roden, 1981).

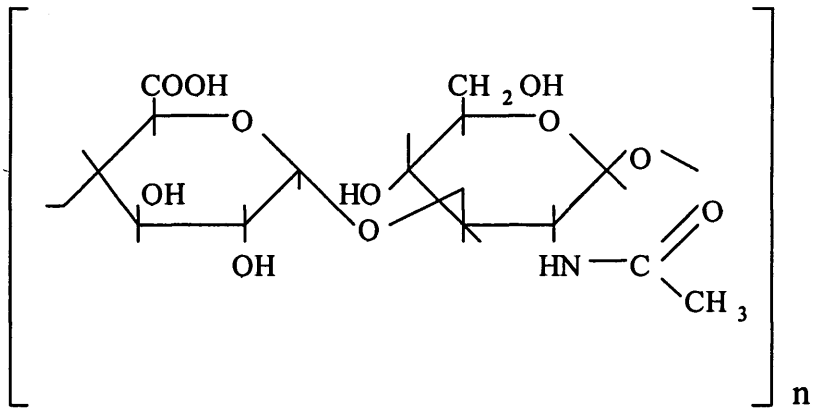
The repeating disaccharide unit undergoes continuous modifications with the addition of sulphate by sulphotransferases, as the GAG chains elongate (Greiling *et al.*, 1972). After sulphation, further changes can occur to the GAG chains, such as the epimerisation of the carboxyl group at position 5 in the hexuronic acid of CS, this causes the carboxyl group within D-glucuronic acid to rotate and lie below the plane of the hexose ring to form L-iduronic acid to produce DS (Hascall and Hascall, 1981).

HA synthesis, which ultimately does not attach to a core protein, is different from other GAG chain synthesis. Synthesis does not occur in the golgi, instead it is linked to the cytoplasmic side of the plasma membrane. HA synthase elongates HA at the reducing end by adding UDP-N-acetylglucosamine or UDP-glucuronic acid (Prehm, 1984). HA is then successively released into the ECM without being bound to a core protein (Mason *et al.*, 1982).

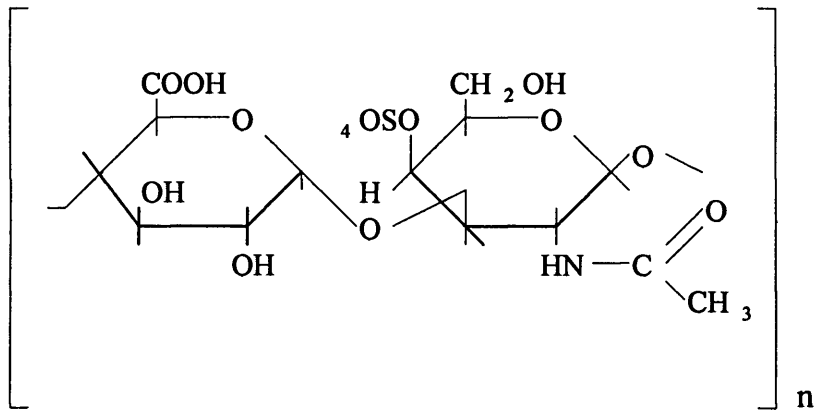
Due to the synthesis process and post-translational modifications, GAG chains display differences, such as the degree and position of sulphation (Neame and Kaye, 2000).

Figure 1.2 Glycosaminoglycan structures

HA



C4S



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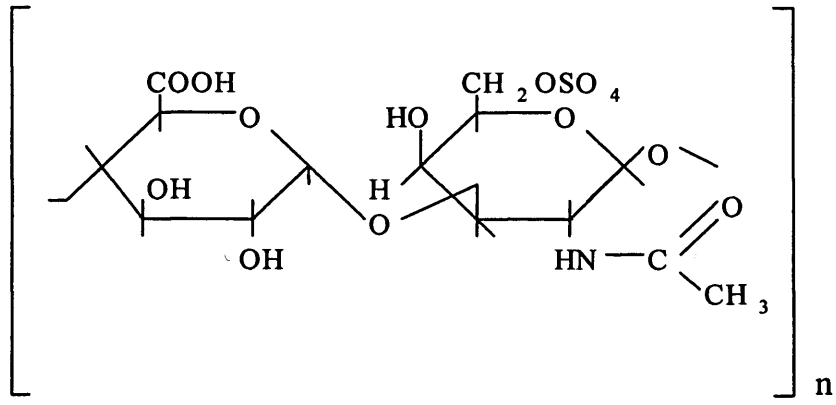
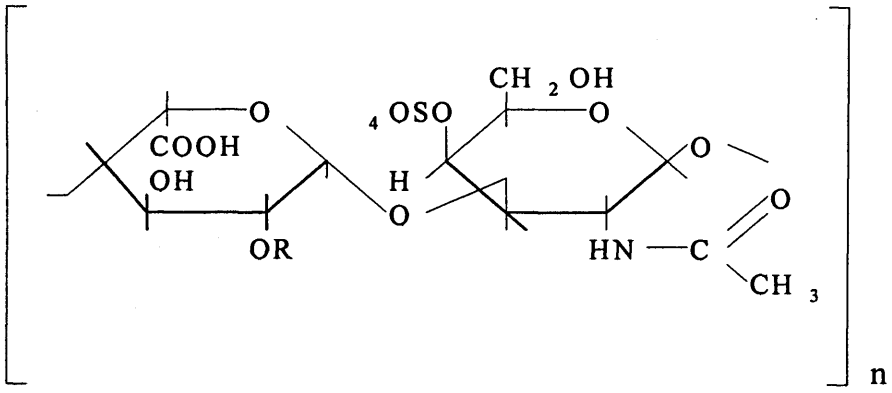


DS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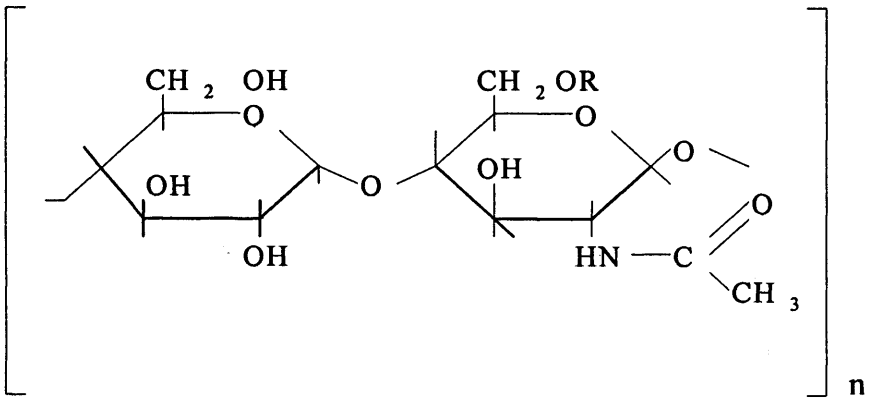
4 OSO
COOH
OH
H
OR
HN —C
CH 3
n

KS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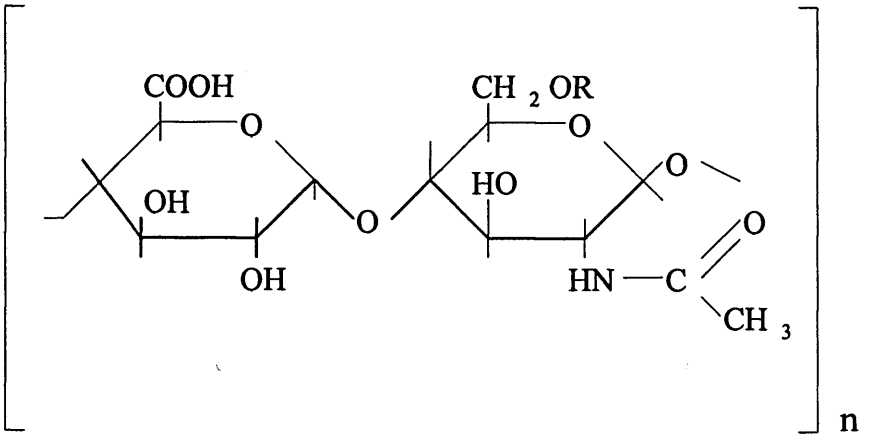
CH 2 OR
OH
OH
OH
HN —C
CH 3
n

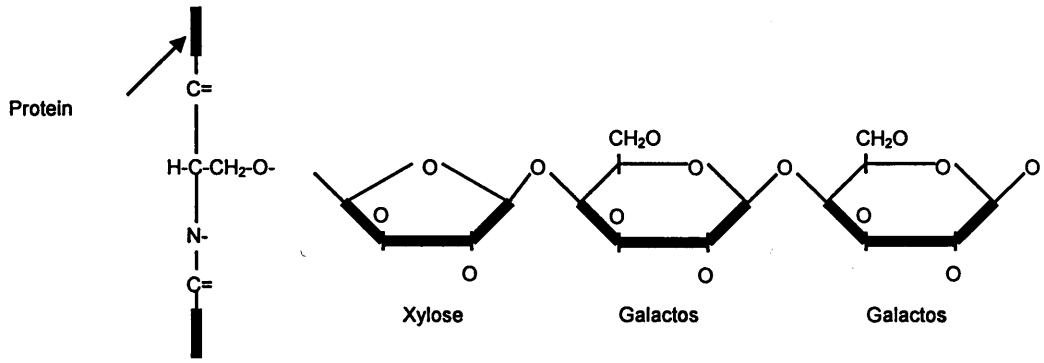
HEP


COOH
CH 2 OR
OH
HO
OH
HN —C
CH 3
n

1.2.3 Glycopeptide linkage and proteoglycan core protein

As mentioned, a trisaccharide sequence of xylose-galactose-galactose forms the linkage region for the attachment between GAGs and PG core proteins, where xylose covalently attaches to the serine residue on the protein core (Roden, 1981). CS, DS, HS and heparin all attach through the reducing terminal of the GAG monosaccharides and the hydroxyl groups of serine residues in the protein core via an O-glycosidic bond. The sulphate and carboxyl groups make these macromolecules highly negative under physiological conditions (Bartold, 1990). The molecular weight of PG core proteins ranges from 10 – 400kDa, and over 100 GAGs can covalently link to a core protein (Kreis and Vale, 1993). A wide range of protein cores serve as acceptors for the initiation and polymerisation of GAG chains. Due to advances in both biochemical and molecular techniques, there has been an emergence of distinct differences between PG families. Therefore, PGs are classified due to their core protein and relative biological function (Iozzo, 1998).

Figure 1.3 Glycopeptide linkage



1.2.4 Proteoglycans

PGs represent a family of macromolecules, ubiquitously extracellular and cell-surface associated. PGs surround the plasma membrane of a cell and constitute part of the substrate upon which the cell attaches and performs its major functions. They comprise some of the most complex and multi-functional molecules of the animal kingdom (Iozzo, 1999).

PGs characteristically carry highly charged, sulphated polysaccharide GAGs. PG families are characterised from the amino acid sequence, the size of the core protein and the nature of the GAG, including its size, sulphation pattern and number of chains attached to the protein core (Iozzo and Murdoch, 1996).

Both structural features of core protein and GAG are important in interactions with cell and matrix components. Overall, PGs provide structural constraints, can function as growth-supportive or suppressive molecules, and possess adhesive and anti-adhesive properties. They also act as major biological filters, promote angiogenesis, and bind, store, and deliver growth factors to target cells during normal development and in pathologic states (Iozzo and Murdoch, 1996).

1.2.4.1 Biosynthesis of proteoglycans

Maintenance of the structural and functional organisation of ECM components requires the correct targeting of proteins to their destinations. This is achieved by the delivery of newly synthesised PGs via the secretory pathway. Newly synthesised proteins in the secretory pathway

encounter several important components such as recognition elements for nascent protein insertion in to the endoplasmic reticulum (ER), molecular surveillance chaperones, endogenous enzyme for post-translational modifications, and routing molecules to deliver the finished protein to the cell exterior (Rothman, 1994).

The biosynthesis of PGs is a strict, complex, highly regulated process, which begins with the formation of the protein core. The biosynthesis of PG core proteins follows that of other proteins. The specific mRNA is transcribed from DNA, which is then used in polysomes as a template for the core protein. The majority of the core proteins are synthesised with one or more asparagine-X-serine (threonine) sites. These are important for the commencement of N-linked oligosaccharide synthesis, which are required for the correct folding of the protein. The N-asparagine linked oligosaccharide is covalently attached to the core protein by a lipid-linked oligosaccharide pre-cursor assembled on dolichol pyrophosphate (Kornfield and Kornfield, 1985). This dolichol moiety is set in the rough endoplasmic reticulum (RER) membrane with the oligosaccharide protruding into the lumen. Specific enzymes (transferases) move the entire oligosaccharide onto the carboxyamino side chain of an asparagine residue in the budding core protein, providing that the asparagine-X-serine (threonine) recognition sequence is available. The dolichol pyrophosphate is released with the formation of an N-glycosylamine bond. Then, the entire molecule is transported through the RER to the golgi body, where it undergoes the addition of sialic acid residues and GAG addition (Lohmander *et al.*, 1986). Those PGs whose primary sequences are known

to contain hydrophobic, N-terminal signals, are removed co-translationally. However mutant proteins with abnormal confirmations frequently fail to pass through the secretory pathway. Therefore, improperly folded proteins are retained in the ER lumen until folded properly or they are destined for degradation (Hammond and Helenius, 1995).

1.2.5 Proteoglycan families

There are several PG families: aggregating PGs, small leucine-rich repeat PGs (SLRPs), cell-surface PGs and orphans.

1.2.5.1 Aggregating proteoglycans

The aggregating PG family are also known as modular molecules integrating structural motifs such as an epidermal growth factor (EGF)-like domain, lectin-like domain, complement regulating protein-like domain, immunoglobulin folds and PG tandem repeats (Fosang and Hardingham, 1989). This family is further divided into two subfamilies; the hyalectans (hyaluronan- and lectin-binding PGs) comprising versican, aggrecan, neurocan, brevican; and the non-hyaluronan-binding PGs, which comprise perlecan and agrin (Iozzo and Murdoch, 1996).

One of the most studied large aggregating PGs is versican, which is expressed by numerous cells in a variety of connective tissues. It has been identified in osteoid predentine, cartilage, placenta, gingiva and tendon (Ruoslahti, 1989; Dours-Zimmermann and Zimmermann, 1994). This is a large extracellular CS PG, with a molecular mass over 1000kDa

(Zimmermann and Ruoslahti, 1989; Iozzo, 1998). The structure of the protein core, which is around 400kDa, consists of several distinct domains. There are two globular domains present at the N-terminus; the amino-terminal globular domain (G1) comprising an immunoglobulin-like domain and a PG tandem repeat form aggregates with hyaluronan. The selectin-like domain (G3) at the carboxy-terminus is composed of EGF-like, lectin-like, and complementary-regulatory protein-like domains. These terminal globular domains can interact with a variety of ligands, including other ECM proteins, as well as components of the cell surface. These diverse binding activities predict a central role for versican in the assembly of the pericellular matrix, which directly and indirectly participates in the regulation of cellular adhesion, proliferation, and migration. There are two extended regions, which bear CS chains (Neame and Barry, 1993), which are believed to serve an anti-adhesive function, and their hygroscopic properties make versican a large space-filling molecule (Yamagata *et al.*, 1993).

1.2.5.2 Small-leucine rich proteoglycans

SLRPs are typically compact proteins with alternating hydrophobic and hydrophilic amino acid residues that harbour multiple amino acid repeats with conserved leucine residues (Iozzo and Murdoch, 1996). Fisher *et al.* (1983) first identified SLRPs as small PGs in bone, which were subsequently found in the ECM of most tissues (Kresse *et al.*, 1994). There are currently nine members of this family with multiple sequence alignment classifying three main groups and three subfamilies. Class I comprise decorin and biglycan, showing the greatest amount of homology

and they are primarily involved in the maintenance and regulation of the ECM in tissues. Class II includes fibromodulin, lumican, keratocan, proline arginine-rich end leucine-rich repeat protein (PRELP), and osteoadherin (Sommarian *et al.*, 1998). Opticin, osteoglycan/mimecan, and epiphycan comprise class III (Iozzo, 1998; Scott *et al.*, 2004).

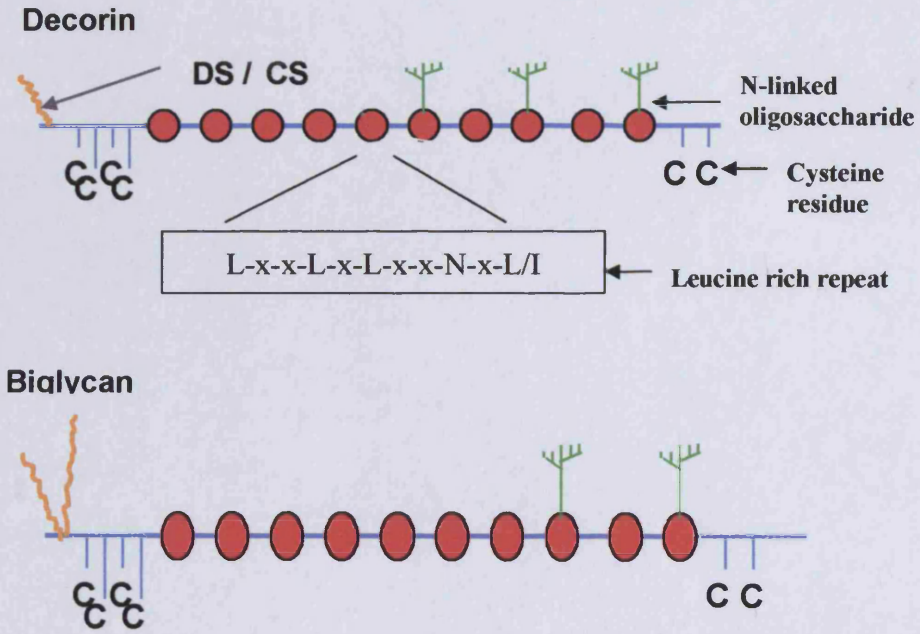
The molecular weight of the protein core for SLRPs ranges from 30-50kDa. The core protein consists of four main regions. There is an amino-terminal region, containing an attachment site for the negatively charged GAGs, a central domain with varying numbers of LRRs (7 to 24 leucine-rich tandem repeat pattern), which also contains consensus sites for the addition of 2 (for biglycan) or 3 (for decorin) N-linked oligosaccharides, and a carboxyl end region. Both the C- and N-terminal contain a number of cysteine residues, which create a loop, stabilised by intra-chain disulphide bonds (see figure 1.4). Variability is shown within the N-terminal due to the covalent attachment of GAG chains (Iozzo and Murdoch, 1996; Matsushima *et al.*, 2000; Neame and Kaye, 2000).

1.2.5.2.1 Decorin and Biglycan

Structure

Decorin and biglycan are extremely important members of the SLRP family, therefore they are the most widely studied. Both are widely distributed in connective tissues, including the periodontium (Kinsella *et al.*, 2004), and both are highly homologous, suggesting their formation by a relatively recent gene duplication event. The genes of these closely

Figure 1.4 General features of small leucine-rich proteoglycans, decorin and biglycan (modified from Iozzo and Murdoch, 1996).



related SLRPs are both composed of 8 exons, with highly conserved intron/extron transitions (Fisher *et al.*, 1991; Danielson *et al.*, 1993). Decorin and biglycan are characterised by the presence of signal peptides, which control intracellular trafficking by targeting the protein core into the ER, these signal peptides are cleaved co-translationally. There are also short propeptides, which are highly conserved and influence the attachment of GAG chains to decorin and biglycan core proteins (Hocking and McQuillian, 1996; Oldberg *et al.*, 1996). Generally, the propeptides are not present on the mature, secreted forms of decorin and biglycan. Regardless of the protein sequence homology, decorin and biglycan are distinct gene products and, to date, their genes are situated on at least two different chromosomes (McBride *et al.*, 1990; Danielson *et al.*, 1993; Gover and Roughley, 1995).

Until recently, the leucine-rich repeat motif was comparable to that of the ribonuclease inhibitor protein. It was thought that the SLRPs folded in a similar horseshoe confirmation, with the inner concave face proposed to provide the sites for protein-protein interactions and therefore responsible for many of the interactions that define SLRP function. X-ray crystallography demonstrated the leucine-rich repeat folded via alternating parallel α-helices and β-sheets, with the parallel β-sheets in the inner concave surface (Kobe and Deisenhofer, 1993; Weber *et al.*, 1996; Kinsella *et al.*, 2004). Contrary to this, biophysical analyses of decorin have recognized the potential presence of a dimeric form in solution. Low angle X-ray scattering data has suggested that the concave structures are involved in dimerisation, potentially making them unavailable for ligand

binding (Scott *et al.*, 2003; Scott *et al.*, 2004). However, Goldoni *et al.* (2004) disputed this, and proposed decorin is a monomer, and that dimerisation is artefactual. In 2006, Scott *et al.* reinforces the notion that decorin is a dimer and also provided evidence that biglycan is also a dimer in solution.

Decorin (also known as PG-1, PG-S1, and PG-40) is found in both mineralised and soft connective tissues, such as bone (Fisher *et al.*, 1983) and gingiva (Bratt *et al.*, 1992). There is one GAG attachment site at the fourth amino acid, serine (Fisher *et al.*, 1983). This is invariably substituted with CS in mineralised tissue (Franzen and Heinegard, 1985; Waddington *et al.*, 1989), or DS in non-mineralised tissue (Heinegard *et al.*, 1985). Similar DS substituted decorin and biglycan have been identified in pre-mineralised matrices of osteoid (Robey *et al.*, 1993) and pre-dentine (Waddington *et al.*, 2003). The molecular weight of the entire glycoconjugate is around 120kDa and the protein core is around 38-45kDa (Krusius and Ruoslahti, 1986).

Biglycan is enriched in bone and other non-skeletal connective tissues (Fisher *et al*, 1983). The molecular weight of the glycoconjugate approximately 200kDa, with a protein core of between 37-45kDa (Fisher *et al.*, 1987). The core protein comprises twelve LRR. At the N-terminus, biglycan has a short domain consisting of 20-30 amino acids that contain two GAG attachment sites, which are located on serine residues at position 5 and 11. As with decorin, the attached GAG depends on the source of

tissue, with CS found in mineralised tissue and DS in soft tissues (Krusius and Ruoslahti, 1986, Fisher *et al.*, 1989).

Functions

Decorin and biglycan exhibit a different pattern of expression and tissue location, which indicates diverse biological functions within the ECM of connective tissues. Both the core protein and the GAG chains of these SLRPs play important roles in the formation and regulation of connective tissue (Iozzo and Murdoch, 1996). There are two principle mechanisms of action thought to contribute to the biological functions of decorin and biglycan. First, they interact with other matrix proteins, including collagens, fibronectin, elastin, and thrombospondin, and influence matrix assembly and fibrillogenesis. Second, considerable evidence suggests that decorin and biglycan can modify the cellular response to growth factors, such as transforming growth factor ß (TGF-β) and EGF (Laine *et* al.2000; Kinsella *et al.*, 2004).

Decorin

Decorin interacts with several matrix molecules, and has a role in the organisation and alignment of the collagen fibril scaffolding. The association between collagen and decorin has been demonstrated by immuno-staining (Pringle *et al.*, 1990; Greve *et al.*, 1990), and by *in vitro* binding studies (Vogel and Trotter, 1987; Sugars *et al.*, 2002). The decorin protein core binds to the collagen at specific points along the fibril and the GAG chain regulates interfibrillar distances (Scott, 1986). Since the interaction between decorin and collagen type I is blocked by the reduction

of the disulphide bonds in decorin, the disulphide-bonded loops of decorin appear to be required for the interaction (Scott and Haigh., 1988). However, the extended LRR region in the central portion of decorin is also required for the interaction with collagen type I (Schonherr *et al.*, 1995).

The targeted disruption of decorin was the first *in vivo* demonstration of the importance of this SLRP for proper collagen fibrillogenesis (Danielson *et al.*, 1997). Mice harbouring a decorin gene disruption were viable, but they had fragile skin with markedly reduced tensile strength compared to their wild-type counterparts. Ultra-structural analysis of these mice revealed abnormal collagen morphology in skin and tendon, with coarser and irregular fibre outlines and sizes, due to abrupt increases and decreases in mass along their axes. In addition to modulating the morphology of the collagen fibrils, decorin deficiency also induces changes in collagen orientation. In the absence of decorin, the collagen fibrils in the periodontal ligament display a random orientation instead of their usual parallel orientation, leading to susceptibility of infection (Hakkinen *et al.*, 2000).

TGF-β is a principal growth factor identified in many tissues, including periodontal tissues (Yamaguchi *et al.*, 1990), regulating cell proliferation in the PDL (Hakkinen *et al.*, 2000), and has important roles in bone remodelling. Decorin is considered a potential anti-fibrotic agent because this SLRP binds TGF-β and potentially sequesters the growth factor. Over-expression of TGF-β in the rodent lung induces fibrosis that can be ameliorated by co-expression of decorin (Kolb *et al.*, 2001). However, the situation is complex, as it has been proposed that the activation of TGF-β

can either enhance or inhibit cellular growth. This phenomenon is dependent on cell type and it has been reported that TGF-β can inhibit epithelial cell growth and enhance mesenchymal cell growth (Sporn and Roberts, 1990).

It has been suggested that complexes between decorin and TGF-β result in the inactivation of this cytokine. Inactivation of TGF-β in Chinese hamster ovary cells resulted in the inhibition of cell growth (Yamaguchi *et al.*, 1990). Markmann *et al.* (2000) used MG-63 osteosarcoma cells to suggest that possible inhibitory effects of TGF-β by decorin may be due to the sequestration of TGF-β to the collagen matrix. An important consideration is whether the PG binds to a biologically active or inactive form of TGF-β. Hildebrand and co-workers (1994) suggested there is only an active form. The pro-peptide of TGF-β is cleaved prior to secretion but remains with the homodimer as latency associated protein (LAP), thus continuing the molecule in a biologically active state. This TGF-β/LAP complex can associate with different components within the ECM, suggested to be awaiting activation (Schonherr and Hausser, 2000).

Biglycan

The primary role of biglycan is similar to that of decorin, as this SLRP binds to a variety of ECM components and modulates cell function. Biglycan interacts with TGF-ß, with consequent up-regulation of biglycan (Kahari *et al.*, 1991), and this may be related to increases in biglycan expression with fibrotic responses such as, glomerulonephritis, pulmonary and hepatic fibrosis. Hausser *et al.* (1994) showed TGF-ß mediated an

increase in biglycan expression in MG-63 osteosarcoma cells, which was inhibited by the presence of decorin.

Despite the core protein sequence of biglycan sharing homology with decorin, its interaction with type I collagen is often debatable and appears to depend upon the ionic environment (Pogany *et al.*, 1994). Miosge *et al.* (1994) found collagen fibrils of human articular cartilage decorated with biglycan. Also, Schonherr *et al.*, (1995) provided evidence indicating a weak interaction between biglycan and purified type I collagen fibrils, suggesting binding is possibly dependent upon ionic strength. Biglycan also interacts with fibronectin (Schmidt *et al.*, 1991), suggesting this protein is important for matrix assembly. Biglycan may fulfil some tissue-specific functions in the vascular system too. Heparin co-factor II inhibition of thrombin is accelerated by intact biglycan (as well as decorin) (Whinna *et al.*, 1993).

A role for biglycan in mineral deposition has also been reported. Boskey *et al.* (1997) showed low concentrations of CS- and DS-biglycan induced apatite formation within a gelatin gel system, whilst DS-decorin had no effect on mineral accumulation compared to the PG free controls. Other studies using recombinant CS-decorin and CS-biglycan indicated that binding to hydroxyapatite crystal (HAP) was greater to biglycan than to decorin (Sugars *et al.*, 2003), thus leading to the proposal that biglycan plays a more predominant role in interacting with HAP and thus regulating mineral deposition (Sugars *et al.*, 2003). Waddington *et al.* (2003) investigated the role of SLRPs during bone formation. DS substituted

biglycan was expressed during cell proliferation, which ceased at early matrix deposition. Biglycan was re-expressed at the onset of mineralisation, substituted with CS. DS substituted decorin expression was associated with early matrix deposition, and continued to the mineralisation stages, substituted with CS. These results indicate the importance of GAG substitution in dictating the role SLRPs play in cell signalling, matrix formation and control of mineral deposition.

1.2.5.3 Cell surface proteoglycans

Cell surface PGs consist of mainly HS-containing PGs such as syndecan, fibroglycan, glypican and CD44 that have been ascribed important functions relating to cell-cell and cell-matrix interactions. Syndecan is around 33kDa and has three distinct domains; a 34 amino acid cytoplasmic domain, a hydrophobic trans-membrane and an extracellular domain containing five potential GAG attachment sites for CS or HS (Saunders *et al.*, 1989). Fibroglycan is an integral membrane PG with a core protein of around 48kDa. It displays widespread homology to syndecan as it has a cytoplasmic, trans-membrane domain (Bernfield *et al.*, 1992). However, fibroglycan has one potential site for N-glycosylation (Bourdon *et al.*, 1987).

1.2.6 Glycoproteins

A variety of glycoproteins have been identified as important ECM components, which differ in their molecular size, structures, functions and distribution. Their diversity and versatility is indicative of their importance in the ECM (Embery *et al.*, 2000).

Glycoproteins are a heterogeneous group of molecules that collectively contain a protein core with attached N- or O- linked oligosaccharide chains that can be branched or linear. Glycoproteins exhibit several structural features, including binding domains such as Arg-Gly-Asp (RGD), which allow interactions of various ECM proteins and cell binding sequences. Therefore, glycoproteins have been allocated roles in cell attachment, cell behaviour, and formation of an organised matrix.

The most significant glycoproteins within periodontal ligament and gingival connective tissue are the adhesive glycoproteins; fibronectin, vitronectin, laminin and thrombospondin. Osteonectin, elastin, and tenascin are also found diffusely within gingiva (Waddington *et al.*, 2001). Specific glycoproteins have also been reported in mineralised tissues, such as, bone sialoprotein, osteonectin, and osteopontin all contain highly conserved regions rich in acidic amino acids. The structurally significant highly negative charge on these molecules lends themselves to potential roles in controlling mineralisation and they provide the resilient hardness of bone (Bartold and Narayanan, 1998).

1.2.7 Catabolism of the extracellular matrix

The ECM is not static: it is remodelled constantly, which implies constant breakdown by proteases, most notably the family of matrix metalloproteases (MMPs) (Bosman and Stamenkovic, 2003). MMPs comprise a large family of zinc-dependent extracellular enzymes that share common structural features, particularly those regions involved in the

regulation of proteolytic activity. MMP-mediated proteolytic processing has several possible roles in dental tissue. MMPs, together with their inhibitors, may act in the process of synthesis and mineralization of dentin and osteoid matrix. They could also be responsible for tissue destruction in pathological injuries such as caries, attrition or pulpal inflammation (Nagase and Woessner, 1999).

Generally, MMPs play a central role in remodelling the ECM, and this is essential for embryonic development, morphogenesis and tissue remodelling. Members of this family include collagenases, gelatinises, stromelysins, and membrane-bound metalloproteinases (MT-MMPs) (Birkedal-Hansen *et al.*, 1993; Nagase and Woessner, 1999). With respect to PGs, remodelling of the ECM is a two-step process; initially there is cleavage at a specific site within the core protein at neutral pH. Then, endocytosis and intracellular digestion of the partially digested PG occurs by lysosomal proteinases. MMP1, 2, 3, 8 and 13 have been identified in the developing tooth tissues and proposed to be part of ECM turnover of the tooth tissues and surrounding area (Tsubota *et al.*, 2002; Maruya *et al.*, 2003).

Many of the secreted MMPs, such as collagenase-1 and –3, stromolysins 1, 2 and 3, and gelatinase–B are not expressed in normal, healthy, resting tissues or their production and activity are maintained at nearly undetectable levels. Some MMPs, including matrilysin, endometase, and MT5-MMP are expressed in healthy tissues, implying roles in homeostasis. In contrast, increased levels of MMP expression are observed

in repair or remodelling processes, in any diseased or inflamed tissue, due to matrix turnover, cell migration, and secreted factors (Pei, 1999; de Coignac *et al.*, 2000).

It is becoming increasingly clear that matrix remodelling is neither the sole, nor common, function of MMPs. They are, after all, proteinases, which can act on an array of proteins. Several studies have demonstrated various MMPs can modulate the activity of non-matrix proteins, such as matrilysin activating the pro-form of α-defensins (Wilson *et al*, 1999). Several MMPs, such as, collengase-1, stromelysin –1 and –3, and matrilysin, among others, directly modulate the activity of several growth factors and chemokines, such as TGF-β (Yu and Stamenkovic, 2000), TNFα (Haro *et al.*, 2000), and FGFs (Levi *et al.*, 1996). In addition, fragments of matrix proteins released by MMP-mediated proteolysis can act as chemoattractants for distant cells. Therefore, MMPs are not only proteinases of matrix catalysis but as extracellular processing enzymes involved in cell-cell and cell-matrix signalling (Parks and Shapiro, 2000).

Tissue inhibitors of metalloproteinases (TIMPs) are a family of secreted proteins that selectively inhibit MMPs (Chang and Werb, 2001). TIMPs regulate the breakdown of extracellular matrix components and play an important role in tissue remodelling and growth, in both physiological and pathological conditions. Four members of the TIMP family have so far been characterised, with all being able to inhibit most active MMPs. Of these, TIMPs 1, 2 and 3 have been implicated in early tooth morphogenesis (Sahlberg *et al.*, 1999; Woessner and Nagase, 2000).

There must be regulation and balance among the production of ECM molecules, the degradation of ECM molecules by MMPs and the inhibition by TIMPs for turnover of ECM molecules in alveolar bone, cementum and periodontal ligament.

1.3 Microbial adherence to and invasion through the extracellular matrix

The structure and function of the periodontium undergo severe stress when the matrix components of periodontal tissues are affected by inflammatory and fibrotic disease (Narayanan and Bartold, 1996). The clinical presentations of periodontitis include the loss of gingival epithelium attachment to the tooth root surface, exposure of the cementum and formation of periodontal pockets adjacent to the root surface. These conditions are symptomatic of PDL destruction and resorption of alveolar bone (Armitage, 1996).

Adherence of microorganisms to host tissue is regarded as the first step in the pathogenic processes leading to infection. In the healthy state, the ECM is not available for bacterial binding. However, exposure of ECM components following epithelial trauma may lead to colonisation and infection by microbes as they interact with various sub-epithelial ECM structures (Ljungh and Wadstrom, 1995; Trelstad, 2004).

PGs are present in the ECM and membranes of virtually all animal cells, and are among the first host macromolecules encountered by pathogenic agents (Menozzi *et al.*, 2002). The versatility of PGs and their GAG chains

capacity for multiple interactions with many different protein ligands gives them the ability to function as multi-purpose "glue" in cellular interactions (Rostand and Esko, 1997). Due to their wide distribution and ease of access, pathogenic bacteria appear to have evolved mechanisms to exploit PGs for their own functions (Menozzi *et al.*, 2002). A large array of glycoproteins, glycolipids, and PGs are known to be targets of bacterial adherence including; fibronectin, collagen, fibrinogen/fibrin, elastin, and laminin, as well as decorin and HS-containing PGs (Joh *et al*, 1999).

Following binding to ECM components, microorganisms may use them as a stronghold for propagating and spreading to other parts of the body (He *et al.*, 2004). Migration is a prerequisite for the spread of infectious agents. Direct or bridge molecule-mediated interactions occur between invaders and host ECM and cells, and may be crucial events for successful infection (Marino *et al.*, 2003).

Overall, the experimental evidence for the adherence of a range of microbes to host ECM proteins is compelling (Rostand and Esko, 1997), and streptococcal *spp.* have been examined in detail (see table 1.2). Decorin, bound to collagen has been shown to be utilised by pathogens for their entry and colonisation of tissues. The spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease, adheres to collagen-associated decorin but does not adhere directly to collagen types I or III (Brown *et al.*, 1999). The conformation of some ECM components, such as fibronectin and vitronectin, differs significantly when the glycoprotein is immobilised in tissues or on surfaces, as opposed to the soluble form.

Microbial adhesins such as Yad A of *Yersinia enterolitica* and P fimbriae of *Escherichia coli* bind immobilised but not soluble fibronectin. This may indicate that these adhesins only recognise an exposed domain when the fibronectin is immobilised in tissues (Westerlund *et al.*, 1991; Schulze-Koops *et al.*, 1993).

Cryptic or hidden sites within native ECM molecules may become activated or exposed upon disassembly or degradation of the ECM component structure. Degradative or proteolytic enzymes may be released by bacteria or by host tissue invading leucocytes. As a biological consequence, the ECM breakdown products may facilitate invasion of target cells by the bacteria (Marino *et al.*, 2003). There is evidence to show that *Trypanosoma cruzi* interacts with host ECM components producing breakdown products that play an important role in parasite mobilisation and infection (Marino *et al.*, 2003).

Adhesin	Approx. Mol. Mass (kDa)	Species	Substrate
sfbl family	70	*S. pyogenes* (group A), group G streptococcus	Fibronectin
sfbl, SOF	113	*S. pyogenes*	Fibronectin
FnbA	117	*S. dysgalactiae* (group C)	Fibronectin
FnbB	122	*S. dysgalactiae*	Fibronectin
Fnb	120	*S. equisimilis* (group C)	Fibronectin
FBP54	54	*S. pyogenes* M-type 5	Fibronectin, fibrinogen
SDH	*39*	*S. pyogenes*	Fibronectin, lyzozyme, myosin, actin
28-kDa antigen	28	*S. pyogenes*	Fibronectin
14-kDa protein	14	*S. anginosus*	Fibronectin
Laminin binding protein	145	*S. gordonii*	Laminin
Collagen adhesin	90	*S. mutans* UA140	Collagen
Collagen adhesin	57	*S. pyogenes* 6414	Collagen (type II)
GAG-BP	9	*S. pyogenes* M-type 12	Heparin, heparan sulphate

Table 1.2 Streptococcal protein adhesins recognise extracellular matrix components (modified from Jenkinson and Lamont, 1997).

1.4 *Streptococcus anginosus* group

The SAG is a collective name for a group of facultative, microaerophilic, Gram-positive cocci. Until recently, the SAG were also known as the *Streptococcus milleri* group and there is a long history of taxonomical confusion. The SAG were first described in 1906 as streptococci that fermented lactose, saccharose, and clotted milk. Later, Lancefield *et al* (1934) characterised these streptococci as possessing beta-haemolysis and the F antigen. In 1943, the species "*Streptococcus MG*" was proposed, which was a group of non-haemolytic (alpha haemolysis) streptococci that were bile resistant and fermented sialicic but not inulin or raffinose. Five decades have passed since Guthof (1956) coined the term *S. milleri* to describe non-haemolytic oral streptococcal species isolated from dental abscesses. However, Colman and Williams (1972) proposed that *S. milleri* consisted of both minute beta-haemolytic streptococci along with non-haemolytic streptococci. Subsequently, Coykendall *et al.,* (1987) proposed the unification of these streptococci into a single species. However, the term *S. milleri* was not accepted by taxonomists (Facklam, 2002).

Through DNA-DNA reassociation studies and ribotyping studies, supported by phenotypic characteristics, it is now regarded that the group consists of: *S. constellatus, S. intermedius* and *S. anginosus*, *S. constellatus* subsp. *constellatus* and *S. constellatus* subsp. *pharyngis.* These species are similar, but show various haemolytic, serological and physiological characteristics (Whiley *et al.*, 1990; Jacobs and Stobberingh, 1995; Whiley *et al.*, 1999; Facklam, 2002).

1.4.1 Commensal to pathogen

The SAG are frequently isolated from humans (Toyoda *et al*, 1995) and are regarded as commensals of mucocutaneous surfaces including the oral cavity, oropharynx, gastrointestinal tract and urogenital tract (Jenkinson and Lamont, 1997). However, members of the normal flora can become important pathogens (Fujiyoshi *et al*, 2001). Under the appropriate conditions, and in the presence of predisposing factors, such as trauma, diabetes, immunodeficiency, malignancy and prosthetic devices (Toyoda *et al*, 1995), previous 'harmless' bacteria can initiate both localised and systemic diseases (Hornef *et al.*, 2002).

1.4.2 SAG infections

In most purulent clinical infections, the SAG are isolated alongside obligate anaerobes. However, they can be isolated alone in pure culture, even after inoculation as part of a mixed culture (Lewis *et al.*, 1988). It has been suggested that the SAG may initiate infection and prepare the way for subsequent colonisation by anaerobic species. Furthermore, a degree of synergy between SAG and anaerobic bacteria has been proposed (Nagashima *et al.*, 1999).

In purulent infections, the SAG is usually isolated as part of a mixed infection usually in combination with Gram-negative strict anaerobes such as *Prevotella* species, *Fusobacterium* species, *Veillonella* species and *Eikenella* species (Van der Auwera, 1985; Shinzato and Saito, 1995). Shinzato and Saito (1995) investigated the synergy between strains of SAG and anaerobic bacteria in infectious disease. They infected mice with a

mixture of *S. constellatus* and *Prevotella intermedia.* When infected individually, the mice showed only 10% mortality compared to 60% when both organisms were present. Evidence from this study also suggested that metabolites of anaerobes act to boost the growth rate of the streptococcus *spp.* by acting as a barrier to any bactericidal action from the host Also, Kuriyama *et al.* (2000) showed that *S. constellatus* behaved synergistically in the presence of *Fusobacterium nucleatum.*

1.4.2.1 Oral infection

A dentoalveolar abscess usually develops by the extension of the initial carious lesion into dentine, and spread of bacteria to the pulp via the dentinal tubules. Once pus formation occurs, an acute or chronic abscess forms and may develop into focal osteomyelitis; or spread into the surrounding tissues (Samaranayake, 2001).

As mentioned, the association of SAG with dental abscess formation was first described by Guthof (1956). Fisher and Russell (1993) examined 45 samples from dental periapical abscesses and identified 15 as *S. anginosus* and one as *S. intermedius*. Okayama *et al.* (2005) examined the abscess-forming ability of native human dental plaque in mice and the microbial features of the infectious locus produced by the plaque. The microbial composition of pus was examined in 17 of 76 abscesses. Of these 17 cases, the majority of isolates belonged to the SAG. *S. anginosus* was the most frequently detected (76.5%), and *S. intermedius* (47.1%) and *S. constellatus* (35.3%) were predominant in abscess samples. Siqueira *et al.* (2002) studied the microbiology of primary root canal infections of 53 teeth. The

most prevalent bacterial species found were members of the SAG. With regard to asymptomatic lesions, the most dominant species were *S. intermedius* (11.5% of cases) and *S. anginosus* (7.7% of cases), whereas *S. constellatus* was the most common species within pus samples (25.9% of cases).

1.4.2.2 Non-oral SAG infections

The SAG are also associated with a range of other infections, including brain and liver abscesses, meningitis, infective endocarditis, obstetrical infections and neonatal sepsis. It has been found that *S. anginosus* is more frequently associated with abdominal and urogenital sites than the other two species, whereas *S. intermedius* was frequently isolated from purulent head and neck specimens and liver abscesses (Bantar *et al*, 1996). *S. constellatus* has been isolated from abdominal and respiratory tract infections (Whiley *et al.*, 1992).

Cardiothoracic infections

The SAG are significant pulmonary pathogens and are associated with thoracic infection, such as pneumonia, purulent empyema and lung abscess (Wong *et al.*, 1995). Thoracic infection resulting from secondary spread due to abscess formation elsewhere within the body has been reported, thus the SAG appear to have a propensity to spread haematogenously via the bloodstream (Molina *et al.*, 1991)

Microaerophilic streptococci account for 10-20% of anaerobic isolates recovered from properly obtained samples of pulmonary infections (Cade

et al., 1999). Wong *et al.* (1995) identified twenty-five cases of pulmonary disease caused by SAG, including 16 empyema, five lung abscesses, and four with both lung abscess and empyema. Predisposing factors were present in 80% of patients and included the following: pneumonia, periodontal disease, excess alcohol intake, previous thoracic surgical procedures, and malignancy. Recently, Woo *et al* (2004) reported SAG-associated cases of infective endocarditis caused by *S. anginosus*. Two out of the 6 cases had had previous oral intervention procedures, such as tooth extraction. However, limited data exists on infective endocarditis due to the SMG (Lefort *et al*, 2002).

Bacteraemia

In a prospective study of bacteraemia caused by SAG, Casariego *et al* (1996) observed 32 patients over a 7-year period. The majority (84.7%) of SMG bacteraemia cases were shown to be monomicrobial. The species isolated were *S. constellatus* (n=14), *S. intermedius* (n=14) and *S. anginosus* (n=4) of the sample population, 69% were male with a mean age of 64 years. One or more underlying diseases or factors were present in 47% of the patients, including neoplasia, diabetes mellitus, chronic alcoholism, chronic renal failure, glucocorticoid therapy and granulocytopenia. In eleven of these patients, a possible origin of infection was odontogenic infection. Other causes were biliary or gastrointestinal disease and/or abdominal surgery, observed in seventeen patients. Tsai *et al.* (2004) reported a case of *S. constellatus* bacteraemia that resulted in the rare complications of myocardial abscess and cerebritis.

Skin and soft tissue infections

Within direct skin and soft tissue injuries, SAG are generally isolated in mixed culture with other aerobic and anaerobic species. Humar *et al.*, 2002, reported three cases of necrotising soft tissue infection, resembling group A streptococcus disease, but which were caused by a β-haemolytic group G streptococcus belonging to the SMG. There was a severe underlying medical condition present in each case.

1.4.3 Pathogenic mechanisms of the *Streptococcus anginosus* group

The pathogenic mechanisms of the SAG are unclear. It is important to note that the outcome of the interaction between a mammalian host and an invading bacterium depends on interplay between a constellation of host defences and microbial virulence factors. These virulence factors include the ability to target and colonise an appropriate tissue surface by specific adhesion, produce toxins, and to resist the immune defences of the host (Klemm and Schembri, 2000).

1.4.3.1 Adherence

The SAG surface properties have been investigated in the context of adherence and surface binding. Importantly, any receptor-binding event may activate complex signal transduction events in the host cell that can have diverse consequences including the activation or alteration of innate host defences or the subversion of cellular processes facilitating bacterial colonisation or invasion. In addition, the binding event may also activate

1.4.3.1.1 Surface-associated proteins involved in adherence

Many bacteria possess adhesins on their surfaces that bind in a stereochemically specific manner to complementary molecules, or receptors, on the tissue surface. Most bacterial species express several types of adhesins (Gibbons, 1989). In many instances, adhesins assemble into hair-like appendages called pili or fimbriae that extend out from the bacterial surface. In other cases, the adhesins are directly associated with the microbial cell surface (Soto and Hultgren, 1999).

Willcox and Knox, (1990) studied the adherence characteristics of the SAG that are possibly associated with pathogenicity and compared them to type strains of other streptococci. They found that abscess related strains adhered to buccal epithelial cells to a greater extent than commensal strains. It was also reported in this study that the SMG were not readily aggregated by human saliva and exhibited low levels of binding to hydroxyapatite. Later, Willcox (1995) and Willcox *et al.* (1995a) reported that all SAG strains studied bound fibronectin via a cell-surface protein. These strains also bound to platelet fibrin or fibrin clots and fibrinogen, and members of the *S. constellatus* species produced thrombin-like activity. Subsequently, a fibronectin receptor from *S. anginosus* strain F4 was characterised as a 14-kDa protein, exhibiting specific binding.

Oral streptococci express a family of structurally and antigenically related surface proteins termed antigen I/II, which are capable of binding to soluble ECM glycoproteins and to host cell receptors. They are also responsible for co-aggregation with other microbes, interaction with

salivary glycoproteins and activation of monocytic cells (Jenkinson and Demuth, 1997; Petersen *et al.*, 2001). *S. intermedius* antigen I/II proteins have been investigated with regard to adhesive properties. Petersen *et al.* (2002) demonstrated antigen I/II of *S. intermedius* was not involved in adhesion to a salivary film under flowing conditions. Also, adhesion to collagen types IV or I was negligible whereas binding to human fibronectin was a common function. Hydrophobicity was unaltered, as measured by water contact angles and zeta potentials (Petersen *et al*, 2002).

Allen *et al.* (2002) studied the adherence of *S. intermedius* and S. *anginosus* adherence in the initial stages of native valve endocarditis. Using an *in vitro* endothelial monolayer model to mimic *in vivo* areas of exposed basement membrane, components of the ECM were utilised as receptors for bacterial adhesion. They demonstrated binding of both SAG species to laminin, fibronectin, fibrinogen, and collagens type I and IV. *S. anginosus* (an IE-associated blood isolate) exhibited a higher level of adherence to all of the matrix components and laminin in particular.

1.4.3.2 Enzyme and toxin production

Production of tissue destroying enzymes and extracellular products have been observed as virulence factors in these bacteria (Willcox *et al*, 1995b; Jacobs and Stobberingh, 1995).

Strauss and Falkow (1977) described a strain of *S. intermedius* from a case of infective endocarditis that produced several extracellular enzymes with

proteolytic activity. The growth of this strain, in conditions mimicking a fibrotic heart lesion, resulted in a 4–8 fold increase in proteinases compared with controls. More recently, intermedilysin (ILY) is a human specific cytolysin, and its distribution amongst the SAG has been studied. Nagamune *et al.* (1996) first recognised ILY as a cytotoxin responsible for haemolysis caused by the SAG. PCR and Southern hybridisation specific for the ILY gene revealed the toxin gene exists only in *S. intermedius* and there is no homologue to the toxin gene in *S. constellatus* or *S. anginosus*. ILY production was found to be greater in isolates from deep-seated infections, such as brain and liver abscesses (6.2- to 10.2-fold, respectively), than in commensal strains from dental plaque or peripheral infection sites. This toxin also affects human red blood cells and causes the lysis of phagocytes. The authors suggested that ILY could be the pathogenic or triggering factor of significance in inducing deep-seated infections with *S. intermedius* (Nagamune *et al.*, 2000).

Other bacterial enzymes include glycosidases, which remove terminal sugar residues from naturally occurring polysaccharides and glycoproteins (Whiley *et al*, 1990). Glycosaminoglycan (GAG) depolymerising enzymes such as hyaluronidase (HAase) degrade connective tissue components. HA contributes to the elastic properties of soft tissues, and is present in many tissues including gingivae tissues and heart valves. HAase production degrades HA and has been detected in *S. intermedius* and *S. constellatus*, with increased levels of activity in the former (Takao *et al.*, 1997). This enzyme is valuable in the discrimination of these species from *S. anginosus*, which is unable to degrade HA (Whiley *et al*, 1990). Shain *et*

al. (1996) suggested that the role of HAase was as a proliferator for the SAG in pyogenic infection due to its destruction of the extracellular matrix whilst releasing carbohydrates useful for bacterial metabolism. *S. intermedius* spreads very rapidly through the soft tissues, presumably because of its ability to produce HAase and streptokinase to break down ground substance and fibrin (Green *et al*, 2001). Poole and Wilson (1979) showed a positive association between HAase production in SAG and haemolysis. HAase production was most frequent in β-haemolytic strains of Lancefield groups A, C and F. Strains of SAG isolated from systemic abscesses and dental plaque produced type IV HAase. Unsworth (1989) showed significant HAase production in SAG strains isolated from abscess sites, and strains from sites of deep seated infection had a higher frequency of HAase production than strains isolated from surface sites. Takao (2003) cloned and expressed hyaluronate genes and reported HAse is possibly anchored to the cell surface in *S. intermedius* and *S. constellatus*.

Chondroitin sulphate depolymerase (CSase) is capable of breaking down the GAG CS in tissue structures to release carbohydrates that can be utilised metabolically, and contribute to the "spreading" of microorganisms. Homer *et al.* (1993) reported that strains of *S. intermedius* produced CSase when the organisms were cultured in the presence of the GAG, suggesting that the enzyme is inducible. Jacobs and Stobberingh (1995) also examined the link between the SAG and CSase production. They demonstrated that only certain strains of *S. intermedius* and *S. constellatus*, isolated from suppurative infection, produced the enzyme. Shain *et al.*, (1996) observed *S. intermedius* readily manufactured

an A form of the enzyme when grown in the presence of chondroitin sulphate A. The production of the enzyme became inhibited in the presence of glucose, as this was more easily broken-down.

Bacterial sialidases are enzymes that cleave terminal non-reducing sialic acid residues from sialoglycoconjugates. This mechanism may represent a virulence factor in the exposure of cryptic carbohydrate binding sites on host cells to which invading organisms may attach (Gibbons *et al.*, 1990). The action of this enzyme may allow the breakdown of the mucosal defence barriers of the host in addition to freeing sialic acid as a fermentable carbohydrate (Corfield, 1992). Beighton and Whiley (1990) detected sialidase activity in all three species of the SAG. However, they did not detect any activity in many other species of oral streptococci such as *S. parasanguis*, *S.salivarius*, *S.vestibularis*, *S. sanguis*, *S. mutans*, *S. mitis* or *S. sobrinus*.

The production of nucleases (DNase and RNase), collagenase and gelatinase is documented for the SAG (Steffen and Hentges, 1981; Marshall and Kauffman, 1981). Most bacteria produce nucleases (DNase and RNase) in order to defend themselves against other species. Jacobs and Stobberingh, (1995) established that of the three SAG species, *S. intermedius* and *S. constellatus* produce nuclease enzymes and that DNase is only detected in those strains isolated from sites of infection. In 1998, Dedkov and Degtyarev isolated novel restriction endonucleases from bacteria of the microflora of human teeth. The main producers were *S. milleri* along with *Actinobacillus suis*. The new enzymes were

isoschizomers of the restriction endonucleases R. HphI, R. SauI, R. NheI, R. MboI and R. SwaI

1.4.3.3 Avoidance of the host response

The way in which a host responds to bacteria is determined by the nature and control of both the innate and adaptive responses. The host response to bacteria and their activities is dominated by the heavy infiltration of neutrophils into the surrounding tissues, which contributes both to the elimination of bacteria and the destruction of host tissue. The innate immune response may be responsible for a significant amount of damage to surrounding tissue. This is via the release of inflammatory mediators and cytokines, which leads to increased inflammation (Seymour and Taylor, 2004).

SAG exhibit a striking propensity for abscess formation (Wanahita *et al*, 2001) and systemic illness, suggesting they are capable of withstanding the host response in order to disseminate within the body (Gossling, 1988). This ability may be related to the pathogenicity of the SAG and hereby follows a summary of the potential mechanisms underlying this important property (Willcox, 1995; Corredoira *et al*, 1998).

Some streptococcal surface structures and extracellular products have been found to possess immuno-protective properties (Edwards *et al.*, 2003). As well as adherent properties, antigen I /II has been shown to display antibody suppressive traits (Jenkinson and Demuth, 1997). Evidence has emerged to show that some strains of SAG have the ability

to produce extracellular products with the ability to reduce the effectiveness of the immune system. Higerd *et al.* (1978) were the first to identify an extracellular product named EP–Si. This protein suppressed fibroblast formation and altered lymphocytic response *in-vivo*. Arala–Chaves *et al.* (1979) expanded on this by examining the extract and found an immunosuppressive non–cytotoxic protein named F3' EP–Si. This protein could induce T–cell suppressor lymphocytes and possess B–cell mitogenic activity (Arala–Chaves *et al.*, 1981). Limia *et al.* (1992) re-named this protein P90. Their studies indicated that treating mice with P 90 made them 50-fold more vulnerable to infection with strains of *S. intermedius*. B–cells obtained from these mice were less able to respond to antigenic challenge, and furthermore, T–cells actively suppressed the specific immune response produced by B–cells. However, a much higher concentration of P 90 was utilised in this study, than *S. intermedius* would actually be able to produce *in vivo*.

Virulent SAG strains appear highly resistant to phagocytosis by human polymorphonuclear neutrophils (PMNs). Wanahita *et al.*, (2002) examined the interaction of SMG with human PMNs. More specifically, the pathogenicity of *S. constellatus* in pulmonary infections was investigated in mice in correlation with the phagocytic killing of the microorganisms by PMNs. Histological inflammation score of the lungs in mice inoculated with RZYK001 (virulent strain obtained from bronchoalveolar fluid of a pneumonia patient) was significantly higher than in mice inoculated with RT4303 and RT6002 (non-virulent strains obtained from saliva of healthy adults). This suggests a possible pathogenic mechanism of *S. constellatus*,

as a virulent strain is less likely to be killed than a non-virulent strain, predisposing it to survive in infection sites (Toyoda *et al*, 1995). Okayama *et al.* (2005) demonstrated all three SAG species, isolated from dental plaque induced abscesses in mice, possessed high anti-phagocytic capacities against human PMNs in the presence of complement. This was in comparison to a strain of *Staphylococcus aureus*, a well-known abscess-inducing pathogen, which was killed. Furthermore, *S. intermedius* produces the human erythrocyte-lytic enzyme ILY, which has the capability to lyse human PMNs (Macey *et al.*, 2001). Such a direct effect of ILY could support bacterial survival by reducing the number of intact PMNs.

Capsules are a well-established virulence factor as encapsulated bacterial cells have increased resistance to cellular phagocytosis and offer a degree of protection against serum antibodies when in the host (Kanamori *et al.*, 2004). A small number of SAG have been isolated with capsules, although their structure is unknown. Brook and Walker (1985) documented that encapsulated SAG strains induced pyogenic abscesses when injected into mice, while strains without capsules required the presence of a capsulated organism to do so. In addition, Lewis *et al.* (1988) demonstrated that encapsulated SAG strains are capable of inducing abscesses in pure culture, and demonstrated the isolation of encapsulated strains from dentoalveolar abscesses. Recently, Kanamori *et al.* (2004) studied the pathogenicity of encapsulated strains of the SAG by examination of the ability to cause subcutaneous abscesses in mice and by phagocytosis and phagocytic killing by human PMNs of the bacteria. Three encapsulated

isolates from patients with pneumonia or lung abscesses induced abscesses in mice, whereas only 2 of 20 unencapsulated isolates from patients with lung abscess or phagocytosis and phagocytic killing of PMNs to a greater extent than the unencapsulated forms.

Platelet aggregation is important in the induction and progression of endocarditis and, maybe in abscess formation, whereby bacteria are localized in a platelet / fibrin clot (Willcox *et al.*, 1994). Willcox *et al.* (1994) reported that Lancefield group C members of the SAG aggregated human platelets. The platelet-interacting substance on the surface of SAG appeared to be proteinaceous as digestion of cells with protease inhibited aggregation, whereas treatment with lipase, periodate or antisera to Lancefield group C polysaccharide had no effect. Willcox (1995) also observed certain strains of the SAG produced thrombin. Thrombin converts fibrinogen to fibrin to produce blood clotting, therefore the SAG may produce clotting to seal off areas of systemic colonisation and leads to a platelet/fibrin clot, effectively producing a pyogenic abscess structure, which in turn serves to offer protection from the circulating host defences.

1.5 Aims of thesis

The above review has highlighted the importance of the ECM components, such as PGs and GAGs, in both healthy and diseased periodontium. Furthermore, the numerous studies on the potential adherence mechanisms and virulence factors of the SAG, suggest that periapical abscesses caused by these bacteria is complex, therefore a better understanding of host-SMG interactions is essential. The aims of this

thesis were investigate the disease process caused by SAG, from initial adherence to host ECM components through to the potential SAG virulence factors that may affect, or be affected, by certain cellular components of host tissues.

The specific aims of this thesis were to;

1) investigate the interactions of SAG, with characterised recombinant decorin and biglycan from a mammalian protein expression system and mixed decorin and biglycan from ovine tissues, utilising real-time bio-specific interactions;

2) observe the effects of SAG on periodontal ligament fibroblasts and endothelial cells;

3) observe the effects of ECM on SAG.

CHAPTER TWO

Chapter 2

Isolation, purification and characterisation of the small leucine-rich proteoglycans, decorin and biglycan.

2.1 Introduction

The SLRPs are a family of ECM molecules characterised by the presence of tandem leucine-rich repeats flanked by disulphides. Within this family, variation is brought about through the number of leucine-rich repeats, amino acid substitutions, and differences in glycosylation (Iozzo, 1997). Most investigations have focused upon the Class I SLRPs decorin and biglycan. These PGs have been ascribed many roles within the ECM. Studies involving decorin have highlighted numerous potential binding domains, such as those for collagen and TGF-β, which are important in matrix formation, cell proliferation, and differentiation (Hildebrand *et al.*, 1994; Schonherr *et al.*, 1995, 1998; Kresse and Schonherr, 2001). Biglycan has not been as widely studied as decorin, but it has been ascribed roles in cellular signalling and mineralisation. The role of biglycan in osteogenesis is apparent (Xu *et al.*, 1998), where it also binds TGF-β and regulates crystal growth (Hildebrand *et al.*, 1994). The use of knockout models has highlighted the importance of both decorin and biglycan in connective tissue formation. The single decorin knockout mouse showed fragile skin and reduced tensile strength (Danielson *et al.*, 1997) and the biglycan knockout mouse possessed an osteoporosis-like phenotype, representing a decrease in bone mass that correlates with the lack of biglycan (Xu *et al.*, 1998). The double knockout mouse produced a

severe phenotype, one that could not breed. The bone phenotype was more severe than for the single knockout mice with dramatic changes in collagen fibril formation (Corsi *et al.*, 2002).

Recent advances in molecular techniques have allowed for the genetic analysis of PGs, including decorin and biglycan. These analyses have provided valuable insights into the functional regulation of PGs. Despite representing different gene products, the primary structure of decorin and biglycan are very closely related. In humans, both SLRPs are encoded by genes composed of eight exons and seven introns. Decorin is mapped to chromosome 12q23 (Vetter et *al.,* 1993) and biglycan is located on Xq28 (Fisher *et al.*, 1991). Of significance in functional studies is that decorin and biglycan genes are highly conserved across different species (Scholzen *et al.*, 1994).

Structural and functional studies of PG domains can be facilitated by the isolation and purification of native PG. Most current procedures for isolation of PGs from mineralised and soft tissues require sequential dissociative extraction and fractionation techniques. Previous investigations involving the extraction of ECM components include anion-exchange and affinity chromatography (Fisher *et al.*, 1987, Waddington *et al.*, 1989, Waddington and Embery, 1991). Due to the highly anionic nature of PGs, anion-exchange chromatography has been extensively used in their purification, particularly for mineralised tissues. The technique relies upon the use of chaotrophic agents and strict anionic conditions to

facilitate the separation of these proteins. A number of further purification procedures have been used as a final purification stage, to ensure the purity of the isolated protein species. These procedures include a second anion exchange chromatography step, gel filtration chromatography or hydroyapatite chromatography. PGs, including decorin and biglycan, have been isolated from many tissues by chromatographic methods, including ovine gingiva and alveolar bone (Moseley, 1996), pig knee menisci (Scott *et al.*, 1997), and predentine, the transitional phase at the predentine-dentine interface, and mineralised dentine (Milan *et al.*, 2005).

In this study, ovine tissues were used to isolate and purify PGs from alveolar bone and gingiva. The choice sample tissue is important in PG analyses. Domestic sheep suffer a naturally occurring form of periodontitis called 'broken-mouth', which can cause the premature spontaneous exfoliation of teeth (Spence *et al.,* 1988). The oral microbial flora in periodontally diseased sheep from many countries is consistent with findings from humans with periodontitis (McCourtie *et* al., 1990; Dreyer and Basson, 1992). Duncan *et al.* (2003) were able to demonstrate that clinical, microbiological (*P. gingivalis*, *P. intermedius* and *F. nucleatum*) and serological characteristics (increased IgG titres to bacterial strains) of sheep oral infections have important similarities to human oral infections, thereby suggesting ovine tissue sources can be utilised for human research.

However, components with similar structures within tissues, such as decorin and biglycan, have proved difficult to isolate alone and hence mixed protein preparations are often obtained, although they have been reasonably well separated from collagen, glycoproteins and phosphoproteins. Hydrophobic separation of these PGs has been unsuccessful for mineralised tissues in past studies (personal communication with R. J. Waddington). Therefore, an alternative method to tissue extraction is the use of recombinant protein expression systems. These systems overcome the problem of mixed protein preparations so relatively pure decorin or biglycan preparations inclusive of the GAG chains can be obtained (Hocking *et al.*, 1996; Ramamurthy *et al.*, 1997; Sugars, 2001). The development of such systems has allowed the synthesis of proteins with an epitope tag, usually six consecutive histidine residues. The purification of these recombinant proteins can therefore utilise nickel affinity chromatography, which is a rapid procedure, achieving a highly purified protein in a single step (Crowe *et al.*, 1996). Many studies have used recombinant expression systems to study the interactions of decorin and biglycan with other ECM components, mainly the interactions with collagen type I (Schonherr *et al.*, 1995; Ramamurthy *et al.*, 1996; Krishnan *et al.*, 1999; Sugars, 2001).

This chapter describes the procedures used for the extraction, purification and characterisation of SLRPs from ovine alveolar bone and gingival tissues. It also describes the purification and characterisation of a previously developed recombinant mammalian protein expression system

(Sugars, 2001), to ensure this system is sustaining the production of large quantities of highly pure intact decorin and biglycan. These purified PGs will be utilised to examine specific interactions with the SAG, studied through biomolecular interaction analysis, in order to produce a successful model for studying host-microbial interactions in relation to infection caused by SAG, primary interactions which could progress into periapical abscess formation.

2.2 Materials and methods

2.2.1 Source of periodontal proteoglycans

Nine-month-old Welsh Mule breed sheep, which had been slaughtered at a local abattoir within the previous 1-2hr, were decapitated and the heads collected from the abattoir on request. The heads were stored on dry ice (BOC Gases Ltd., Surrey, UK) on the journey to the Cardiff Dental School and then stored at -20°C, until required for dissection. The gingiva was dissected 1-2mm below the gingival-tooth junction from around the upper and lower dentition and sliced into smaller pieces with a scalpel. The ovine gingival tissue exhibited no signs of gingival recession or inflammation and therefore was regarded as relatively healthy. The molar and premolar teeth were extracted from both the maxillae and mandibles using a dental drill with a circular saw, followed by excision with a surgical chisel and hammer. The alveolar bone was removed from around the roots of the extracted teeth and from the immediate areas around the sockets of these teeth using a surgical chisel and hammer. All the alveolar bone and gingival tissue collected was stored at -20°C until required.

2.2.2 Isolation of non-collagenous extracts from alveolar bone

The PGs were isolated from the ovine alveolar bone according to the method of Waddington and Embery (1991). Non-collagenous material was removed from small alveolar bone pieces with 4M guanidinium chloride ($GuCl_2$), 0.05M sodium acetate, pH 5.9. The cleaned alveolar bone was successively washed twice in ethanol, to dehydrate the tissue, followed by

diethyl ether for 10min, to remove lipids. The alveolar bone pieces were left at room temperature for the residual solvent to evaporate.

The alveolar bone was immersed in liquid nitrogen and powdered in an oscillating ball mill. The powdered bone was placed in dialysis sacs (Scientific Instrument Centre Ltd, Hampshire, UK), together with 10% EDTA (trisodium salt) solution, pH 7.45. The dialysis sacs with a molecular weight cut off of 12-14kDa were suspended in 5l of 10% EDTA solution, pH 7.45, containing the protease inhibitors benzamidine hydrochloride (5mM), iodoacetic acid (1mM) and N-ethylmaleimide (5mM), and left at 4°C for 13 days to demineralise, with 2 changes of the EDTA solution. EDTA was removed from the sample by exhaustive dialysis against 5l of double distilled water (ddH_2O) with protease inhibitors (as above) for 4 days, changing the ddH_2O twice a day, and a final day of ddH_2O with no protease inhibitors. The demineralised bone was recovered by lyophilisation.

The crude non-collagenous tissue components containing the PG were extracted into 4M $GuCl_2$, 0.05M sodium acetate buffer, pH 5.9, containing protease inhibitors (as above), at 4°C for 48h under constant agitation. The extract was centrifuged at 1800g for 15 min. The supernatant, containing the non-collagenous proteins, was recovered and dialysed against ddH_2O with protease inhibitors (as above) followed by 1 day with ddH_2O only and then recovered by lyophilisation.

2.2.3 Isolation of non-collagenous extracts from gingival tissue

The gingival tissue was briefly washed in phosphate buffered saline (PBS) then sequentially washed twice in ethanol, followed by diethyl ether for 10 min and left at room temperature for the remaining solvent to evaporate. The non-collagenous gingival tissue components were extracted into 4M $GuCl_2$, 0.05M sodium acetate buffer, pH 5.9, containing protease inhibitors (as in section 2.2.2) at 4°C for 48h under constant agitation. The extract was centrifuged at 1800g for 15 min and the supernatant, containing the non-collagenous proteins, was dialysed against ddH_2O followed by 1 day with ddH_2O only, and then recovered by lyophilisation.

2.2.4 Purification and identification of proteoglycans from ovine and gingival tissue extracts

2.2.4.1 Q-Sepharose Anion Exchange Chromatography

The crude alveolar bone and gingival tissue non-collagenous protein extracts were dissolved in 7M urea, 0.05M sodium acetate buffer, pH 6.8, at a concentration of 10mg/ml. Samples (1ml) of the dissolved tissue extracts were applied to a Q-Sepharose column (Sigma), integrated into a fast performance anion exchange chromatography system (Amersham Pharmacia Biotech, UK), and eluted with 7M urea, 0.05 M sodium acetate buffer, pH 6.8. The bound material was selectively eluted with an increasing linear sodium chloride gradient to a concentration of 1M, at a rate of 2ml/min. The eluant was continuously monitored at 280nm and 4ml fractions were collected over a 240ml period. Larger fractions were pooled according to the protein peaks identified in the 280nm profile.

The pooled fractions were dialysed against ddH_2O with protease inhibitors (as in section 2.2.2) and recovered by lyophilisation. Samples were stored at -20°C until required. Those fractions containing PG were characterised using dot blot analysis for CS (section 2.2.4.2), and SDS-PAGE (section 2.2.4.3) followed by silver staining (section 2.2.4.4).

2.2.4.2. Immuno-identification of PG-rich fractions by glycosaminoglycan dot blot analysis

To determine the PG-rich fractions obtained following chromatography, each pooled fraction was subjected to dot blot analysis. Pooled protein fractions were examined by dot blot analysis to quickly determine those samples that contained the CS GAG chains. 1µl of each of the pooled fraction samples (10mg/ml) were directly dotted onto a PVDF membrane (Hybond™ C, Amersham Pharmacia Biotech). The membrane was blocked in 10% non-fat milk (Marvel™), in TBS (10mM tris-HCl, pH 7.4 and 0.9% sodium chloride), for 30 min to prevent non-specific binding. Subsequent incubation in mouse anti-CS-56 (detailed in table 2.1) diluted in 3% non-fat milk in TBS for 30 min, was followed by three 5min washes of TBS. The membrane was subsequently incubated with a secondary antibody, anti-mouse IgM (detailed in table 2.1), conjugated to alkaline phosphatase, diluted in 3% non-fat milk in TBS, for 1 h. The membrane was washed for a further three times in TBS, 10min each wash.

Immunoreactivity was visualised by incubation of the membrane with NBT/BCIP chromogenic substrate (Promega). Briefly, the membrane was submerged with 10ml alkaline phosphatase buffer (100mM Tris, 100mM

sodium chloride, 5mM magnesium chloride, pH 9.55) containing 66µl NBT substrate (50mg/ml nitroblue tetrazolium in 70% dimethylformamide) and 33µl BCIP substrate (50mg/ml 5-bromo-4-chloro-3-inodyl phosphate in dimethylformamide) (Promega). Dark bands were allowed to develop (10-20min) and the reaction stopped by washing in ddH_2O. Membranes were air-dried.

2.2.4.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was utilised to determine the molecular weight of the purified PG and to assess the purity of the samples. This technique was performed on the Phast™ System (Amersham Pharmacia Biotech), which provided a rapid separation of small sample volumes, providing highly reproducible and highly sensitive results.

PG samples were lyophilised and dissolved to a concentration of 10mg/ml in dissociating sample buffer (0.062M tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-β-mercaptoethanol, 0.002% bromophenol) and samples were heated to 100°C for 5min.

3cm^2 preformed 4-15% gradient gels (Amersham Pharmacia Biotech) were positioned horizontally onto 70µl ddH_2O on a separation bed, pre-cooled to 16°C. SDS buffer strips (Amersham Pharmacia Biotech) were placed into the anode and cathode compartments and 1µl samples were applied to the applicator comb and inserted into the applicator arm. Each gel

contained the separation of a molecular weight marker (Sigma Chem. Co.) containing carbonic anhydrase (29kDa), ovalbumin (45kDa), bovine serum albumin (66kDa), phosphorylase B (97.4kDa), β-galactose (116kDa) and myosin (205kDa). Two separation phases were carried out, an initial low output current (100V, 1mA, 1W, 4Vh), followed by a higher output step (250V, 10mA, 3W, 66Vh).

2.2.4.4 Silver staining

Silver staining of polyacrylamide gels is a highly sensitive visualisation technique that allows for the detection of protein in the nanogram range. A commercially prepared Phast System Silver Stain Kit (Amersham Pharmacia Biotech) was used for this procedure. Gels were washed with 10% ethanol / 5% acetic acid for 6min, with a change of solution after 2min. The gels were transferred to fixing buffer (4% background reducer (described below), 0.05% glutaraldehyde, 28.5% ethanol, 1.5% isopropanol, 3% sodium acetate, 56% ddH_2O) for 6min and then returned to ethanol / acetic acid solution for 8min, with the solution changed after 3min. Gels were rinsed twice in ddH_2O (2min each wash) before placing in 0.4% silver nitrate solution for 6.5min. Two subsequent washes 30sec washes with ddH_2O were followed by incubation in developer solution (0.013% formaldehyde in 2.5% sodium carbonate) for 1min. Gels were placed in fresh developer until bands appeared. Development was stopped by replacing the solution with background reducer (0.016% sodium thiosulphate, 0.355% Tris) for 2min. Gels were then fixed in 2% glycerol for 1hr and left to air dry.

2.2.4.5 Resource-Q Anion Exchange Chromatography

Previous studies have indicated that PGs co-elute with other matrix proteins such as soluble collagens and α_2HSG protein (Sugars, 2001). To further purify the PGs from any remaining anionic glycoconjugate species, the separated alveolar bone and gingival PG rich fractions were redissolved in 7M urea, 0.05M Tris-HCl buffer, pH 6.8, to a concentration of 5mg/ml. 1ml samples were applied to a Resource Q column (Pharmacia) integrated into a fast performance anion exchange chromatography system (Pharmacia). Unbound material eluted at room temperature with 7M urea, 0.05M Tris-HCl buffer, pH 6.8. The bound material was removed with an increasing linear sodium chloride gradient to a concentration of 2M. The eluant was continuously monitored at 280nm and 2ml fractions were pooled over a 35ml period. Larger fractions were pooled according to the 280nm profile produced. The samples were then dialysed against ddH_2O (as in section 2.2.2), recovered by lyophilisation, and PG-rich fractions identified through dot blot analysis (section 2.2.4.2) and SDS-PAGE analysis (section 2.2.4.3) followed by silver staining (section 2.2.4.4).

Table 2.1. Antibodies utilised in immuno-detection of ovine gingival and alveolar bone proteoglycans, recombinant proteoglycans and chondroitin sulphate glycosaminoglycan

Primary antibody	Epitope		Secondary antibody	Reference
Anti-decorin **LF-113** (Recombinant decorin)	Raised against sequence IIPVDPDNPLIMC located in the N-terminal of mouse decorin		Goat anti-rabbit IgG whole molecule conjugated to horse radish peroxidase/ alkaline phosphatase	Fisher *et al.*, 1995
Anti-decorin **LF-122** (Ovine decorin)	Recombinant protein including propeptide		Goat anti-rabbit IgG whole molecule conjugated to horse radish peroxidase/ alkaline phosphatase	Fisher *et al.*, 1995
Anti-biglycan **LF-106** (Recombinant biglycan)	Raised against sequence VPDLDSVTPTFS located in the N-terminal of mouse bilycan		Goat anti-rabbit IgG whole molecule conjugated to horse radish peroxidase/ alkaline phosphatase	Fisher *et al.*, 1995
Anti-biglycan **LF-51** (Ovine biglycan)	Raisd against sequence GVLDPDSVTPTYSA-[BSA]		Goat anti-rabbit IgG whole molecule conjugated to horse radish peroxidase/ alkaline phosphatase	Fisher *et al.*, 1989
Anti-chondrotin sulphate **CS-56**	Monoclonal antibody specific for CS-containing PG		Goat anti-mouse IgM conjugated to alkaline phosphatase	Anvur & Geiger, 1984

2.2.5 Isolation of recombinant proteoglycans

A previously established HeLa cell line (a human epithelial carcinoma cell line) transfected with vectors carrying complementary DNA (cDNA) for either decorin or biglycan were used. Briefly, murine cDNA for decorin and biglycan were cloned into a mammalian protein expression vector, pcDNA3.1 myc/his, followed by liposomal transfection into the HeLa cells. The vector facilitated the expression of either recombinant decorin or biglycan synthesised with a fusion of six-histidines (His-tag), attached to the C-terminus (Sugars, 2001).

HeLa cells were cultured in Eagles Minimum Essential Medium (EMEM) with Earles Balanced Salt Solution (EBSS), supplemented with 2mM glutamine, 1% antibiotic/antimycotic solution and 2% foetal bovine serum (FBS). The cells incubated at 37°C, in 5% carbon dioxide (CO_2), with the media changed every two days, retained and stored at -20°C until required.

2.2.5.1 Purification of recombinant proteoglycans by poly-histidine tag nickel affinity chromatography

The presence of the His-tag allowed for purification by nickel affinity chromatography. HiTrap chelating 1ml columns (Amersham Pharmacia Biotech) were used to purify recombinant decorin and biglycan directly from tissue culture media. The lyophilised sample was reconstituted to a concentration of 10mg/ml in 0.02M sodium phosphate buffer, pH 7.4 containing 0.5M sodium chloride.

HiTrap columns were prepared for use by washing with 5ml ddH_2O to remove any traces of 20% ethanol (used later as storage buffer), using a peristaltic pump (Pharmacia LKB) at a flow rate of 1ml/min. The columns were charged by elution with 500µl of 0.1M nickel sulphate (Sigma Chemical Co) followed by a further wash of 5ml ddH_2O. After charging the column, a blank run containing no protein sample, was performed to elute any unbound metal ions that may be eluted during the desorption stage. The column was equilibrated with 10ml 0.02M sodium phosphate, 0.5M sodium chloride, pH 7.4 (Sigma Chemical Co.), followed by the application of 1ml of sample to the column. The column was then washed with 10ml of 0.02M sodium phosphate, 0.5M sodium chloride and 10mM imidazole, pH 7.4 (Sigma Chemical Co.). The His-tag containing protein was then eluted with 5ml 0.02M sodium phosphate, 0.5M sodium chloride and 500mM imidazole, pH 7.4. Imidazole was included within the wash buffer and the elution buffer to reduce the non-specific binding and to facilitate high elution purity. The column was regenerated with 0.02M sodium phosphate, 0.5M sodium chloride, pH 7.4.

The samples were dialysed against ddH_2O (as in section 2.2.2) and recovered by lyophilisation. The samples were analysed by dot blot for CS-GAG (section 2.2.4.2), to identify those fractions containing PG.

2.2.5.2 Further purification of recombinant proteoglycans

Further purification of the isolated recombinant PGs, was performed by anion exchange using 1ml Resource Q column as previously described (section 2.2.4.5). Recombinant PGs were dissolved in 7M urea, 0.05M tris-

HCl buffer, pH 6.8, to a concentration of 1mg/ml, and applied to the Resource Q column, and pooled fractions were obtained, according to the 280nm profile. Samples were dialysed against ddH_2O (as in section 2.2.2), recovered by lyophilisation and PG rich fractions identified by dot blot analysis (section 2.2.4.2).

2.2.6 Protein standardisation

A protein (BCA) assay kit (Pierce Endogen Ltd., Cheshire, UK) was used to determine the amount of protein present in the PG-rich fractions. The BCA assay was performed in 96 well microplates. Working reagent was freshly prepared in 50:1, reagent A:B (supplied in kit). Albumin standards (supplied within kit) were prepared to give a 50μg/ml to 1200μg/ml concentration range. 10μl of either the standard or sample was pipetted (in duplicate) into a well and 190μl of the working reagent added to each of the standards or samples and mixed thoroughly. The plate was incubated at 37°C for 30min, after which, the plate was cooled to room temperature and the absorbance taken at 540nm (Microplate autoreader, Bio-Tek Instruments). The mean absorbance readings of the standards were plotted against protein concentration and the protein concentration (μg/ml) of each sample determined. Each PG-rich fraction protein content was then adjusted accordingly to each method, by appropriate dilution in ddH_20.

2.2.7 Characterisation of the purified intact proteoglycans and core proteins

At each stage of the purification process, pooled protein fractions of alveolar bone, gingiva and recombinant PGs were examined to identify the

PG-rich fractions. These fractions were then analysed to characterise the purity of both the intact PG and the molecular size of the core protein, using SDS-polyacrylamide gel electrophoresis and Western blot analysis.

2.2.7.1 Release of the proteoglycan core protein

To isolate the core proteins of alveolar bone and gingiva, the samples were dissolved at 10mg/ml, with 0.1M tris-HCl buffer, 0.03M sodium acetate, pH 8. In order to digest the GAG chains from the core protein, an equal volume of 2 units/ml chondroitinase ABC (protease free, Seikagku Corporation, Tokyo), in the above buffer, was added and incubated at 37°C for 1-2h.

The digested core protein products were separated from the chondroitinase ABC and GAG fragments using gel filtration chromatography on a pre-packed Sephadex 75 HR 10/30 column (Amersham Pharmacia Biotech.), with a molecular weight fraction range of 3-70kDa. The separation was carried out at room temperature, incorporated into and FPLC system (Amersham Pharmacia Biotech.). The void volume (V_0) and the total bed volume (V_t) of the column were calculated by applying a solution of 2mg/ml blue dextran 2000 (Sigma Chemical Co.), and 0.5% p-nitrophenol (spectroscopic grade, BDH), to the columns. The elution profiles for V_0 and V_t were determined by monitoring the 1ml fractions at 254nm. Samples of digested PG (500µl) were applied at a concentration of 5mg/ml and eluted with 4M urea, 0.05M Tris-HCl, pH 8, containing 0.35M sodium acetate, at a flow rate of 1ml/min. The absorbance was monitored and 1ml fractions were collected. Based on elution profiles, protein fractions were

pooled, dialysed against ddH_2O with protease inhibitors (as described in section 2.2.2) and lyophilised. The pooled fractions were characterised by SDS-polyacrylamide gel electrophoresis (see section 2.2.4.2), followed by silver staining (see section 2.2.4.3) and Western blot analysis (detailed in section 2.2.7.2).

2.2.7.2 Immuno-identification of proteoglycans by Western blot analysis

Western blot analysis was performed to further confirm the identification of the proteins within the PG-rich and digested samples. The samples were separated by SDS-PAGE, each gel containing the separation of a pre-stained molecular weight marker (as described in section 2.2.4.3).

Gels were electroblotted using the Phast System electroblot assembly. The polyacrylamide gel, nitro-cellulose membrane and six pieces of filter paper were equilibrated in transfer buffer (25mM Tris HCl buffer in 20% methanol, containing 192mM glycine, pH 8.3) for 5min. Three pieces of filter paper were placed onto the anode of the electroblotting apparatus and the nitro-cellulose membrane applied on top of filter paper. The gel was removed from the Perspex backing by a wire cutter, and the gel placed on top of the membrane. The gel was then covered with another three pieces of filter paper. The cathode was applied above the 'transfer sandwich' and electroblotted with an electrical gradient of 20V, 25mA, 1.0W, 5.0vH, for 30min.

Following electroblotting, non-specific protein binding was blocked with 5% non-fat milk (Marvel™) in TBS at room temperature for 1h, with constant agitation. The membrane was then incubated with the relevant primary antibody to PG (as described in table 2.1) diluted in 3% non-fat milk in TBS for 1h at room temperature, with constant agitation. The membrane was washed three times for 5 min with TBS, and re-incubated with the appropriate secondary antibody (as described in table 2.1) diluted in TBS containing 3% non-fat milk for 1h, with constant agitation. The membrane was re-washed with TBS, three times for 10min.

Throughout this study, two methods were employed to visualise the immuno-reactivity of the tissue PGs, ECL Plus Western Blotting Detection Reagent kit (Amersham Pharmacia) and alkaline phosphatase chromogenic detection (Promega). During the final washes, the visualisation reagents were prepared according to manufacturers' instructions. For ECL, 10ml of chemiliminescent reagent was added to the membrane for 1min at room temperature. Once completed, the excess reagent was removed and the membrane was wrapped in SaranWrap®. The membrane was then placed in a film cassette, protein side up, and taken to a dark room where a sheet of autoradiographic film (hyperfilm-ECL) was placed on top. The autoradiographic film was exposed for up to 20min, after which films were developed using an automatic developer (Curix 60, Agfa Gevaert N. V., Germany). For alkaline phosphatase detection, the membrane was incubated with 10ml alkaline phosphatase buffer, 66µl NBT substrate and 33µl BCIP substrate (Promega) (as described in section 2.2.4.2).

2.2.7.3 Glycosaminoglycan characterisation

GAG components from ovine tissues or recombinant cell lines were examined by cellulose acetate electrophoresis (Waddington, 1988). Prior to electrophoresis, GAG chains were released from the core protein by treatment with a non-specific protease (type XIV) (Sigma Chemical Co.). An equal volume of the PG sample (10mg/ml) was mixed with an equal volume of non-specific protease (Sigma) (10mg/ml) in 0.2M tris-HCl, 10mM calcium chloride, pH 7.5 and incubated at 55°C for 20h. The samples were subsequently recovered by lyophilisation and reconstituted with ddH_2O to the equivalent of the original starting volume of the PG sample.

Cellulose acetate electrophoresis was performed using cellulose diacetate sheets (Electrafor, Cellulose diacetate, Shandon Southern Ltd). Sheets were immersed in running buffer (0.2M calcium chloride buffer, pH 7.2) and positioned centrally in the electrophoresis tank (Shandon Southern Ltd). Electrical contact was made using filter paper wicks (Whatman chromatography paper). 2µl samples were applied onto the electrophoresis sheets at an origin 6.5cm from the anode end of the sheet, over 0.5cm lanes, each sample being 0.5 –1.0 cm apart and 1.0cm from the edge (in order to prevent drag effects). Sheets were run at 0.6mA per cm width for 4.5h. A GAG standard mixture (Sigma Chemical Co.) containing hyaluronan (HA), dermatan sulphate (DS), chondroitin 4-sulphate (C4S) and chondroitin 6-sulphate (C6S) was included on each electrophoretic run.

The separated GAGs were visualised by staining with 0.05% w/v Alcian blue 8 GX (Gurr Biological Stain, BDH) in 3% acetic acid, containing 0.05M magnesium chloride, pH 3.9, for 15 min. The sheets were destained with 1% acetic acid with 0.05M magnesium chloride, pH 3.9 with several changes. Sheets were washed in ddH_2O and air-dried.

Specific enzyme digestion of glycosaminoglycan chains

Individual GAG species were characterised based on their susceptibility to chondroitinase ABC and AC. Chondroitinase ABC (*Proteus vigaris*) (Sigma) depolymerises both glucuronic acid and iduronic acid containing GAG, such as C4S, C6S, DS and HA. The enzyme was diluted to 0.2units/ml in 0.1M Tris-HCl, pH 8.5. Equal volumes of the protease digested sample were digested with an equal volume of chondroitinase ABC (working dilution) at 37°C for 20h. Chondroitinase AC (*Flavobacterium heparinium*) (Sigma) depolymerises glucuronic acid containing GAG only at the β(1-4) bond, such as C4S, C6S and HA but not DS, KS, or HS. Chondroitinase AC was diluted to 0.2units/ml in 0.1 Tris-HCl, pH 7.1 and incubated with equal volumes of the protease digested sample for 37°C for 20h. Samples were separated by cellulose acetate and stained with alcian blue, as above.

2.3 RESULTS

2.3.1 Purification and characterisation of proteoglycans from the isolated alveolar bone and gingival tissue extracts

Initially, the non-collagenous proteins were extracted from the alveolar bone and gingival tissues into 4M $GuCl_2$. These protein extracts were then separated using anion exchange chromatography. This method allowed for the bulk preparation of ECM components from mineralised and soft tissues, to yield a PG-rich fraction of approximately 85-90% purity.

2.3.1.1 Q-Sepharose anion exchange chromatography purification of ovine alveolar bone tissue extracts

Figure 2.1 (a) is a typical 280nm elution profile for $GuCl_2$ extracts of alveolar bone by Q-Sepharose anion exchange chromatography. From these profiles, six major protein fractions (I-VI) were identified and pooled. These fractions were examined by dot blot analysis (figure 2.2) to ascertain the PG-rich peak. A large amount of material was identified in fraction IV for ovine alveolar bone extracts, which eluted with approximately 0.3M NaCl. There was also a small quantity of material present in fraction V (figure 2.2), which eluted at about 0.4M NaCl. Therefore, fractions IV and V were pooled to form the alveolar bone PG-rich fraction

The PG-rich fraction derived from alveolar bone was subsequently examined by SDS-PAGE, with protein bands stained with silver (figure 2.3). Also shown are the separations of the core proteins obtained following digestion with chondroitinase ABC. SDS-PAGE obtained for

the intact fraction IV showed bands ranging from 200kDa to 29kDa, with prominent bands at 98-, 70- and 45kDa. However, streaking was a noted feature when separating these intact alveolar bone PGs, which led to poor resolution. Streaking is a common occurrence when separating intact PG molecules and is believed to be due to the heterogeneous nature of the attached GAG chains. Following digestion with chondroitinase ABC, alveolar bone PG-fraction, silver staining revealed a band at 45kDa. Bands observed below 45kDa were assumed to be products of core protein degradation.

Western blot analysis for decorin and biglycan in the alveolar bone PG-rich extract are shown in figures 2.4 (a) and (b), respectively. For decorin, apparent visualisation was noted from 120kDa- 29kDa. Following digestion, clear bands are visible at 45- and 50kDa. Immuno-reactivity of biglycan in intact alveolar bone was observed from 200-29kDa. Upon digestion with chondroitinase ABC, there are noticeable bands at about 45kDa. The presence of a core protein band at 45kDa is similar to previous reports of the core protein molecular weights for the alveolar bone decorin and biglycan from rabbit, sheep and human sources (Oosawa, 1986, Waddington and Embery, 1991, Moseley, 1996). Comparison of SDS-PAGE with Western blot analysis of the alveolar bone PG-rich fraction reveals there are additional bands present in the former, which are non-reactive to decorin or biglycan antibodies, indicating the presence of co-purifying proteins.

2.3.1.2 Q-Sepharose anion exchange chromatography purification of ovine gingival tissue extracts

Figure 2.1 (b) illustrates a typical elution profile of gingival proteins separated by Q-Sepharose chromatography. As for alveolar bone, gingival proteins were pooled and the PG-rich fraction identified by SDS-PAGE followed by silver staining, and Western blot analysis for decorin and biglycan.

Following silver staining, a high proportion of protein was identified in fraction IV for the gingival extract (figure 2.5), which was eluted at approximately 0.3M NaCl. The intact gingival PG-rich fraction showed bands ranging in molecular weight from 200-29kDa. Streaking was evident due to the presence of the GAG chain. Following digestion with chondroitinase ABC, the gingival fraction IV revealed a prominent band at 45kDa. However, there were also a number of bands below 45kDa, considered as degradation products of digestion.

Figures 2.6 (a) and (b) show the presence of decorin and biglycan, respectively, following Western blot analysis of the gingival PG-rich. There are a number of bands with a range of molecular weights. For decorin, there were visible material at approximately 120-, 98- and 66kDa. Also visible was a doublet at 45- and 50kDa. After digestion with chondroitinase ABC, higher molecular weight bands were not visible. However, a double band was visualised at 45- and 50kDa and there were bands visible at about 30- and 15kDa. The presence of a core protein band in the 40-55kDa region is similar to previous reports of the decorin core

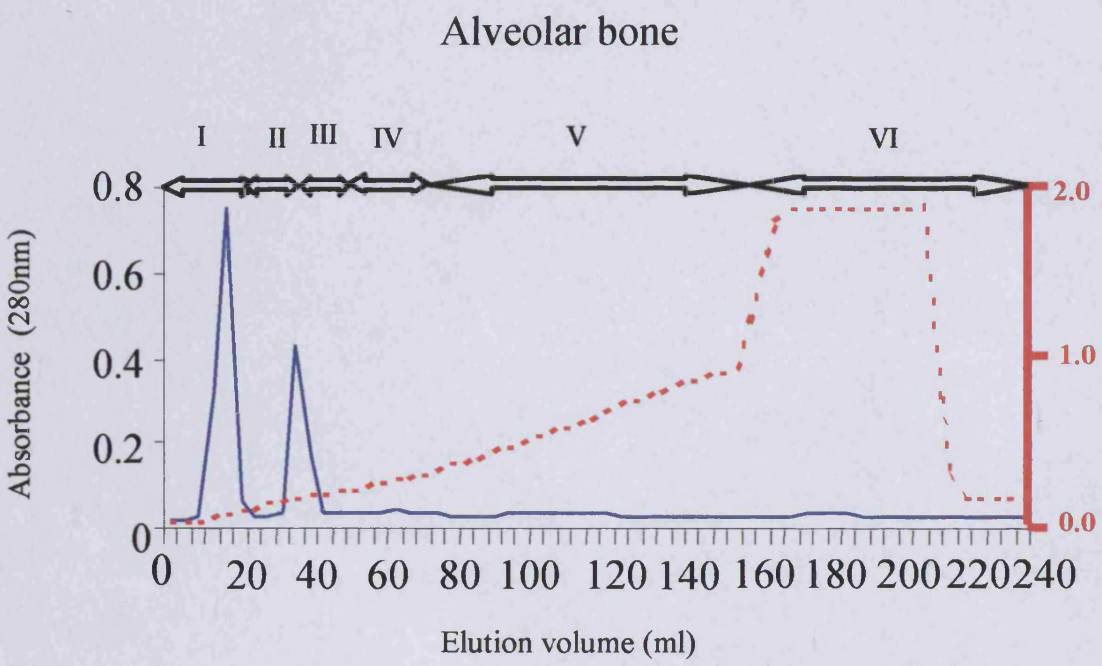
protein weight from bovine and ovine gingival tissues (Rahemtulla, 1992, Moseley, 1996). Stained materials below 45kDa are probable degradation products.

Biglycan immuno-reactivity within the gingival PG-rich fraction showed bands from 200kDa to 29kDa. The digestion of the gingival PG with chondroitinase ABC produced several band regions at around 45kDa and below. Bands detected below 45kDa were regarded as core protein degradation products. The additional bands at 66kDa observed in SDS-PAGE were not immuno-reactive to either decorin or biglycan verifying the presence of co-purifying proteins.

The presence of the GAG chain conjugated to the intact alveolar bone and gingival PG was confirmed by cellulose acetate electrophoresis followed by alcian blue staining of these separated GAG components, as judged by their electrophoretic mobility. Chondroitin sulphate was found within the PG-rich fraction of alveolar bone (figure 2.7a) and dermatan sulphate was predominant in the PG-rich fraction IV of gingiva (figure 2.7b).

Figure 2.1 Typical elution profiles for the separation of (a) alveolar bone and (b) gingival connective tissue extracts by anion exchange chromatography, on a Q-Sepharose column. _____ absorbance (280nm) - - - NaCl gradient (2M)

(a)



(b)

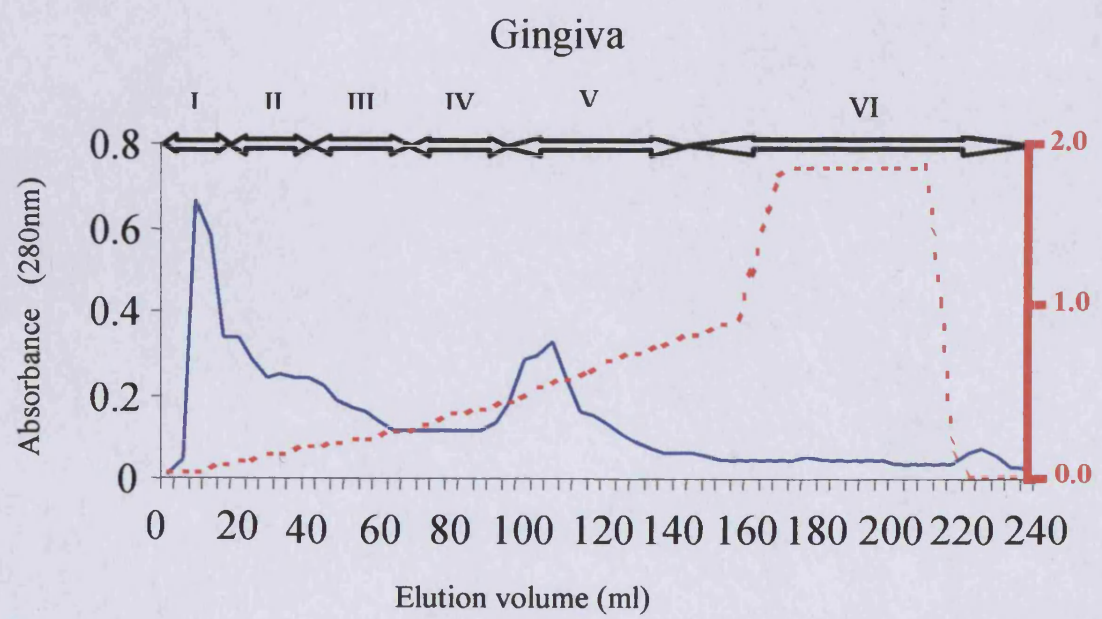


Figure 2.2. Dot blot analysis for the identification of chondroitin sulphate in alveolar bone fractions following Q-Sepharose chromatography

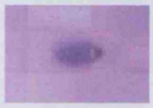
Fraction	Immuno-reactivity	
	Gingiva	Alveolar bone
I		
II		
III		
IV		
V		
VI		

Figure 2.3 SDS-PAGE of alveolar bone PG-rich fraction following Q- Sepharose chromatography

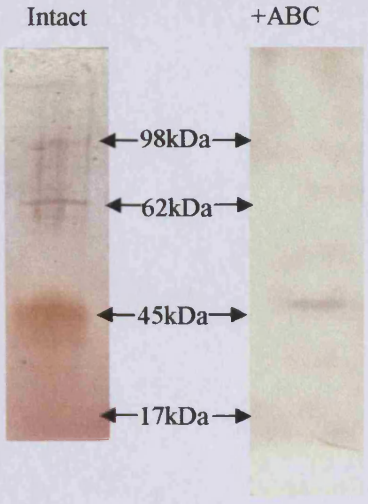


Figure 2.4 Western blot analysis of alveolar bone PG-rich fraction following Q-Sepharose anion exchange for (a) decorin and (b) biglycan

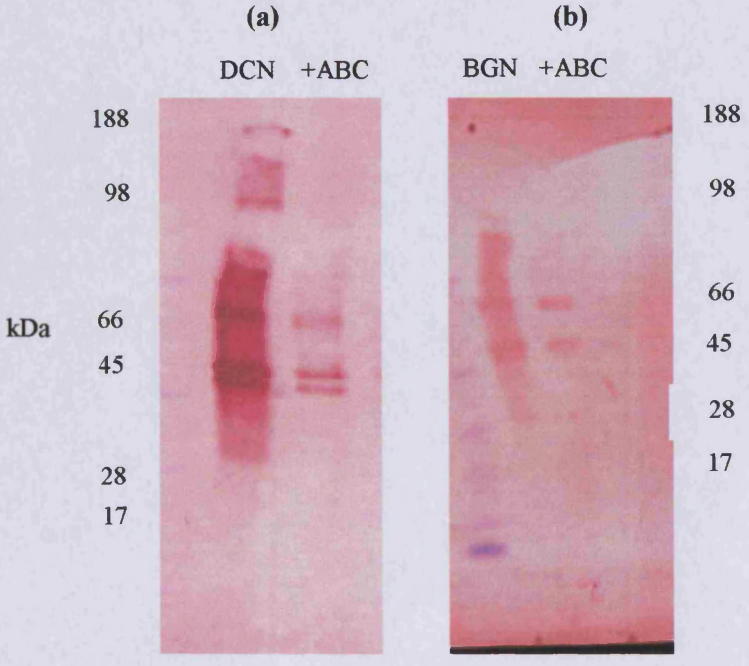


Figure 2.5 SDS-PAGE of gingiva (fraction IV) following Q-Sepharose chromatography

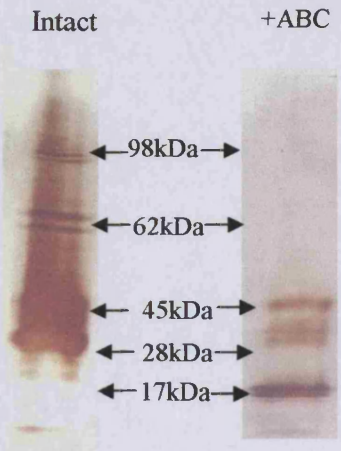


Figure 2.6 Western blot analysis of gingival tissues (fraction IV) following Q-Sepharose anion exchange for (a) decorin and (b) biglycan

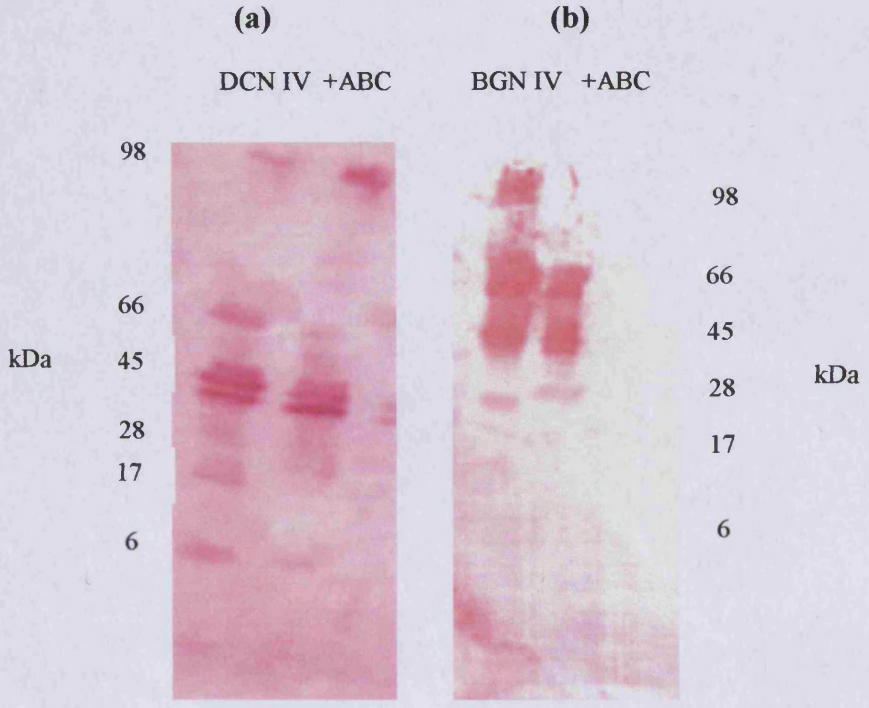
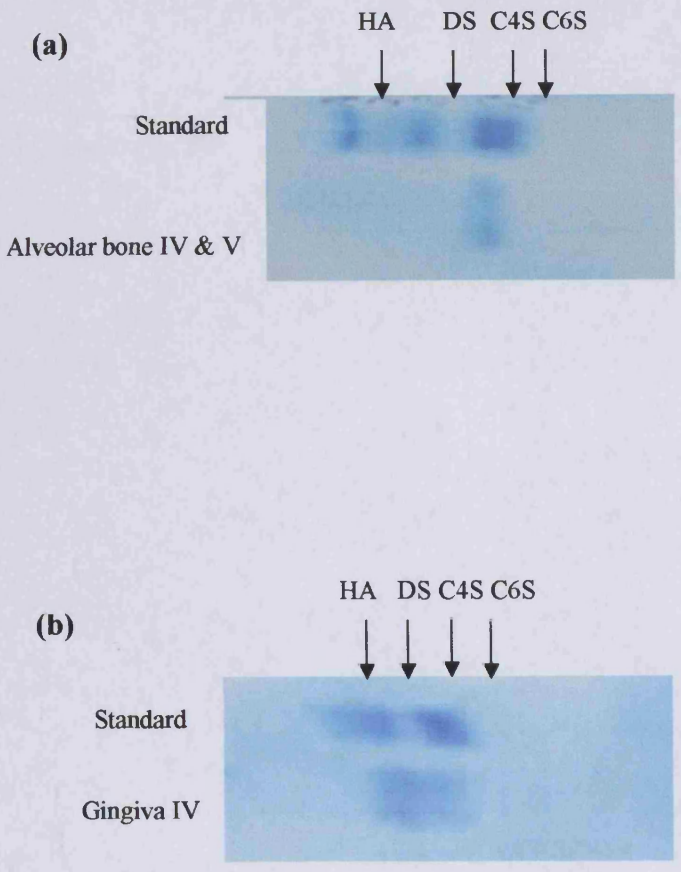


Figure 2.7 Cellulose acetate electrophoresis of (a) alveolar bone and (b) gingival glycosaminoglycans



HA = Hyaluroan

DS = Dermatan sulphate

C4S = Chondroitin-4-Sulphate

C6S = Chondroitin-6-Sulphate

2.3.1.3 Resource Q anion exchange chromatography purification of ovine tissue proteoglycans

To remove co-purifying eluting components from the PG-rich fractions of ovine alveolar bone and gingival extracts, a second anion-exchange method was employed. Figures 2.8 (a) and (b) illustrate typical 280nm elution profiles for the further purification of PGs derived from ovine alveolar bone and gingiva, respectively. Initial peaks (fractions I and II) produced are assumed to be collagens and other glycoproteins, supporting the necessity for, and the accomplishment of, a further purification step. Four major fractions were resolved for both alveolar bone and gingiva. The PG-rich fractions were identified through SDS-PAGE followed by silver staining and Western blot analysis for decorin and biglycan. Fraction III was PG-rich for both alveolar bone and gingiva, eluted at 0.35-0.4M NaCl.

Figure 2.9 shows the protein profile for alveolar bone following Resource Q chromatography. Protein stained material was identified in a molecular weight range of 200 – 45kDa. There was a streaking effect, again due to the nature of the GAG attached, illustrating the effects of the heterogeneous nature of the GAG attachment. Also, there were less prominent bands visible compared to the initial purified PG-rich fraction. When digested with chondroitinase ABC, a single band was visible at approximately 45kDa. Western Blot analysis of intact fraction III of alveolar bone, for both decorin and biglycan (figure 2.11), show single bands at about 45kDa for each PG, indicating the presence of a mixture of decorin and biglycan within the purified alveolar bone extract following

two purification steps. The absence of additional bands subsequent to silver staining indicates the removal of any co-purifying proteins from the initial purification step. Electrophoretic separations by SDS-PAGE of the intact gingival PG-rich fraction (III), followed by silver staining are shown in figure 2.10. The molecular weight of stained material ranged from about 120- to 45kDa, with a double band present at 45- and 50kDa. Streaking was present but there was a loss of protein bands compared to initial SDS-PAGE following Q-Sepharose chromatography, particularly a band at 66kDa, thereby suggesting successful further purification of gingival PGs. Following digestion with chondroitinase ABC, a single band was visible at 45kDa. Western blot analysis of the digested gingival PG-rich fraction for decorin and biglycan are shown in figure 2.12. Immuno-reactivity was apparent for both PGs, with a band observable at approximately 45kDa. This indicates that the material present within the gingival sample was a mixture of decorin and biglycan, without the presence of co-purifying proteins.

The presence of the GAG chains within the intact alveolar bone and gingival PG were confirmed by alcian blue staining (figure 2.13), with CS in alveolar bone PG-rich fraction and HA and DS in the gingival PG-rich fraction. Following digestion with chondroitinase AC, staining was absent from the alveolar bone PG-rich fraction whereas DS remained present within the gingival PG-rich fraction (data not shown). Staining was absent from both alveolar bone and gingival PG samples following treatment with chondroitinase ABC (data not shown), demonstrating the ability of this enzyme to digest the GAG chains within these samples.

Figure 2.8 Typical elution profiles for the further purification of (a) alveolar bone and (b) gingival extracts by anion exchange chromatography, on a Resource Q column. ____absorbance (280nm) - - -NaCl gradient (2M)

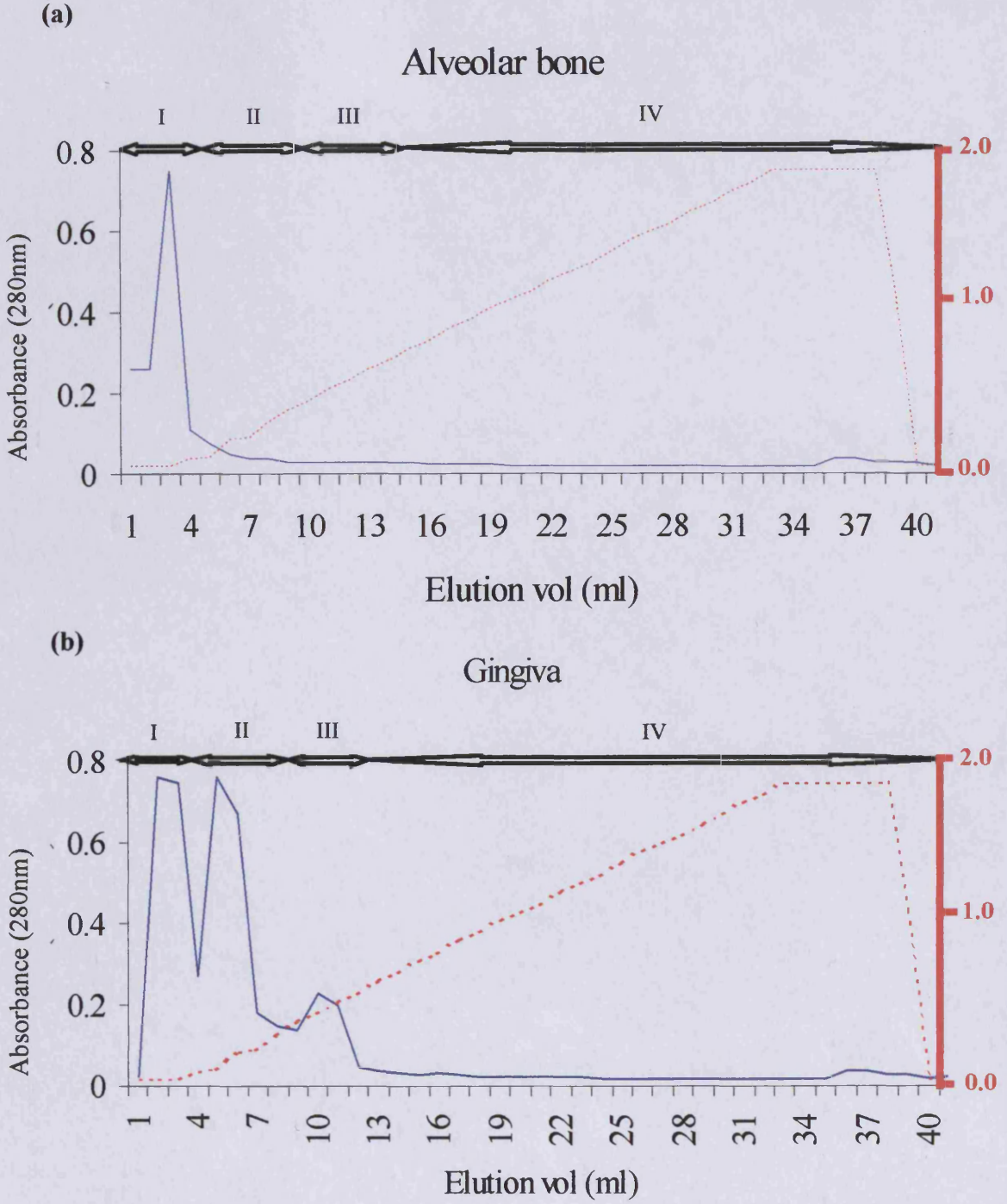


Figure 2.9 SDS-PAGE of alveolar bone (fraction III) following Resource Q chromatography

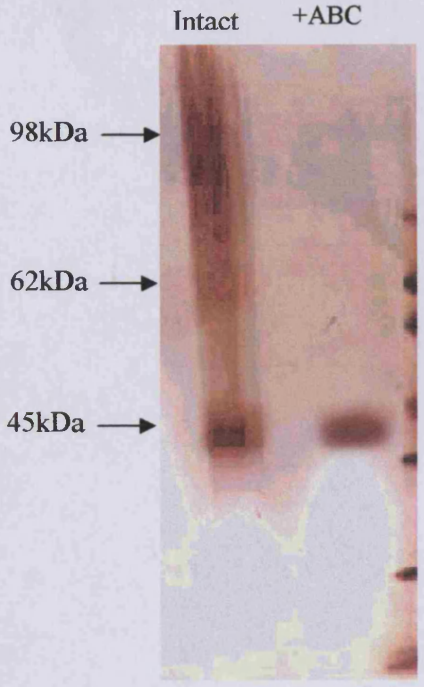


Figure 2.10 SDS-PAGE of gingiva (fraction III) following Resource Q chromatography

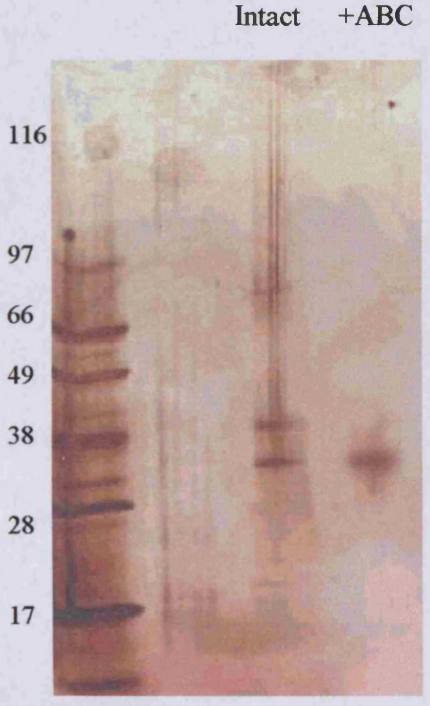


Figure 2.11 Western blot analysis of digested alveolar bone tissue (fraction III) for decorin and biglycan, following Resource Q anion exchange

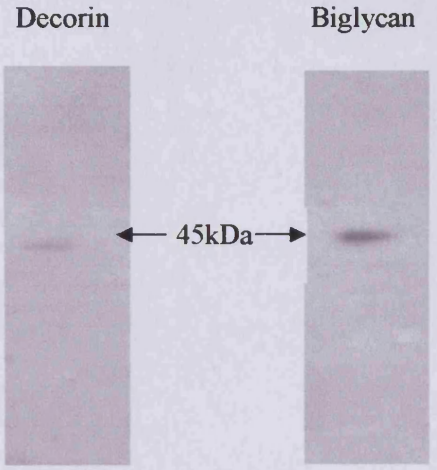


Figure 2.12 Western blot analysis of digested gingival tissue (fraction III) for decorin and biglycan, following Resource Q anion exchange

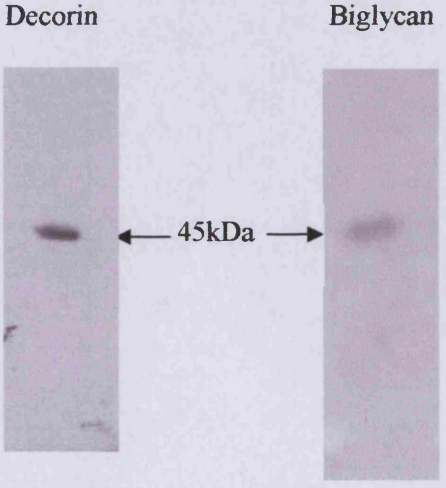
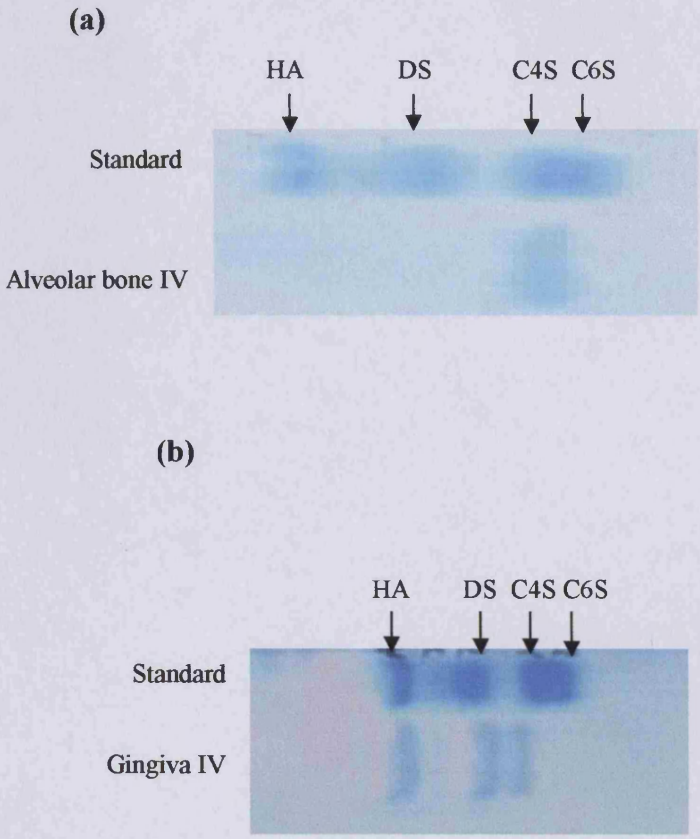


Figure 2.13 Cellulose acetate electrophoresis of (a) alveolar bone fraction IV and (b) gingiva fraction IV



2.3.2 Purification and characterisation of recombinant proteoglycans

2.3.2.1 Nickel affinity chromatography purification of recombinant proteoglycans

Initially, the culture media obtained from the HeLa cells transfected with pcDNA3.1 myc/his decorin or biglycan were subjected to purification by nickel affinity chromatography on a HiTrap Chelating column. This method exploited the presence of the His-tag present on the C-terminal of the expressed protein. Nickel affinity chromatography allowed for the rapid throughput of protein and achievement of a good degree of purity, equivalent to that obtained by Q-Sepharose anion exchange chromatography (Sugars, 2001).

Pooled samples containing the recombinant PGs were identified following monitoring through absorbance at 280nm, together with their immuno-reactivity to the monoclonal antibody (CS 56) using dot blot analysis (figure 2.14). The intact, pooled PG-rich fraction was subjected to SDS-PAGE followed by silver staining (figure 2.15). Recombinant biglycan demonstrated protein material from 200kDa to 17kDa, with prominent bands visible at 98-, 66- and 45kDa. For recombinant decorin, stained material was visible from 200-29kDa, with noticeable bands at 66- and 38kDa. The attached CS-GAG chain is probably responsible for the streaking effects observed, producing the higher molecular weight material. Co-purifying proteins were assumed to be present due to the extra bands detected within the recombinant samples (Sugars, 2001) thus the need for a further purification step.

Figure 2.14 Dot blot analysis for chondroitin sulphate in recombinant decorin and biglycan-rich fractions following nickel affinity chromatography

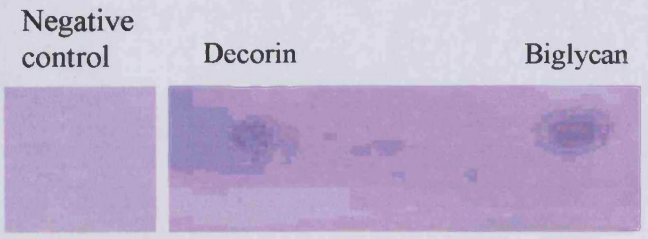
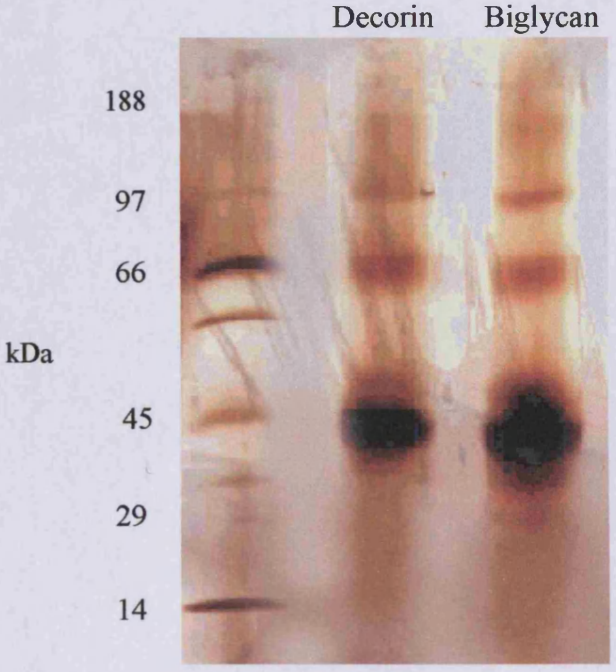


Figure 2.15 SDS-PAGE of intact recombinant decorin and biglycan following initial purification by nickel affinity chromatography



2.3.2.2 Resource Q anion exchange purification of recombinant proteoglycans

Following resource Q anion exchange of recombinant PGs six major fractions were resolved for the recombinant PGs (figure 2.16). Initial peaks (fractions I and II) were assumed to be elution of collagen and co-purifying proteins, reinforcing the need for a second purification step. Fraction IV, for both recombinant PGs, eluted at 0.3-0.35M NaCl, were determined to be PG-rich subsequent to examination by SDS-PAGE followed by silver staining and Western blot analysis for decorin and biglycan. Digestion of recombinant biglycan and decorin with chondroitinase ABC are shown in figure 2.17 (a). Protein stained material was determined to be approximately 45kDa for recombinant biglycan and about 38kDa for recombinant decorin. In both samples, a single protein band was apparent, thereby suggesting the presence of an isolated protein species.

The presence of either recombinant biglycan or decorin was confirmed following immuno-identification with anti-biglycan (LF-106) and anti-decorin (LF-113), respectively (figure 2.17b). Following chondroitinase ABC digestion, strong identification was observed at about 45kDa for recombinant biglycan, and 38kDa for recombinant decorin, confirming the presence of a single species for each recombinant PG without the presence of any co-purifying proteins. Both recombinant biglycan- and decorin-rich fractions were found to be CS-rich, following dot blot analysis probing with the CS56 antibody (figure 2.18).

Figure 2.16 Typical elution profiles for the further purification of (a) recombinant biglycan and (b) recombinant decorin by Resource Q column ____absorbance (280nm) - - -NaCl gradient (2M).

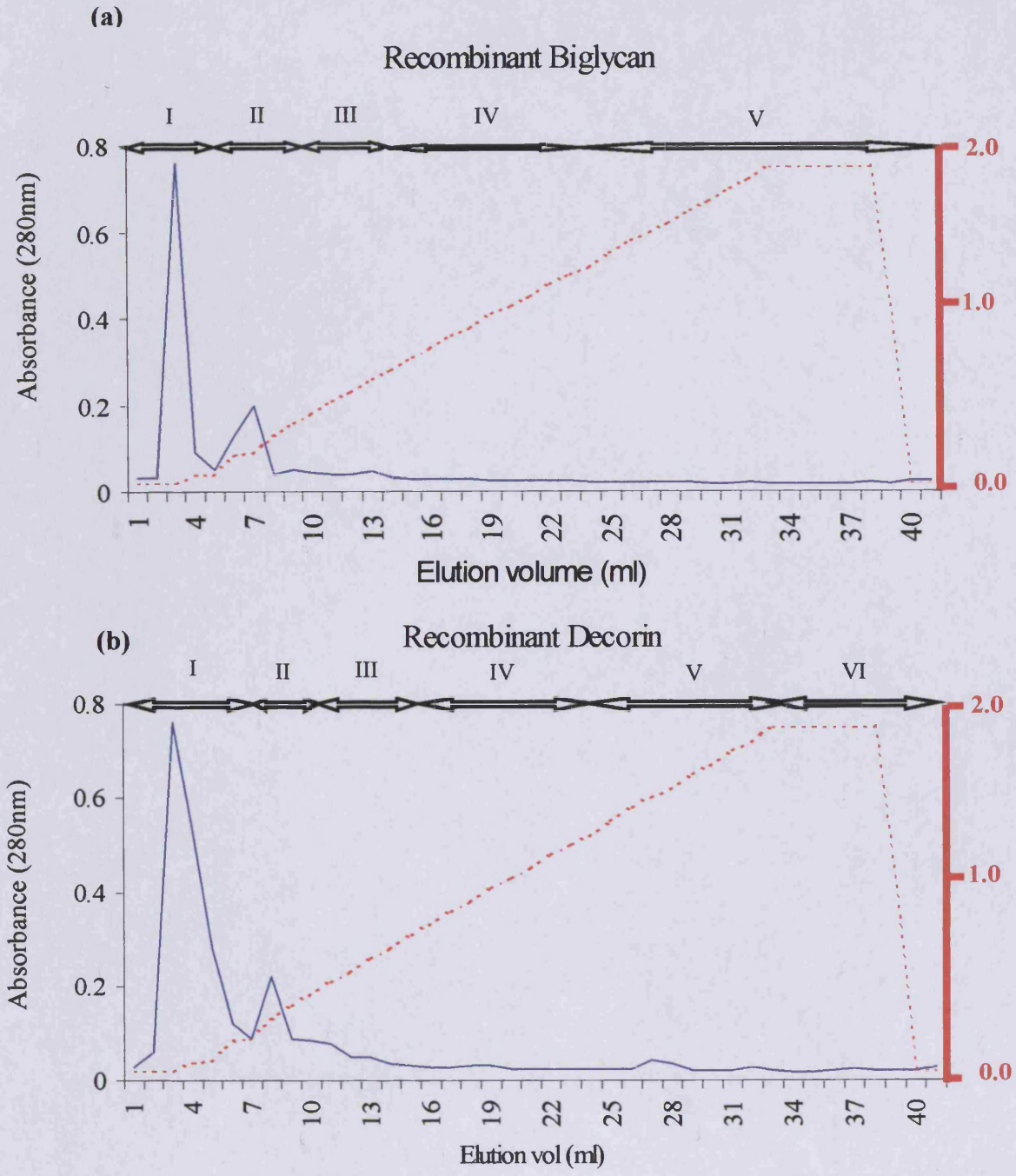


Figure 2.17 SDS-PAGE followed by (a) silver staining and (b) Western blot analysis of digested recombinant PGs (fractions IV), following Resource Q chromatography

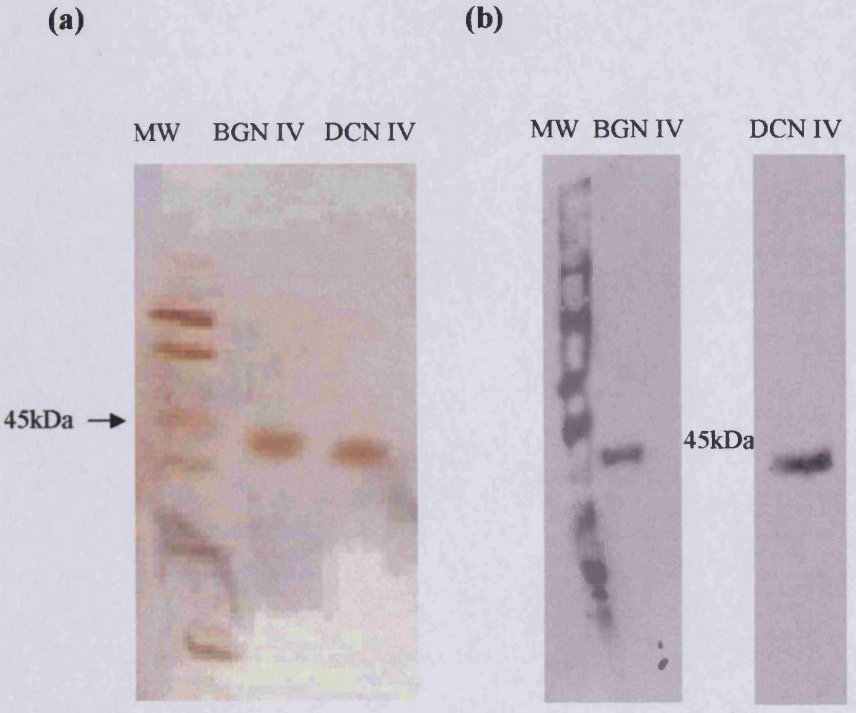
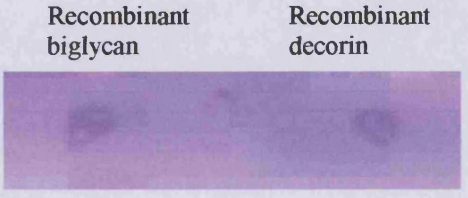


Figure 2.18 Dot blot analysis for chondroitin sulphate within recombinant biglycan and decorin rich fractions



2.4 Discussion

This chapter has focused upon the isolation, purification and characterisation of ovine alveolar bone and gingival PGs, resulting in a mixed population of both decorin and biglycan that are glycosylated with respect to the presence of GAG chains. In addition, a mammalian expression system allowed for the expression, purification and characterisation of single protein species of recombinant decorin and biglycan, which are glycosylated through attachment of CS GAG chains.

Anion exchange is a well-established technique used for the purification of tissue proteins, including those from mineralised tissues (Waddington *et al.*, 1989, 1991). The highly anionic nature of the PG lends itself to the procedure. There is a necessity to consider a number of conditions in the purification of PGs. The denaturing conditions of guanidinium chloride (GdnHCl) extraction serves to disaggregate proteins during purification, releasing most non-covalent bonds to supply high yields with nominal degradation of PGs. Also, in order to inhibit PG susceptibility to proteases present within the tissue, purification processes were carried out in the presence of protease inhibitors, at low temperatures which are unfavourable for proteases.

In these analyses, the PGs were extracted from ovine alveolar bone and gingiva using 4M GdnHCl, followed with extensive purification via anion exchange chromatography. The primary purification of ovine tissues at a concentration of 10mg/ml was performed using a Q-Sepharose column facilitating bulk preparation of the PGs. The intact PG-rich fractions for

alveolar bone were analysed by SDS-PAGE and showed a number of protein bands with molecular weights ranging from 200- to 29kDa. Following digestion with chondroitinase ABC, a prominent band was visible at around 45kDa. Additional bands were assumed to be co-purifying proteins such as α_2HSG (70kDa) that eluted due to their interactions with the proteins of interest. The co-purification of α_2HSG with other ECM components isolated from mineralised tissues has previously been shown. Mizuno *et al.* (1991) demonstrated the presence of α_2HSG with osteopontin from rat bone, and Milan *et al.* (1999) showed co-elution of α_2HSG within a dentine phosphoprotein fraction, isolated from rat incisors. Immuno-probing with a decorin antibody (LF 122), known to cross-react with sheep (Fisher *et al.*, 1995), and a biglycan antibody (LF51) (Fisher *et al.*, 1989) confirmed the presence of the core protein of the two SLRPs within ovine alveolar bone. Following chondroitinase ABC digestion of the ovine PG-rich fractions, immuno-detection of the decorin and biglycan showed intense staining at around 45kDa, corresponding to SLRPs in previous reports (Waddington and Embery, 1991; Moseley, 1996).

SDS-PAGE of gingival tissues following initial purification revealed a number of bands ranging from approximately 120-29kDa. A doublet was apparent at 45- and 50kDa, which remained visible following digestion. Immuno-detection for biglycan revealed a prominent band at about 45kDa, which is concurrent with previous reports. Immuno-detection for decorin revealed the presence of the doublet core protein. The presence of a

decorin core protein doublet of 45-48kDa has been reported, representing glycoforms containing either two and three *N*-linked oligosaccharides, respectively (Hocking *et al.*, 1996; Seo *et al.*, 2005). Seo *et al.* (2005) analysed the N-terminal amino acid sequence for the doublet core protein. They reported that the amino acid sequences of both core protein glycoforms were identical and matched exactly with the predicted sequence for intact decorin. Not all bands, notably a band at 66kDa (evident in SDS-PAGE), were immuno-reactive with anti-decorin or anti-biglycan suggesting the presence of co-purifying proteins.

Due to potential co-purifying proteins, the ovine tissue PG-rich fractions from the initial bulk purification were subsequently subjected to a second anion-exchange step in order to achieve a highly purified protein preparation. The samples were applied to a Resource Q column at a low concentration and eluted over an expanded sodium chloride gradient, thereby attaining a high level of purity. Analysis of the alveolar bone PG-rich fraction by SDS-PAGE revealed a protein species that migrated from 200- to 29kDa. Following digestion with chondroitinase ABC, there was staining at approximately 45kDa. The identity of the PG species present within the gingival PG-rich fraction was confirmed as a mixture of decorin (LF122) and biglycan (LF51) following Western blot analysis. Chondroitinase ABC digestion revealed single bands at 45kDa for decorin and biglycan, confirming the presence of these SLRPs, as a mixed population, within the ovine gingival preparations. Cellulose acetate electrophoresis established the presence of CS within the alveolar bone PG-rich fraction and DS was the predominant GAG within the gingival

PG-rich fraction. These findings are in agreement with many studies that have isolated CS in mineralised tissues (Fisher *et al.*, 1983, Waddington *et al.*, 1989) and DS in soft tissues (Heinegard *et al.*, 1985). HA was also present within gingival tissues. As previously mentioned, HA is found in many tissues and fluids, including umbilical cord, synovial fluid and muscle, and is a major component of the ECM especially in soft tissues (Laurent and Fraser, 1992), including gingiva. Overall, these results were in accordance with previous characterisation of PGs isolated from ovine alveolar bone and gingiva (Moseley, 1996), bovine gingival tissues (Rahemtulla, 1992) and human alveolar bone (Waddington and Embery, 1991).

The recombinant PGs were isolated slightly differently to ovine PGs, in that initial bulk purification was via nickel affinity chromatography followed by a second FPLC anion exchange procedure. The His-tag comprising six-histidine residues allowed for the initial purification. These residues are uncharged at physiologically pH (Crowe *et al.*, 1996), thus the His-tag rarely affects the structure or function of the tagged proteins, and there is no need to remove it after purification (Linder *et al.*, 1992). A number of studies that have used tagged proteins leave the His-tag intact in functional studies, including recombinant decorin interacting with TGF-β (Shi *et* al., 2006) and SLRPs interacting with collagen fibrils (Geng *et al.*, 2006).

Following initial purification, SDS-PAGE analysis of the PG-rich fractions revealed a number of protein bands with molecular weights

ranging from 200- to 29kDa, with an intense band at approximately 45kDa, for both recombinant PGs. Other protein bands ranging from 200kDa to less than 29kDa were accredited to undigested PG material, degradation products and co-purifying proteins.

αHSG is a known co-elutant with recombinant PGs due to the addition of foetal bovine serum to the culture system (Sugars *et al*, 2002). This component was used at the relatively low concentration of 2%, as HeLa cells fail to survive for prolonged periods in serum-free conditions (Sugars, 2001). The strong interactions between PGs and co-purifying proteins may be due to an electrostatic affinity considering the highly anionic conditions utilised in previous separation procedures (Sugars, 2001). As described earlier, the co-purification of αHSG with ECM components of mineralised tissues has been noted. Decorin is renowned for its non-covalent interactions with other ECM components, facilitated via functional groups within β-sheets (Iozzo, 1999). The proposed open horseshoe configuration provides a general binding area for biological ligands, accommodating reversible interactions with matrix components such as collagen (Schonherr *et al.,* 1995) and TGF- β (Hildebrand *et al.*, 1994).

Due to the co-purifying proteins, recombinant PGs were subjected to a second fast performance anion exchange step to obtain single species samples. Initial identification of recombinant decorin and biglycan, which were substituted with a CS chain, was through dot blot analysis for CS.

This finding is similar to the GAG component isolated from mineralised tissues such as bone and dentine (Fisher *et al.*, 1983, Waddington *et al.*, 1989, Bartold, 1990). Subsequent identification was via SDS-PAGE followed by silver staining and Western blot analysis of recombinant decorin (LF 113) and biglycan (LF 106), which showed material at approximately 45kDa. Overall, these results support the findings of Sugars (2001), and provide evidence that the transfected HeLa cells are stable and continuing to produce intact recombinant decorin and biglycan with CS GAG chains attached.

In initial preparations of PG from tissue sources and recombinant cell lines, there was a continuous detection of a band at approximately 98kDa, following immuno-reactivity for decorin and biglycan. The horse-shoe monomeric structure of these SLRP core proteins is based on the α-helix rich ribonuclease inhibitor and it is believed to bind collagen monomers through its inner β-sheet face (Weber *et al.*, 1996). However, it has recently been reported that decorin and its protein core can have molecular masses nearly double the size of those previously published (i.e. ~38kDa) suggesting a dimeric structure (Scott *et al.*, 2003). At present, there is debate as to whether this SLRP exists as a monomer (Goldoni *et al.*, 2004) or as a dimer (Scott *et al.*, 2003). According to Scott *et al.* (2003), the inner concave face of decorin facilitates dimerisation. This suggests that the higher molecular weight bands of the core proteins at approximately 98kDa, for decorin and biglycan, may be due to dimer formation. If this is correct, it is possible that the dimer structured SLRPs may bind to one or more ligands, which could lead to an additional function, compared to the

monomeric structured proteins. However, Goldini *et al.* (2004) provide strong evidence that decorin monomers are the biologically active forms and that decorin dimers are an *in vitro* artefact generated by removing water and salts, which causes non-specific self-association during the freeze-drying step. Indeed, the major difference in experimental design between Goldini *et al.* (2004) and Scott *et al.* (2003) is that the latter authors extensively dialysed their samples against water and subsequently freeze-dried them. Also, the former authors experimental conditions kept decorin proteoglycan and glycoprotein preparations in solution under non-denaturing conditions. The dimeric protein core preparations showed reduced biological activity on interaction with collagen suggesting this conformation was not suited to interact with cell surface receptors and trigger a biological response.

Goldini *et al.* (2004) also reported that biglycan protein core does not oligomerise. However, Scott *et al.* (2006) demonstrated a crystal structure of the biglycan dimer and have shown evidence that dimerisation is fundamental for the folding and stability of this SLRP. They reported biglycan becomes a dimer in solution the same way as decorin, by apposition of the concave inner surface of the LRR domains, and crystallises as a dimer. This group used GdnHCl to denature biglycan, and they demonstrated that it is completely reversible following removal of the GdnHCl. They also revealed that biglycan has some unique features, including a histidine-lined pocket that is likely to provide a binding site for a complex anion.

It was deemed necessary in the current study to perform chromatography separation procedures using 7M urea during anion exchange to ensure dissociation of the protein of interest and co-purifying proteins. The use of this dissociative agent throughout the purification procedures are of particular interest as the use of chaotrophic agents have been questioned by certain studies (Ramamurthy *et al.*, 1996; Krishnan *et al.*, 1999). However, these studies utilised circular dichromism (CD) to investigate the secondary structure of recombinant decorin purified in the presence or absence of chaotrophic agents. They suggested that chaotrophic agents may have an effect on the tertiary structure of decorin, which results in the unfolding of the protein. In addition, extensive dialysis failed to assist correct re-folding following removal of these dissociative agents. However, these studies did not confirm the removal of co-purifying proteins such as soluble collagens or α-HSG, which are likely to influence the resulting spectra.

The current study shows the successful purification of both mineralised- and soft- tissue PGs and recombinant decorin and biglycan, which closely resemble the biochemical characteristics of SLRPs from mineralised tissues. The results confirmed the necessity for thorough purification to obtain highly purified protein sources. The presence of decorin and biglycan has been confirmed through extensive characterisation analyses, which have also shown the PGs to be highly glycosylated. These purified PGs will be used in subsequent studies to establish a model for interactions between SLRPs and the *S. anginosus* group, in relation to the manifestation of periapical abscesses.

CHAPTER THREE

Chapter 3

Interactions of *Streptococcus anginosus* group with extracellular matrix components

3.1 Introduction

Bacterial attachment is an essential step for colonisation of commensal and pathogenic bacteria (Soto and Hultgren, 1999). Many bacteria are known to possess components, called "adhesins", on their surfaces that bind to molecules, or "receptors", on or within tissue (Gibbons, 1989). It is proposed that binding of oral streptococci is a multifactorial process involving both specific and non-specific receptors. Specific binding is a high-affinity, saturable interaction between the cell and binding surface. Conversely, non-specific binding is considered to be a non-saturable, generalised, low-affinity reaction (Staat and Peyton, 1984). The bacterial surface attachments that bear adhesins include fimbriae, fibrillae, flagella, capsule, outer membrane, and other appendages (Gibbons, 1989; Whittaker *et al.*, 1996).

Over time, many microorganisms have adapted to exploit cell surface glycoconjugates as receptors for attachment. Studies have shown that a variety of microbes, including staphylococci and streptococci, express surface components that recognise and bind to ECM components. Joh *et al.* (1999) proposed a model describing how staphylococci and streptococci employ microbial adhesins known as microbial surface components recognising adhesive matrix molecules (MSCRAMMs). These molecules bind fibronectin and form a molecular bridge to integrins

on the host cell, thus facilitating bacterial adherence and inducing internalisation.

A number of studies have identified the ability of SAG to bind to a range of cellular and extracellular components. These include binding to various glycoproteins, including fibronectin, laminin, and collagen (Willcox *et al.*, 1995; Allen and Hook, 2002; Petersen *et al.*, 2002), co-aggregation with *Actinomyces spp.* (Eifuku *et al.*, 1990), haemagglutination with human and animal erythrocytes (Yamaguchi *et al.*, 1993), and adherence to glass and saliva-coated hydroxyapatite (Eifuku-Koreeda *et al.*, 1991; Yakushiji and Inoue, 1992). However, to date, there is no evidence to suggest the SAG adhere to the ECM components, PGs; an interaction which could have a major influence on the progression of infection with the SAG.

The methods used to assay bacterial adherence include scanning electron microscopy of the target surfaces (Vacca Smith and Bowen, 2000), scintillation counting of adherent radio-labelled bacteria (Scannapieco *et al.*, 1995; Tarsi *et al.*, 1997), colony forming unit counting of adherent and/or non-adherent fractions on an agar plate in a suitable medium (Visca *et al.*, 1989), and spectrofluorometric micro-assays (Gaines *et al.*, 2003). Many of these techniques yield information on defined events in a complicated series of host-microbial interactions. Some bacterial adhesins exhibit differential affinities for matrix proteins in soluble versus immobilised states, presumably due to phase-related conformational changes of the ligands. Thus, the solid phase ECM adherence assay may mimic conditions present in tissue more closely than the soluble phase

assay (Allen *et al*, 2002). However, recent developments in surface plasmon resonance (SPR) technology have provided us with a technique to further our understanding of host-microbial interactions.

SPR is a physical process based on the detection of a small refractive index change on a gold surface modified with molecular recognition agents. As the target molecule binds to the immobilised recognition molecule, there is an increase in the surface concentration of the target molecule, which results in measurable changes in the refractive index (Biacore AB, 1993).

The BIAcore biosensor has been utilised to study collagen- and fibronectin-binding adhesins on the cell surface of Gram-positive bacteria of *S. aureus*, where MSCRAMMs mediate the adherence to host cells (Patti *et al.*, 1993 and 1995). Collagen I or fibronectin were immobilised to sensor chips and the binding characteristics of the MSCRAMMs were evaluated. Immobilised fibronectin has also shown binding with different binding proteins from *S. aureus* and *S. epidermidis*. Holmes *et al.* (1997) and Wann *et al.* (2000) identified the fibronectin binding adhesin from *S. aureus* using SPR, and Kawashima *et al.* (2003) utilised SPR to study the interaction of oral streptococci, including *S. sanguis* and *S. mutans,* and salivary components. SPR technology has also been used in studies of adhesion of Gram-negative bacteria to ECM components. Medina and Fratamico (1998) reported the binding interactions of immobilised *E. coli* 0157:H7 cell surface components with ECM proteins and GAGs, HA and CS, and later with type I collagen (Medina, 2001). Also, Nakamura *et al.*

(1999) studied the binding kinetics of the *Porphyromonas gingivalis* fimbriae with immobilised human ECM proteins (elastin, laminin, type I collagen, vitronectin) using SPR and reported that the *P. gingivalis* fimbriae bound to all ECM components.

As previously mentioned the majority of SMG adherence studies have been restricted to various glycoproteins and other bacterial cells. However, decorin and biglycan are also important considerations due to their ability to interact with a range of other ECM components including TGF-β and collagen. Chapter 2 detailed decorin and biglycan purification from ovine alveolar bone and gingival tissues, and recombinant decorin and biglycan from a mammalian expression system. The aim of the current investigation was to examine the interactions between commensal and disease isolates of SAG with decorin and biglycan by SPR analysis. More specifically, the objectives were:

- to investigate any differential binding properties, both inter-species and same related species of the SMG to PGs;
- observe whether the SMG exhibit differential binding to intact PGs compared to PGs devoid of their GAG chains, and;
- to relate any findings to the potential role of SAG in establishing bacterial inflammatory infections, such as abscesses formation.

3.2 Materials and methods

3.2.1 Bacterial source

The isolates of the SAG studied are shown in table 2.3 and were selected from the culture collection of the Oral Microbiology Unit, Cardiff School of Dentistry. Plaque samples and swabs taken from the dorsum of the tongue were obtained from healthy adult individuals. Pus samples were collected by needle aspiration from subjects presenting with dentoalveolar abscesses at Cardiff School of Dentistry. Those isolates from extra-oral pyogenic infections were kindly donated by the PHLS (UHW, Cardiff, UK). The isolates have been characterised extensively by a range of phenotypic and genotypic methods (Bartie, 2000). Following routine maintenance on blood agar (LabM, Bury, UK) with 5% (v/v) defibrinated sheepblood (BA; TCS Microbiologoical Ltd, Buckingham, UK), Gram positive, catalase negative cocci exhibiting reduced growth under aerobic conditions were subjected to glycosidic and hyaluronidase assays, rapid API ID 32 Strep, and SDS-PAGE of whole cell proteins. All phenotypic properties correlated with a reference identification system (Whiley *et al.*, 1990). Haemolysis, sugar utilisation and possession of Lancefield antigens tended to be associated with individual species. Macrorestriction fingerprinting following *Sma*I and *Apa*I digestion revealed each strain had a unique profile but despite this genetic diversity, there was a persistent characteristic contained within the profiles that defined each individual species to create species specific clusters (Bartie, 2000).

3.2.2 Preparation of bacteria

Bacteria were sub-cultured on Fastidious Anaerobic Agar (FAA) (Lab M™ International Diagnostic Group plc, Bury, UK), supplemented with 5% v/v defibrinated sheep blood (TCS Bioscience Ltd., Buckingham, UK), and purity was confirmed regularly. Bacterial cells were inoculated into 10ml of Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK), and subsequently incubated overnight, in an anaerobic cabinet (10% v/v CO_2, 20% v/v H_2, 70% v/v N_2), at 37°C (Don Whitley Scientific Ltd., Shipley, UK), until the stationary growth phase. The latter had previously been determined by a series of preliminary growth curves experiments.

Bacterial cells were harvested by centrifugation at 3000g for 10 min and washed twice in filtered, degassed HBS-EP buffer (0.01M Hepes, 0.15M NaCl, 0.003mM EDTA, pH 7.4, containing 0.005% surfactant P20). The cells were then sonicated (60W, 10s), to disrupt the streptococcal chains, and re-suspended in HBS-EP buffer to an $OD_{(550nm)}$ = 0.5 (the OD had previously been deemed as approximately 1 x 10^8 cfu/ml, determined by serial dilutions, plated onto FAA plates, and colonies subsequently counted following an overnight incubation at 37°C, as above).

Species	Reference	Clinical source
S. constellatus	350/96	Dentoalveolar abscess
S. constellatus	F436	Lung abscess
S. constellatus	322/95	Dentoalveolar abscess
S. constellatus	48C	Tongue
S. constellatus	34C	Tongue
S. intermedius	HW13	Dentoalveolar abscess
S. intermedius	127/95	Dentoalveolar abscess
S. intermedius	447/96	Dentoalveolar abscess
S. intermedius	11C	Tongue
S. intermedius	30C	Tongue
S. intermedius	84C	Plaque
S. anginosus	43586/96	High vaginal swab
S. anginosus	39/2/14A	Unknown
S. anginosus	670/95	Dentoalveolar abscess
S. anginosus	16C	Plaque
S. anginosus	19C	Plaque
S. anginosus	43C	Tongue

Table 3. 1. Identity and source of the SAG isolates used in this study.

3.2.2 Preparation of source materials – Small leucine-rich proteoglycans

Chapter 2 described the isolation, purification and characterisation of tissue-derived SLRPs and recombinant SLRPs. The PGs were subjected to two purification procedures and underwent numerous characterisation procedures, in order to determine the exact nature of the PG core proteins and constituent GAG chains. The alveolar bone and gingival preparations contained a mixture of decorin and biglycan substituted with chondroitin sulphate and dermatan sulphate GAGs, respectively. Both the recombinant PGs (decorin or biglycan) contained chondroitin sulphate. Samples of the intact PGs were digested with chondroitinase ABC and subjected to a further purification step in order to obtain the core proteins alone.

3.2.3 Surface Plasmon Resonance analysis

The interactions between mixed SLRP preparations from ovine tissues and recombinant decorin and biglycan with the SAG isolates were investigated by real-time biomolecular interaction analysis (BIA), using surface plasmon resonance technology on a BIAcore® 3000 system (BIAcore, Uppsala, Sweden). Real-time BIA monitors the formation and dissociation of biomolecular complexes on a sensor surface as the interactions occur. A direct binding assay was performed whereby the SLRPs (ligands) were immobilised on a sensor surface and a solution containing the bacteria (analyte) were passed over the surface. The signal was produced in resonance units (RU). The SPR signal obtained in each binding cycle was recorded as a sensogram, which is a real-time pattern, plotted in RU versus time, in seconds. The guidelines of the BIAapplications handbook (1993)

were followed for the preparation of the sensor surfaces, binding techniques and interpretation of the sensograms and are described below.

3.2.3.1 Sensor chip

The BIAcore sensor chip is composed of an optically flat glass with a gold film deposited on one side, which is covered with a covalently bound carboxylated matrix, to which the ligand is bound. A number of chips are available, which vary according to the surface capture ligand for subsequent covalent binding. A number of chips were tested in the present study, such as the CM5, CM3 and C1 chips (BIAcore AB), in order to produce appropriate immobilisation levels of PGs.

Before use, the sealed sensor chip pouch was allowed to equilibrate to room temperature for 1hr. The sensor chip was then docked into the BIAcore® 3000 apparatus and the system was primed with HBS-EP buffer. Variations in reflectance characteristics between sensor chips may affect the SPR signal, therefore 500µl of BIAnormalisation solution (70% w/w glycerol in water) (BIAcore AB) was passed through the system and used to create a standardised total reflection.

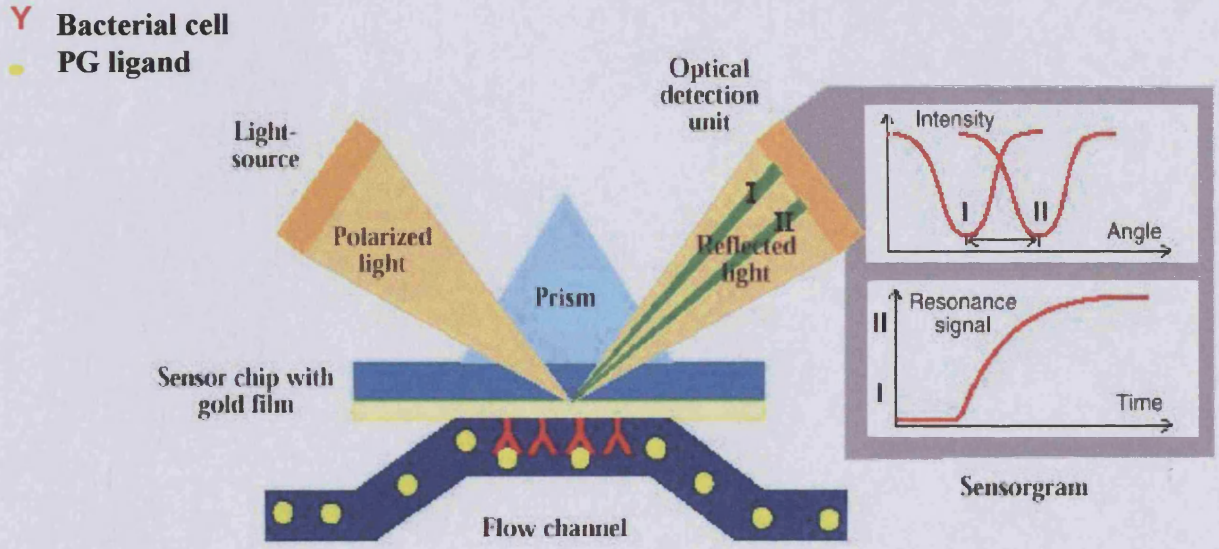


Figure 3.1 Principles of Biacore technology (BIAcore handbook)

3.2.3.2 Immobilisation conditions of proteoglycan ligands

Immobilisation typically shows a pH optimum at a value that is dependent on the nature of the ligand. Uncharged amino groups on the ligand are required for the coupling reaction. Due to the high isoelectric points of the intact ligands (recombinant decorin, pI = 8.87; recombinant biglycan, pI = 6.63 (Sugars, 2001), they were diluted to a concentration of 150μg/ml in 10mM phosphate buffer, pH 7.1 (0.05% monosodium phosphate, 0.17% disodium phosphate), for the immobilisation process.

For immobilisation of deglycosylated PGs, 150μg/ml of deglycosylated PGs (following chondroitinase ABC digestion) were diluted in 10mM sodium acetate buffer (BIAcore AB) at various coupling pH's (pH 4.5, 5 and 5.5), in order to find the best pH to achieve preferred immobilisation levels. In addition, the coupling buffer 2M $GuCl_2$ with sodium acetate buffers (pH 4.5-5.5) (Zhang *et al.*, 2002) were also tested.

The sensogram was initiated and 20μl of the diluted PG sample was run over the flow cell surface at 10μl/min. The level of immobilisation suited for SMG-PG interaction purposes was achieved through amine coupling.

3.2.3.3 Amine coupling immobilisation

Ligands (PGs) were immobilised on the sensor chip surface using amino coupling. The surface preparation 'wizard' was utilised to couple the ovine decorin and biglycan preparations, recombinant decorin and biglycan, and their corresponding core proteins, onto the chip surface. The surface was

first eluted with 10µl/min continuous flow of HBS-EP buffer, and the flow rate was maintained at 10µl throughout the immobilisation procedure.

Flow cell 1 was used as an in-line reference for flow cell 2, where the blank surface was activated by injecting 70µl of 400mM N-ethyl-N'- (3-diethylaminoprophyl) carbodiimide (EDC) and 100mM N-hydroxysuccinimide (NHS) solution, in equal quantities. After activation, 35µl of 150µg/ml of ligand, in appropriate buffer, was applied to the surface. The system was washed through with HBS-EP buffer to remove any loosely associated ligand. Finally, residual NHS esters were then inactivated with 70µl of 1M ethanolamine hydrochloride, pH8.5.

3.2.3.4 Suitable surface regeneration conditions

Regeneration conditions were established to maintain the activity of the ligand whilst ensuring the removal of all the interacted material. To test the surface, the SAG isolates (OD_{550} = 0.5) were injected across the chip at 20µl/min and an interaction with the ligands was observed in the sensogram. A number of regeneration solutions were investigated to dissociate the interactions between the bacteria and PGs. These included 100mM NaOH, pH 9.5, 1M NaCl, pH 6.5, and 4M GdnHCl, pH 5. The injection period was for 1min and the flow rate maintained at 20µl/min.

3.2.3.5 SPR measurement conditions

Preliminary studies showed binding parameters of SAG interactions with PGs were affected by flow rates above 20µl/min. Therefore, the flow rate

of the sensogram was maintained at 20µl/min with HBS-EP buffer. Following a steady sensogram baseline, bacteria were injected at 20µl/min for 3 minutes, at 25°C.

In this study, the term adherence was taken as the entire process of attachment and accumulation i.e. both initial cell-to-surface adherence and the subsequent cell-to-cell adherence, of bacterial cells (Eifuku-Koreeda *et al.*, 1991). Therefore, the amount of adherence was observed as the difference in RU value from before injection of cells (binding response at baseline) to the end of injection. After the binding cycle, a dissociation phase of 5min was permitted to enable the sensogram to return to baseline (running buffer only was allowed to flow over the sensor surface). If bacteria remained bound to the sensor chip, the surface of the chip was regenerated by exposure to 100mM NaOH, pH 9.5, for 1min (as determined in section 3.2.3.4). The chip was allowed to re-equilibrate between injections by the application of HBS-EP buffer for 5min. Each isolate was analysed in triplicate, in a randomised order.

3.2.4 SPR control – fibronectin

The SAG are known to bind to fibronectin (Willcox *et al.*, 1995), therefore fibronectin was used as a control. 150µg/ml fibronectin was immobilised to the sensor surface (as in section 3.2.3.3) following pH scouting (see section 3.2.3.2) with sodium acetate buffers. SPR measurement conditions for the interaction with SAG were as those in section 3.2.3.5.

3.2.5 Statistical analysis

The RU data obtained was assessed by analysis of variance (ANOVA) using SPSS 12. The data was evaluated both in terms of inter-species and inter-strain differences, for each substrate studied. Differences were considered significant at $p<0.05$ and data expressed as the average value of triplicate assays $\pm$ SD.

3.3 Results

3.3.1 Surface plasmon resonance

A total of 9 clinical SAG isolates and 8 oral isolates from healthy individuals were tested for their *in vitro* adherence to ECM PGs commonly present throughout the gingival and alveolar bone tissues. The adherence profiles were determined using a surface plasmon resonance biosensor, within the BIAcore® 3000 system, allowing interactions to be monitored between unlabelled reactants.

3.3.2.1 Immobilisation levels

The C1 chip was preferred for immobilisation of PGs following preliminary studies to establish which sensor chip would be appropriate for SAG interactions with PGs. The C1 chip has a carboxymethylated surface without a dextran matrix and is useful when studying interactions where the interaction partner (analyte) is large, such as bacteria. The CM5 and CM3 chips also have carboxymethylated surfaces with varying lengths of dextran chains attached, which in this study, appeared to interfere with the interaction.

PGs were immobilised onto the C1 sensor chip using amine coupling as seen in figure 3.2. Following a stable baseline with HBS-EP buffer, the immobilisation process started with the injection of EDC/NHS solution, to convert the carboxymethyl groups on the sensor chip surface to active esters. The intact PGs were injected in phosphate buffer (pH 7.1). For deglycosylated PGs, the most appropriate immobilisation levels were

obtained using sodium acetate coupling buffer pH 5.5, compared to other buffers (data not shown). The free (uncharged) amino groups of the PG sample formed covalent bonds with the active esters, and the residual esters were subsequently blocked (deactivated) with ethanolamine.

The amount of immobilisation was represented as an increase in RU between the start and end of the immobilisation process. A response of 1000RU corresponds to a shift of 0.1° in the resonance angle, which represents a change in surface protein concentration of about 1ng/mm^2. Table 3.2 shows the immobilisation levels obtained for intact ovine and recombinant PGs and their core proteins, indicating apparent binding to the chip surface. Immobilisation levels were between 302-350 RU, corresponding to 0.3 – 0.35ng/mm^2.

The fibronectin control produced a larger RU level when immobilised probably due to this ligand being less negatively charged than the test PG ligands. 4515RU corresponds to approximately 4.5ng/ml.

Figure 3.2. An example of a typical immobilisation procedure (intact gingival proteoglycans approx. 308RU). The sensor surface was immobilised with intact PG or its corresponding core protein. A sensogram is a plot of continuous real-time biniding of captured molecules (RU) vs. time (s).

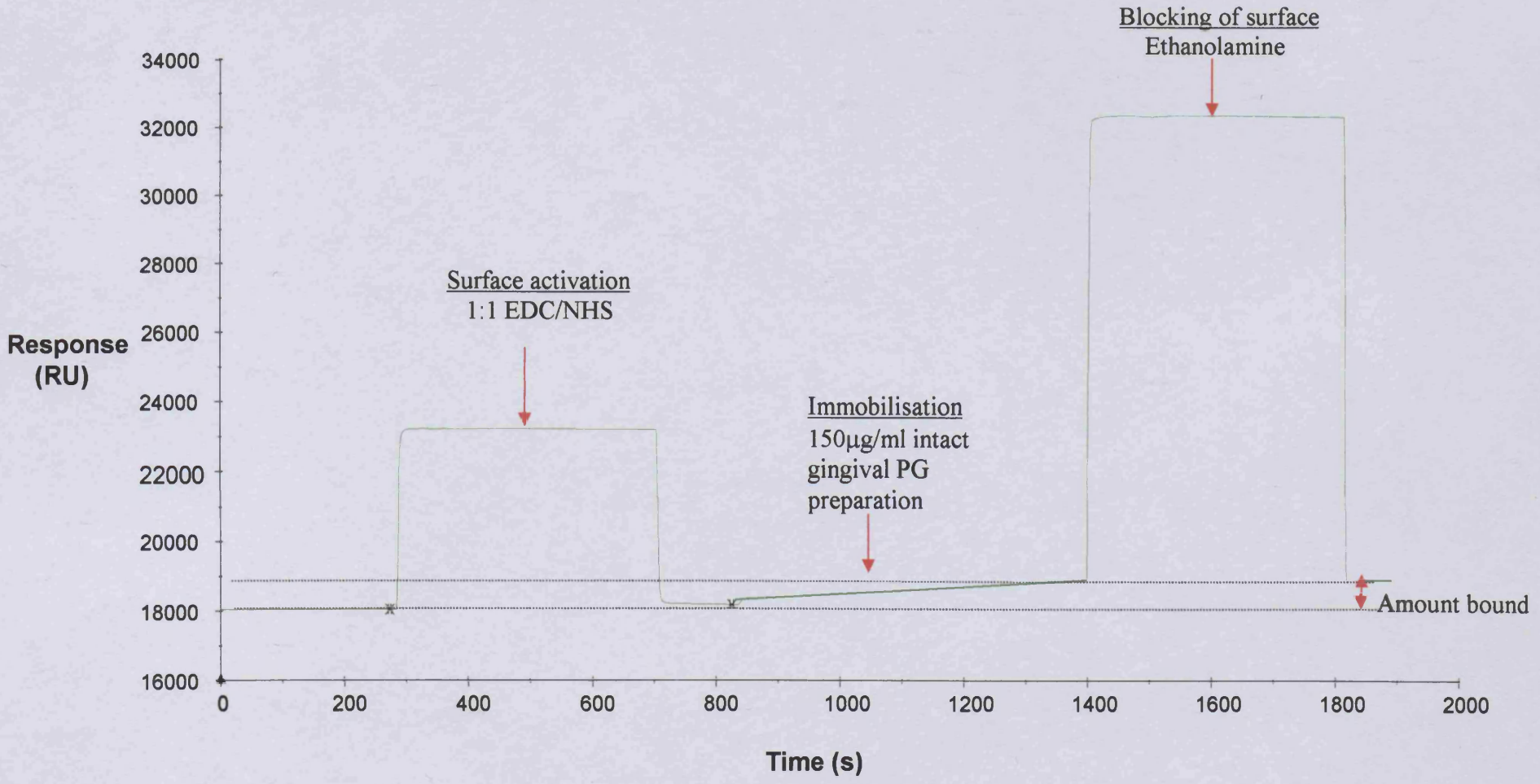


Figure 3.3. Immobilisation of fibronectin.

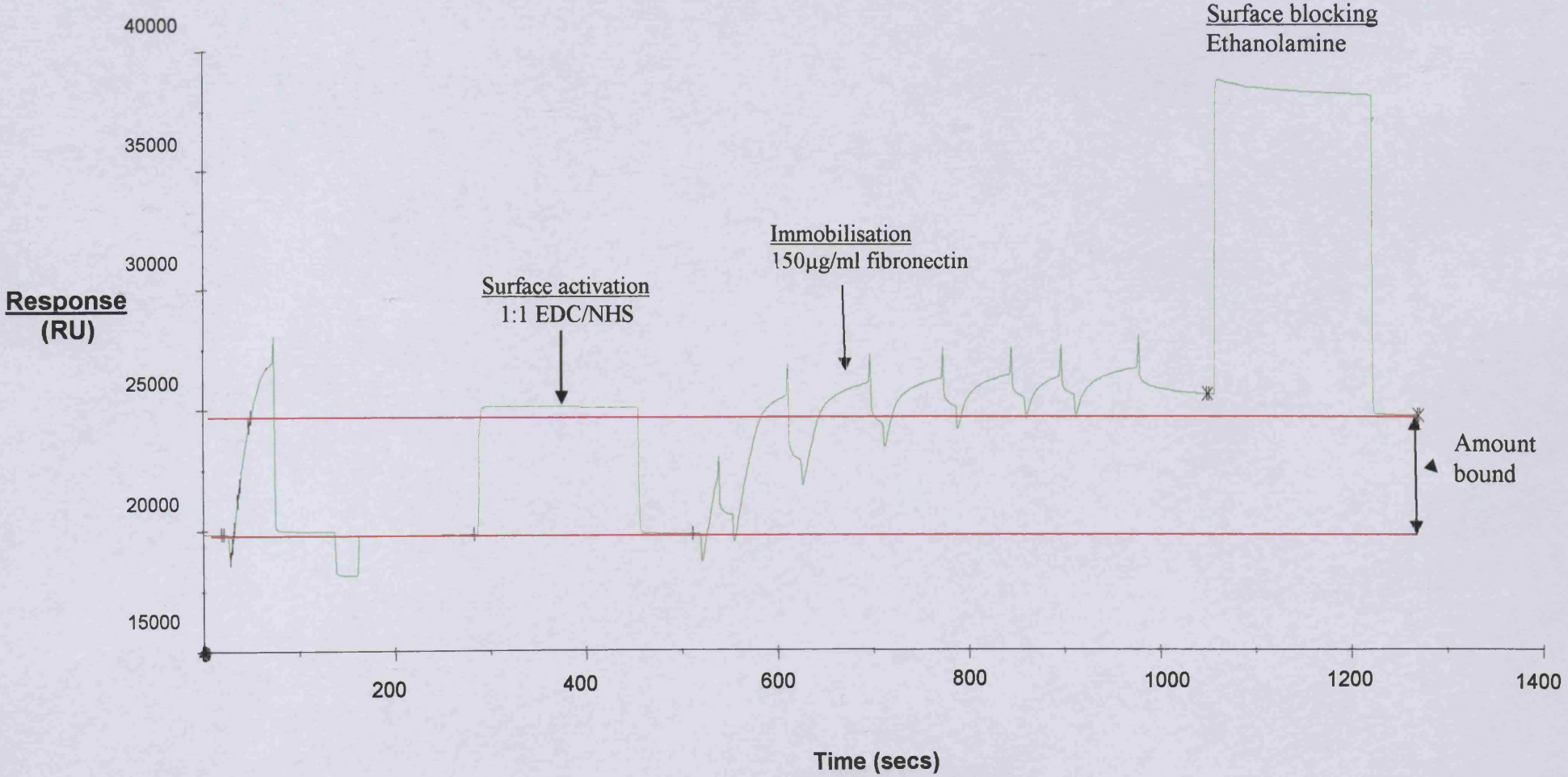


Table 3.2. Immobilisation levels of the intact proteoglycans and their corresponding core proteins to the C1 sensor chip.

Ligand	Immobilisation level (RU)
Intact:	
Recombinant decorin	317
Recombinant biglycan	313
Gingiva	308
Alveolar bone	302
Deglycosylated (core protein):	
Recombinant decorin	350
Recombinant biglycan	327
Gingiva	318
Alveolar bone	337
Fibronectin	4515

3.3.2.2 Establishment of regeneration conditions

An important detail to enable repeated analysis was the determination of the regeneration conditions following the interaction of SAG with the immobilised PGs. Large amounts of SAG remained on the chip surface following regeneration with 1M NaCl, pH 6.5, whereas regeneration with 4M GdnHCl, pH5, resulted in a RU value that had decreased below the original baseline, suggesting removal of all bacteria but also of some ligand. However, subsequent re-binding of SAG to the chip achieved similar RU levels to previously obtained levels, thereby proposing that the surface chip had not been damaged (data not shown). Regeneration of the chip with 100mM NaOH, pH 9, for 1min resulted in the majority of SAG being removed. If there was any residual bacteria, the chip was subjected to a second exposure with 100mM NaOH, pH 9, for 1min.

3.3.2.3 Interaction of SAG with proteoglycans

SAG solutions (OD = 0.5), in their stationary phase following incubation for 18hr, were injected onto the sensor chip immobilised with PGs. Figure 3.3 shows an overlay of typical sensograms obtained from SAG isolates interacting with intact PGs (gingival PGs). The sensogram records specific interactions, recorded as the difference between the start and end of injection. During SAG interactions with PGs, 'spiking' was a common occurrence, with many peaks visible throughout the course of the assay. This phenomenon may be due to noise, as the size of the immobilised

ligand is small (~ 45kDa) (Huber *et* al., 2004), but it is more likely to be due to cell-cell interactions of the SAG strain, which build up on the PG chip surface and subsequently become removed in 'clumps' with the running buffer. This clumping effect is a common occurrence in streptococci, and may be a biological determinant of infection with SAG (Willcox *et al.*, 1988). The mean RU (±SD) for triplicate interactions are shown in table 3.3 and figure 3.4 (a-d).

With the BIAcore 3000 system, it is relatively simple to measure binding responses with a maximum of less than 50RU (BIA handbook), therefore most of the responses in these studies are sufficient to show host-microbial interactions are taking place. However, for the purpose of this study, low binding was considered for strains exhibiting RU values of 50 or less, moderate binding was considered as 51-200RU, and RU values over 200 were considered as high binding strains.

For *S. anginosus* strains exhibited low binding RU values (4 - 16RU) or no binding to intact recombinant decorin and biglycan. All *S. anginosus* strains bound to intact gingival proteoglycans ranging from 39-206 RU. 3/6 strains (two commensal - 19C and 16C and one pathogen - 39/2/14A) bound to alveolar bone PGs ranging from 4 – 32 RU.

All *S. constellatus* strains bound to intact recombinant decorin (4.2 – 775.6 RU) and gingival PGs (53.2 – 439.5RU), with strain 322/95 (dentoalveolar abscess) exhibiting statistically significant binding to recombinant decorin (775.6RU), compared to strains within the same species and with strains from the other SMG species. For intact recombinant biglycan and alveolar bone, 4/5 and 3/5 strains bound, respectively, with no statistically significant binding.

S. intermedius strains demonstrated the most significant binding to intact PGs. For recombinant decorin, 5/6 strains bound, with 30C (tongue) and 127/95 (dentoalveolar abscess) exhibiting statistically significant binding ($p<0.05$) compared to strains within the *S. intermedius spp. S. intermedius* strains produced 100% binding to intact recombinant biglycan and gingival PGs. Statistically significant binding was shown by 30C to intact recombinant biglycan (1378.1 RU), and 30C and 84C (plaque) exhibited significant binding to gingival PGs (1532.2 and 1133.7 RU, respectively). This significant binding was in comparison with other *S. intermedius* strains and between *S. anginosus* and *S. constellatus* strains to the same PGs. For alveolar bone PGs, 4/6 *S. intermedius* strains bound, producing values ranging from 64 – 155 RU. Of these, 84C demonstrated significant binding (155 RU) when compared to strains from within the *S. intermedius* species interacting with alveolar bone PGs.

Figure.3.4. A typical sensogram of the response differences in resonance units (RU) between the two flow channels (Fc2-Fc1) when various SMG isolates were injected randomly over intact gingival PGs (_ 19C; _ F436; _ 322/95; _ 48C; _ 39/2/14A).

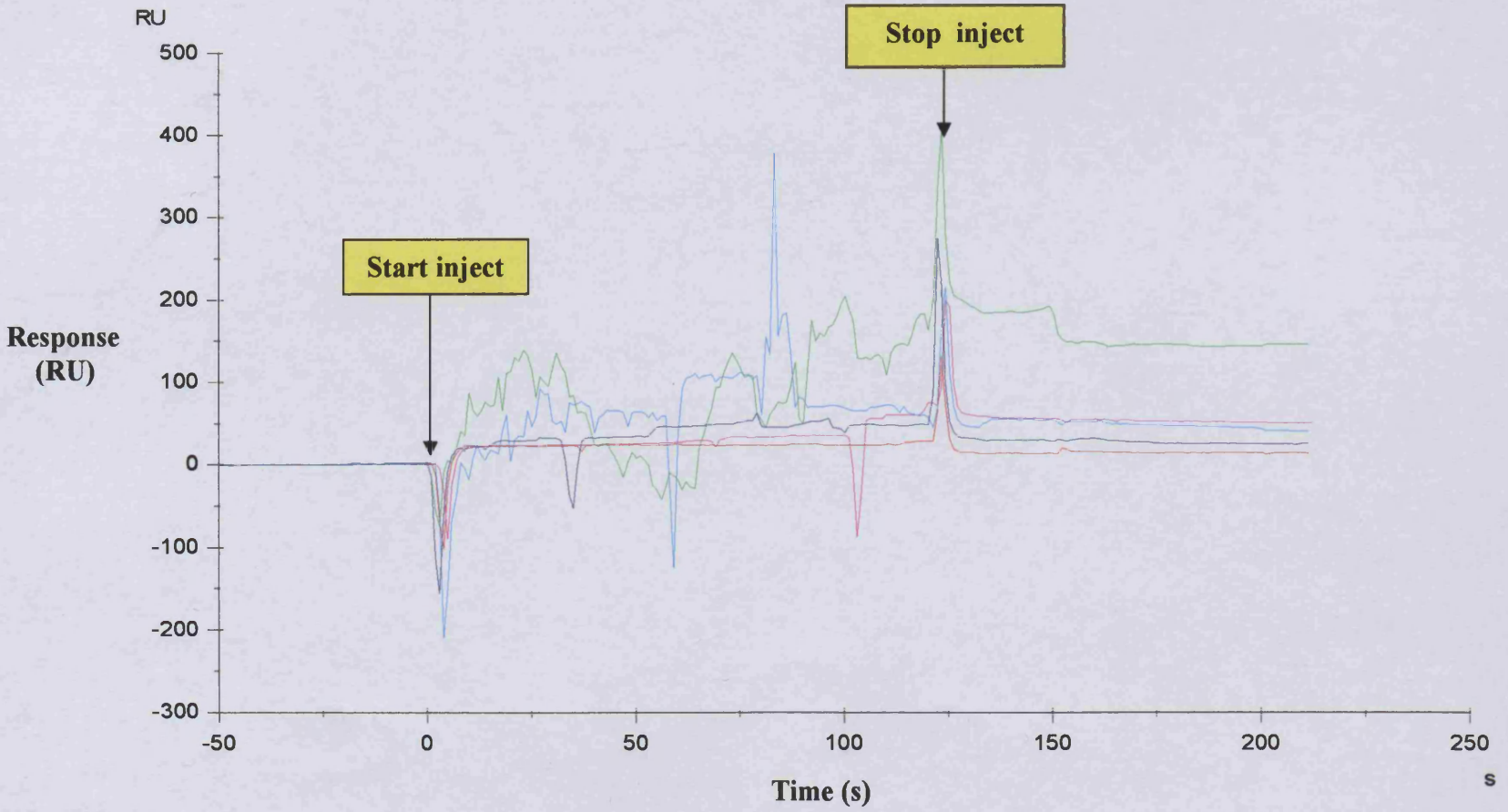
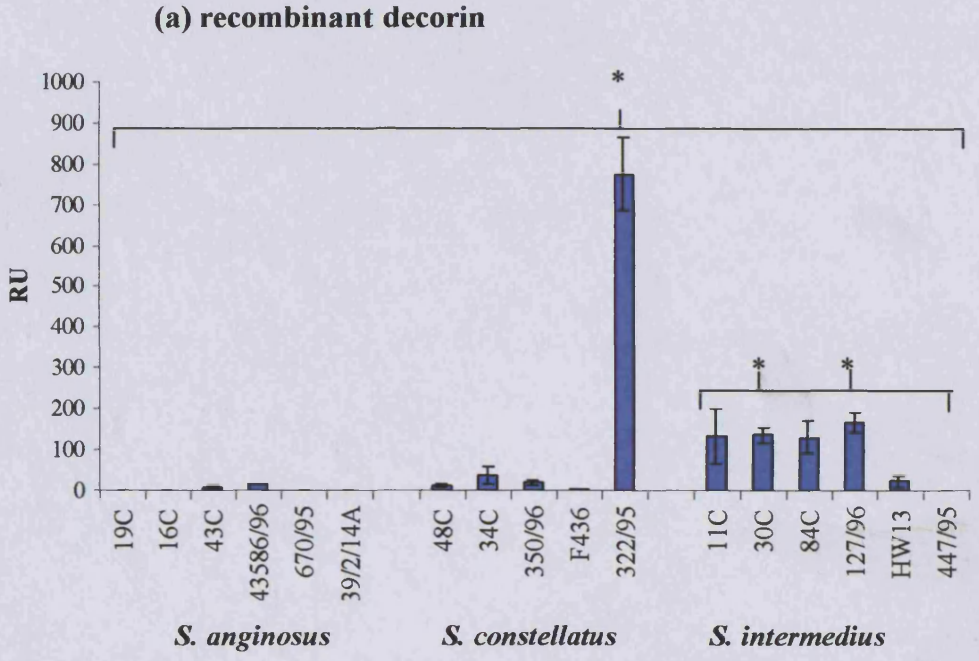


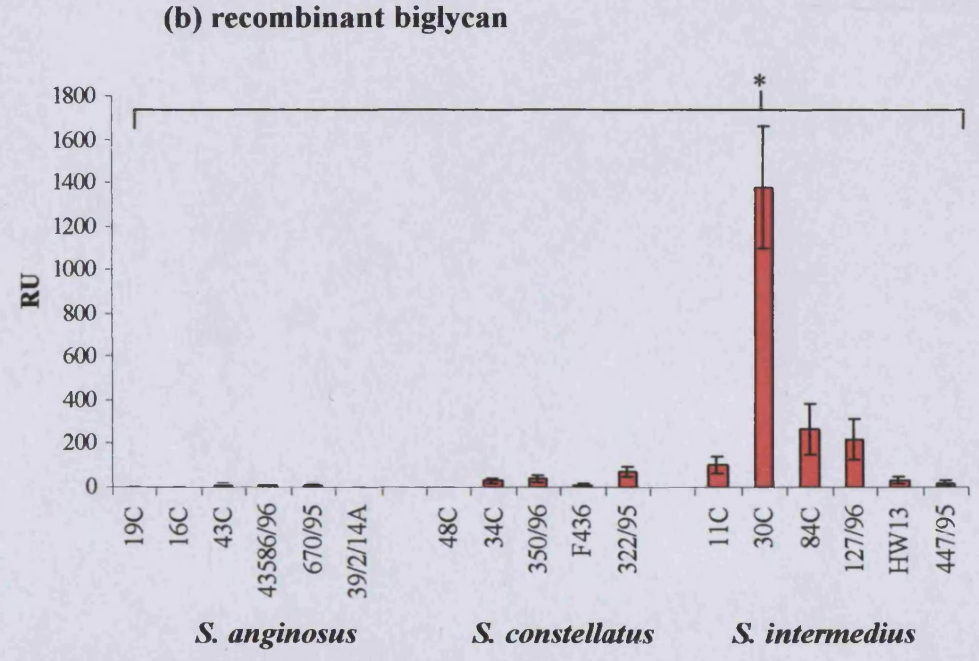
Table 3.3 Average binding (in RU), for three independent assays (± SD), of commensal and pathogenic strains of *S. anginosus*, *S. constellatus* and *S. intermedius* to recombinant proteoglycans, ovine proteoglycan preparations, and their corresponding core proteins.

Strain	Recombinant decorin		Recombinant biglycan		Gingiva		Alveolar bone	
	Intact	Core protein	Intact	Core protein	Intact	Core protein	Intact	Core protein
S. anginosus								
19C	NBD	NBD	NBD	NBD	41.7 ± 25.1	NBD	32.9 ± 20.2	NBD
16C	NBD	NBD	NBD	NBD	95.8 ± 45.8	NBD	4.7 ± 1.4	NBD
43C	9.2 ± 4.9	NBD	9.6 ± 2.9	NBD	39.9 ± 3.4	NBD	NBD	NBD
43586/96	16.9 ± 1.2	NBD	4.6 ± 1.5	NBD	145.7 ± 59.7	NBD	NBD	NBD
670/95	NBD	NBD	9.0 ± 5.5	NBD	206.2 ± 79.0	NBD	NBD	NBD
39/2/14A	NBD	NBD	NBD	NBD	60.5 ± 23.1	NBD	27.9 ± 1.9	NBD
S. constellatus								
48C	11.9 ± 3.9	NBD	NBD	NBD	119.5 ± 66.1	NBD	NBD	NBD
34C	38.4 ± 21.2	NBD	28.5 ± 11.8	NBD	54.8 ± 25.8	NBD	NBD	NBD
350/96	19.4 ± 5.8	NBD	40.6 ± 13.7	NBD	204.4 ± 135.9	NBD	26.6 ± 13.4	NBD
F436	4.2 ± 1.1	NBD	7.7 ± 4.9	NBD	53.2 ± 23.3	NBD	11.2 ± 3.1	NBD
322/95	775.6 ± 90.1*	NBD	72.4 ± 24.9	NBD	439.5 ± 148.7	NBD	16.9 ± 5.1	NBD
S. intermedius								
11C	134.4 ± 67.1	NBD	102.9 ± 37.7	NBD	139.8 ± 72.9	NBD	31.9 ± 15.9	NBD
30C	136 ± 17.2*	NBD	1378.1± 284.7*	NBD	1532.2 ± 114.0*	74.3 ± 58.9	112.4 ± 50.1	NBD
84C	130.5 ± 40.2	NBD	266 ± 114.7	NBD	1133.7 ± 115.7*	NBD	155 ± 8.1*	NBD
127/95	168.1 ± 23.8*	NBD	219.5 ± 91.5	NBD	258.1 ± 66.0	42.4 ± 3.9	NBD	NBD
HW13	23.6 ± 14.9	NBD	29.8 ± 17.3	NBD	147.3 ± 63.3	25.0 ± 5	64.0 ± 13.1	NBD
447/95	NBD	NBD	19.1 ± 8.9	NBD	184.6 ± 44.6	NBD	NBD	NBD

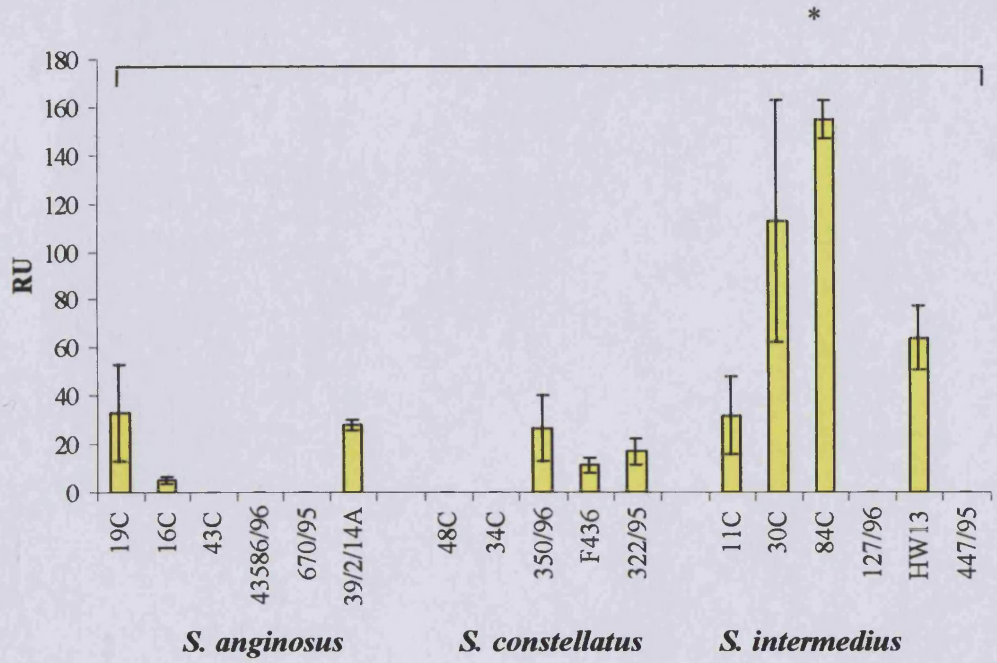
* $p < 0.05$ Commensal isolates NBD = No binding detected

Figure 3.5 Bar charts illustrating SAG binding, in resonance units (RU), to (a) recombinant decorin, (b) recombinant biglycan, (c) alveolar bone proteoglycans, and (d) gingival proteoglycans. Each bar represents the mean of three independent binding assays ± SD; * <p=0.05

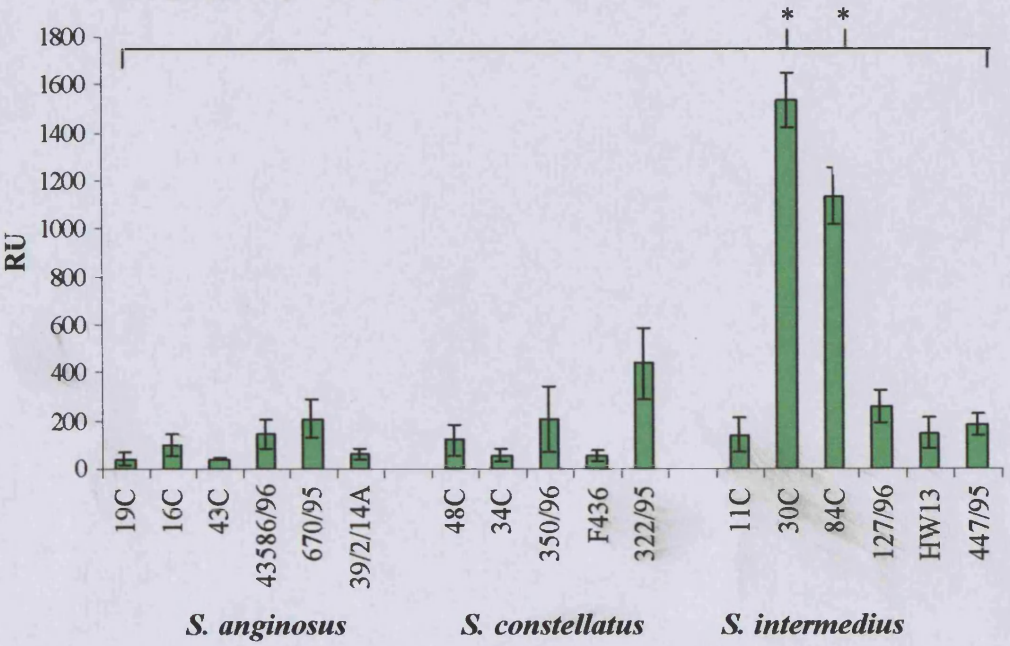




(c) alveolar bone proteoglycans



(d) gingival proteoglycans



Overall, 59% of the SAG strains, including commensal and pathogenic isolates, bound to alveolar bone PGs, 70% of strains bound to intact recombinant decorin, 76% bound to recombinant biglycan, 100% of the SMG bound to intact gingival PGs. However, increased standard deviations within triplicate assays for the SAG isolates with PGs causes some isolates to appear insignificant. These high deviations maybe due to a decreased activity in the ligand because of the time course of the assay and moderately harsh regeneration conditions. Important to note is the majority of strains that bound to intact PGs did not dissociate immediately from the immobilised sensor chip following the end of injection (see figure 3.3). However, subsequent regeneration with NaOH (one or two regeneration cycles) removed the bound bacteria and the sensor chip surface was returned to baseline.

There was a severe lack of bacterial binding when the chip was immobilised with the core proteins of recombinant decorin, biglycan and ovine alveolar bone and gingival PGs (see table 3.3). There was no binding of *S. anginosus* and *S. constellatus* strains to any of the core proteins. For *S. intermedius* strains, there was no binding to recombinant PG core proteins or alveolar bone core proteins. However, several *S. intermedius* strains bound to gingival PG core proteins; 30C (74RU), 127/95 (42RU), and HW13 (25RU). The lack of binding to core protein alone suggests that the GAG chain is an important factor in PG-SAG interaction analysis.

Figure 3.6 Interaction of SAG with fibronectin.

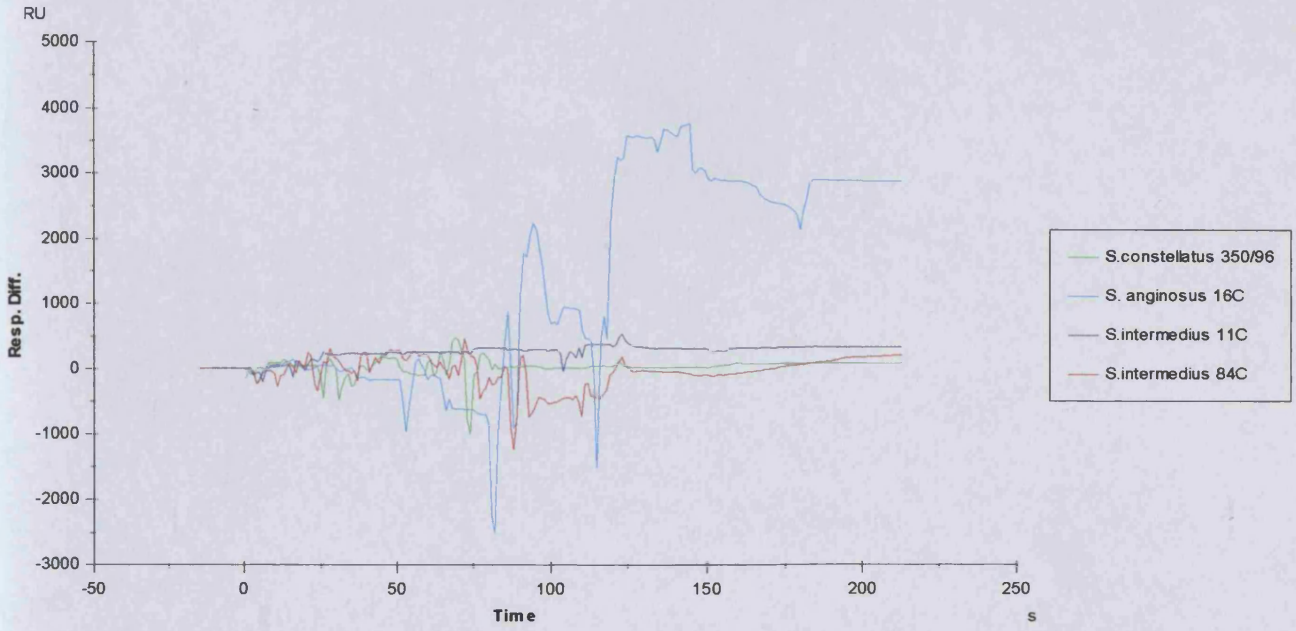


Table 3.4 Average binding (in RU), for three independent assays (± SD), of commensal and pathogenic strains of *S. anginosus*, *S. constellatus* and *S. intermedius* to fibronectin.

S. constellatus	RU±SD	*S. intermedius*	RU±SD	*S. anginosus*	RU±SD
34C	82.6±5.2	11C	109.5±17.2	16C	2170.2±245.3
48C	104±26.3	30C	1270.8±115	19C	289±23.4
F436	60.4±13.1	84C	158.9±23.5	43C	32.8±15.2
350/96	75.1±14.6	127/95	452±48.2	39/2/14A	32.9±12.4
322/95	283±46.1	HW13	251±32.1	43586/96	56.2±5.7
		447/95	180.5±23.1	670/95	45.8±26.3

3.3.2.4 Interaction of SAG with fibronectin control

Fibronectin was used as a control for SAG binding as interactions between this group of bacteria to this extracellular glycoprotein are already established (Willcox, 1995). All SAG isolates in this study bound to fibronectin albeit in varying amounts from 32 – 2170 RU. Of particular interest, *S. anginosus* commensal isolate 16C was most significant with 2170RU, other significant binding was *S. intermedius* 30C (1250RU). This control study suggests that SAG binding interactions with intact SLRPs are relatively specific.

3.4 Discussion

Adhesion of microbes to host components is a critical initial step in the establishment of bacteria as commensals or in the initiation of infection. An increasing number of microbes have been shown to depend upon extracellular matrix components for adhesion to host cells and tissues (Rostand and Esko, 1997; Wadstrom and Ljungh, 1999; Menozzi *et al.*, 2002). Therefore, the interactions between isolates of the SAG, from a range of sources, with intact and deglycosylated PGs were investigated.

This study has established suitable conditions for the successful analysis of host-SAG interactions utilising SPR technology within a BIAcore system. This technique allows for analysis of many aspects of the host-microbial interaction process, in particular early interactions between bacteria and surfaces in a range of seconds, without the use of labelling interactants. It was clear from the results that there is a difference in the binding abilities of the different species of the SAG. Generally, *S. anginosus* strains exhibited low binding to intact PGs (below 50RU) compared to *S. constellatus* and *S. intermedius* strains. *S. constellatus* strains exhibited low binding to intact alveolar bone PGs, moderate binding to gingival PGs, and low to moderate binding to intact recombinant PGs, however, strain 322/95 did produce statistically significant binding to intact recombinant decorin. For *S. intermedius*, the majority of strains produced moderate to high binding to all intact PGs, with strains 127/95, 30C and

84C producing statistically significant interactions. Interestingly, 30C also exhibited significant binding to the fibronectin control. All of the SAG strains bound to gingival PGs, with an average of 75% binding to recombinant PGs and just over half of strains (59%) binding alveolar bone PGs. In considering the binding patterns of the SAG, it is recognized that different species of the SAG are associated with different clinical manifestations (Clarridge *et al.*, 2001), which may govern their binding characteristics.

The reason for the significant differential binding characteristics between commensal and pathogenic strains of *S. intermedius* is unknown, but it may indicate an adaptation of commensal isolates to become better pathogens. Whiley *et al.* (1990) examined 157 strains of SAG in relation to disease. Of the 58 strains originating from supra-gingival plaque of healthy individuals, 66% were *S. intermedius*, 24% were *S. constellatus* and 10% were *S. anginosus*. Of the 6 strains isolated from sub-ginigival plaque, 5 strains were *S. constellatus* and one was *S. anginosus*. The ability of these strains to interact with the ECM of the oral tissues is paramount for the survival of these bacteria to initiate infection. There are numerous studies that have observed commensal interactions. Allen *et al.* (2002) examined the ability of 55 clinical blood and 21 oral viridans streptococci (VS), which included *S. anginosus* and *S. intermedius* isolates, to adhere to individual purified ECM including laminin, fibronectin and collagen type I

and IV. This study reported ECM adherence is a common trait among all VS, whether isolated from the bloodstream or from the healthy oral cavity. Also, Okayama *et al.* (2005) examined the abscess-forming ability of native dental plaque in mice and anti-phagocytic properties of microbial isolates following injections of supra-gingival dental plaque. From the thirteen microorganisms detected from pus samples, the majority of isolates belonged to the SAG, suggesting plaque itself is a source of infectious pathogens that cause abscess formation.

One of the fundamental parameters of using SPR technology is the purity of the investigated (immobilised) compound. In this study, highly purified PGs were used to examine their role as potential binding sites that mediate adhesion of oral bacteria. As well as single protein species of recombinant decorin and recombinant biglycan, this study also investigated the interaction of SAG with a mixed decorin and biglycan population present within the gingival and alveolar bone matrices.

SLRPs are renowned for their binding abilities to numerous components of the ECM and growth factors, providing the migration and signals for governing cell function (Yamaguchi *et al.*, 1990; Schonherr *et al.*, 1995; Kinsella *et al.*, 2004), and also being involved in the maintenance and healing of periodontal tissues, following infection by periodontopathogens. These interactions involve defined regions within the SLRP protein core

and their GAG chains. Potential binding to these areas by SAG could mediate adherence and colonisation to the host tissues, and might modulate or trap the functional interactions of SLRPs within the ECM.

Some bacterial adhesins exhibit differential binding for matrix proteins in soluble versus immobilised states, presumably due to phase related conformational change of ligands. Thus, the solid phase ECM adherence assay may mimic conditions present within tissue more closely than the soluble phase (Allen *et al.*, 2002). Within BIAcore analysis, cell-surface multivalent ligands such as, highly glycosylated PGs, are often tethered by a single attachment point. *In vitro,* it is difficult to immobilise multivalent ligands at a single site due to their heterogeneity. Thus, it is unknown whether the binding site on the PG is 'hidden' or shielded away from the bacterial (analyte) receptor. Therefore, random covalent coupling was utilised in these studies to immobilise the PGs. This method yields a surface displaying an ensemble of multivalent ligand orientations (Gestwicki *et al.*, 2002). The low immobilisation levels obtained in this study were probably related to the use of a C1 sensor chip devoid of a dextran matrix. Therefore, the surface has a lower hydrophilic character and has a much lower immobilisation capacity than CM dextran chips. Dextran matrix chips were utilised in pilot studies but the matrix appeared to interfere with the interaction. However, the use of the C1 chip was preferable since mass transport, steric hindrance, surface crowding,

aggregation and rebinding events were minimised. Zhang *et al.* (2002) covalently immobilised heparin and the structurally related heparan sulphate, the most acidic polysaccharides in the human body, on the C1 bio-chip. They achieved immobilisation levels of approximately 300RU due to the addition of 2M guanidine to the sodium acetate buffer (pH 4), which provided sufficient charge shielding. However, the addition of 2M guanidine in this study did not result in higher immobilisation levels to those successfully achieved without 2M guanidine.

Some of the interactions observed for the SAG with PGs appear to show moderate to high binding values, but they do not appear statistically significant due to the high standard deviations incurred. This maybe due to the regeneration of the chip resulting in possible loss of ligand function after many binding cycles (50-100 times). Also, as binding is not as specific in some interactions, other tissue matrix proteins such as fibronectin, fibrin, laminin and collagen may well provide alternative colonisation sites and involve other binding proteins on the bacterial surface. The SaG are known to bind fibronectin (Wilcox *et al*, 1995a), thereby promoting indirect cell adhesion. While this interaction may be relevant for adhesion to host cells surroundings where this ligand is nearby, it seems probable that these bacteria could also possess a mechanism whereby adhesion can occur in the absence of this circulating ligand. The data from this study suggests that one such mechanism of SAG

binding to ECM components, in particular decorin and biglycan, could be via the GAG chain, as within this study there was only binding of several *S. intermedius* strains to deglycosylated PGs.

GAGs are renowned for their functions as attachment sites for cellular adhesion molecules and matrix molecules, stabilising tissue structure and surrounding structures such as matrices. Beside these endogenous functions, invading microorganisms, such as parasites, bacteria and viruses also utilise GAGs for invasion. They serve as primary attachment sites for these microbes or their effector molecules before they eventually enter the cells. It is assumed that binding of bacteria to various GAGs depends on electrostatic interactions between the negatively charged sulphate groups of the GAG chains and positively charged regions of the ligand (Rostand and Esko, 1997). Leong *et al.* (1998) found that the recognition of GAG chains by the spirochete *Borrelia burgdorferi*, previously shown to bind to heparin, HS, and DS, as well as decorin, depended upon both charge and chain length of the GAG (Guo *et al.*, 1995; Leong *et al.*, 1995). Parveen *et al.* (1999) reported charge was not the sole determinant for binding of *B. burgdorferi*, as it did not recognise all GAG chains, and the spectrum of GAGs that were efficiently bound varied among different strains. Thereby suggesting GAG binding by this particular bacterium displays an element of specificity (Leong *et al.*, 1998). Therefore, the SMG-PG interactions within this study may be dependent not only on electrostatic attractions,

but also on specificity due to the presence of iduronic acid residues in the DS-GAG chain.

This investigation proposes that the SAG have significantly increased binding to DS containing PGs from the gingival tissues, compared to CS GAG chains in the decorin and biglycan mixture from alveolar bone and recombinant PGs. CS and DS differ mainly in the epimerisation of uronic acid. The CS GAG chain is composed of a linear polysaccharide assembled as disaccharide units containing *N*-acetyl-galactosamine and hexuronic acid that are linked together by β-glycosidic (β1,4 or β1,3) linkages. DS is structurally related to CS, however the uronic acid of DS is iduronic acid, whilst CS contains glucuronic acid. Iduronic acid is also present in significant amounts in heparin and HS. The presence of iduronic acid causes an increase in the flexibility of these GAG chains, a property that has been shown to be important for GAG interactions with other proteins (Casu *et al.*, 1988). *S. pyogenes* was found to bind more to DS than CS, which was mediated through surface associated M proteins (Frick *et al.*, 2003). Also, Srinoulprasert *et al.* (2006) demonstrated *Penicillium marneffei* conidia adherence to DS and heparin but not to C4S, C6S or HA. This study suggested that binding was via the iduronic acid of the polysaccharide chains, as the conidia were unable to adhere to HA confirming that glucuronic acid is not involved in adhesion.

In conclusion, this study has shown that certain commensal and disease isolates of the SAG are more capable than other commensal and disease SAG strains in binding to the GAG chain attached to the SLRPs, decorin and biglycan, found within gingival and alveolar bone tissues. This is a potential novel pathogenic mechanism of the SAG that is likely exploited during very early stages of colonisation, and maybe a pathogenic determinant in the production of abscess formation and other infections caused by this group of bacteria. Characterisation of these interactions between matrix proteins and their corresponding SMG binding proteins would be important in understanding the mechanism of SMG colonisation with a view to preventing or controlling infection.

CHAPTER FOUR

Chapter 4

Periodontal ligament cellular response to *Streptococcus anginosus* group bacterial supernatants

4.1 Introduction

Dentoalveolar infections are pus-producing infections that result from infection around the apicies of teeth. Bacteria gain access to this site either from the pulp, secondary to dental caries or via the periodontal route between the gingivae and the teeth (Samaranayake, 2001). Bacteria frequently isolated from dentoalveolar abscesses include the microaerophilic streptococci, the SAG and the presence of this group of bacteria in the root canal system is well documented (Sundqvist, 1992; Ozaki *et al.*, 1994; Fouad *et al.*, 2003). The root canal space is in open communication with the periapical tissues (PDL, cement and alveolar bone) via the apical foramen. Bacterial metabolites and toxic products arising from bacteria present within the root canal diffuse into the periapical tissues and evoke inflammatory responses (Love and Jenkinson, 2002). The SAG are frequently encountered within supra- and sub-gingival plaque (Whiley and Beighton, 1991; Rawlinson *et al.*, 1993; Okayama *et al.*, 2005), and entry into gingival tissues with subsequent colonisation and infection, often result in exposure of PDL cells to microbial products as well as to proinflammatory cytokines liberated from neighbouring tissue (Agarwal *et al.*, 1998).

The PDL is a complex, vascular, and highly cellular soft tissue. It is a physically small but functionally important tissue, particularly in terms of

tooth support, proprioception and regulation of alveolar bone volume (McCulloch *et al.*, 2000). The PDL contains a wide range of cells including cementoblasts, osteoblasts, fibroblasts, myofibroblasts, endothelial cells, nerve cells and epithelial cells. Fibroblasts are abundant and are responsible for maintaining PDL integrity and for the regeneration of the periodontium, via the metabolism of ECM components (McCulloch *et al.*, 2000). Fibroblasts attach to the substratum of the ECM via surface receptors for structural matrix components such as, collagen and fibronectin. This attachment is essential for cell migration and organisation of the extracellular fibrillar matrix (Cho and Garant, 2000).

The SLRPs play an important role in the maintenance and fundamental integrity of the PDL. As discussed in chapters 1 and 2, both decorin and biglycan have a role in connective tissue matrix formation, growth regulation, and they also bind certain growth factors (Ruoslahti, 1989). Many studies have identified the presence of PGs, including SLRPs, within the ECM of healthy PDL. Larjava *et al.* (1992) carried out a biochemical analysis of newly synthesised PGs by PDL cells obtained from healthy human periodontium and studied the distribution of PGs in different culture compartments (medium, cell membranes and ECM). They found that human PDL cells express a number of PGs, including decorin, which were predominant in the culture medium. Hakinnen *et al.* (2000), through immunohistochemical analysis, reported that decorin is consistently expressed throughout the PDL during development, and Qian *et al.* (2004) demonstrated biglycan and decorin expression in healthy rat periodontium. Matheson *et al.* (2005) showed co-expression of decorin,

biglycan, fibromodulin and lumican in human and mouse gingival and PDL connective tissues where they colocalized with collagen fibril bundles. The predominant GAG constituent within PDL appears to be DS (Larjava *et al.*, 1992), with others including HA and CS (in an over-sulphated form).

Generally, bacteria and their constituents (i.e. lipopolysaccharide, lipoteichoic acid, enzymes) have been proposed to be responsible for the destruction of periodontal attachment and bone (Lamont and Jenkinson, 1998). PDL cells are capable of responding directly to oral microbial challenges or indirectly, following activation of the host immune response. They can alter the composition of connective tissue in several ways, namely, synthesis of inflammatory mediators, their receptors and antagonists; fibroblast proliferation; collagen synthesis; and synthesis of proteolytic enzymes, including MMPs and their corresponding inhibitors (Havemose-Poulsen and Holmstrup, 1997).

Alterations in the ECM components are reported at sites of periodontal disease. Several periodontopathic bacteria, including *P. gingivalis* and *Treponema denticola*, have been reported to suppress tissue regeneration *in vivo* not only by activating host defence mechanisms but also directly via a suppression of growth and differentiation of human PDL fibroblasts and a reduction in ECM collagen (Marquez-San Miguel *et al.*, 2003). The expression of type I collagen mRNA has been demonstrated to be reduced within both gingival and PDL tissues during periodontal disease (Larjava

et al., 1989). Other immunochemical studies have demonstrated the appearance of CS-decorin and CS-biglycan metabolites in gingival crevicular fluid associated with advanced with periodontal disease, which has been further shown to arise as a consequence of alveolar bone destruction (Waddington *et al.*, 1998).

Surprisingly, little is known about the direct influence of the SAG bacteria on the biological activity of PDL cells. This chapter aims to investigate the effects of SAG supernatants on PDL cell growth, differentiation and ECM synthesis, as the nature of these interactions are likely to have a major influence on the progression of disease. More specifically, this chapter was initiated with a view to determining the possible roles of decorin and biglycan PGs within the PDL during infection with the SMG. The specific aims were, therefore, to examine PG expression from cultured PDL cells in the absence and presence of SMG supernatants. These PGs were examined at both the mRNA level and through the identification at the protein level of the parent species and metabolites extracted from the surrounding medium compartment. These observations will provide a better understanding of the exact functions of PGs during PDL cell response in the presence of pathogenic agents associated with periapical abscess formation.

4.2 Materials and methods

4.2.1 Bacterial culture and supernatant preparation

SAG strains showing high levels of adherence in the previous chapter were selected for further studies. These were *S. constellatus* strains 322/95 and 34C, *S. intermedius* isolates 127/95 and 30C, and *S. anginosus* isolates 43586 and 43C (see table 3.1 for source). Bacteria were grown on 5% blood agar anaerobically for 18hr, and subsequently sub-cultured into 10ml Brain Heart Infusion (BHI) broth. Broth cultures were incubated to an OD (550nm) = 0.5 under anaerobic conditions at 37°C. 5ml of bacterial suspension was added to 45ml of fresh BHI broth and incubated anaerobically until stationary phase (approximately 8hrs) (1 x 10^8 cfu/ml).

Bacteria were pelleted at 3, 500g for 10 min. The supernatants were filtered with a 0.2μm filter, and the concentration of the supernatant adjusted to 10ng/ml – 1μg/ml, with culture media, using the BCA protein assay kit (section 2.2.6) and stored at -70°C until required.

4.2.2 Preparation of periodontal ligament-like cells

4.2.2.1 Source of cells

Periodontal ligament-like cells were obtained by enzymatic digestion of soft tissue adherent to alveolar bone dissected from 28 day-old male Wistar rats. Incisions were made at both corners of the mouth, cutting through skin and muscle. The front molars were forced apart using a sharp scalpel and the muscle and other soft tissue around the jawbone was

excised. The molars and incisors were carefully removed leaving the bone. Alveolar bone lining the teeth was removed, detaching any remaining gingiva then dissected into 1mm^2 pieces.

Rat bone chips were incubated in a 1:1 mixture of 1mg/ml collagenase (Type IV, Sigma Chemical Co.) and 4.5U/ml elastase (Type IV, Sigma Chemical Co.), for 2 hours at 37°C with vigorous agitation every 15 min to release the PDL. The enzyme mixture, containing the released cells was removed, 2ml of serum-containing media (Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) and antibiotics mixture (100 units/ml penicillin G, 10 mg/ml streptomycin sulphate, 25mg/ml amphotericin B) (Sigma Chemical Co.), was added to inhibit the collagenase and elastase activity, and cells were pelleted by centrifugation at 1000g for 5min.

The supernatant was discarded and another 2ml of serum-containing media was added to wash the cells, which were gently pelleted again by centrifugation at 1000g for 5min. The cells were aseptically transferred to a T-75 tissue culture flask, incubated at 37°C, in a humidified atmosphere of 5% CO_2. After 24hr, the media was changed to remove any dead cells, after which the media was changed every 2 days.

4.2.2.2 Cell culture conditions

On reaching confluence, PDL-like cells were treated with trypsin to obtain a homogeneous cell suspension for secondary cultures. The existing media

was removed and the cells washed twice in PBS followed by the addition of 2ml 0.01% trypsin (Invitrogen Ltd.), which was incubated at 37°C in 5% CO_2 for 10 min. An equal volume of media was added to neutralise the action of the enzyme and the mixture centrifuged at 1000g for 5min. The supernatant was decanted and the pellet re-suspended in 1ml of serum-containing media.

PDL-like cells were counted using a haemocytometer and reseeded at a 1 x 10^4 cells/cm^2 in a T-75 flask and incubated at 37°C in 5% CO_2. Both serum-containing media with and without 50µg/ml ascorbic acid were used, in order to establish the most favourable growth conditions, according to an MTT assay (as described in 4.2.3.1).

4.2.3 Characterisation of periodontal ligament-like cells

To determine the characteristics of the PDL-like cells and to establish the number of times cells could be successfully passaged for experimental use following trypsinisation, MTT (as described in 4.2.3.1) and alkaline phosphatase (ALPase) activity (described in section 4.2.3.2) were measured at every passage.

4.2.3.1 Cell growth

PDL-like cell growth was assessed following the uptake of MTT formazan by viable cells. The MTT assay is a colorimetric, short-term suspension culture assay, which functions by measuring the metabolic activity of living cells. The tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), is taken up into cells and reduced by

a mitochondrial dehydrogenase enzyme to yield a purple formazan product which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells results in the liberation of the product, which can readily be detected using a simple colorimetric assay. The intracellular reduction of MTT is thus indicative of the normal functional biochemistry of energy-requiring mitochondrial enzyme reactions and, more broadly, provides a means to estimate the number of viable cells.

PDL-like cells from passage 1 to 5 were seeded into 6 well culture dishes at 1 x 10^4 cells/cm^2, and incubated with 2ml of DMEM containing 10% FBS and antibiotics for 24h. On day 0, media was removed and replaced with fresh media and every 24hr after day 0, 500µl of media was removed and 500µl 5mg/ml MTT (Sigma Chemical Co.), in PBS, was added and incubated for 4h at 37°C in 5% CO_2. The media was removed and replaced with 500µl lysing buffer (0.5M NN Dimethylformamide, 20% SDS; Sigma Chemical Co.), and incubated for 3h at 37°C, after which, the colour was released from the cells by mixing thoroughly, by pippetting. 100µl of sample was subsequently transferred to a 96 well plate (in triplicate) and the absorbance read at 540nm (Microplate Autoreader; Bio-Tek instruments). This procedure was repeated on days 2, 3, 4, and 5, to monitor the expansion of cell numbers with time in the culture media.

4.2.3.2 Alkaline phosphatase activity

Alkaline phosphatase enzymes are a specialised group of glycoproteins usually associated with bone matrix, detected in mineralised matrices and

linked with the external membranes of osteoblasts (Robey, 1993). No specific marker is available at present, however, many studies have used ALPase as an effective marker since it is thought that PDL-like cells are osteogenic in nature (Lekic *et al.*, 200; Saito *et al.*, 2002). Therefore, ALPase activity was utilised in the present study to characterise the extracted PDL-like cells.

PDL-like cells were cultured as described above, trypsinised and reseeded at $1x10^4$cells/cm^2 in 24-well plates, and incubated with 1ml of serum-containing media. Cells were allowed to settle for 24h, after which (day 0), the media was changed every 24h.

Following re-seeding of the cells (passage numbers 1-5), on days 2, 5, 7, 10, and 13, the media was removed from three of the wells in the culture plates and the remaining adherent cells were treated with 2.5ml 0.1% Triton X-100, 50mM Tris-HCl buffer, pH 7.2, for 15min at 37°C. The PDL-like cellular supernatants, containing solubilised cell components, were removed and buffer-exchanged using PD-10 columns (Amersham Life Sciences, Buckinghamshire, U.K.). PD-10 columns are pre-packed, disposable columns containing Sephadex® G-25 (bed volume 9.1ml) for rapid desalting and buffer exchange. Manufacturers' instructions suggest protein yields are greater than 95%, with salt contamination less than 4%. The columns were equilibrated with 25ml elution buffer (1M diethanolamine, 0.5mM magnesium chloride, pH 9.8). Once equilibrated,

the 2.5ml samples were allowed to run into the column and protein fractions were eluted with 3.5ml of elution buffer.

The alkaline phosphatase assay is based on that of Walter and Schutt (1974), in which *p*-nitrophenyl phosphate is dephosphorylated by alkaline phosphatase to generate *p*-nitrophenol, detected by an increase in absorbance at 405nm.

p-Nitrophenyl phosphate + H_2O	**Alkaline phosphatase** →	*p*-Nitrophenol + phosphate

The reaction was performed as a continuous spectrophotometric rate determination, with the increase in absorbance determined over 5 min. To 90µl of eluted sample, 10µl of freshly prepared 150mM *p*-nitrophenyl phosphate (Sigma Chemical Co.) solution was added. The activity of alkaline phosphatase present was calculated by applying the mean absorbance readings to the following equation:

$$\textbf{ALP activity (units)} = \frac{\textbf{Abs/min x TV}}{\textbf{18.45 x LP x SV}}$$

TV = Total volume (ml)
SV = Sample volume (ml)
18.45 = Millimolar absorptivity of p-nitrophenol at 405nm
LP = Lightpath (1-cm)

The assay was performed in triplicate and the mean activity of alkaline phosphatase (activity units/10^4 cells) for each time point was calculated.

4.2.4 Periodontal ligament cellular response to *S. anginosus* group supernatants

4.2.4.1 Viable cell count

The possible cytotoxic effect on PDL-like cells at a range of SAG supernatant concentrations was assessed by trypan blue staining. This dye is taken up by all cells, but only viable cells are able to pump the dye out. Therefore, this dye is only present within non-viable cells.

PDL-like cells were seeded in 24 well plates at a concentration of 1 x 10^4 cells/ml with 1ml media and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24hr. 500µl media was removed and 500µl of fresh media containing bacterial supernatants were added at various concentrations (20, 50, 100, 500ng/ml, 1, 10, 50, 100, 200, 500µg/ml). BHI broth was also used as a control. After 24 and 48hr incubation, the cells were washed three times with PBS and subsequently incubated with 1ml 0.4% trypan blue stain for 1min. The stain was removed and cells washed once with PBS. The amount of blue stained cells and non-stained cells were immediately counted under an inverted microscope with viability greater than 95% deemed to be acceptable.

4.2.4.2 Effect of *S. anginosus* group supernatants on periodontal ligament cell growth

The influence of SAG supernatants on PDL-like cell growth was assessed by MTT assay (see section 4.2.3.1). Following determination of viability counts for PDL-like cells with a range of supernatant concentrations, there was a survival rate of less than 95% for cells cultured with supernatant

concentrations above 50ng/ml. Therefore, for the MTT assay, PDL-like cells were cultured with 0ng/ml or 20ng/ml of SAG supernatants from day 0, and every 24h thereafter, for 5 days.

4.2.4.3 Effect of *S. anginosus* group supernatants on periodontal ligament cell alkaline phosphatase activity

PDL-like cells were prepared as in section 4.2.3.2, with the addition of 0ng/ml or 20ng/ml of SAG supernatants on day 0. The assay was carried out on days 2, 5, 7, 10 and 13.

4.2.4.4 Effect of *S. anginosus* group supernatants on mRNA expressions of decorin and biglycan by periodontal ligament cells

PDL-like cells were examined for decorin and biglycan mRNA expression in the presence and absence of 20ng/ml of each SAG supernatant. Cells were seeded at 1 x 10^4 cells/cm^2 in 6-well plates with media and at day 0, the cells were cultured in serum containing media supplemented with or without the supernatant, and changed every 24h. On days 2, 5, 7, 10, and 13, the media was removed and the cells washed three times in PBS, ready for subsequent extraction.

4.2.4.4.1 RNA extraction

Isolation of total RNA from PDL cells was carried out using a Qiagen RNeasy Mini kit (Qiagen Ltd, West Sussex, UK). The extraction steps were carried out according to the manufacturers' instructions. All steps were performed quickly, at room temperature, to minimise RNA

degradation. Contamination with genomic DNA was eliminated using a DNA-free kit (Promega, UK), according to the manufacturers' instructions.

4.2.4.4.2 Quantification of total RNA by UV spectrophotometry

2µl of total RNA was diluted in 98µl of RNase-free water (Qiagen Ltd), and the absorbance read on a GeneQuant II 'DNA calculator' (Amersham Biosciences). RNase free water was used to provide a blank reading and the absorbance value of each RNA sample was read at wavelengths of 260nm (E260) and 280nm (E280). The concentration of RNA (µg/ml) was determined using:

[RNA µg/ml] = Absorbance (E260) x 40 (RNA factor) x 50 (dilution factor)

The ratio of E260 divided by E280 gave an indication of the purity of the RNA obtained, with a ratio of approximately 1.8 suggesting highly pure RNA. Values between 1.6 and 2.1 were considered normal; samples above or below these values were discarded.

4.2.4.4.3 Reverse-transcription of total RNA

500ng (equivalent volume in µl) of total RNA starting material or water control was placed into a 0.2ml thin-walled eppendorf. To each sample was added 5µl x 10 thermophilic DNA polymerase buffer (100mM Tris-HCl, 500mM KCl and 1% Triton®X-100), 5µl 10mM dNTPs, 10µl 25mM magnesium chloride, 1µl 40U/µl RNase inhibitor, 2.5µl 500ng/ml random

hexamers, and 1µl 200U/µl reverse transcriptase (Promega, Southampton, UK). The final volume was made up to 50µl with nuclease-free DEPC (diethylpyrocarbonate, Sigma Chemical Co.) treated water. Addition of nuclease-free water in replacing the constituents described above provided a non-RT control.

All samples were vortexed, centrifuged for 1min at 8, 000g and incubated at 25°C for 10 min, 42°C for 60 min and 95°C for 5 min, for 37 cycles, to generate cDNA. The samples were subsequently cooled to 4°C and stored at -20°C until required for PCR.

4.2.4.4.4 Polymerase chain reaction (PCR)

PCR was performed to amplify gene sequences relating to decorin and biglycan. Levels were compared to the housekeeping gene glyceraldehyde-3-phosphate (GAPDH). Primer sequences, optimal magnesium concentrations and annealing temperatures are displayed in table 4.1.

The RT products (cDNA), water and non-RT (RNA) control samples were added to a PCR mixture containing corresponding sense and antisense primers in order to amplify a specific part of the gene of interest. PCR mixtures were prepared on ice by adding 1µl 0.2mM dNTPs, 2.5µl 1µm sense primer, 2.5µl 1µm antisense primer, 5µl x1 thermophilic DNA polymerase buffer (100mM Tris-HCl, 500mM KCl and 1% Triton®X-100), 0.5µl 2.5U Taq DNA polymerase, 3µl magnesium chloride 2µl cDNA / non-RT (RNA) / water control, making the final volume up to

50µl with nuclease-free water. All samples were vortexed, then centrifuged for 1min at 8, 000g. Cycling parameters were 94°C for 3 min, 35 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 5 min. Samples were cooled to 4°C and stored at -20°C until required.

Table 4.1 Oligonucleotide primer sequences and specific conditions for PDL PCR (Roberts, 2004).

	Primer sequence	Magnesium concentration	Annealing temperature
GAPDH forward primer **GAPDH reverse primer**	5' - TCCCTCAAGATTGTCAGCAA - 3' 5' - AGATCCACAACGGATACATT - 3' 307 bp product	2mM	58°C
Biglycan forward primer **Biglycan reverse primer**	5' - CCTCCAGCACCTCTATGCTC - 3' 5' - ACTTTGAGGATACGGTTGTC - 3' 204 bp product	3mM	58°C
Decorin forward primer **Decorin reverse primer**	5' - CAATAGCATCACCGTTGTGG - 3' 5' - CCGGACAGGGTTGCTATAAA - 3' 262 bp product	3mM	58°C

4.2.4.4.5 Detection of PCR products by agarose gel electrophoresis

PCR products were examined by agarose gel electrophoresis containing ethidium bromide, which is a fluorescent chemical that binds nucleic acids between base pairs in a double-stranded DNA molecule.

5x TBE buffer was prepared by adding one sachet of TBE (Sigma Chemical Co.) to 1 litre of ddH_2O and autoclaved. 2% agarose gels were prepared by mixing 1g of general-purpose agarose (Bioloine Ltd, London, UK) to 10ml 5-x TBE and 40ml ddH_2O. The gels were heated until the agarose had completely dissolved and cooled to approximately 56°C. 5µl of ethidium bromide (500µg/ml) (Sigma Chemical Co.) was added to the gel, mixed thoroughly and poured into a gel-casting tray and allowed to set for 10min.

A 100 base-pair marker (Promega) was prepared by mixing 10µl of marker with 2µl of 6 x loading buffer (Promega). 10µl samples were added to 2µl of x 6 loading buffer (Promega), then molecular weight marker and samples were loaded onto gels and run at 75V for 40 min in 0.5 x TBE electrophoresis running buffer. The gels were subsequently visualised under UV light and the gel images captured and semi-quantified using Bio-Rad Quantity One (Bio-Rad Lab., Hertfordshire, UK).

4.2.4.5 Extraction and identification of proteoglycans from conditioned cell culture medium

PDL cells were re-seeded at 1 x 10^4 cells/cm^2 and cultured in the presence and absence of 20ng/ml SMG supernatant. PDL cell culture media (in T25 flasks) was removed on days 2, 5, 7, 10 and 13 and dialysed, as described in section 2.2.2, against ddH_2O. The fully dialysed extracts were lyophilised and stored at -20°C until needed.

4.2.4.6 Immuno-detection of small leucine-rich repeat proteoglycans

PGs present within the tissue culture media from PDL-like cells, cultured in the absence and presence of 20ng/ml of SAG supernatants, were characterised on the basis of their immuno-reactivity to the polyclonal antibodies, anti-decorin (LF113) and anti-biglycan (LF106), by Western blot analysis (see section 2.2.7.2)

4.2.4.7 Glycosaminoglycan detection by cellulose acetate electrophoresis

The PDL-like media samples, supplemented with or without 20ng/ml of SAG supernatant, were examined for the presence of GAG chains using cellulose acetate electrophoresis (section 2.2.7.3).

4.2.4.8 Immunolocalisation of collagen type I

Due to the well-established presence of collagen type I within the PDL ECM, and the literature of evidence demonstrating the influence of decorin on collagen fibrillogenesis, the effects of SMG supernatants upon collagen type I within the PDL ECM were examined.

PDL-like cells were reseeded in 6 well plates at 1 x 10^4 cells/cm^2 and cultured in serum-containing media at 37°C in 5% CO_2 for 24h. The media was changed at day 0, after which the media (with and without supernatant) was changed every 24h. On days 2, 5, 7, 10, and 13, the media was removed and the slide fixed in 2% formaldehyde for 30min then stored in PBS at 4°C until sampling was complete.

Prior to staining, the slides were washed in PBS. Normal horse serum (Sigma Chemical Co.) was placed in each well and left for 10min to block background staining. This was removed and the cells incubated with mouse monoclonal anti-collagen type I (Sigma Chemical Co.), at 1:2,000 in 0.5% horse serum in PBS for 1hr at room temperature. The wells were then washed with 0.5% Tween-20 PBS, three times for 5min. Cells were incubated with rabbit anti-mouse IgG FITC conjugate secondary antibody at 1:100 (Sigma Chemical Co.) for 30min at room temperature. Slides were observed directly under a fluorescent microscope (at x40 objective).

4.2.5 Statistical analysis

The Student unpaired two-tail T-test (Instat Package, Graph Pad software) was performed to obtain *p*-values for analysis of differences between cells cultured with and without SMG supernatants. $p<0.05$ was determined as statistically significant.

4.3 Results

4.3.1 Optimisation of cell culture conditions

To establish the optimal culture conditions for PDL-like cells, culture media with and without ascorbic acid was examined. Figure 4.1 illustrates the unfavourable effects of ascorbic acid on PDL-like cells. Therefore, subsequent analysis of PDL-like cells were carried out without ascorbic acid in the growth media.

4.3.2 Passage effects on cell growth and alkaline phosphatase activity

In order to establish the optimal number of times PDL-like cells could be passaged until they lost their initial characteristics, PDL-like cells were subjected to an MTT and ALPase activity assay. Figure 4.2 shows the effects of passaging on PDL-like cells. Average MTT values (absorbance at 540nm) over five days in culture were taken from sets of cells from passages 1-5. These average MTT absorbance values were subsequently compared to average MTT assay results (over a five-day culture period) of PDL-like cells from passage 1. It appeared that, on average, PDL-like cells became less viable after passage 4. Therefore, PDL-like cells for subsequent assays were taken from passages 2-4. ALPase activity (data not shown) was also decreased in PDL-like cells from passage 5 compared to the activity of those cells from earlier passages.

Figure 4.1. The effects of ascorbic acid (50µg/ml) in serum-containing culture media for PDL-like cells. * Significant difference ($p<0.05$)

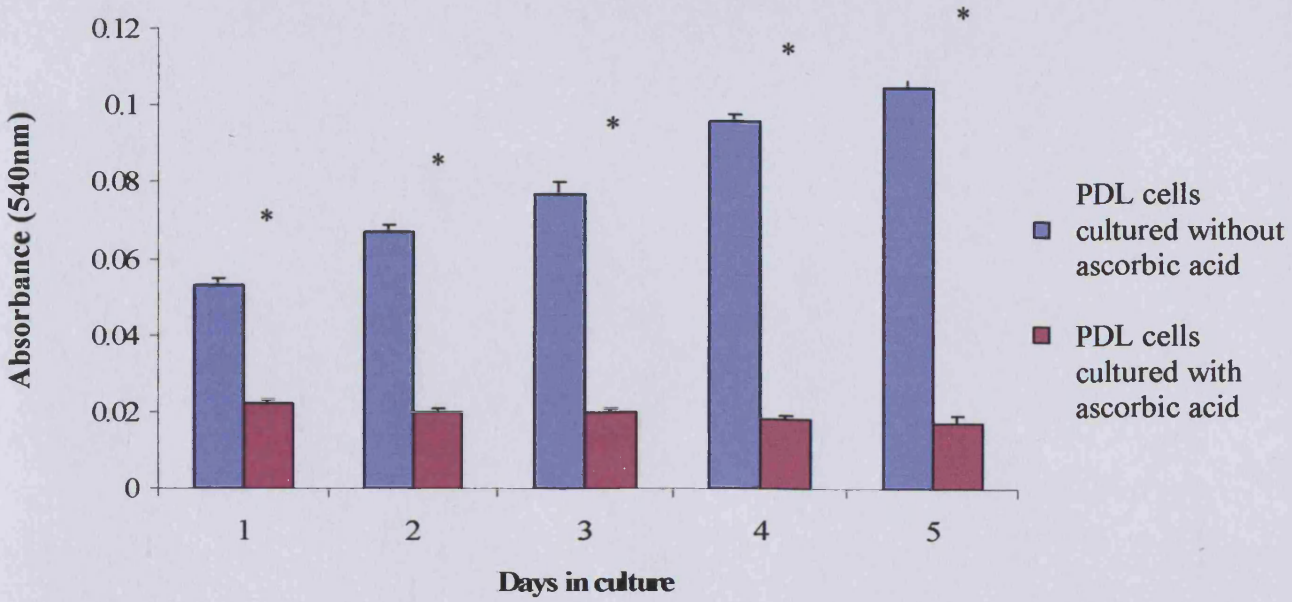
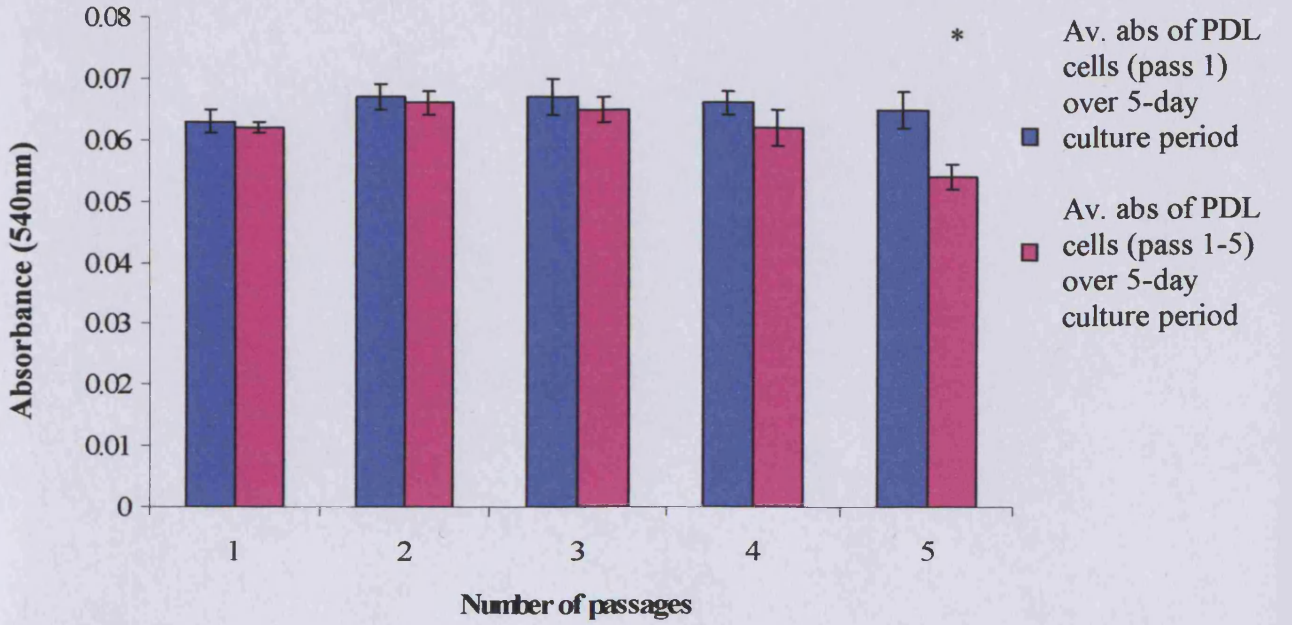


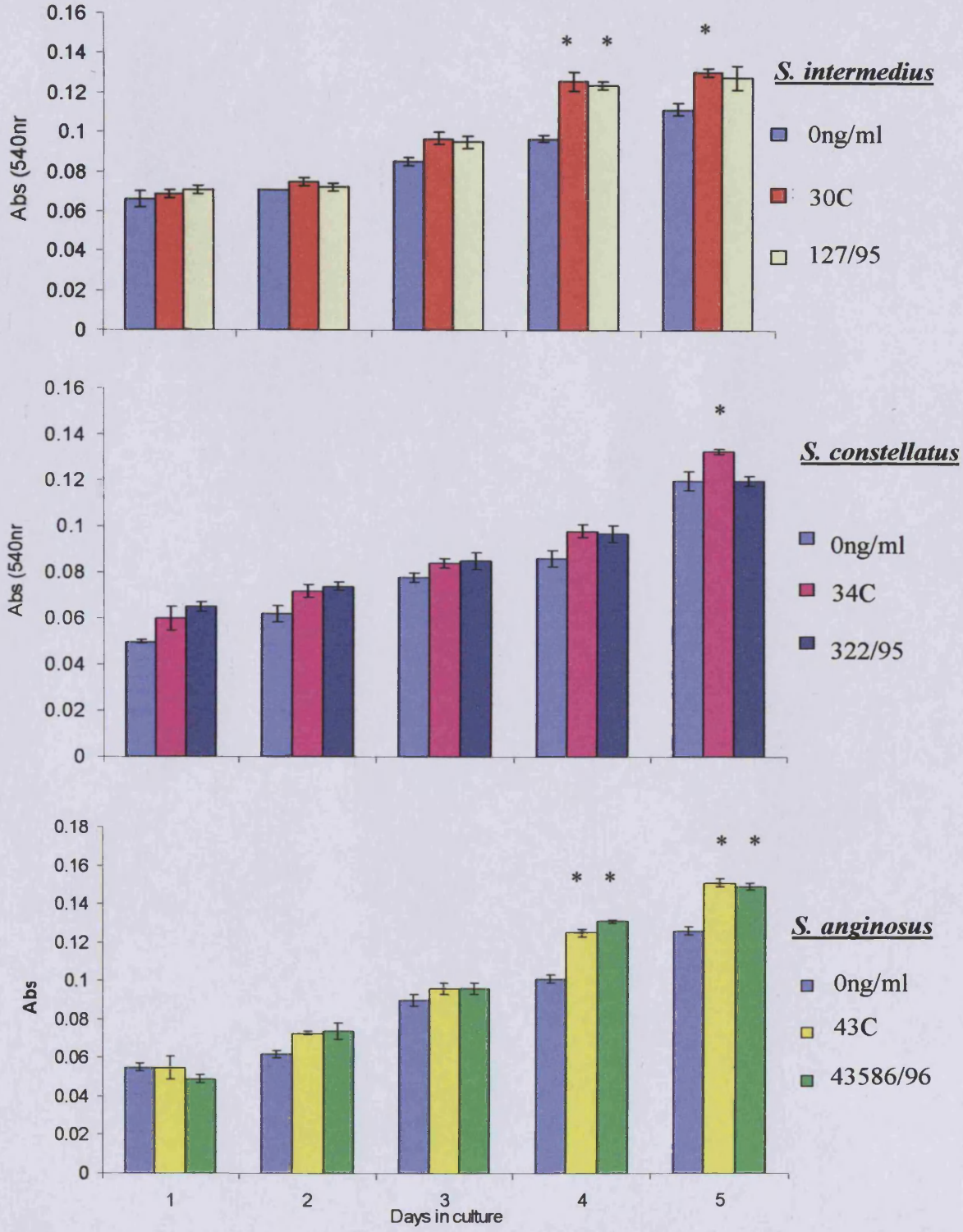
Figure 4.2. The effects of passaging on PDL-like cell growth. * Significant difference ($p<0.05$)



4.3.3 Viable cell count

Following culture of PDL-like cells with 0 - 500µg/ml of SAG supernatants (one commensal and one pathogen from *S. constellatus, S. intermedius* and *S. anginosus*), the cells were stained with trypan blue to obtain cell viability counts. 100 cells were viewed and the percentage of non-viable cells (blue stained) and viable cells (unstained) were counted. The percentage of viable cells for each supernatant were calculated from the cell count, where a cell viability count greater than 95% was acceptable. The cells cultured in the presence of 0ng/ml and 20ng/ml of all SMG supernatants demonstrated a viability of 95-98%. Cells incubated with 50ng/ml had a viability count of 90%, cells cultured with 100ng/ml or more of SAG supernatants had viabilities of 80% and less. Also noted at concentrations above 20ng/ml of bacterial supernatant were a number of cytoxic effects, appearing appreciably different from control cells, including rounding up of cells (data not shown). Therefore, 20ng/ml was used in further studies.

Figure 4.3 The influence of SAG supernatants on PDL-like cell growth. * Significant difference ($p<0.05$) between cells co-cultured with 0ng/ml and 20ng/ml of supernatant



4.3.4 The effects of *S. anginosus* group supernatants on periodontal ligament cell growth

The influence of SAG supernatants at a concentration of 20ng/ml on viable cell numbers was investigated using the MTT assay (figure 4.3). This assay revealed all commensal and pathogenic SAG strains demonstrated the same behaviour toward PDL-like cell growth, by increasing PDL cell growth in comparison to those PDL-like cells cultured without SAG supernatants. For all strains, there was an increase in PDL-like cell growth from day 1 to day 5, with statistically significant PDL-like cell growth on day 4 ($p<0.05$), with 10-20% increases, and on day 5 ($p<0.05$), with up to 15% increases compared to PDL-like cells cultured without SAG supernatants.

4.3.5 The effects of *S. anginosus* group supernatants on periodontal ligament cell alkaline phosphatase activity

Figure 4.4 illustrates the effect SAG supernatants have upon membrane-bound alkaline phosphatase activity. The PDL-like cells were cultured at day 0 in the absence or presence of individual supernatants and were subsequently assessed for ALPase activity at different intervals for 13 days. PDL cells cultured in the presence of *S. intermedius* commensal (30C) and pathogenic (127/95) supernatants showed a significant increase (25%) in ALP activity on day 5 in comparison to PDL-like cells cultured without superntants. However, this initial increase in activity did not continue and by day 13 the activity was less than the activity produced by PDL-like cells without supernatant.

For *S. constellatus* supernatants (34C and 322/95), there was a statistically significant increase in ALPase activity on day 5 compared to the activity of PDL-like cells without supernatant. However, on days 7 and 10 this activity began to decrease compared to cells without supernatant. On day 13 of culture, the ALP activity in PDL-like cells incubated with *S. constellatus* supernatants were statistically significantly decreased ($p<0.05$) compared to PDL-like cells without supernatant.

Both *S. anginosus* supernatants produced statistically significant decreases in ALPase of PDL-like cells on days 5 (~30% decrease) compared to those cells cultured without supernatant. Further statistically significant decreases were also observed for the *S. anginosus* commensal strain (43C) on days 10 and 13 (approximately 35% decrease). The pathogenic strain (43586/96) had no significant difference on the ALPase activity of PDL-like cells in the later stages of culture.

Figure 4.4 The influence of SAG supernatants on PDL alkaline phosphatase activity. * Significant difference ($p<0.05$) between cells co-cultured with 0ng/ml and 20ng/ml of supernatant

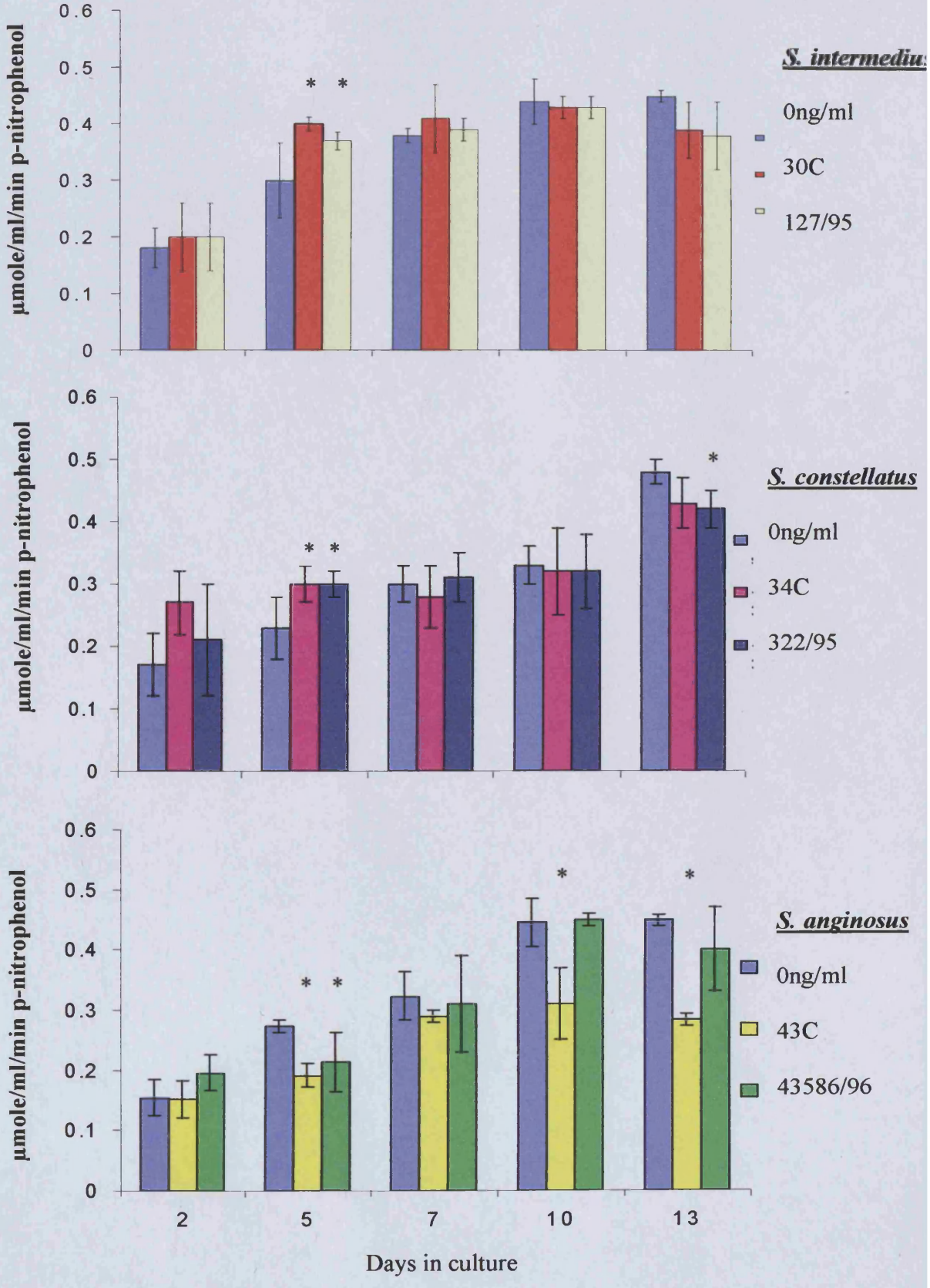


Figure 4.5. Typical effects of SAG supernatants on mRNA expressions for biglycan and decorin, standardised against GAPDH (example shown is *S. constellatus* 322/95).

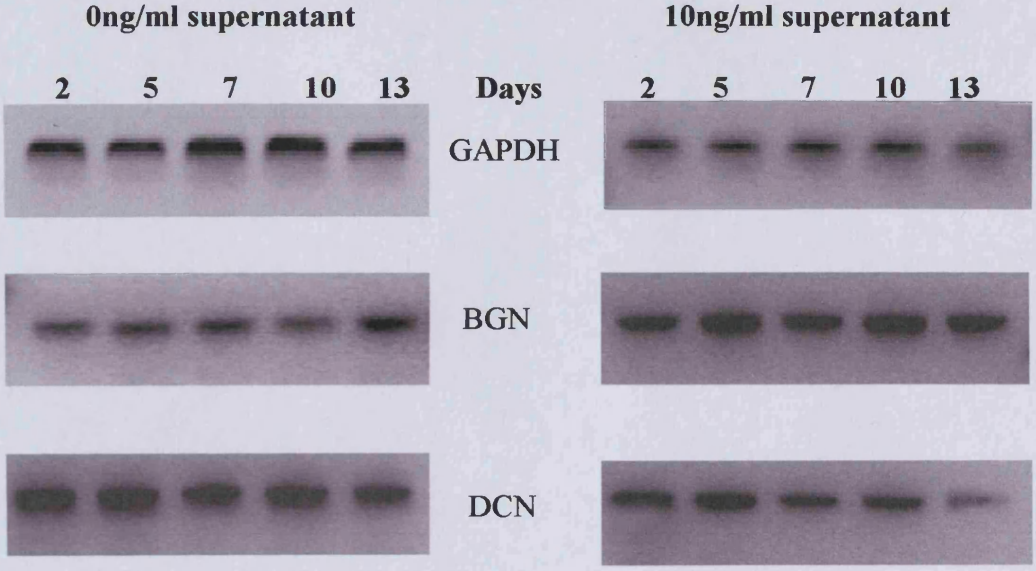
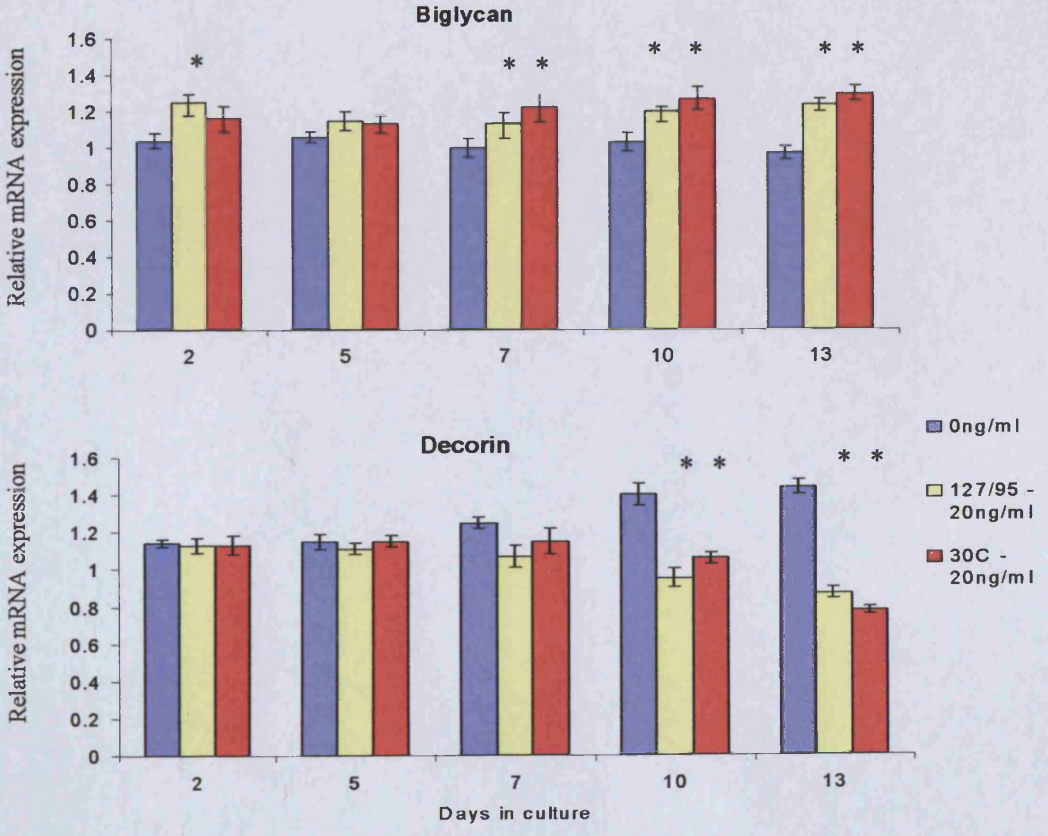
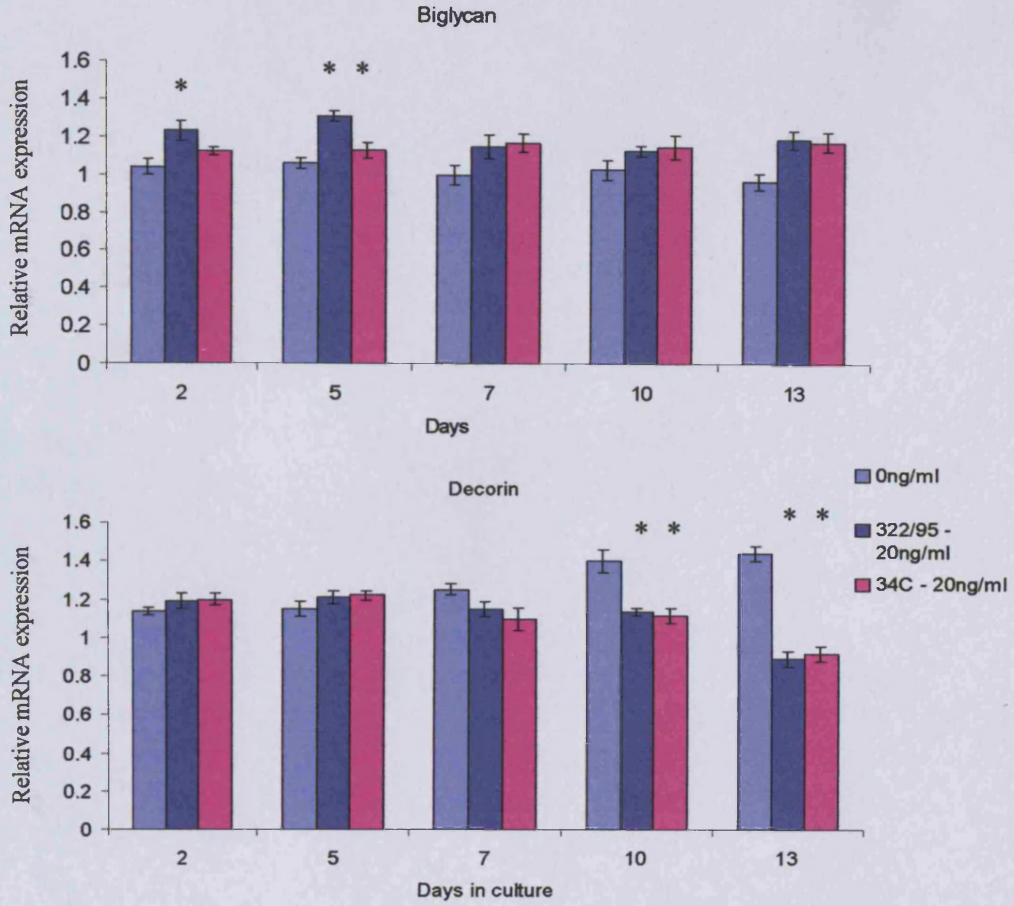


Figure 4.6. The effects of (a) *S. intermedius* (b) *S. constellatus* and (c) *S. anginosus* supernatants on mRNA expression of biglycan and decorin. * Significant difference ($p<0.05$) between PDL-like cells cultured in the absence and presence of 20ng/ml SAG supernatant.

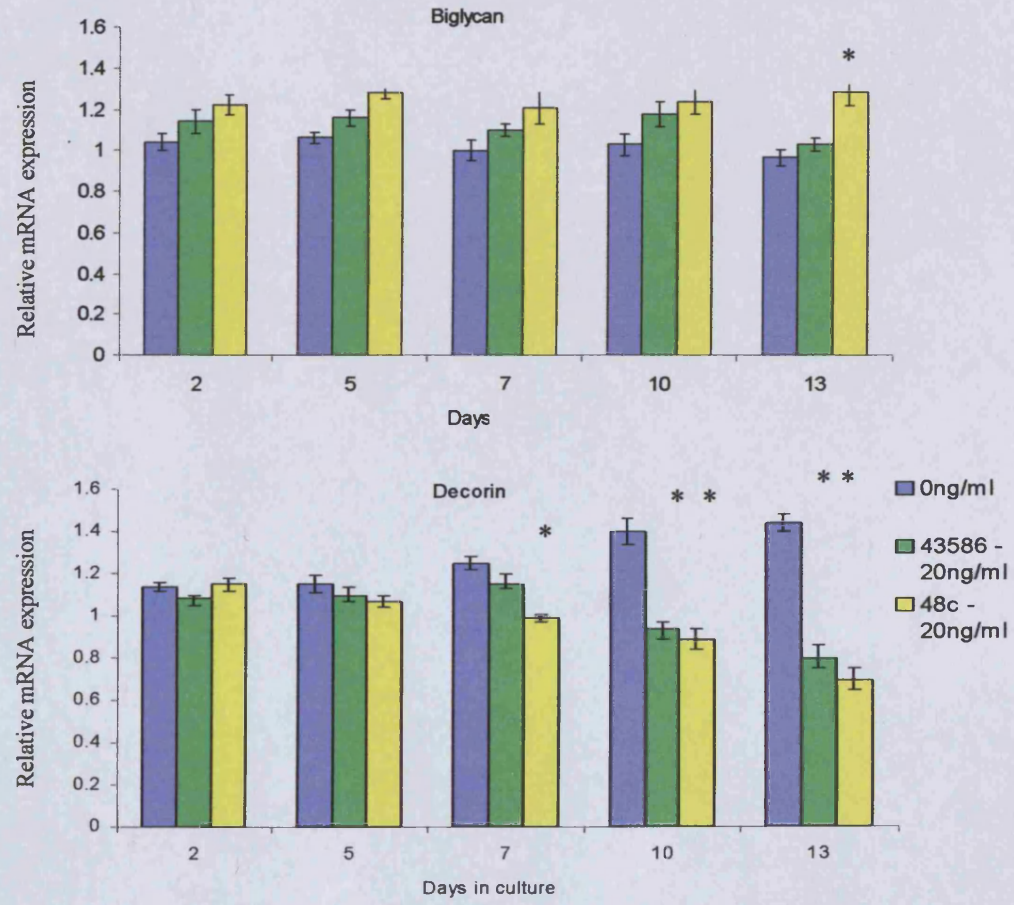
(a)



(b)


Biglycan
Relative mRNA expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
5
7
10
13
Days
Decorin
Relative mRNA expression
Days in culture
0ng/ml
322/95 - 20ng/ml
34C - 20ng/ml

(c)


Biglycan
Relative mRNA expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
5
7
10
13
Days
Decorin
Relative mRNA expression
Days in culture
0ng/ml
43586 - 20ng/ml
48c - 20ng/ml

4.3.6 The effects of *S. anginosus* group supernatants on biglycan and decorin expression by periodontal ligament cells

Figure 4.5 demonstrates the mRNA expressions for biglycan (204bp) and decorin (262bp) by PDL cells cultured in absence and presence of SAG supernatants. These expressions were normalised against GAPDH and the relative mRNA expressions for these SLRPs are illustrated in figure 4.6 (a-c).

Figure 4.6 (a) shows the relative mRNA expression of biglycan and decorin by PDL-like cells following co-culture with or without *S. intermedius* isolates. In the absence of supernatant, biglycan mRNA expression was detected at a steady rate throughout culture. Following exposure of PDL-like cells to either commensal or pathogenic *S. intermedius* supernatants, biglycan expression of PDL-like cells appeared to produce statistically significant increases from as early as day 2 and continued to increase biglycan (compared to cells without supernatants) throughout culture until day 13. The increase in biglycan expression by PDL-like cells appeared to be of a similar trend for each of the commensal and disease isolates of *S. constellatus* shown in figure 4.6 (b) and *S. anginosus* shown in figure 4.6 (c).

Figure 4.6 (a-c) also show the relative decorin mRNA expression by PDL-like cells cultured in the absence and presence of SAG supernatants. Decorin expression appeared to increase throughout culture in the absence of supernatants. However, on exposure to commensal or pathogenic strains

of *S. intermedius*, *S. constellatus* and *S. anginosus*, the relative mRNA expression of decorin decreased in comparison to PDL-like cells cultured in the absence of supernatant. For both *S. intermedius* supernatants, there were statistically significant ($p<0.05$) decreases in decorin levels on days 10 and 13 (35% and 50% decrease, respectively). Both commensal and pathogenic strains of *S. constellatus* produced the same effect on PDL decorin expression, showing significant decreases ($p<0.05$) on days 7, 10 and 13 (10-, 15- and 45%, respectively), in comparison to the relative amount of decorin mRNA expression by PDL-like cells cultured without these supernatants. Also, the commensal and pathogenic strain of *S. anginosus* acted in the same manner as *S. constellatus* strains, by producing significantly less decorin mRNA on days 7, 10 and 13 when compared to PDL-like cells cultured without supernatants.

4.3.7 Immuno-identification of small leucine-rich repeat proteoglycans

Following sample digestion with chondroitinase ABC, typical Western blots identifying the PG species present within media extracts of PDL-like cells, subsequent to culture in the absence and presence of SAG supernatants are shown in figure 4.7 (a), and figure 4.7 (b) respectively.

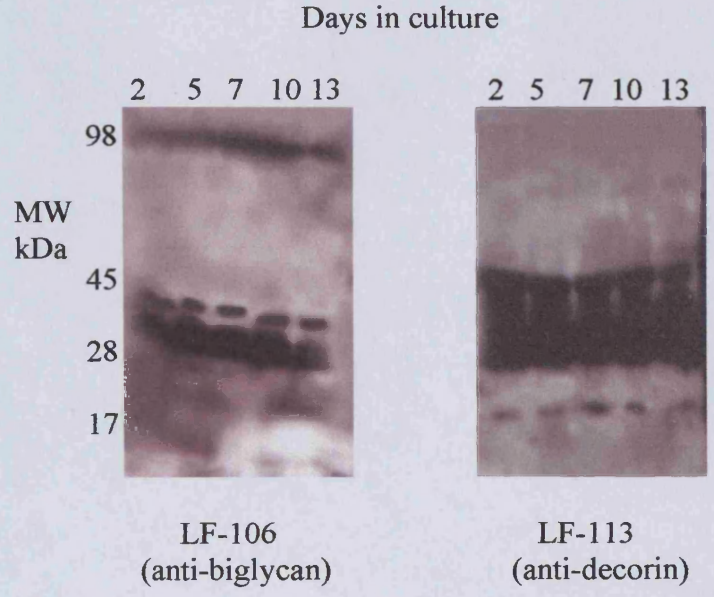
In the absence of SAG supernatant, LF 106, which detects a sequence in the terminal of biglycan, produced an intense immuno-response from day 2 which continued throughout culture until day 13, with a band recognisable at approximately 45kDa. Other observable bands were present including a strong band at about 98kDa, and a range of bands between 45-28kDa.

The immuno-detection of decorin within PDL-like cells was carried out by utilising LF-113, which has an epitope to the sequence in the N-terminal of the decorin core protein. In the absence of SAG supernatants, this antibody produced intensely stained bands at approximately 38- and 45kDa from day 2 through to day 13, with other bands detected at 28- and 17kDa.

Following co-culture with supernatants, immuno-detection for biglycan showed a band was evident at approximately 45kDa throughout culture, with other bands detectable from approximately 28- to 6kDa. Decorin immuno-detection for PDL-like cells cultured with SAG supernatants produced visible bands at approximately 45kDa, however, there was less intense staining when compared to decorin detection from cells without supernatants. Staining at 45kDa appeared to decrease from day 7, in the presence of SMG supernatant, until it was undetectable at day 13. Other bands at 28- to 6kDa were visible and did not decrease in reactivity throughout culture.

Figure 4.7. Typical Western blot analysis, following chondroitinase ABC digest, for the immuno-identification of biglycan and decorin synthesised into the media by PDL-like cells (a) without SAG supernatant (b) with 20ng/ml SMG (322/95) supernatant.

(a) 0ng/ml supernatant



(b) 20ng/ml SAG supernatant (322/95)

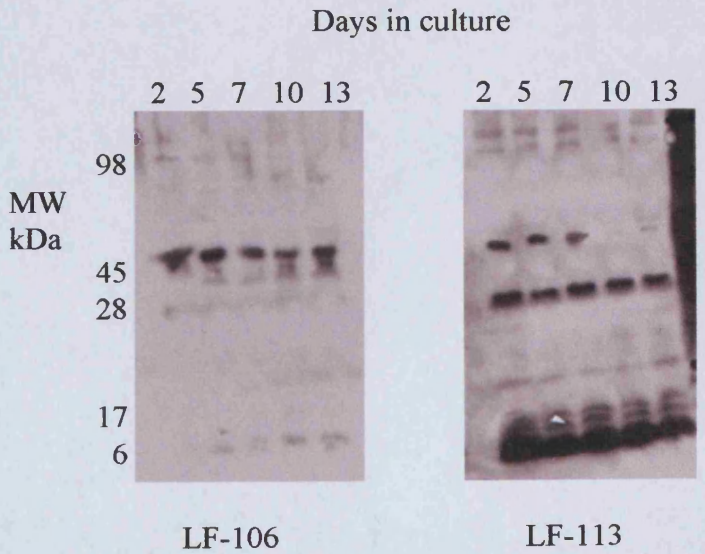
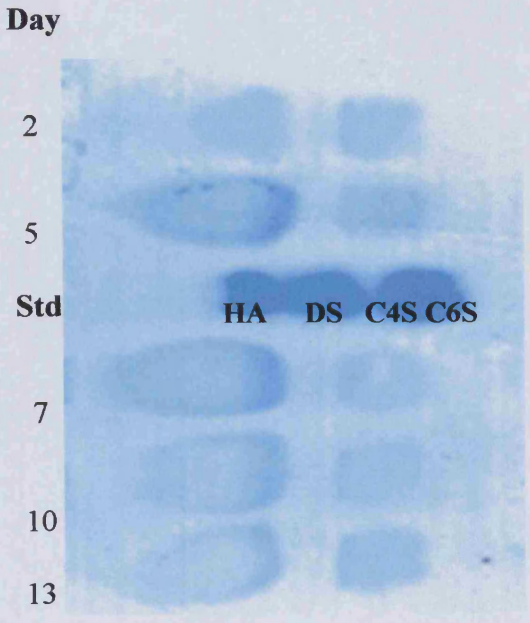
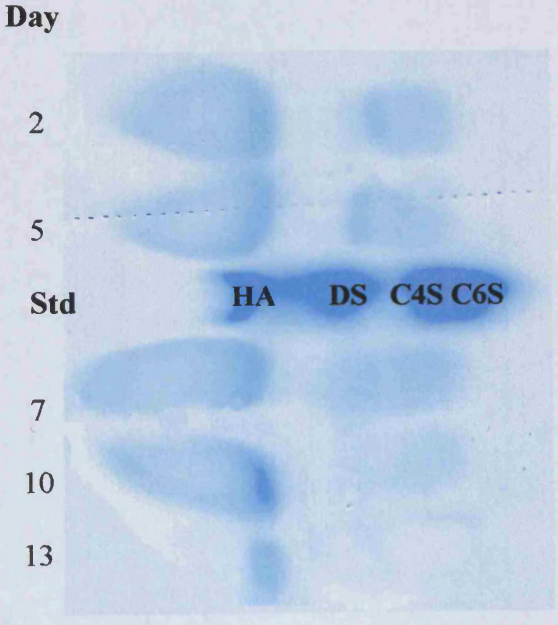


Figure 4.8. Typical glycosaminoglycan profiles produced by PDL-like cells with (a) no supernatant (b) *S. constellatus* or *S. intermedius* isolates, and (c) *S. anginosus* isolates.

(a) 0ng/ml SAG supernatant

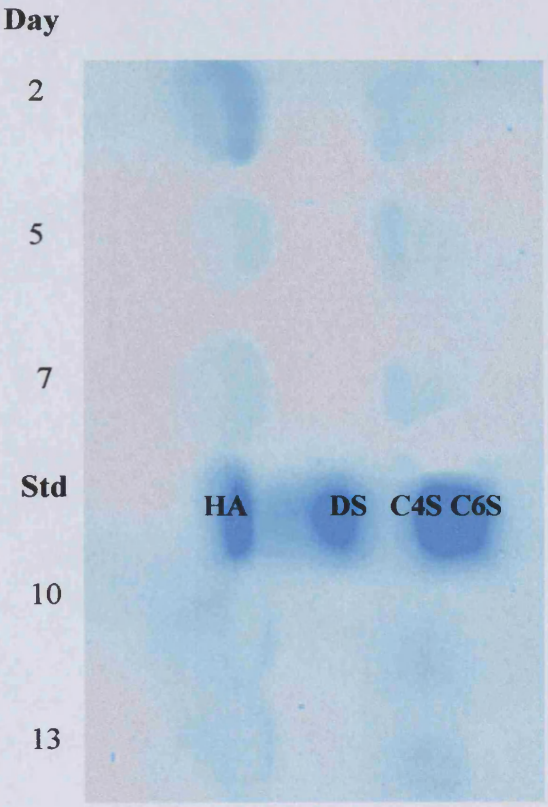


(b) 20ng/ml *S. constellatus / S. intermedius* supernatants



Std = Standard

(c) 20ng/ml *S. anginosus* supernatants



4.3.8 The effects of *S. anginosus* group supernatants on periodontal ligament cell glycosaminoglycan synthesis

The GAG chains were released from the PG media samples by protease digestion and analysed by cellulose acetate electrophoresis. Identification was judged on the distance of sample GAG migration compared to the GAG standard mixture.

A typical GAG separation profile from PDL-like cells cultured without SAG supernatants is shown in figure 4.8 (a). Cellulose acetate electrophoresis revealed the predominant presence of HA, DS and CS throughout culture in the absence of SAG supernatants, with an average of 35%, 32% and 33% of the total GAG content, respectively.

GAG analysis of PDL-like cell media following co-culture with commensal and pathogenic strains of *S. constellatus* and *S. intermedius* showed a similar pattern, illustrated in figure 4.8 (b). There was a gradual decrease in DS and CS from day 2 to day 13, with average decreases of 32% down to 21%. However, the intensity of HA increased when PDL-like cells were exposed to *S. constellatus* and S. *intermedius* supernatants. This increase in HA from day 2 through to day 13, averaged from 36% of the total GAG content to 47%.

Figure 4.8 (c) illustrates that, in the presence of commensal and pathogenic *S. anginosus* supernatants, HA was present from day 2 to day 7, after which it appeared to decrease in intensity. CS and DS levels also

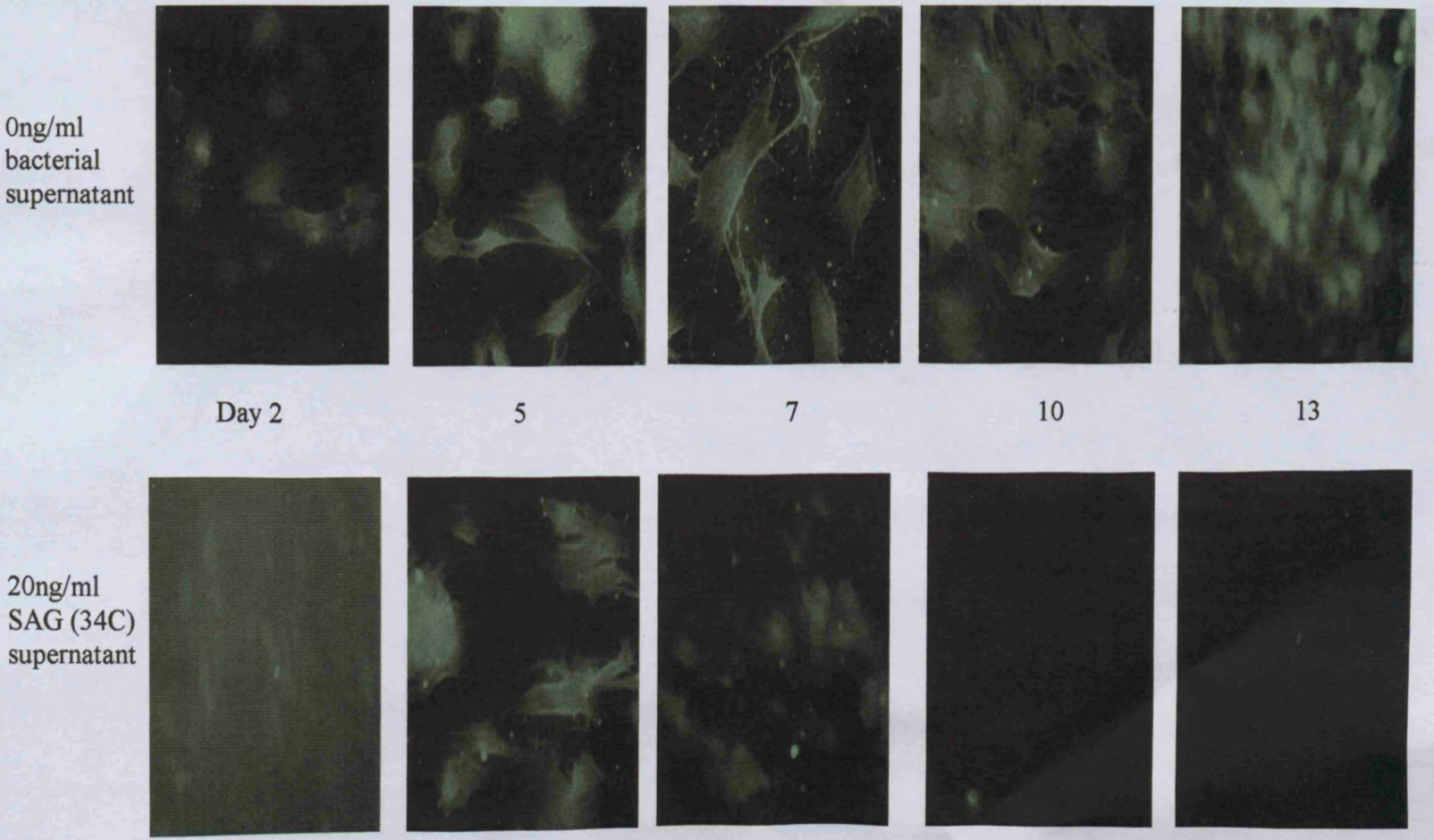
decreased over the culture period with an average presence of 33% on day 2 to 29% on day 13 for both GAGs.

In order to confirm the identification of the iduronic acid-containing GAGs, specific enzyme digestion with chondroitinase AC was carried out (data not shown). There was apparent DS detection for PDL-like cells on days 2, 5, 7, 10 and 13 when co-cultured with all SAG supernatants albeit, decreasing in proportions as the time course progressed. Digestion with chondroitinase ABC (data not shown), which depolymerises both glucuronic acid and iduronic acid containing GAGs, demonstrated no identifiable GAG, thus indicating the presence of HA, DS and CS in all samples.

4.3.9 The effects of the *S. anginosus* group supernatants upon collagen within the periodontal ligament cell extracellular matrix

The PDL cells were immuno-stained for collagen type I, in order to observe the effects of SAG on ECM formation. Figure 4.9 demonstrates the presence of collagen type I within the normal matrix (0ng/ml) and the typical effects of supernatant upon collagen type I within the PDL matrix. In the absence of SAG supernatant, there is an increase in collagen from day 2 until day 13. However, in the presence of SAG supernatant there was a decrease in immuno-staining from day 2 until day 7, after which there was no staining of collagen. All supernatants from commensal and pathogenic strains of SAG appeared to behave in the same manner.

Figure 4.9. Typical immuno-localisation of collagen within periodontal ligament cells extracellular matrix following culture in the absence (0ng/ml) and presence (20ng/ml) of SAG supernatant (example shown is *S. constellatus* 34C).



4.4 Discussion

The present study has investigated the effect of commensal and pathogenic supernatants of *S. constellatus, S. intermedius* and *S. anginosus* on PDL-like cells, with regards to cell growth, differentiation and matrix synthesis. By analysing gene expression and identification of the corresponding protein secreted by the cells, the results firmly indicate a differing pattern of expression for decorin and biglycan by PDL-like cells when exposed to SAG, with potential effects upon collagen within the matrix. The results obtained from this study provide strong evidence to suggest that SAG are potentially capable of damaging the ECM of the PDL.

Initial culture conditions for PDL-like cells were determined by assessing cell viability in the presence or absence of ascorbic acid. Ascorbic acid is a known co-factor and anti-oxidant that stimulates the transcription, translation and post-translational processing of collagen in connective tissue cells (Francesshi, 1992). In the present study, the addition of ascorbic acid failed to show any benefits as the PDL cells were more viable in the absence of ascorbic acid. Also, PDL cells cultured without ascorbic acid did not fail to produce a collagenous matrix, as demonstrated by later collagen immuno-detection studies. There are previous studies that have used ascorbic acid in the conditioned culture media of PDL cells and there are studies that have not used ascorbic acid. Most notably, ascorbic acid has been added to PDL culture media in numerous studies in order to demonstrate the potential osteogenic potential of these cells (Marquez-San Miguel *et al.*, 2003; Shiga *et al.*, 2003; Ishikawa *et al.*, 2004).

Crude bacterial supernatants were used as a representative of a combination of factors released by the bacterial cells. Bacterial supernatants have been utilised in a number of studies to observe effects upon host cells, as secreted toxins play an important role in bacterial pathogenesis. Sukeno *et al.* (2005) used *S. intermedius* supernatants to observe the effects of secreted toxins upon liver cells. This group also demonstrated culture supernatants of *S. intermedius* mirrored the results obtained using live bacteria with regard to the effects on hepatic cells. Other studies include Zhang and Maddox (2000) who used crude supernatants of coagulase negative staphylococci to study cytoxic activity in bovine mastitis and Ohkusa *et al.* (2003) induced experimental ulcerative colitis by *Fusobacterium varium* by exposing Vero cells to culture supernatants.

The MTT assay has previously been used to establish the effect of oral bacteria on cell growth and proliferation of periodontal cells (Yamasaki *et al.*, 1998; Roberts, 2004). Within this study, the cell growth of PDL-like cells appeared to increase when co-cultured with 20ng/ml of all SAG supernatants, particularly from days 3 to 5. A possible explanation for the increase compared to PDL-like cells cultured without SAG supernatant is the initiation of an inflammatory response. The increase in this study is in agreement with a number of studies that have detected an increase in cell growth when certain cells are in contact with bacterial extracts/products. Through histopathology and immuno-staining analysis, it was reported that

infection with the bacterium *Citrobacter rodentium* increased epithelial cell proliferation (Newman *et al.*, 2001) as does *Helicobacter pylori* in mucosal cells (Lynch *et al.*, 1999), with both of these bacteria being linked to colonic and gastric cancer. Roberts (2004) demonstrated an increase in rat alveolar bone-like cell growth and proliferation, via the MTT assay, when these cells were co-cultured with *P. gingivalis* lipopolysaccharide, compared to cells cultured without this bacterial product. Also, Belibasakis *et al.* (2002) found *Actinobacillus actinomycetemcomitans*, which possesses a cytolethal distending toxin (cdt), inhibited proliferation of gingival fibroblasts and PDL cells without affecting their viability, with no evidence of lysis or apoptosis detected. Total protein synthesis remained unaffected, and cells exhibited an elongated and distended cell body, suggesting cdt may compromise tissue homeostasis in the periodontium.

SAG had a profound effect upon the ALPase activity of PDL-like cells, with an overall decrease in ALPase activity by all SAG supernatants compared to cells cultured without supernatants. The marked decrease in ALPase activity following culture with SAG suggests that this group of bacteria could target the osteoblast-like cell type or their progenitor cell within the heterogeneous cell population of the PDL. Marquez-San Miguel *et al.* (2003) showed that extracts from a range of periodontopathic bacteria, such as *P. gingivalis* and *T. denticola*, caused the suppression of ALP activity and reduced the collagen content of human periodontal fibroblasts. Marquez *et al.* suggested the extracts from these Gram negative bacteria contain not only soluble proteins, but also butyric acid,

propionic acid and collagenases that may have a direct influence on periodontal fibroblasts. Murata *et al.* (1997) studied the effects of various oral-related bacteria, including *P. gingivalis, A. actinomycetemcomitans*, and *Streptococcus* spp. on bone formation of MC3T3-E1 cells (immature embryonic cells with osteoblast-like activity). *P. gingivalis* and *A. actinomycetemcomitans* decreased the ALPase activity in a dose-dependent manner, whereas *Streptococcus* spp., including *S. milleri*, did not influence the ALPase activity in of MC3T3-E1 cells. In the current study, the relative inhibition of ALPase activity of PDL-like cells following bacterial application suggests that products present within the SAG supernatants could target mature osteoblast-like cells within the heterogeneous population of PDL cells, rather than immature cells of this type.

The relative mRNA expression of biglycan and decorin from PDL-like cells co-cultured with SAG supernatants showed an increase in biglycan and a decrease in decorin compared to the relative mRNA expressions of these SLRPs from PDL-like cells cultured without SAG supernatants. These results were supported by immuno-detection of the SLRPs. The detection of protein at approximately 45kDa, in both experimental conditions correspond to the core protein of both SLRPs. Other detectable proteins larger or smaller than 45kDa may represent a combination of the intact PG and PG metabolites, formed in the process of catabolism and/or remodelling of the PDL matrix.

Differences seen in expression between decorin and biglycan maybe due to the different roles they play within the matrix and their being located differently. Biglycan has a pericellular location while decorin is bound to the collagen network (Bianco *et al.*, 1990). In the presence of SAG supernatant, biglycan expression significantly increases from day 2 through to day 13 compared to those PDL-like cells cultured in the absence of supernatants. This strong detection of biglycan could indicate a number of roles for this PG, emphasising a function in cell signalling, possible related to cellular growth (Chen *et al.*, 2002). Waddington *et al.* (2003) identified periods of decorin and biglycan expression within a primary rat bone cell culture model. Analysis of biglycan mRNA levels revealed expression during phases related to cell proliferation. Biglycan has also been implicated as a pro-inflammatory factor, by acting as an endogenous ligand of TLR4 and TLR2, which mediate innate immunity and increase TNF-α (Schaefer *et al.*, 2005).

There are a number of studies that suggest SLRPs, particularly decorin, can directly or indirectly influence cell growth, via epidermal growth factor receptor (EGFR) or TGF-ß. In transformed cells, such as A431 squamous carcinoma cells, decorin elicits growth suppression by binding to EGFR. This interaction induces phosphorylation of the EGFR, which leads to activation of the mitogen-activated protein kinase (MAPK) pathway, up-regulation of p21 cyclin-dependent kinase inhibitor expression (leading to cell cycle arrest), and an increase in the intracellular Ca^{2+} level (De Luca *et al.*, 1996; Santra *et al*, 1997; Moscatello *et al*, 1998;

Iozzo, 1999). However, within PDL cells, blocking of the EGFR tyrosine kinase activity did not prevent decorin-induced growth inhibition and p21 expression, suggesting an alternative or additional signal transduction pathway within these cells, which are heterogeneous in nature (Hakkinen *et* al., 2000). Biglycan is not capable of activating the EGFR (Moscatello *et al.*, 1998).

The ability of decorin and biglycan to complex with TGF-ß isoforms suggests another mechanism via which these SLRPs influence cell growth. Altered patterns of expression of SLRPs in the presence of SAG supernatants is likely to involve TGF- ß, although this can only be hypothesised at the present time.

A change in production of PGs and/or their side-chains may influence the fibroblast, either directly or indirectly, through modulating growth factor and cytokine activity. An important example of this is the abrogation of the activity of TGF-β by decorin (Hildebrand *et al.*, 1994). Another possible explanation for the observed increase of biglycan and decrease in decorin following exposure to SAG supernatants could be through the ability of these SLRPs to bind TNF-α, (Tufvesson and Westergren-Thorsson, 2002). One possibility is that decorin can function as a reservoir for sequestering TNF-α out in the ECM. In inflammation, there is an increase in TNF-α expression, as well as a decrease in decorin, that reduces this resevoir, resulting in an additional release of TNF-α. Partial proteolysis of decorin due to the action of MMPs could be an explanation

for releasing immobilised TNF-α. Furthermore, the up-regulation of biglycan, which at its pericellular location binds TNF-α, could augment a higher concentration of TNF-α closer to the cell, promoting association of the cytokine to its cell surface receptors (Tufvesson and Westergren-Thorsson, 2002). Overall, the observations of SLRPs within the current study support the hypothesis that there are different roles for SLRPs within the inflamed PDL and that SAG supernatants may have consequences upon cell growth through modulation of SLRPs.

Immuno-localisation studies in the present chapter demonstrated an increase in collagen content in a time-dependent manner for those PDL-like cells cultured without SAG supernatants. However, for PDL-like cells cultured with SAG supernatants, there was a marked decrease in collagen content throughout the culture period. The ability of SLRPs to interact with collagen and facilitate collagen fibril formation have been proposed as having important roles in the requirement of a collagenous framework, associating with fibrillar collagens and regulating collagen fibrillogenesis (Vogel and Trotter, 1987; Sugars *et al.*, 2003). Structural organisation of collagen may also be important for the defence against periodontal disease, because in certain conditions abnormal collagen fibrils associate with increased susceptibility to periodontal disease. Matheson *et al.* (2005) studied the role of SLRPs to regulate collagen fibril and fibril bundle formation in periodontal tissues. To assess the function of SLRPs they studied periodontal tissues of mice harboring targeted deletions of decorin, fibromodulin or lumican genes and lumican and fibromodulin double

knockout mice using histological and electron microscopic methods. They demonstrated SLRPs were co-expressed in human and mouse gingival and periodontal ligament connective tissues where they co-localized with collagen fibril bundles. Teeth in the knockout animals were fully erupted and showed normal gross morphology. Targeted deletion of decorin, fibromodulin, and lumican resulted in abnormal collagen fibril and fibril bundle morphology that was most evident in the periodontal ligament. This indicated that decorin, fibromodulin and lumican co-ordinately regulate the fibrillar and suprafibrillar organisation of collagen in the periodontal ligament. In periodontal disease, the breakdown of the collagen-rich connective tissue matrix parallels a decrease in the level of decorin in inflamed connective tissue (Oksala *et al.*, 1997). The immuno-localisation results obtained in this study along with the observed decrease in decorin expression suggests that SMG supernatants have an unfavourable effect on matrix formation and assembly. It is possible that abnormal collagen organisation, because of decorin deficiency, may lead to increased susceptibility to periodontal disease (Hakkinen *et al*, 2000) and undermine periapical integrity. However, it is also known that the SAG secrete collagenases, therefore the decreasing effect observed with decorin could be a secondary event following bacterial degradation of collagenase. Overall, aberrant ECM expression may enhance inflammatory tissue breakdown and encourage perapical abscess formation.

With regard to the GAG moiety, PDL-like cells cultured in the absence of SAG supernatants showed a constant production in HA, CS and DS,

suggesting these GAGs encourage cell development and matrix formation. GAGs are important in regulating assembly of collagen fibrils. Small PGs substituted with CS isolated from cartilage were reported to promote fibrillogenesis (Vogel and Trotter, 1987). Studies using recombinant decorin substituted with CS chains have also shown that both the core protein and GAG chain are capable of promoting collagen fibrillogenesis (Sugars *et al.*, 2003). Following co-culture with SAG supernatants, the nature of the GAG moiety of PDL cells appeared to be affected. For *S. constellatus* and *S. intermedius* supernatants, there was an increase in HA proportions whereas there was a decrease in CS and DS proportions. However, *S. anginosus* supernatants did not appear to effect HA as the other supernatants, but *S. anginosus* isolates did decrease CS and DS proportions. The altered GAG expression may suggest that matrix remodelling is being inhibited, hence delaying favourable conditions for tissue repair.

The decreasing proportions of CS and DS may be due to the observed decrease in decorin within the PDL matrix, particularly for the decrease observed in DS proportions. Although biglycan is increased within this study it may not be glycosylated, as various forms of this SLRP have previously been reported (Hocking *et al.*, 1996; Tufvesson *et al.*, 2002). However, bacterial degradation of GAGs cannot be discounted at this time, as the hydrolytic enzyme production of SMG is well established. Chondroitin sulfatase activity has been observed within the SMG, particularly *S. intermedius* and *S. constellatus* (Homer *et al.*, 1993; Jacobs

and Stobberingh, 1995), which provides a plausible explanation for the decrease in CS proportions observed in PDL-like cells cultured with SAG supernatants. In addition, the decrease in CS within cells co-cultured with supernatants observed in this study supports the independent effects of SAG supernatants on ALP-positive cells, as osteoblast-like cells are likely to carry the CS GAG chain. Although there was a clear decrease in the total GAG content of CS following culture with SAG supernatants, CS presence did not disappear completely. This is possibly due to the presence of larger PGs, such as versican, abundantly expressed by PDL cells (Larjava *et al.*, 1992), however, the detection of this large CSPG was not conducted in this study. The presence of versican in PDL is well-established and roles in other tissues in the initial formation of the ECM have been proposed (Zimmerman and Ruoslahti, 1989). Therefore, the reduction in CS by PDL-like cells in the present study may suggest a reduction in versican synthesis, which in turn may effect matrix formation.

The data presented within this chapter suggests that the SAG may alter ECM components and their synthesis directly via a suppression of PDL differentiation or via a reduction or destruction of the ECM. It is of interest to note that SAG supernatants can potentially modulate various functions of PDL-like cells, including those of decorin and biglycan, present in host tissues. However, it is only possible to hypothesise the consequence of altered PG levels at this time.

CHAPTER FIVE

Chapter 5

Endothelial cellular response to *Streptococcus anginosus* group supernatants

5.1 Introduction

Dentoalveolar infections, including dentoalveolar abscesses, can develop by the extension of the initial carious lesion into dentine, and spread of bacteria to the pulp via the dentinal tubules. They can also develop via the perapical tissues (PDL, cementum and alveolar bone) due to open communication of these tissues to the root canal space and vice versa (Love and Jenkinson, 2002).

The pulp and surrounding periodontal tissues are extremely vascularised. Blood vessels are fundamentally composed of endothelial cells, which inter-connect to form the tubes that direct and maintain blood flow and tissue perfusion (Hanahan and Folkman, 1996). They are vital for the proper functioning of blood vessels by aiding in the regulation of blood flow, inhibition of smooth muscle cell growth, and resisting thrombosis (Bermophl *et al.*, 2005).

The functional state of endothelial cells (quiescent or angiogenic) is regulated by a number of growth factors and cytokines (Roberts and Sporn, 1989). However, and of relevance to this thesis, the functional state of endothelial cells appears to correlate with the expression of specific SLRPs (Schonherr *et al.*, 2001). Resting endothelial cells *in vivo* as well as

confluent cells *in vitro* constitutively express biglycan, which becomes up-regulated when endothelial cell migration is stimulated (Jarvelainen *et al.*, 1991; Kinsella *et al.*, 1997). Nelimarkka *et al.* (1997) made similar observations using a cell line derived from human umbilical vein endothelial cells. This group demonstrated that, when forming monolayer cultures typical of macrovascular endothelial cells, this cell line expresses and synthesise detectable amounts of biglycan, but not decorin. However, it has been demonstrated that decorin is expressed in human capillary endothelial cells during inflammation when angiogenesis occurs (Schonherr *et al.*, 1999).

As well as constitutively expressing SLRPs, endothelial cells synthesise HSPGs, including perlecan (Saku and Furthmayr, 1988), members of the cell surface associated PGs, syndecan and glypican (Mertens *et al.*, 1992). Growth factors and cytokines can regulate PG synthesis in endothelial cells, particularly TGF-β. This process appears to be cell density-dependent; TGF- β promotes the synthesis of both perlecan and biglycan when the cell density is high whereas only biglycan synthesis is stimulated when the cell density is low. Furthermore, GAG chains in biglycan are longer at a high cell density (Kaji *et al.*, 2000).

When challenged by bacteria, the endothelium responds by presenting a thrombotic surface and increasing vessel permeability, thereby allowing local adherence, activation and extravasation of inflammatory cells. Angiogenesis and vascular remodelling are important features of many

chronic inflammatory diseases. Angiogenesis can be initiated in pathological circumstances in order to repair vessels and preserve tissue structure and function. However, it can also contribute to the pathology of diseases such as periodontal and pulpal disease, by exacerbating the inflammatory conditions (Ezaki *et al.*, 2001). Rajashekhar *et al.* (2006) reported endothelial expression of a transmembrane form of the pro-inflammatory cytokine TNFα activated the endothelium, enhancing sprout formation independently of angiogenic growth factors.

In view of the importance of endothelial cell response to bacterial challenge, the aim of this chapter was to observe the response of a human aortic endothelial cell line to SAG supernatants. More specifically, the objective was to determine the role of SAG in endothelial cellular growth, the effect on migratory properties, and the expression of decorin and biglycan PGs within endothelial cells.

5.2 Materials and methods

5.2.1 Endothelial cell culture conditions

EA.hy926 cells were generated by immortalisation of human umbilical vein endothelial cells (HUVECs) (Edgell *et al.*, 1983). HUVECs were fused with the immortalised human lung epithelial carcinoma cell line A549 and retained the signalling characteristics of HUVECs. In this study EA.hy926 cells (a kind gift from Dr Elke Schonherr/Lorna Fiedler) were grown in MCDB 131 media (Difco) supplemented with 10% fetal bovine serum (FBS) (Difco), glutamine (Difco), antibiotics mixture (100 units/ml penicillin G, 10 mg/ml streptomycin sulphate, 25mg/ml amphotericin B) (Sigma Chemical Co.) and 1 x hypoxanthin/aminopterin/thymidine (HAT) (Invitrogen).

5.2.2 Bacterial strains

The bacterial strains used in this study were the same as those used in chapter 4: *S. constellatus* 322/95 and 34C; *S. intermedius* 127/95 and 30C; *S. anginosus* 43586/96 and 43C (for original sources see table 3.1). The supernatants were prepared as in section 4.2.1.

5.2.3 Endothelial cellular response to *S. anginosus* group supernatants

5.2.3.1 Viable cell count

Similar to section 4.2.4, EA.hy926 cells were seeded as a monolayer, at a concentration of 1 x 10^5 cells/ml in 24 well plates with 1ml media and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for

24h. 500µl media was removed and 500µl of fresh media containing bacterial supernatants were added at various concentrations (10, 50, 100, 500ng/ml, 1, 10, 50, 100, 200, 500µg/ml, 1mg/ml – determined using the BCA protein assay as described in section 2.2.6) and diluted with working media. BHI broth was used in place of the bacterial supernatant, as a control. After 24 and 48 hr of incubation, cell growth was determined by trypan blue dye exclusion. The assay was carried out in triplicate and the highest concentration at which all the cells exhibited cytopathic effects was recorded as the cytotoxic level.

5.2.3.2 Effects of *S. anginosus* group supernatants on endothelial cell growth

The influence of SAG supernatants on endothelial cell growth was assessed by MTT assay (as described in section 4.2.3.1). Following determination of viability counts for endothelial cells with a range of supernatant concentrations, there was a survival rate of less than 95% for cells cultured with supernatant concentrations above 500ng/ml. Therefore, for the MTT assay, endothelial cells were cultured with 0ng/ml or 100ng/ml of SAG supernatants from day 0, and every 24h thereafter, for 5 days.

5.2.3.3 Effects of *S. anginosus* group supernatants on endothelial cell migration

A migration assay was performed using endothelial cell cultured as spheroids according to Korff and Augustin (1998) and Lorna Feidler

(personal communication). EA.hy926 cells (1000/100µl), were suspended in Waymaouth medium supplemented with 5% heat-inactivated FCS, and 30% methyl cellulose (Sigma). Cells were seeded at 100µl/well of a 96-well round bottom hydrphobic plate (Griener Bio-One Ltd, UK), and incubated for 48h at 37°C, 5% CO_2. After incubation, all suspended cells associated to form a single spheroid per well, of equal size and shape. Using cut-off 1ml pipette tips, each spheroid was removed, transferred to individual 15ml tubes (Greiner Bio-one, Gloucestershire, UK), and pelleted by centrifugation at 1000rpm for 2 min. The medium was exchanged for Waymouth medium supplemented with 0.5% heat-inactivated FCS, without (control) and with 100ng/ml of SAG supernatant, a final volume was maintained at approximately 100µl per spheroid. 100µl of each spheroid and culture media (with or without 100ng/ml SAG supernatants) was transferred to one well of a flat bottomed 96-well hydrophilic plate (Cellstar, Griener), with a minimum of 12 wells for each supernatant. Images were captured at 6-72h at 40x magnification using an Olympus CK2 microscope using a Nikon E995 digital camera. Spheroid height and width at the indicated time-points were measured on Adobe Photoshop (Adobe Systems Incorporated), using the ruler tool and the average and standard deviation for each variable and time-point was calculated. Spheroids cultured with 100ng/ml SAG supernatants were statistically compared to spheroids cultured without supernatants (see section 5.2.4).

5.2.3.4 Effects of *S. anginosus* group supernatants on decorin and biglycan mRNA expressions of endothelial cells

EA.hy926 cells, cultured as monolayers, were examined for decorin and biglycan mRNA expression in the presence and absence of 100ng/ml of each SAG supernatant. At day 0 the cells were seeded at 1 x 10^5 cells/ml cultured in MCDB 131 serum containing media supplemented with or without the supernatants and continued to be cultured as in section 5.2.1.

On days 1, 3, 5, and 7, the media was removed and the cells washed three times in PBS. RNA was subsequently extracted as in section 4.2.4.4.1. The total RNA was quantified by UV spectrophotometry as described in section 4.2.4.4.2. Total RNA was reverse-transcribed (see section 4.2.4.4.3), followed by PCR (see section 4.2.4.4.4). For EA.hy926 endothelial cells, the primer sequences for decorin, biglycan and the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) are displayed in table 5.1 along with the optimal magnesium concentration and annealing temperatures. The detection of PCR products was performed by agarose gel electrophoresis as described in section 4.2.4.4.5.

Table 5.1. Oligonucleotide primer sequences and specific conditions for EAhy.926 PCR.

		Magnesium concentration	Annealing temperature
GAPDH forward primer	5' – GTCAGTGGTGGACTGACC - 3'	2mM	51°C
GAPDH reverse primer	5' – ACCTGGTGCTCAGTGTAGCC - 3'		
	123 bp product (Strazynski *et al.*, 2004)		
Biglycan forward primer	5' – CCCTCTCCAGGTCCATCCGC - 3'	2mM	63°C
Biglycan reverse primer	5' – GAGCTGGGTAGGTTGGGCGGG - 3'		
	480 bp product (Chen *et al.*, 2002)		
Decorin forward primer	5' – AGCTGAAGGAATTGCCAGAA - 3'	2mM	51°C
Decorin reverse primer	5' – TGGTGCCCAGTTCTATGACA - 3'		
	132 bp product (Strazynski *et al.*, 2004)		

5.2.3.5 Extraction and identification of proteoglycans from conditioned cell medium

EA.hy926 cells were seeded at a density of 1 x 10^5 cells/ml in T25 flasks (Griener), in MCDB 131 serum containing media supplemented with or without 100ng/ml SMG supernatants. Culture medium was removed and collected on days 1, 3, 5, and 7 and dialysed against ddH_2O, as in section 2.2.2. The dialysed media samples were lyophilised and stored at -20°C until required. For the immuno-detection of SLRPs, the tissue culture media from EA.hy926 cells were characterised on the basis of their immuno-reactivity to the polyclonal antibodies, anti-decorin (LF113) and anti-biglycan (LF106), by Western blot analysis (as described in section 2.2.7.2)

5.2.3.6 Glycosaminoglycan detection by cellulose acetate electrophoresis

The EA.hy926 media samples, supplemented with or without 100ng/ml of SAG supernatant, were examined for the presence of GAG chains using cellulose acetate electrophoresis (see section 2.2.7.3).

5.2.4 Statistical analysis

The Student unpaired two-tail T-test (Instat Package, Graph Pad software) was performed to obtain *p*-values for analysis of differences between cells cultured with and without SMG supernatants. $p<0.05$ was determined as statistically significant.

5.3 Results

5.3.1 Viable cell count

Following culture of EA.hy926 cells with 0-1mg/ml of SAG supernatants (one commensal and one pathogen from each of the three species; *S. constellatus, S. intermedius* and *S. anginosus*), the cells were stained with trypan blue to obtain cell viability counts. 100 cells were viewed and the percentage of non-viable cells (blue stained) and viable cells (unstained) were counted. The percentage of viable cells for each supernatant was calculated from the cell count, a cell viability count greater than 95% was acceptable. The cells cultured in the presence of 0ng/ml and 100ng/ml, of all SMG supernatants, demonstrated a viability of 95-98%. Cells incubated with over 500ng/ml had viability counts of less than 80%. Therefore, 100ng/ml of bacterial supernatant was used in further studies.

5.3.2 Effects of *S. anginosus* group supernatants on endothelial cell growth

The influence of SAG supernatants at a concentration of 100ng/ml on viable endothelial cell numbers was investigated using the MTT assay. All strains of SAG species exhibited the same behaviour, namely, the growth of endothelial cells increased following culture with 100ng/ml SAG supernatants (figure 5.1). Statistically significant differences were observed on days 3 and 4 with average growth increases of 30% in comparison to cells cultured without supernatant. The largest stimulatory effects were observed on day 5 (average 40-50% increases).

Figure 5.1. Effects of SAG supernatants on EA.hy 926 cell growth. * Significant difference ($p<0.05$) between cells co-cultured with 0ng/ml and 100ng/ml of supernatant.

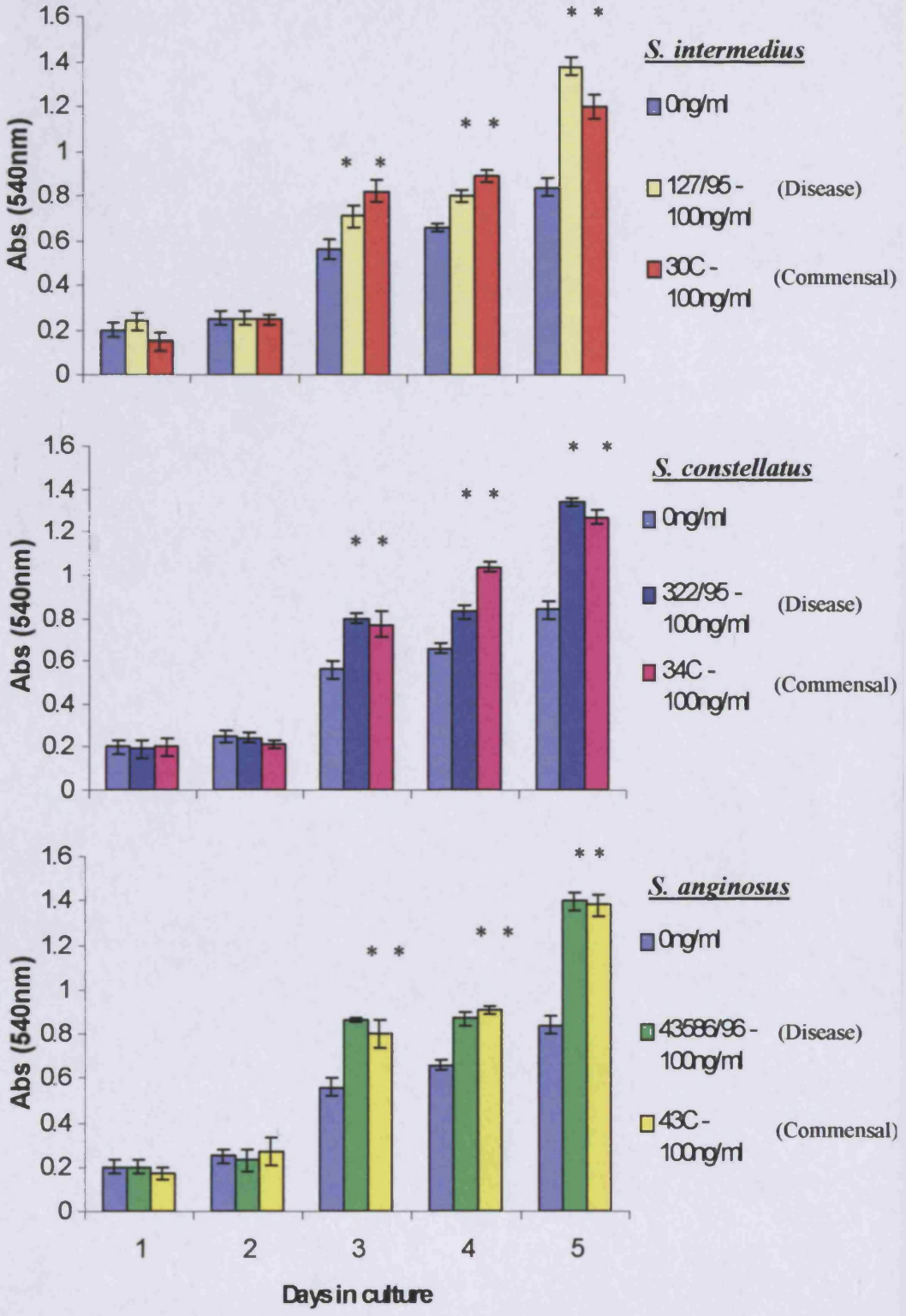
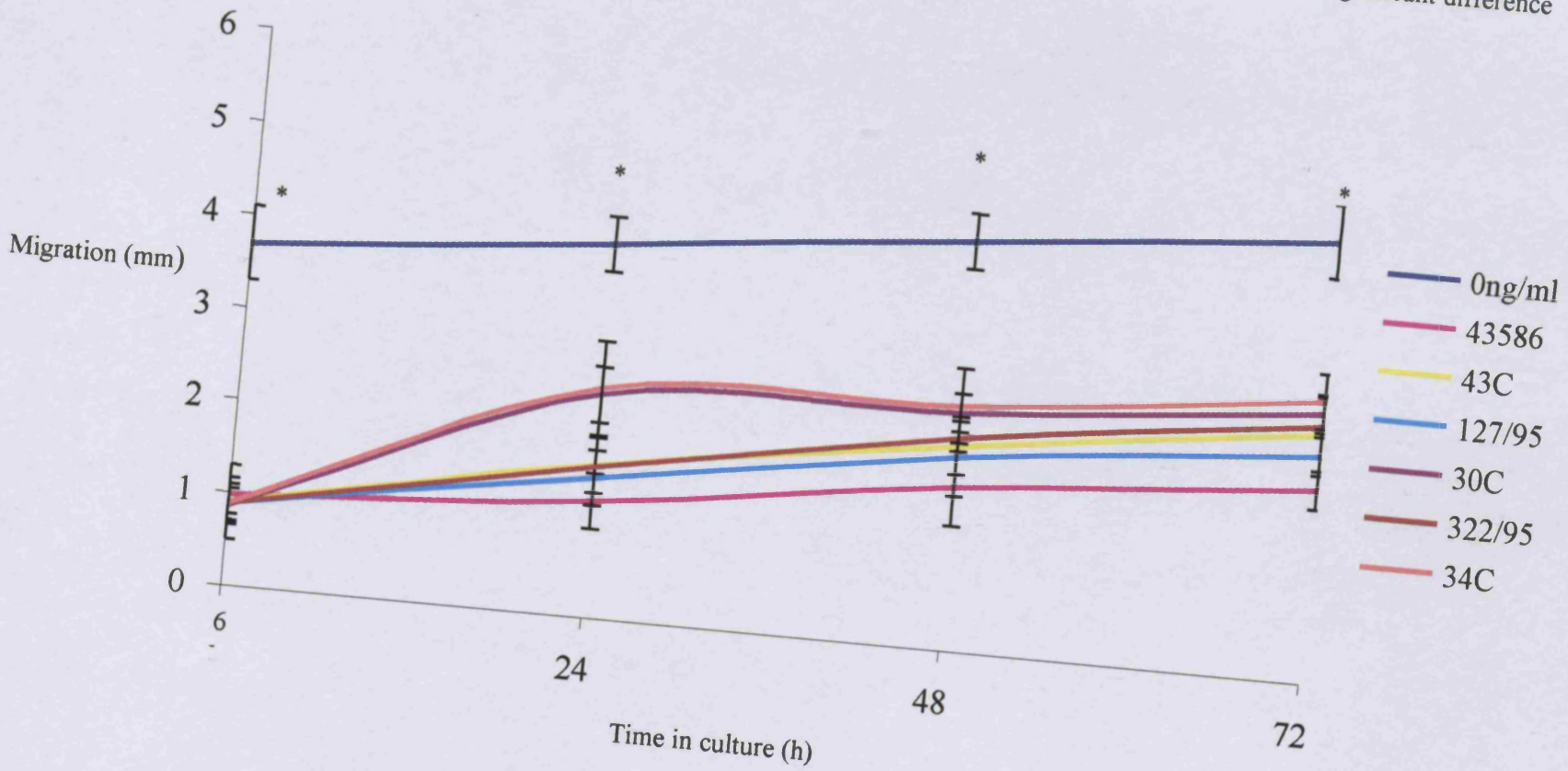


Figure 5.2. Migration of EA.hy926 cell spheroids in the absence and presence of SAG supernatants. * Significant difference ($p<0.05$) between cells co-cultured with 0ng/ml and 100ng/ml of supernatant



5.3.3 Effects of *S. anginosus* group supernatants on endothelial cell migration

Figure 5.2 illustrates the effects of SAG supernatants on the migration properties of endothelial cells. The cells showed statistically significant migration (2- to 3-fold higher) in the absence of SAG supernatants from 6h through to 72h, compared to cells cultured with supernatants. At 6h, the migratory distances of cells cultured without supernatant were, on average, 28% more than those cells co-cultured with SAG supernatants. At 24h, the migratory distance rose to a 40% increase compared to cells co-cultured with SAG supernatants, and at 48h there were increases between 30 and 60% for the migration of cells without supernatants.

There appeared to be a difference between commensal and disease SAG isolates. For supernatants derived from disease strains (43586/96 and 127/95), cell migration appeared to be less compared to commensal supernatants assayed. However, this was deemed not to be statistical significant within our sample population.

Figure 5.3 (a) Biglycan and decorin mRNA expression from EA.hy926 cells (example shown is *S. intermedius* 127/95)

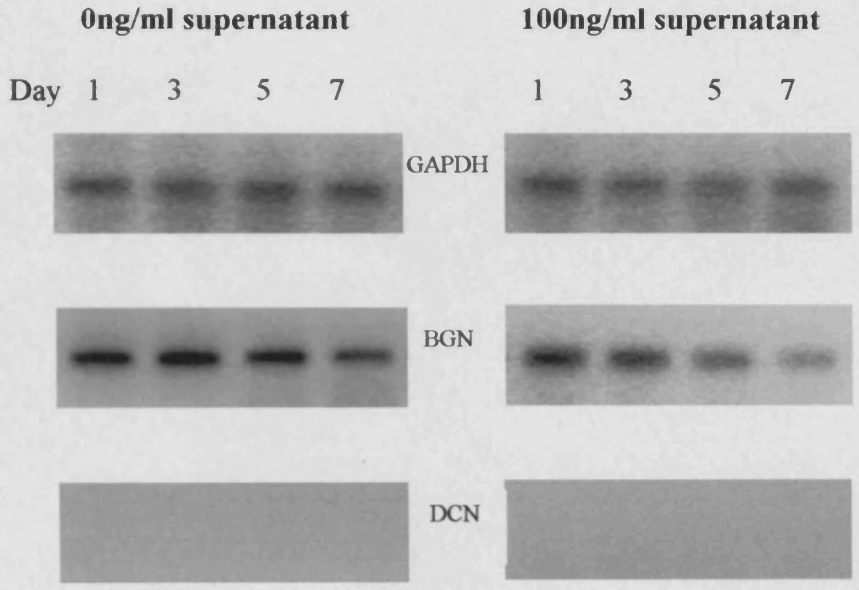
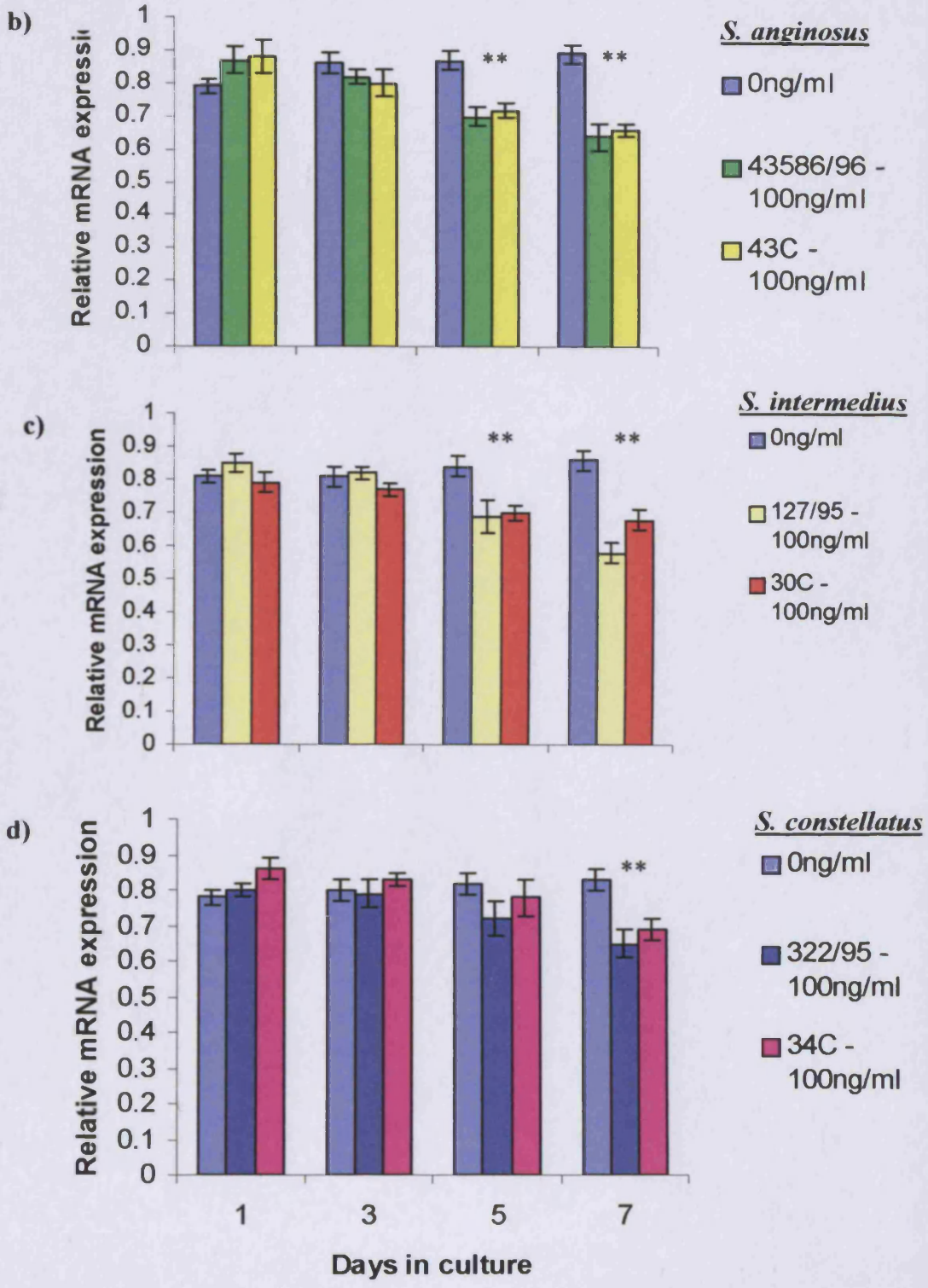


Figure 5.3 (b-d). Relative biglycan mRNA expression. * Significant difference ($p<0.05$) between cells co-cultured with 0ng/ml and 100ng/ml of supernatant



5.3.4 Effects of *S. anginosus* group supernatants on biglycan and decorin expression by endothelial cells

Figures 5.3 (a-d) show the mRNA expressions for biglycan and decorin by endothelial cells cultured in the presence and absence of 100ng/ml SAG supernatants. In the absence of bacterial supernatants, biglycan mRNA expression was detected as early as day 1, with a band identifiable at 204bp. This detection remained constant throughout culture until day 7. However, in the presence of SAG supernatants, the expression of biglycan was strong on day 1 but detection subsequently decreased. On days 5 and 7 significantly reduced ($p<0.05$) levels of biglycan mRNA were evident compared to cells cultured without supernatants, demonstrating an average decrease of 20% on day 5, and 30% on day 7. Decorin mRNA expression was not apparent in endothelial cells cultured in the absence or presence of SAG supernatants.

5.3.5 Immuno-identification of small leucine-rich proteoglycans

Western blot analyses identifying the PG species produced by EA.hy926 cells, without and with SMG supernatant (322/95) are shown in figure 5.4 (a) (anti-biglycan) and figure 5.3 (b) (anti-decorin).

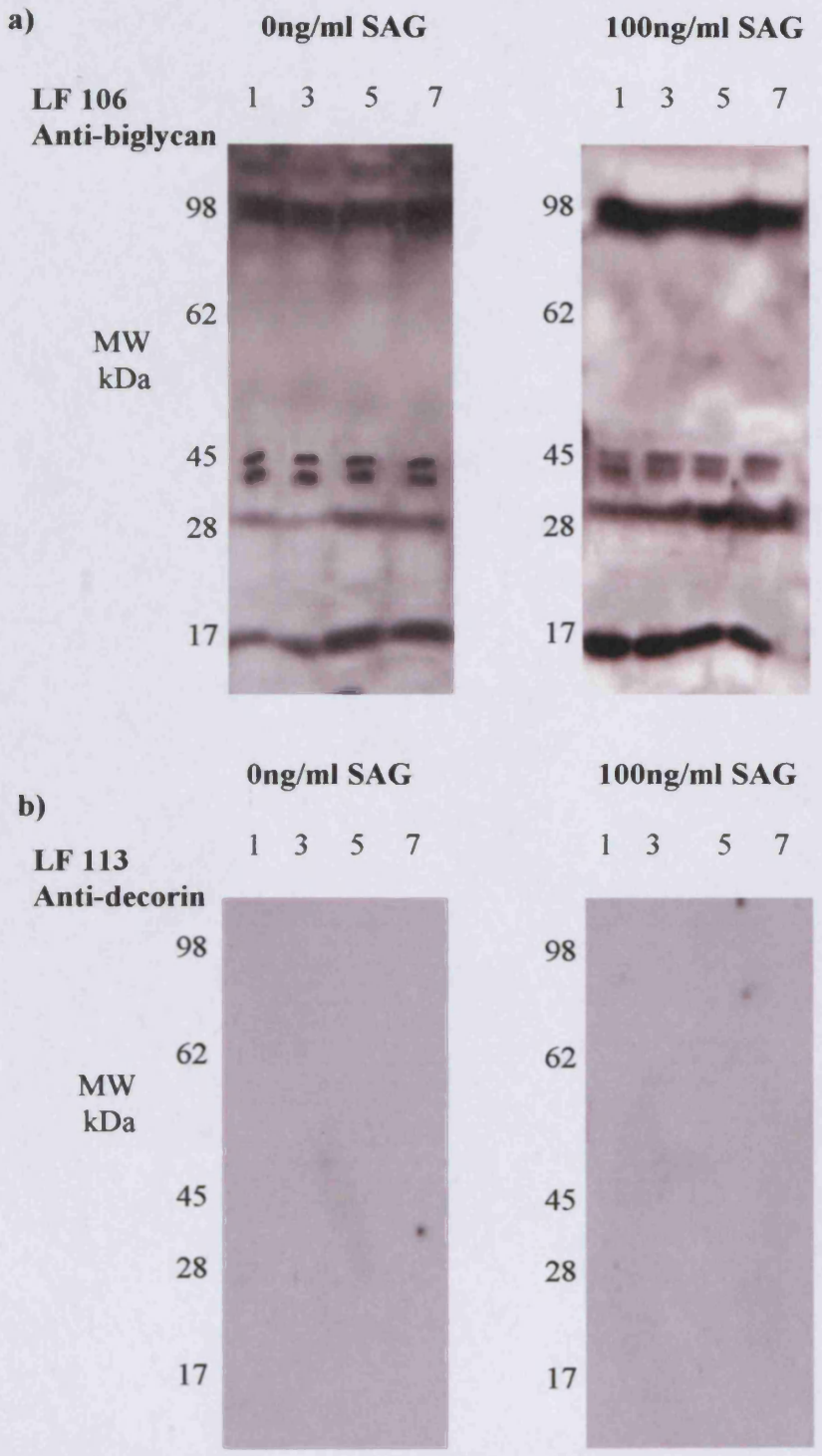
In the absence of supernatant, LF 106, which detects a sequence located in the N-terminal of biglycan, demonstrated immuno-reactivity from day 1. A positive reactivity using this antibody continued throughout culture, until day 7. A number of bands were revealed from 200kDa down to 17kDa. Sharp bands were visible at 98kDa and a doublet at approximately 45kDa,

which became much more intensive following the action of chondroitinase ABC. Other bands were visible below 38kDa, and were regarded as degradation products of biglycan.

In the presence of SAG supernatants, biglycan detection was also detected at day 1 and continued throughout co-culture until day 7, thus biglycan expression appeared to be the same as for biglycan expression by cells cultured without supernatant. As with immuno-reactivity for cells cultured without supernatants, there was strong detection at 98kDa and a doublet at approximately 45kDa. There were also bands detected below 38kDa, again regarded as degradation products. There were no evident differences in biglycan immuno-detection between strains of the SAG.

Immuno-detection for decorin with LF-113, whose recognition site rests in a sequence in the N-terminal of decorin, did not produce an immune-reactive response in cells cultured in the presence or absence of SAG supernatant. This result is in accordance with the mRNA results observed within this study.

Figure 5.4 Western blot analysis of (a) biglycan and (b) decorin by EA.hy926 cells in the absence and presence of SAG supernatants (example shown is *S. constellatus* 322/95).



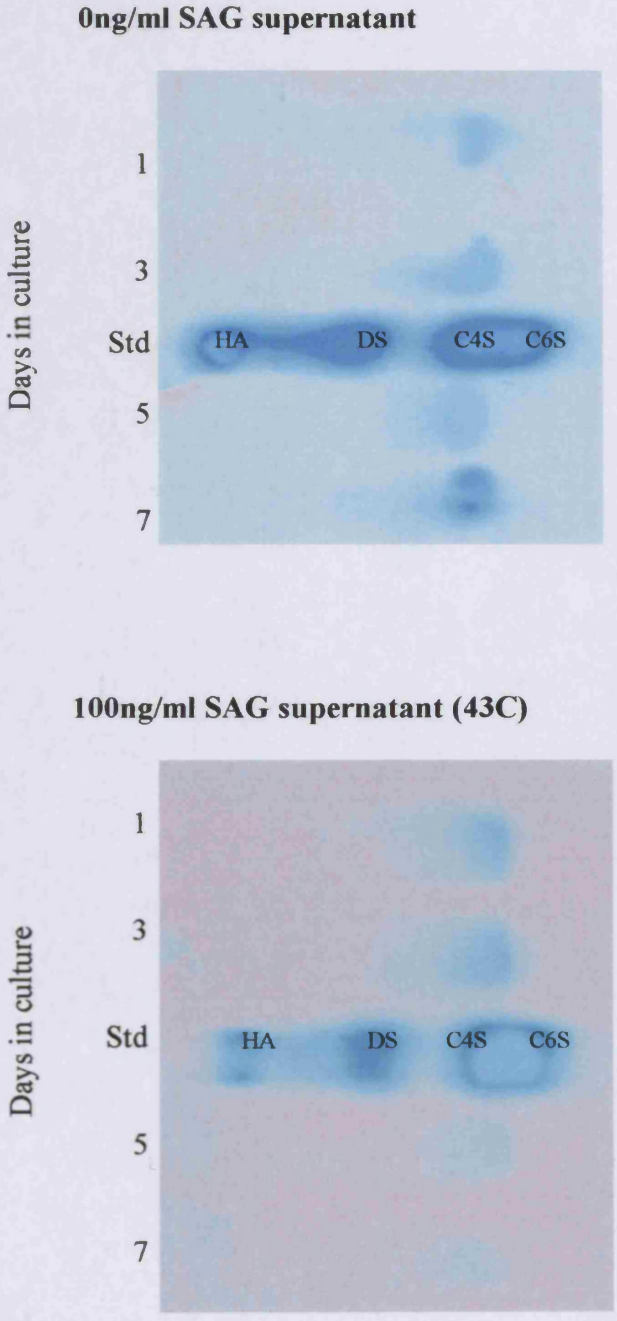
5.3.6 Effects of *S. anginosus* group supernatants on endothelial cell glycosaminoglycan composition

The GAG chains were released from the PG samples by protease digestion and analysed by cellulose acetate electrophoresis (figure 5.5). GAGs were judged by distance of migration compared to the standard GAG mixture. Cellulose acetate electrophoresis detected CS as the exclusive GAG, on each day in culture for EA.hy926 cells in the absence and presence of SAG supernatants. GAG presence was quantified by measuring staining intensities (Bio-Rad Quantity One).

For cells in the absence of SAG supernatants, there was a steady increase in the level of CS from day 1 to day 7. For those cells co-cultured in the presence of SAG supernatant, CS was detectable at day 1 until day 7, but appeared to decrease throughout culture in comparison to cells cultured without supernatant. The differences in CS presence between cells with and without SAG supernatants increased from 30% on day 3 upto 80% on day 7. All SAG supernatants behaved in the same manner toward CS proportions.

Following specific enzymatic digestions with chondroitinase AC and chondroitinase ABC (data not shown), there were no identifiable GAGs present, thereby verifying the presence of CS and the absence of DS and HS in all samples.

Figure 5.5 Cellulose acetate electrophoresis of glycoaminoglycans produced by EA.hy926 cells in the absence and presence of 100ng/ml of SAG supernatants (example shown is *S. anginosus* 43C)



5.4 Discussion

The present study investigated the effect of SAG supernatants on the temporary appearance of PGs, decorin and biglycan, and GAGs synthesised by EA.hy926 endothelial cells, relating their expression in the presence or absence of an inflammatory pathological condition, such as a dental abscess or periapical disease. By observing gene expression and the detection of the corresponding protein secreted by these cells, the results assist in ascribing roles for these PGs during the development of normal and aberrant endothelial cell matrix formation, and describe potential mechanisms via which SAG could potentially orchestrate tissue destruction during inflammatory disease.

Within the current study, it appeared there was an increase in EA.hy926 endothelial cell growth when cells were cultured in the presence of SAG supernatant. Angiogenesis and vascular remodelling are features of many chronic inflammatory diseases (Ezaki *et al.*, 2001). Proliferation and migration of endothelial cells and the subsequent establishment of a stable monolayer are critical events in the repair of injured vessels and in angiogenesis and vasculogenesis, and tissue repair (Kinsella *et al.*, 1997). Ezaki *et al.* (2001) reported that the rate of tracheal endothelial cell proliferation and the size of blood vessels increased rapidly in C3H mice following injection with *Mycoplasma pulmonis*, which ultimately lead to chronic inflammatory airway disease. Cell proliferation peaked at 5 days, at a level 18 times the pathogen-free value, declined sharply until day 9, but remained at approximately 3 times the pathogen-free value for at least

28 days. Although cell growth was increased in the current study following culture with SAG supernatants the migration assay demonstrated that the migratory properties of cells cultured in the absence of SAG supernatants exhibited greater migration, compared to cells cultured in the presence of SMG supernatants. Cell proliferation and migration are controlled by multiple factors, such as fibroblast growth factor (FGF), TGF-β, and cell adhesive interactions mediated by integrins. Therefore, initiation of cell migration may involve a change in cell-matrix remodelling and altered matrix receptor expression, which are processes directed in part by growth factors (Kinsella *et al.*, 1997).

Within the current study, there was detection at both the mRNA and protein level for biglycan, but not decorin, from EA.hy926 cells cultured in the absence of SAG supernatants, which is in agreement with other studies (Jarvelainen *et al.*, 1991; Kinsella *et al.*, 1997; Nelimarkka *et al.*, 1997). For cells cultured in the presence of SAG supernatants, biglycan mRNA expression appeared to decrease at days 5 and 7 compared to cells cultured without supernatants. However, the Western blot analysis investigating the corresponding protein produced under the same conditions, showed strong immuno-detection of biglycan throughout the culture period. Although this finding cannot be fully explained, it may give an indication of the turnover and persistence of the intact biglycan molecule and its metabolites. For both conditions, the band observed at 45kDa is in accordance with the core protein molecular weight of biglycan from endothelial cells (Kinsella *et al.*, 1997). The doublet observed may be due to differences in

glycosylation, as this has been observed for decorin, with glycoforms containing two and three *N*-linked oligosaccharides (Hocking *et al.*, 1996; Seo *et al.*, 2005). Also, biglycan isoforms with differences in polysaccharide substitution and core protein have been identified in human lung fibroblasts (Tufvesson *et al.*, 2002), and Jarvelainen *et al.* (1991) reported two forms of biglycan produced by bovine endothelial cells. The band at 98kDa may be a biglycan dimer (Scott *et al.*, 2006). The detection of biglycan at molecular weights greater or lower than 38kDa through Western blot analysis may represent a combination of the intact PG and PG metabolites, formed in the process of catabolism or remodelling of the matrix.

Many studies have reported the production of SLRPs from endothelial cells, including EA.hy926 cells. As mentioned, the functional state of endothelial cells (quiescent or angiogenic) correlates with the expression of specific SLRPs (Schonherr *et al.*, 2001). Resting endothelial cells *in vivo* as well as confluent cells *in vitro* constitutively express biglycan (Jarvelainen *et al.*, 1991). Biglycan is a major PG of the ECM, the functions of which are only beginning to be understood. EA.hy926 cells express and synthesise detectable amounts of biglycan, but not decorin, when forming monolayer cultures, typical of macrovascular endothelial cells *in vivo* (Nelimarkka *et al.*, 1997).

When diseases evolve slowly, as may be the case in the gradual initiation of infection of the SAG, the accompanying changes in the microvasculature would seem to be similarly gradual (Ezaki *et al.*, 2001),

which could explain the difference between the levels of biglycan at the mRNA and protein levels. Decreases in biglycan levels were observed by Nelimarkka *et al.* (1997). This group reported the net synthesis of biglycan was markedly decreased following endothelial cell culture with TNF-α. Several other growth factors and cytokines, including interferon-gamma and IL-1ß, did not modulate biglycan expression of EA.hy926 cells. However, Kinsella *et al.* (1997) reported an increase in both biglycan core protein synthesis and biglycan proteolytic processing, resulting in the accumulation of a 20kDa N-terminal biglycan fragment in the medium of wounded cultures through SDS-PAGE. This group also reported that biglycan RNA steady-state levels selectively increased 2- to 3-fold after wounding.

It is also apparent that biglycan has different effects depending on the cell type, and these effects are varied depending upon cell conditions. Shimizu-Hirota *et al.* (2004) produced mice which constitutively over-expressed biglycan in the vascular smooth muscle, to examine the effects on vascular pathology. They reported enhanced vascular smooth muscle cell proliferation and migration due to biglycan. However, it was noted that the proliferative effects of biglycan appeared to depend on cell type, as biglycan caused a decrease in proliferation in cultured endothelial cells in contrast to the effects on vascular smooth muscle cells (VSMC). Similarly, biglycan increased migration in VSMCs but not in endothelial cells. Concerning other cells, biglycan has been suggested to stimulate growth and differentiation of brain microglial cells, whereas in pancreatic cancer

cells biglycan suppressed proliferation (Kikuchi *et al.*, 2000; Weber *et al.*, 2001).

It has been demonstrated, by Northern blot analysis, that decorin is expressed in human capillary endothelial cells during inflammation when angiogenesis occurs (Schonherr *et al.*, 1999). The apparent lack of decorin detection within cells at both the mRNA and protein level for cells cultured with SAG supernatants illustrates the complexity of decorin expression and suggests that the cells are not under going angiogenesis, which would diminish the repair mechanisms following disease (Jarvelainen *et al.*, 1991).

The decrease in CS-GAG detection is parallel with the decrease in mRNA expression for biglycan but it is not in accordance with the continuous detection of biglycan at the protein level, suggesting that the SAG supernatants could degrade the GAG chain within the endothelial cell matrix before affecting the core protein. GAG degradation is quite likely due to the hydrolytic enzyme production of SAG. More specifically, the decreasing proportions of CS correspond to CSase activity produced by SAG, particularly *S. intermedius* and *S. constellatus* (Homer *et al.*, 1993; Jacobs and Stobberingh, 1995).

The effects of oral streptococci upon the endothelium have been studied in some detail due to their association with infective endocarditis. However, these studies have focused on the adherence and invasion of endothelial

cells (Parham *et al.*, 2003; Stinson *et al.*, 2003). Under normal conditions, a controlled cellular response to bacterial products protects the host from infection. Important in determining the consequences of bacterial colonisation and occurring early in the interactive process, is the induction of pro- or anti-inflammatory cytokines in response to bacterial products. Gram-positive bacteria may act as cyto-stimulating cell components. Ag I/II of SAG may mediate their ability to stimulate IL8 production in epithelial cells and of IL8 and IL6 in endothelial cells (Vernier *et al.*, 1996).

The data from the present study demonstrates that SAG supernatant modulates a number of functions of the endothelial cell line EA.hy926, most notably cell migration and ECM synthesis. Considering the proposed roles for SLRPs, such as those in migration, these changes may have important consequences on the pathogenesis of disease caused by the SAG, including formation of periapical abscesses and subsequent systemic spread of SAG.

CHAPTER SIX

Chapter 6

Streptococcus anginosus group virulence factor induction due to periodontal ligament extracellular matrix proteins

6.1 Introduction

The mechanism by which the SAG change from harmless commensals to virulent pathogens is unclear but likely to be complex. Successful infection requires that the bacteria gain access to, persist in, and replicate in normally privileged sites within the host. Moreover, they must produce certain factors that result in a level of host damage that perturbs homeostasis (Boyce *et al.*, 2004).

SAG appears to produce an array of glycoprotein- and glycosaminoglycan-degrading enzymes, which may contribute to the virulence of this species. It has been suggested that glycosidase production may play a nutritional role for the SAG. The release of carbohydrates from host glycoproteins is essential for SAG proliferation (Beighton *et al.*, 1994). Among the other potential SAG virulence factors are the glycosaminoglycan-degrading enzymes, including hyaluronidase (HAase) and chondroitin sulphate depolymerase (CSase), which cleave the hexosaminyl-hexuronyl linkages of hyaluronic acid (HA) and chondroitin 4 and 6 sulphates. The resulting tissue destruction may subsequently facilitate the spread of bacteria through tissues (Homer *et al.*, 1993; Jacobs and Stobberingh, 1995). Homer *et al.* (1993) reported that strains of S. *intermedius* and *S. constellatus* degraded HA, whilst only strains of *S.*

intermedius produced CSase. These results were confirmed by Jacobs and Stobberingh (1995), although they also observed CSase activity in *S. constellatus*. Sialidases hydrolyse the release of sialic acid from a variety of glycoconjugates and play significant roles in the pathogenesis of a number of important diseases. Beighton and Whiley (1990) demonstrated sialidase activity in *S. intermedius* but not in *S. anginosus* or *S. constellatus*.

Of the SAG, *S. intermedius*, often highlighted the most important pathogen due its predilections for defined body sites and the fact that it is an early coloniser of the oral biofilm, and as such regarded as a commensal. But it is also a serious pathogen associated with deep-seated purulent infections including abscesses in the liver and brain (Whiley *et al.*, 1992). *S. intermedius* has been implicated as an occasional causative agent of infective endocarditis, which secretes a human-specific pore-forming cytolysin, intermedilysin (ILY) (Nagamune *et al.*, 1996). It has been shown that ILY directly causes cell membrane damage in erythrocytes and in cell lines derived from major organs involved in deep-seated infections associated with *S. intermedius* (Nagamune *et al.*, 1996), and may represent an important virulence factor of this species (Nagamune *et al.*, 1996; Jacobs *et al.*, 2000). It has been shown that the production of this cytotoxin in isolates from deep-seated abscesses is 6.2-10.2-fold higher than in strains considered to be commensals, for example, isolated from dental plaque. However, no differences in the production levels of other

potential virulence factors such as hyaluronidase were found (Nagamune *et al.*, 2000).

Under certain growth conditions, certain streptococci have been shown to regulate the expression of adhesion and virulence associated genes at the transcriptional level (Jenkinson and Lamont, 1997). Previous studies have established that certain growth conditions can enhance bacterial virulence factors. Fibronectin binding protein, *prt*F1, in group A streptococci is up-regulated by atmospheric oxygen, while its M protein levels decrease (Fogg *et al.*, 1994). Also, the presence of collagen in growth medium up-regulates the expression of cell surface platelet aggregation-associated protein in *S. sanguis* (Erickson and Herzberg, 1995). Therefore, bacteria that experience dramatic environmental changes often regulate gene expression in order to maintain optimal phenotypic properties (Jenkinson and Lamont, 1997).

The aim of this chapter was to determine the range of degradative enzyme activities produced by a selected group of the SAG, and whether this profile was affected following contact with host ECM components. As *S. intermedius* appears to have exceptional virulence factors, an additional aim was to investigate the production of ILY production from commensal and disease strains of *S. intermedius*, before and after contact with host ECM proteins.

6.2 Materials and methods.

6.2.1 Preparation of periodontal ligament extracellular matrix proteins

PDL-like cells were obtained as in section 4.2.2 and 4.2.3. Briefly, PDL-like cells from 28 day old male Wistar rats were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin G, 10 mg/ml streptomycin sulphate, 25mg/ml amphotericin B) (Sigma Chemical Co.). On reaching 95% confluence, cell culture media was exchanged for media as above but with 0.2% FBS. PDL-like cells were cultured under these conditions for 48h, the media was subsequently removed, dialysed (as in section 2.2.1.1) and lyophilised. Samples were stored at -20°C until required. The amount of protein within the PDL-like ECM preparation was determined using the BCA assay (see section 2.2.6).

6.2.2 Bacterial growth and incubation with periodontal ligament extracellular matrix proteins

The bacterial strains utilised within this study were *S. anginosus* National Type Culture Collection (NCTC) 10713 and *S. constellatus* NCTC 11325, *S. constelltus* 322/95 and 34C, *S. intermedius* 127/95 and 30C, and *S. anginosus* 43586/96 and 43C (original source in table 3.1). *S. intermedius* strains 11C, 30C, 84C, 127/95, 447/95, HW13 (see table 3.1), were utilised in further studies of virulence factors.

Each bacterial isolate was grown on blood agar at 37°C, in an anaerobic cabinet (10% v/v CO_2, 20% v/v H_2, 70% v/v N_2) (Don Whitley Scientific Ltd., Shipley, UK), until purity was confirmed. Bacteria were inoculated into BHI broth at OD_{550nm} = 0.2 and cultured anaerobically for 18h. 1ml of broth was removed from the overnight culture and subsequently added to 9ml of fresh BHI broth. The fresh culture broth was subsequently grown, under anaerobic conditions, until mid-log phase (approximately 4h). Following these initial growth conditions, the bacteria were incubated for a further 4h, anaerobically, with or without 250μg/ml of protein collected in the media from serum-starved (2% FBS) PDL cells. This procedure was carried out in duplicate and 1ml aliquots of bacterial suspensions were utilised for subsequent analysis of virulence factors.

6.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

10μg of each bacterial suspension, cultured with or without 250μg/ml of protein, was separated by SDS-PAGE (see section 2.2.4.3), and stained with Coomassie brilliant blue solution (50% methanol, 0.05% Coomassie brilliant blue, 10% acetic acid, and 40% ddH_2O) for 1h. The gel was subsequently destained (50% methanol, 10% acetic acid, 40% ddH_2O), until visualisation of the molecular weight marker was evident.

6.2.4 API-ZYM

API ZYM (bioMerieux, Marcy-l'Etoile, France) is a semi-quantitative micro-method designed for the identification of enzymatic activities. The technique is applicable to microorganisms. It allows the systematic and rapid study of 19 enzymatic reactions using very small quantities. The system consists of a strip with 20 cupules, the base of which contains the enzymatic substrate and its buffer (see table 6.1). This base allows contact between the enzyme and both soluble and insoluble substrate. API ZYM permits the detection of enzymatic activities in a complex, non-purified sample, from which more precise spectrophotometric or electrophoretic methods would be unsuitable. The test provides a semi-quantitative estimate of enzymatic production.

1ml of bacterial suspension, cultured with and without 250μg/ml ECM proteins was diluted with distilled water to equate to 5-6 MacFarland. The enzyme strip was assembled as per Manufacturers' instructions. Briefly, 65μl of bacterial suspension was added to each cupule, and incubated for 4hr at 37°C. Following incubation, 1 drop of ZYM A and 1 drop of ZYM B reagents (supplied in kit) were added to each cupule, and the colour left to develop for 7-10min.Using the reading table provided (table 6.1), a value ranging from 0-5 was assigned, corresponding to the colours developed: 0 corresponding to a negative reaction, 5 to a maximum intensity. Due to subjectivity, two individual readers were used.

Table 6.1. API ZYM reading table

No.	Enzyme assayed for	Result	
		Positive	Negative
1	Control	Colourless of the	or colour of sample
2	Alkaline phosphatase	Violet	
3	Esterase (C4)	Violet	
4	Esterase lipase (C8)	Violet	
5	Lipase (C14)	Violet	
6	Leucine arylamidase	Orange	
7	Valine arylamidase	Orange	
8	Cysteine arylamidase	Orange	
9	Trypsin	Orange	Colourless
10	α-chymotrypsin	Orange	or very pale
11	Acid phosphatase	Violet	yellow
12	Napthol-AS-BI-phosphohydrolase	Blue	
13	α-galactosidase	Violet	
14	β-galactosidase	Violet	
15	β-glucuronidase	Blue	
16	α-glucosidase	Violet	
17	β-glucosidase	Violet	
18	N-acetyl-β-glucosaminidase	Brown	
19	α -mannosidase	Violet	
20	α -fucosidase	Violet	

6.2.5 Determination of chondroitinase and hyaluronidase activity

Methods were taken from Tipler and Embery (1985). Autoclaved BHI broth (Gibco) was supplemented with of chondroitin-4-sulphate (Whale cartilage, type A, Sigma Chem. Co.) and hyaluronic acid (Bovine umbilical cord, grade 1, Sigma Chem. Co.) to give a final concentration of 400μg/ml. Chondroitin-4-sulphate was readily soluble, whereas the hyaluronic acid required constant

agitation for 2h at room temperature. Both GAG were sterilised using 0.45μm pore size filters.

The inoculum used for each test was the growth harvested at mid-log phase. 1ml of bacterial suspension, with and without ECM, was added to 4ml of HA-C4S-medium, in duplicate. The broth was incubated under anaerobic conditions for 7 days. After incubation, the broth was removed from the cabinet and 1ml of each broth subjected to centrifugation at 3000g for 10 min. 20μl of each supernatant were added to 4ml of distilled water and the UV absorbance of each diluted sample was read at 232nm against non-inoculated broth (negative controls). Those samples showing U.V. absorption at 232nm, which is the characteristic U.V. absorbance of the unsaturated bond formed following the elimination reaction of HA and CS, were considered positive for the hyaluronidase and chondroitin sulphatase.

6.2.6 Isolation of bacterial total RNA followed by RT-PCR

Isolation of total RNA from bacterial cells was carried out using a Qiagen RNeasy Mini kit (Qiagen Ltd, West Sussex, UK). The extraction steps were carried out according to the manufacturers' instructions. All steps were performed quickly, at room temperature, to minimise RNA degradation. Total RNA was quantified as described in section 4.2.8.2. The protocol used for RT-PCR of bacterial total RNA was the same as that used for PDL cells (see section 4.2.8.3).

6.2.7 Quantification of intermedilysin production by *S. intermedius*

6.2.7.1 Intermedilysin polymerase chain reaction

PCR of the ILY gene was carried out according to the method of Nagamune *et al.* (2000). The reaction volume of 50µl contained 1µl of bacterial DNA, 0.25mM deoxyribonucleoside triphosphate (Promega), 1U *Taq* DNA polymerase (Promega), 20pmol of ILY forward primer (5'-AACACCTACCAAACCAAAAGCAGC-3'), 20pmol of ILY reverse primer (5'-ACTGTGGATGAAGGGTTGTTCCCC-3'), 50mM KCl, 10mM Tris-HCl and 1.5mM $MgCl_2$. The PCR program was as follows; initial denaturation at 95°C for 5min, followed by 30 cycles of a denaturation step at 95°C for 1min, an annealing step at 55°C for 1min, and an extension step at 72°C for 2min. The expected amplified fragment size was 1,463bp. *S. anginosus* (43586/96) was used as a negative control.

6.2.7.2 16S rRNA polymerase chain reaction

The 16S rRNA gene of *S. intermedius* isolates were amplified in order to quantify the amount of ILY expression. PCR amplification of 16S rRNA genes was performed in a reaction volume of 50 µl, consisting of 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 1.5 U Taq DNA polymerase, 5 µl 10x PCR buffer (all reagents from Promega), 5 µl (between 50 fg and 50 ng) of extracted DNA as template, and 0.5 µM each primer. The primer pair used was 27F (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3'), specific for the

domain (Dymock et al., 1996; synthesized by Invitrogen).

A touchdown protocol was used whereby, in the first cycle, denaturation was performed at 94°C for 6 min, primer annealing was performed at 65°C for 1 min, and extension was performed at 72°C for 2 min. In subsequent cycles denaturation was performed for 1 min and the annealing temperature was decreased by 2°C each cycle for 11 cycles, after which 25 cycles were carried out under the same conditions. In the final cycle, extension was performed for 12 min.

6.2.7.3 Detection of PCR products by agarose gel electrophoresis

PCR products resulting from amplification of the ILY gene within *S. intermedius* commensal and disease strains were separated as in section 4.2.8.5.

6.2.8 Statistical analysis

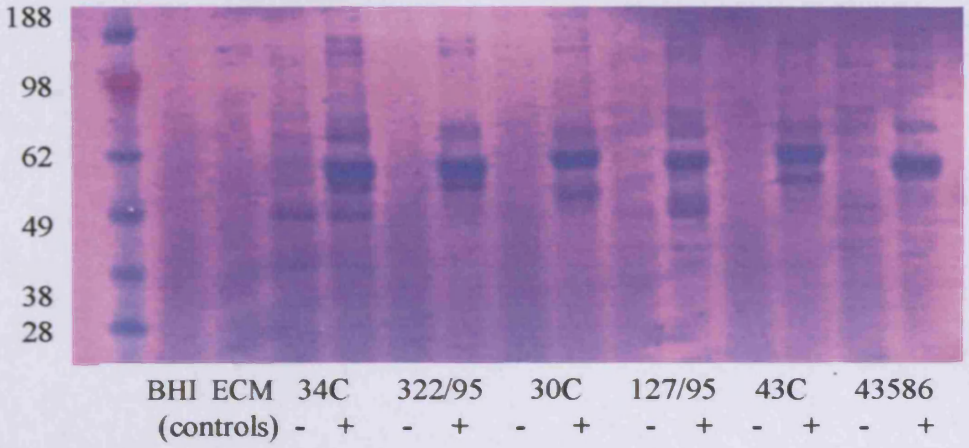
The Student unpaired two-tail T-test (Instat Package, Graph Pad software) was performed to obtain *p*-values for analysis of differences between cells cultured with and without SMG supernatants. A p value of <0.05 was deemed as significant.

6.3 Results

6.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of *S. anginosus* group supernatants following exposure to matrix proteins

SDS-PAGE followed by Coomassie blue staining of bacterial supernatants cultured in the absence or presence of ECM proteins from PDL cells revealed a number of bands ranging from 200kDa to 28kDa (see figure 6.1). For each isolate cultured with the ECM proteins, there was an extra intense band at approximately 55kDa, which was not present in the ECM alone (no bacteria added) control. Both commensal and disease isolates appeared to produce a double band at approximately 188kDa, following incubation with the ECM proteins.

Figure 6.1. SDS-PAGE followed by Coomassie blue staining of bacterial supernatants cultured in the absence and presence of ECM proteins



BHI = Brain heart infusion broth (no bacteria)
ECM = Extracellular matrix components (250µg/ml) (no bacteria)
-/+ = without / with 250µg/ml ECM

Table 6.2 (a) API ZYM of SAG isolates co-cultured without extracellular matrix components

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ECM (alone)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NCTC 11325 (*S. constellatus*)	-	+ (4)	-	-	-	+ (5)	-	-	-	+ (1)	+ (3)	+ (1)	-	-	-	+ (2)	+ (2)	-	+ (1)	-
322/95 (*S. constellatus*)	-	+ (4)	+ (1)	-	-	+ (5)	+ (2)	+ (3)	+ (2)	+ (1)	+ (5)	+ (3)	-	-	-	+ (2)	+ (2)	-	-	-
34C (*S. constellatus*)	-	+ (5)	+ (1)	-	-	+ (5)	+ (3)	+ (2)	-	+ (3)	+ (5)	+ (2)	-	-	-	+ (2)	+ (4)	-	-	-
127/95 (*S. intermedius*)	-	+ (5)	-	-	-	+ (5)	+ (3)	+ (3)	+ (2)	+ (3)	+ (5)	+ (3)	-	+ (3)	-	+ (2)	+ (2)	+ (2)	+ (2)	-
30C (*S. intermedius*)	-	+ (1)	-	-	-	+ (2)	+ (2)	+ (1)	-	-	+ (2)	+ (2)	-	+ (1)	-	-	+ (1)	+ (1)	+ (3)	-
NCTC 10713 (*S.anginosus*)	-	+ (5)	+ (2)	-	-	+ (5)	+ (2)	+ (4)	+ (2)	+ (3)	+ (5)	+ (2)	+ (3)	+ (2)	-	+ (3)	+ (3)	-	+ (2)	-
43586/96 (*S.anginosus*)	-	+ (3)	-	-	-	+ (5)	+ (4)	+ (3)	+ (1)	+ (2)	+ (5)	+ (2)	+ (1)	-	-	+ (1)	+ (1)	-	-	-
43C (*S.anginosus*)	-	+ (1)	-	-	-	+ (4)	-	-	-	+ (1)	+ (1)	+ (3)	-	-	-	+ (2)	-	-	-	-

1 = Control
2 = Alkaline phoshatase
3 = Esterase (C4)
4 = Esterase lipase (C8)
5 = Lipase (C14)
6 = Leucine arylamidase
7 = Valine arylamidase
8 = Cysteine arylamidase
9 = Trypsin
10 = α-chymotrypsin
11 = Acid phosphatase
12 = Napthol-AS-BI-phosphohydrolase
13 = α-galactosidase
14 = ß-galactosidase
15 = ß-glucuronidase
16 = α-glucosidase
17 = ß-glucosidase
18 = N-acetyl- ß-glucosaminidase
19 = α-mannosidase
20 = α-fucosidase

Table 6.2 (b) API ZYM of SAG isolates co-cultured with extracellular matrix components

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ECM	-	- ▼	-	-	-	-	-	-	-	-	-	- ▲	-	-	-	-	-	-	-	-
NCTC 11325 (*S. constellatus*)	-	+ (2)	- ▼	-	-	+ (4)	-	-	-	+ (1) ▲	+ (3)	+ (3) ▲	-	-	-	+ (3) ▲	+ (2)	-	+ (2)	-
322/95 (*S. constellatus*)	-	+ (4)	+ (1)	-	-	+ (5)	+ (2)	+ (3)	+ (2) ▲	+ (3)	+ (5)	+ (5) ▲	-	-	-	+ (4)	+ (3)	-	-	-
34C (*S. constellatus*)	-	+ (5)	-	-	-	+ (5)	+ (3)	+ (3)	+ (3)	+ (3)	+ (5)	+ (5)	-	-	-	+ (4) ▲	+ (5)	- ▲	- ▲	-
127/95 (*S. intermedius*)	-	+ (5) ▲	-	-	-	+ (5) ▲	+ (3)	+ (4)	+ (2) ▲	+ (3) ▲	+ (5) ▲	+ (4) ▲	-	+ (4) ▲	-	+ (4) ▲	+ (3) ▲	+ (5) ▲	+ (4)	-
30C (*S. intermedius*)	-	+ (4)	-	-	-	+ (5)	+ (3)	+ (2)	+ (2)	+ (3)	+ (5)	+ (4) ▲	-	+ (4)	-	+ (3)	+ (3)	+ (5)	+ (4)	-
NCTC 10713 (*S. anginosus*)	-	+ (4) ▲	+ (2)	-	-	+ (5)	+ (2)	+ (3)	+ (2)	+ (3) ▲	+ (5)	+ (5) ▲	+ (3) ▲	+ (3)	-	+ (3) ▲	+ (2) ▲	-	+ (2) ▲	-
43586/96 (*S. anginosus*)	-	+ (5) ▲	-	-	-	+ (5)	+ (4)	+ (4)	+ (2)	+ (4) ▼	+ (4)	+ (4)	+ (3)	-	-	+ (4) ▲	+ (3)	- ▲	+ (3)	-
43C (*S. anginosus*)	-	+ (3)	-	-	-	+ (4)	-	-	-	-	+ (2)	+ (4)	+ (1)	-	-	+ (4)	-	+ (3)	-	-

▲ increase in enzyme production in SAG isolates following co-culture with ECM components

▼ decrease in enzyme production in SAG isolates following co-culture with ECM components

6.3.2 Production of enzymes by the *S. anginosus* group in the absence and presence of extracellular matrix proteins

Table 6.1(a) and (b) indicate the enzymes produced by SAG, using the API ZYM assay, following incubation in the absence (table 6.1 a) and presence (table 6.1 b) of PDL ECM proteins.

The three species within the SAG differed in their abilities to produce an array of enzymes. All strains were negative for the control (number 1), showing the strip in use was producing valid results. All strains produced alkaline phosphatase activity (enzyme no. 2), under both growth conditions, with *S. intermedius* 30C, *S. anginosus* 43586/96 and 43C showing large increases in activity after exposure to ECM proteins. There was negligible activity for esterase (no. 3) and no activity of esterase lipase (no.4) or lipase (no. 5) for any strain. Disease and commensal strains of each SAG species produced all arylamidase enzymes (no. 6, 7, and 8), in the absence and presence of ECM proteins, except for 43C, which produced only leucine arylamidase. Also, there was an increase in trypsin (no. 9) activity following co-culture with ECM proteins for 34C (*S. constellatus*) and 30C (*S. intermedius*).

Numerous glycosidases were produced by the under both conditions. However, following co-culture with the ECM proteins, there were notable increases in certain glycosidase production for a number of the isolates compared to the non co-cultured condition. These isolates included both *S. anginosus* strains (43586 and 43C) for α-galactosidase (no. 13), and *S.*

intermedius (127/95 and 30C) for β-galactosidase (no. 14). For α-glucosidases (no. 16), there were increases in all strains, and there were increases for all strains except 43C for β-glucosidase (no. 17). 127/95, 30C and 43C each showed increases for N-acetyl-β-glucosaminidase (no. 18), and for α-mannosidase (no. 19) activity there were increases in 127/95, 30C and 43586. No activity was observed for α-fucosidase in any isolate.

6.3.3 Degradation of hyaluronic acid and chondroitin-4-sulphate by the *S. anginosus* group

Figure 6.2 illustrates a typical peak at 232nm, showing the breakdown of hyaluronic acid and chondrotin-4-sulphate. Table 6.3 shows the production of HAase and CSase from the SAG. HAase activity was restricted to *S. constellatus* and *S. intermedius* strains. This activity was observed in both the absence and presence of ECM proteins. CSase activity was demonstrated in both disease and commensal isolates for *S. constellatus* and *S. intermedius*, but not in either disease or commensal isolates of *S. anginosus*. However, the peak produced by *S. constellatus* and *S. intermedius* for CSase was less pronounced than the peak produced for HAase.

Figure 6.2. Typical spectra produced by the SAG enzymes acting on GAG substrates

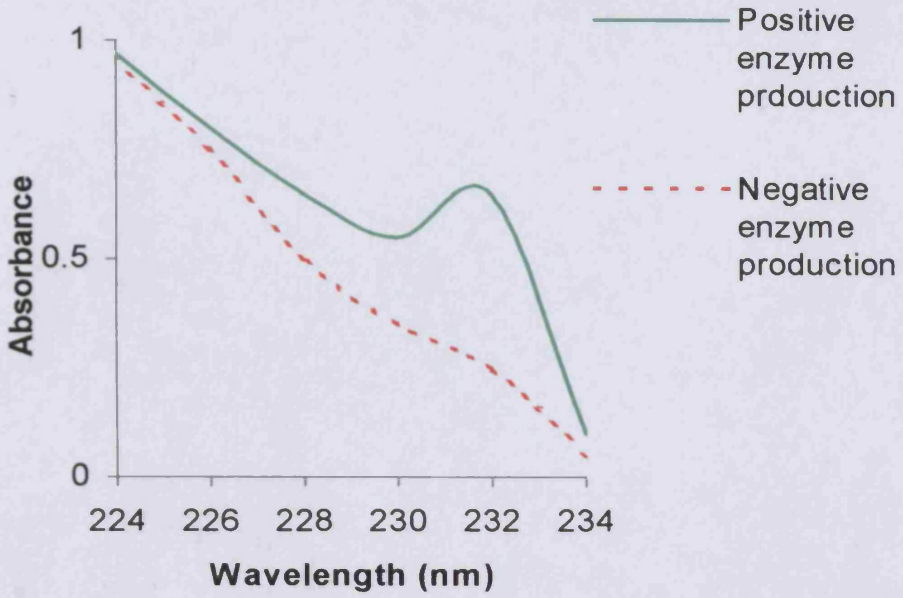


Table 6.3. Hyaluronidase and chondroitin sulphate depolymerase activity of SAG isolates

	Hyaluronidase		**Chondroitin – 4 - sulphate**	
	without ECM	with ECM	without ECM	with ECM
(*S. anginosus*) **NCTC 10713**	-	-	-	-
(*S. constellatus*) **NCTC 11325**	+	+	+	+
(*S. constellatus*) **322/95**	+	+	+	+
(*S. constellatus*) **34C**	+	+	+	+
(*S. intermedius*) **127/95**	+	+	+	+
(*S. intermedius*) **30C**	+	+	+	+
(*S. anginosus*) **43586/96**	-	-	-	-
(*S. anginosus*) **43C**	-	-	-	-

6.3.4 mRNA expression of ILY from *S. intermedius* co-cultured with and without periodontal ligament ECM proteins

Figures 6.3 and 6.4 shows the expression of ILY mRNA within *S. intermedius* strains when cultured without or with ECM proteins, normalised against the bacterial 16S mRNA gene. For commensal strains 11C, 30C and 84C, ILY expression is less in the absence of ECM proteins compared to ILY expression in the presence of ECM proteins ($p < 0.05$).

ILY is present in disease isolates 127/95 and HW13 when cultured with and without ECM proteins in similar amounts, whereas 447/95 did not produce ILY in either culture condition situation.

Figure 6.3. ILY expression by *S. intermedius* isolates in the absence and presence of extracellular matrix components

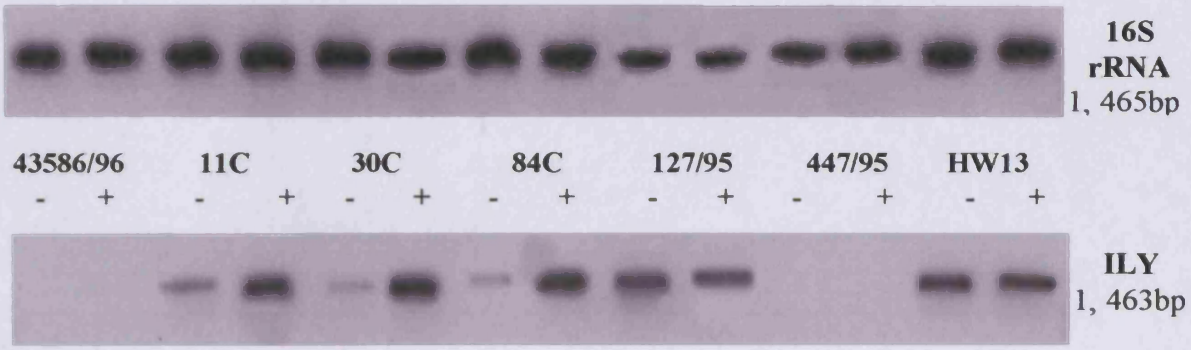
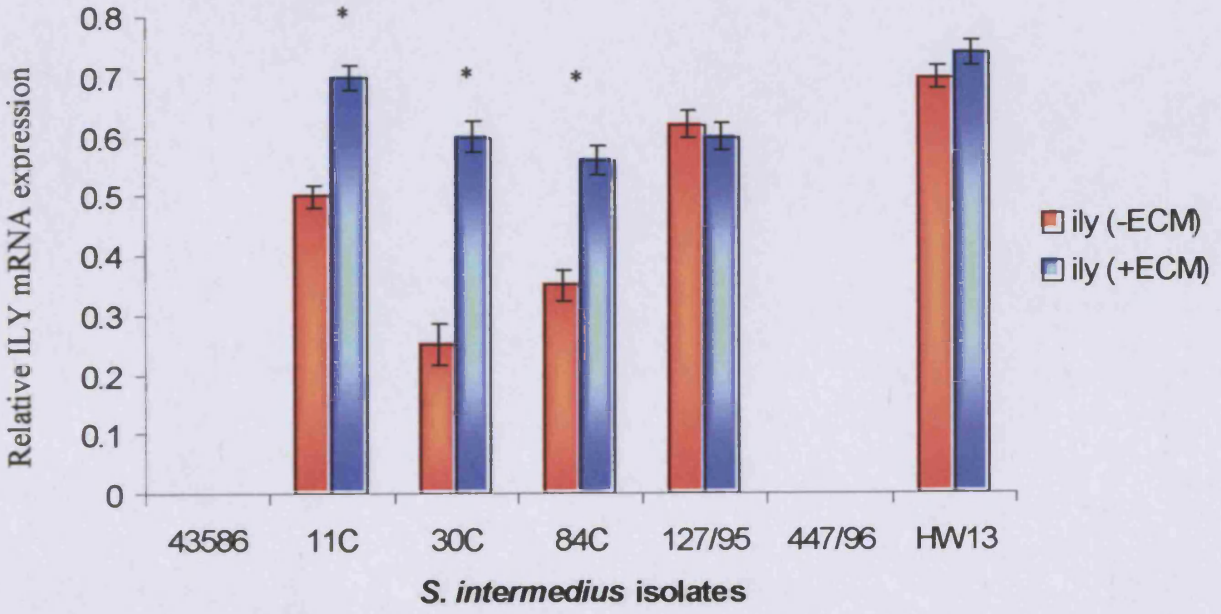


Figure 6.4. Relative expression of intermedilysin by *S. intermedius* isolates cultured in the absence and presence of extracellular matrix proteins, normalised to the expression of the 16S rRNA gene



C = Commensal isolate

* $p<0.05$

6.4 Discussion

Following attachment to specific sites within tissues, the SAG would need to be able to grow and divide successfully. The ECM is rich in nutrients in the form of glycoproteins and other components, which, if the bacteria can degrade them, are potential sources of both amino acids and sugars (Willcox *et al.*, 1995b). It has been shown that members of the SAG produce proteolytic activity and glycosidases (Homer *et al.*, 1990; Whiley *et al.*, 1990; Willcox *et al.*, 1995b) which may be important in ECM degradation.

Utilising a panel of well-established biochemical tests, this study evaluated the differences in the potential virulence factors of the SAG strains following exposure to PDL-like ECM proteins. In this study, SDS-PAGE of the SAG supernatants allowed for a rapid comparison of proteins produced by isolates cultured with and without ECM proteins without the expense required for more specialised techniques (Sacilik *et al.*, 2000). SDS-PAGE, followed by Coomassie blue staining, of total extracellular proteins within the bacterial supernatants, revealed a range of molecular bands under both culture conditions. However, in the presence of ECM proteins, a number of extra bands were present for all SAG isolates, in particular an intense band at approximately 55kDa, which was not present in controls. A number of potential virulence factors have already been reported within the SMG. These include: L-cysteine desulfhydrase (44kDa); fibronectin binding protein (14kDa); amylase binding protein (12.5kDa); intermedilysin (54kDa); antigen I/II (160kDa); hyaluronidase

(~120kDa) and dipeptidyl peptidase (84kDa) (Willcox *et al.*, 1995a; Nagamune *et al.*, 2000; Petersen *et al.*, 2001; Yoshida *et* al., 2002; Takao, 2003; Fujimura *et al.*, 2005). The protein observed within this study may be derived as part of a transport system for ECM proteins or as the consequence of release upon cell death and disintegration (Willcox *et al.*, 1995).

The API-ZYM assay demonstrated that a number of enzymes produced by commensal and disease strains of the SAG increased following culture with host ECM proteins. Overall, marked increases were observed in alkaline phosphatase activity, as well as trypsin, α-glucosidase and N-acetyl-ß-glucosaminidase. The SAG strains utilised in the current study showed similar enzyme production to SAG isolates in previous studies. However, the current study also showed that the activity of many enzymes were increased following SAG exposure to the host ECM, which may have potential pathogenic implications *in vivo*.

Glycosidase activity is of great value for invading bacteria. Within the current study, numerous glycosidase activities appeared to increase following bacterial co-culture with ECM proteins, particularly α- and β-glucosidase. Willcox *et al.* (1995b) studied the degradative enzymes produced by oral streptococci, in particular, the *S. sanguis* group and the SMG. They reported that *S. anginosus*, *S. intermedius* and the majority of *S. constellatus* isolates produce β-glucosidase, whereas only *S. constellatus* and *S. anginosus* produce α-glucosidase. They also reported α-

galactosidase activity was restricted to *S. anginosus* and the majority of *S. anginosus* and all of the *S intermedius* isolates produced β-galactosidase. Willcox *et al.* (1995b) also reported that all isolates of the SAG they tested produced lysine arylamidase activity. *S. constellatus* produced activity against Val-Pro-Arg-7-Amido-4-methyl-coumarin (AMC; a synthetic substrate for thrombin-like activity), and *S. anginosus* and *S. constellatus* produced activity against Ile-Glu-Gly-Arg-AMC (for factor Xa activity). The expression of trypsin-like proteolytic activity is considered a plausible potential virulence factor and has been reported in other periodontopathogens, such as *Porphyromonas gingivalis* (Wyss, 1992).

The production of α-mannosidase within the current study appeared to increase in both *S. intermedius* isolates and was induced in *S. anginosus* (strain 43586/96) following co-culture with ECM proteins. Homer *et al.* (2001) assayed the production of mannosidase activity by all currently recognised species of human viridans group streptococci, in which bacterial growth was dependent on the degradation of RNase B and subsequent utilisation of released mannose. They reported that mannosidase activity was produced by *S. intermedius* and, in addition to its α-(1→3), α-(1→6), and β-(1→4) mannosidase activities, this isolate also produced *N*-acetylglucosaminidase activity. Both *S. intermedius* and *S. anginosus* showed β-mannosidase activity when synthetic substrates were used.

It is generally accepted that bacteria catabolise GAGs by an eliminase enzyme reaction leading to the formation of unsaturated products, whereas mammalian systems are unable to manufacture such enzymes and rely on a hydrolytic cleavage. Within the current study, HAse and CSase production by commensal and disease SAG strains following exposure to ECM proteins was no different to the enzyme production of the SAG strains exposed to the substrate medium only. However, this study demonstrates that the enzyme is induced following initial contact with HA and CS and continuously produced, which would have profound effects upon the host.

The 232-nm assay has been proved sensitive compared to the commonly used plate method (Tipler & Embery, 1985). The production of hyaluronidase by *S. constellatus* and *S. intermedius* strains in this investigation is in accordance with other studies (Homer *et al.*, 1993; Jacobs and Stobberingh, 1995). HAase production may enable cells to gain entry into the body by breaking the epithelial /cell matrix integrity (Willcox *et al.*, 1995), then subsequently to breakdown the matrix when inside the host. As mentioned, HAase is able to breakdown HA, however, some of these enzymes are also able to cleave CS, albeit at a slower rate (Kreil, 1995). Important to note in this study, there was CSase activity in commensal and pathogenic isolates of *S. constellatus* and *S. intermedius*. Previous studies have also shown CSase activity for *S. constellatus* and *S. intermedius* (Homer *et al.*, 1993; Jacobs and Stobberingh, 1995; Shain *et al.*, 1996), however, Jacobs and Stobberingh (1995) reported CSase activity tended to be more associated with infection-related strains.

Therefore, it is difficult to establish the action of bacterial HAase and CSase on the tissues of the periodontium. These GAGs are prominent in both the non-mineralised tissues and mineralised tissues of the periodontium. It must also been borne in mind that GAGs, such as CS, are also found as part of PGs, which subsequently interact with collagen to form a supporting fibrous network common to all types of connective tissues. The structural integrity of periodontal tissues is lost during infection, as such events have detrimental effects upon the ECM components of the underlying periodontal tissues, including collagen, PGs and HA (Tipler & Embery, 1985).

SAG enzymatic activity, in particular glycosidase activity, may not only change the function of the ECM components of the host, but they could also release products, such as carbohydrates, which would play a role in the persistence of the SAG at infection sites. Whether SAG enzymatic activity is the same *in vivo* is yet to be determined, and further biochemical characterisation of enzyme activity and the substrate specificity of the enzyme will be required to elucidate the mechanism by which degradation is achieved.

The detection of commensal and disease strains of *S. intermedius* ILY mRNA levels within the current study is of particular interest. Following co-culture with the ECM protein preparation, the commensal isolates increased ILY production significantly. ILY was present in 2 of 3 disease isolates (127/95 and HW13) when cultured in the absence and presence of

ECM proteins; and expression was slightly increased following co-culture with ECM. The failure to detect ILY in *S. intermedius* isolate 447/95 is not unprecedented given that Nagamune *et al.* (2000) detected the gene in all strains of *S. intermedius* except for two that were phenotypically and genetically atypical.

ILY was previously found to be produced by an *S. intermedius* strain from a human liver abscess (Nagamune *et al.*, 1996). More recently, Nagamune *et al.* (2000) revealed that the toxin exists only in *S. intermedius* and no homologue to the toxin gene is distributed in other members of the SAG. Moreover, isolates from deep sites, such as brain and liver abscesses, produce ILY at levels much higher than that of strains from dental plaque or peripheral-site infections. However, there was a lack of significant correlation in this respect for other enzymatic activities (CSase, HAase, and sialidase). This indirectly highlights the importance of ILY, perhaps as a primary, or triggering, pathogenic factor, to ultimately cause deep-seated infections (Nagamune *et al.*, 2000). Although there is no clear view of the regulation mechanism for ILY expression there may be two types of strains: weak (or poorly inducible) or strong (or potentially strongly inducible) within *S. intermedius*. It has been postulated if an individual carrying a strongly ILY-producing strain in their dental plaque experiences trauma within their oral cavity, the strain may enter their bloodstream and more readily cause a deep-seated infection (Nagamune *et al.*, 2000). In studies with PMN cells, which are important components of the innate immune system, Macey *et al.* (2001) demonstrated that ILY appears not to

act as a chemotactic agent but can modulate PMN functions, such as stimulation of phagocytosis and can cause PMN cell death. Sukeno *et al.* (2005) observed that ILY is essential for *S. intermedius* invasion of human hepatoma HepG2 cells, and that secretion of ILY within host cells is essential for subsequent host cell death. It has been suggested that when a strain of *S. intermedius*, producing ILY, enters the bloodstream from its normal habitat possibly due to oral injury, it can evade killing by PMNs due to the activity of its secreted ILY, and can thereby reach remote infection sites. Subsequently, surviving bacterial cells are bound to host cells at these sites (e.g. brain or liver), and are able to invade the host cells and spread to neighbouring cells in the tissue through ILY action, and other virulent enzymatic action, which may eventually form a necrotic region. Finally, macrophages within the tissue and/or PMNL migrating to the site of infection result in the formation of an abscess, within which *S. intermedius* will persist, aided significantly in its defence by ILY activity (Sukeno *et al.*, 2005).

The aim of chapter 6 was to study a number of well-known virulent enzymes produced by the SAG and to observe whether these enzymes were regulated upon contact with host ECM components. ILY is a crucial virulent enzyme associated with *S. intermedius*, an important member of the SAG due to its tendency to cause severe abscess formation (as mentioned previously). Therefore, ILY was seen as a key enzyme to study in the pathogenesis of these bacteria. Since this study has reported an

increase in the protein expression of ILY, further tests can be warranted to observe if the functional activity of these bacteria is also increased.

The data from the present study shows the successful *in vitro* expression of enzyme and toxin production of the SAG, and it appears that the protein expression by SAG may be regulated by the environmental conditions they are exposed to. The results confirmed the necessity to compare this virulence factor production in normal and diseased states i.e. without and with ECM proteins present. The presence of these potential virulence factors is confirmed through API-ZYM analyses, which has supported previously determined enzyme characteristics of the SAG, whilst also suggesting other enzyme expressions are increased following exposure to host ECM. ILY production by *S. intermedius* strains has been confirmed through PCR analysis, providing evidence for the concept that the expression of this toxin within commensal isolates is up-regulated on contact with the ECM. These studies have provided important information on possible virulence factors, which may operate during initial SAG-ECM interactions, and their subsequent roles throughout infection with this group of bacteria in relation to periapical disease and systemic spread.

CHAPTER SEVEN

Chapter 7

General discussion

The aims of this thesis were to investigate host-microbial interactions associated with the *Streptococcus anginosus* group (SAG) within the periapical tissues, the consequence of the interaction on host cells, and to observe subsequent effects of the host ECM upon the bacteria. An attempt was made to relate these results to our current understanding of periapical infection, namely abscess formation, by the SAG and to enhance our perception of SAG mechanisms of ECM destruction.

Purification of both ovine tissue and recombinant PGs was central to the biomolecular interaction analysis. A number of well-established chromatography and anion exchange purification steps were carried out in order to eliminate co-purifying PGs such as collagen and α_2 HSG (Sugars *et al.*, 2003). The denaturant guanidinium chloride was utilised throughout purification, which served to disaggregate proteins, without grossly changing the structure of the PG (Scott *et al.*, 2006). Following robust characterisation of PGs, this study showed the successful purification of a mixed population of decorin and biglycan substituted with CS and DS in alveolar bone and gingiva, respectively. We were also able to purify and characterise transfected HeLa cells transfected to over-express recombinant decorin or biglycan substituted with CS, producing appropriate material for subsequent studies.

Both decorin and biglycan, which are ubiquitously expressed within alveolar bone and gingival tissues (Bratt *et al.*, 1992; Fisher *et al.*, 1993), are implicated in a wide range of functions including collagen fibril formation (Vogel and Trotter, 1987; Greve *et al.*, 1990), and have been shown to interact with growth factors (Hildebrand *et al.*, 1994; Kinsella *et al.*, 2004). PGs are also reported to act as ligands for bacterial attachment (Rostand and Esko, 1997; Joh *et al*, 1999), behaving as a 'foothold' for subsequent propagation. The BIAcore® surface plasmon resonance system enabled successful immobilisation of purified PGs, and the establishment of a model protocol for the analysis of specific interactions of commensal and pathogenic strains of the SAG with intact PGs and their corresponding core proteins. The apparent lack of binding to the core protein alone suggested that the SAG mediated binding through the GAG chain attached to the PG. There was greater binding to intact gingival PGs compared to other intact PGs indicating that, if binding is via the GAG chain, then it is likely to be the DS-GAG chain. This result is similar to that observed for *B. burgdorferi*, the causative agent of Lyme disease, which also binds to DS (Leong *et al.*, 1998). It is apparent that these initial binding studies of the SAG have uncovered numerous areas worthy of further investigation. Analysis of more strains from the SAG is necessary in order to confirm the results from this study. Other fundamental studies are required to understand the biophysical nature of bacterial attachment to host tissue components. Further work could involve competitive-inhibition studies, where decorin or biglycan antibodies, or antibodies for the GAG chain present

on the sensor chip surface, can be added to the analyte solution and passed across the ligand. In addition, reversing the current situation by using bacteria as the ligand and PGs as the analyte would be useful as a confirmatory experiment. Although a consideration *in vivo*, such an approach would allow us to remove factors relating to steric hindrance due to agglutination of bacteria, allowing us to look directly at PG-bacteria interactions.

Initial attachment within the host tissues may lead to destruction of surrounding periapical tissues, including the PDL. This tissue may be affected by bacteria via the dental pulp, due to entry through the dentinal tubules, following dental caries. Infection finally reaches the periapex, which is in close proximity to the PDL. Work presented in chapter 4 provided information on the expression of decorin and biglycan, and the GAG components synthesised by the PDL following exposure to the SAG. Collectively, the data indicated increased biglycan levels and decreased decorin levels in response to bacterial exposure. These results highlight previous suggestions proposing different roles for these SLRPs, including inflammatory roles for biglycan (Schaefer *et al.*, 2005), and a misassembled collagenous matrix due to decreased decorin (Hakkinen *et al.*, 2000). Commensal and pathogenic strains of the SAG also had detrimental effects upon the GAG moiety, with decreasing proportions of CS and DS. This decrease could possibly be an effect of the SAG resulting in a change in PG profile, as a consequence of altered post-translational synthesis of the GAG leading to shorter chain length.

Equally, it could be due to the GAG-depolymerising enzymes produced by this group of bacteria. Overall, the changing GAG profile would affect the functional integrity of host tissue and tissue remodelling.

The SAG influenced cellular activity of PDL cells in other ways. Both commensal and pathogenic strains of the SAG increased PDL cell growth whilst decreasing their alkaline phosphatase activity, indicating the diverse effects these bacteria produce. This would have potential consequences upon tissue repair by interfering with cell differentiation. Since this study used primary PDL-like cells, it can be considered as being of greater relevance to periodontal disease *in vivo* in possibly paralleling mechanistic avenues involved during PDL destruction.

Infections with the SAG are likely to involve interaction with endothelial cells, as the periapical tissues are highly vascularised. Evidence provided within this study indicated that commensal and pathogenic strains of the SAG had debilitating effects on the ability of endothelial cells to synthesise a stable matrix. The SAG affected a number of parameters including increasing cell growth, decreasing migration and decreasing biglycan synthesis along with a corresponding decrease in CS-GAG detection. The lack of decorin production suggests that the endothelial cells were not undergoing angiogenesis (Schonherr *et al.*, 1995), although the cells were subjected to potential SAG infection conditions, thereby stressing the importance of SLRPs and their

biological roles within the ECM. Overall, the results would suggest that the net endothelial ECM loss observed may be due to both increased destruction in combination with a decrease in angiogenic properties.

The combined effects on different cell types reported in chapter 4 and 5 emphasise the complexity and multifactorial nature of destruction by the SAG. It is of interest to note that SAG supernatants can potentially modulate various functions of PDL-like cells, including those of decorin and biglycan, present in host tissues. It is possible that cells cultured in the presence of any member of the SAG could be stimulated to secrete cytokines in response to pathological products of this group of bacteria. PDL cells are known to express certain pro-inflammatory cytokines during periodontal inflammatory conditions (Quintero *et al.*, 1995; Agarwal *et al.*, 1998). Once synthesised, these cytokines may amplify the host immune response, exerting autocrine/paracrine effects. In furthering our understanding of SAG pathogenesis, it would be advantageous to observe if members of the SAG could also modulate host immune functions, including those of IL-1β and IL-6, and various cytokines, such as TGF-β and TNF-α.

It is well established that the SAG produce a number of degradative enzymes (Homer *et al.*, 1993; Jacobs and Stobberingh, 1995; Willcox *et al.*, 1995b). Interestingly, data presented within this thesis suggests SAG enzymatic virulence factors, such as glycosidases, increase following exposure to ECM

proteins synthesised by PDL-like cells. Other interesting results were observed for commensal strains of *S. intermedius* with regard to intermedilysin (ILY) expression. There was a 2- to 3-fold increase in ILY expression following exposure to ECM proteins. Other streptococci have been shown to regulate expression of virulence-associated genes at the transcriptional level following exposure to different environmental conditions (Fogg *et al.*, 1994; Erickson and Herzberg, 1995; Jenkinson and Lamont, 1997). This increased virulence would have dire consequences for the host by breaking down the matrix and altering tissue repair.

In conclusion, it is known that one way the SAG (within supra- or subgingival plaque) can gain entry into host periapical tissues is via dentinal tubules, whenever dentin is exposed in the oral cavity, due to a number of factors including carious lesions and dental trauma.

Once the bacteria reach the root canal space they can come into open communication with the periapical tissues (including PDL) via the apical foramen. If SAG were to persist and invade the host they could become established within periapical tissues. Evidence from this thesis suggests the SAG recognise ECM components present within the host tissues, which would enable the SAG to gain a foothold within the host and stimulate bacterial adherence and growth within these tissues.

Subsequently, SAG metabolites and toxic products, such as hyaluronidase and chondroitin sulfatase (as seen in chapter 6), could diffuse throughout the periapical tissues evoking a number of inflammatory changes to the host ECM, including alteration of host ECM synthesis. In situations where tissues have been damaged, observed within the PDL matrix in chapter 4, periapical disease can manifest and if host defences are inadequate abscess formation can occur.

This thesis has enhanced our knowledge on the host-microbial interactions associated with the SAG in relation to periapical disease. It provides new potential roles of initial adhesion, in the establishment of infection. Furthermore, data has been presented that may provide additional explanations for the subsequent infections the SAG could cause within these tissues, such as abscess formation. The importance of such information enhances the fundamental knowledge of the SAG, which may lead to novel strategies for management and, more importantly, prevention of disease.

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