

Impact of bidirectional relationships between streptococcus anginosus group and host tissue matrix components on cellular activity: role in establishment of infection

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Abstract

This paper investigates pathogenic mechanisms of the *Streptococcus anginosus* group (SAG) of bacteria which influence the biological activity of periodontal ligament (PDL) cells, endothelial cells and also how matrix proteins produced by these host cells influence bacterial virulence factors. Isolates of SAG species, designated *S. anginosus*, *S. constellatus* and *S. intermedius*, were derived from healthy commensal and clinical pathogenic infection sites. SAG culture supernatants contained multiple protein components which differed between isolates. All SAG supernatants increased cellular proliferation and decreased decorin synthesis and collagen assembly by PDL cells and reduced endothelial cell migration. SAG isolates responded differently to extracellular matrix (ECM) components synthesised by PDL cells, but there was an overall notable increase in hydrolytic enzyme activity and in the production of the cytotoxin intermedilysin by *S. intermedius*. Collectively, the results indicate that both commensal and pathogenic SAG isolates were capable of impairing the ability of PDL cells and endothelial cells to make functional vascularised tissue. Reduced decorin synthesis is likely to have a major impact on cell signalling, angiogenesis and matrix assembly. Furthermore, ECM components produced by PDL cells were differentially capable of moderately increasing SAG enzymic activity, leading to subtle ECM modifications. The impact this bidirectional effect has on the tissue remodelling process is discussed.

Keywords: *Streptococcus anginosus* group, periodontal ligament, mesenchymal cells, endothelial cells, decorin

Introduction

Members of the *Streptococcus anginosus* group (SAG) of microaerophilic bacteria can exist as part of the commensal microflora of the body, particularly within the oral cavity, or as pathogens in polymicrobial orofacial infections such as dentoalveolar and periodontal abscess [1-5] and thoracic infection, such as pneumonia, purulent emphysema and lung abscess [6]. Often described as pioneer microorganisms in the establishment of infection, it has been proposed that the SAG bacteria prepare the micro-environment for further colonisation by other anaerobic bacterial species [7,8]. SAG have a tendency for haematogenous spread leading to the establishment of deeper-seated infections such as brain and liver abscesses [9].

The periodontal ligament (PDL) consists principally of a type I and type III collagen fibre network, providing structural support and attachment of the tooth within its socket. The formation of these collagen fibres is regulated by decorin which is a small leucine rich proteoglycan, the N terminal of which is conjugated by one dermatan sulphate glycosaminoglycan chain [10,11]. Decorin has been demonstrated to interact with tropocollagen and guide lateral fusion in fibril formation *in vitro* [12,13]. Of note, targeted deletion of decorin in mice resulted in irregularly sized and shaped collagen fibril bundles, many with an over-large morphology, which was most evident in the PDL [14]. Decorin has also been proposed to influence cell signalling

and proliferation of both PDL and endothelial cells either by direct binding to cell surface receptors [14-17] or by binding of growth factors in the matrix and regulating their release [11,18]. As a consequence of the constant physical forces placed on this tissue, collagens within the PDL have amongst the highest turn-over rates of all connective tissues within the body, [19] resulting in the constant renewal of the periodontal fibres, the alveolar bone proper and the anchoring Sharpey's inserts. Cells within the PDL are thus capable of a rapid response, facilitating an extremely high turnover of the extracellular matrix (ECM) proteins [20]. Within this scenario, mesenchymal progenitor cells within the PDL proliferate, migrate and ultimately differentiate, leading to the synthesis of new collagen fibres, orchestrated by proteins such as decorin. In addition, high proliferation of endothelial cells and angiogenesis in the PDL is apparent during these periods of repair, together with signalling factors such as vascular endothelial growth factor (VEGF) and prostaglandin E₂ from endothelial cells which are proposed to promote PDL progenitor cell osteogenesis [21]. Alterations to the biological activity of these cells would therefore have a sharp deleterious impact in maintaining a functional tissue.

In considering the establishment of infection, our previous studies have demonstrated that SAG isolates favour binding to the dermatan sulphate glycosaminoglycan chains conjugated

to decorin and biglycan, along with fibronectin, which are all predominant in soft tissues of PDL and skin. Adherence was lower for the chondroitin sulphate conjugated decorin and biglycan which is predominant in osseous tissues, suggesting a preference for colonisation of soft connective tissues [22]. Numerous streptococcal species have also been shown to bind to heparin sulphate, which are components of cell surface proteoglycans such as syndecan, via microbial surface cell recognition adhesion matrix molecules (MSCRAMMs) [23-26]. These host-microbial interactions lead to the initiation of reciprocated processes whereby factors from eukaryotic cells are able to engage with and change the activity of prokaryotic cells and *vice versa*. This ability is recognised as a crucial first step in the development of infection, although neither the factors involved in evoking these responses, nor the cellular responses have been investigated in any detail. The aims of this study were therefore to characterise the effect of soluble components from various SAG isolates on PDL cells and endothelial cells in considering their biological activities towards synthesis of new periodontal tissues. Conversely, the effect of the extracellular components synthesised by PDL cells in altering the pathogenic activity of the SAG isolates was also investigated. Further, this study compared the responses of SAG isolates recovered from healthy body sites, regarded as part of the normal commensal microflora, and clinical infections in an attempt to consider factors causing pathogenic induction.

Materials and Methods

Isolation and culture of periodontal ligament-like cells

PDL cells were obtained from 1 mm² alveolar bone pieces dissected from bone lining the teeth of 28 day-old male Wistar rats. Bone pieces were incubated with 1 mg/ml collagenase, 4.5 U/ml elastase (Type IV, Sigma Chemical Co.), 2 h, 37°C. Released cells were decanted from the bone chips, mixed with equal volume of Dulbecco's modified Eagle's medium (DMEM) (Gibco), 10% fetal bovine serum (FBS), 100 units/ml penicillin G, 10 mg/ml streptomycin sulphate, 25 mg/ml amphotericin B (Sigma Aldrich) and recovered by centrifugation at 1000 g, 5 min. Cells were seeded at 10⁴ cells/cm² in T-75 tissue culture flasks and cultured in the above DMEM media, 37°C, 5% CO₂. Cells were utilised at passage 2-4.

Endothelial cell source and culture

Human endothelial cell line EA.hy926 represents an immortalised cell line generated by fusion of human umbilical vein endothelial cells with the immortalised human lung epithelial carcinoma cell line A549, retaining the signalling characteristics of human umbilical vein endothelial cells [27]. Cells were cultured in MCDB 131 medium (Invitrogen) supplemented with 10% (v/v) foetal calf serum, 100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin.

Bacterial sources and supernatant preparation

SAG isolates were collected from clinically healthy sites (suffixed with C) and sites of infection. SAG had previously been characterised and designated as *S. constellatus* (322/95, source dentoalveolar abscess; 34C, source tongue), *S. anginosus* (43586/96, source high vaginal swab; 43C, source tongue) and *S. intermedius* (127/95, HW13, source dentoalveolar abscess; 30C, 11C source tongue; 84C, source plaque) using a phenotypic identification scheme [28]. Bacteria were anaerobically cultured in 10 ml Brain Heart Infusion (BHI) medium at 37°C, to an OD 550_{nm} = 0.5. Five ml of bacterial suspension was added to 45 ml of fresh BHI and incubated to stationary phase (8 h; ~10⁸ colony forming units (cfu)/ml). Bacteria were harvested by centrifugation at 3500 g for 10 min. Supernatants were passed through a 0.2µm filter and protein concentrations determined using a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc, IL, USA).

Influence of bacterial supernatant on PDL and endothelial cell viability

PDL and EA.hy926 cells were seeded in 24 well plates at 10⁴ cells/ml and cultured with 1 ml of their respective media, 37°C, 5% CO₂ for 24 h. Media (500 µl) was replaced with fresh media containing bacterial supernatants, to give a final concentration of 10 ng - 500 µg protein/ml of media (determined by the BCA assay). For uninoculated control cultures, 500 µl of media was replaced with 500 µl of PBS. At 24 and 48 h, cells were washed with phosphate buffered saline (PBS), stained with 0.4% trypan blue and the percentage of viable (non-stained) cells was immediately determined by microscopic enumeration. Sub-toxic levels, where cell viability was greater than 95% were determined as <20 ng/ml for PDL cells and <100 ng/ml for EA.hy926 cells, for each bacterial isolate. These sub-toxic levels were used in all subsequent analysis to assess effects on host cell biological behaviour.

Influence of bacterial supernatant on PDL and endothelial cell growth

PDL and EA.hy926 cells were seeded in 24 well plates at 10⁴ cells/ml in their respective media. After 24 h, media was replaced and cells were cultured with media supplemented with either 0 ng/ml or 20 ng/ml (PDL cells) or 0 ng/ml or 100 ng/ml (EA.hy926 cells) of the respective SAG supernatants (day 0). Cell growth was determined using an MTT assay (R&D Systems Europe, Oxon, UK) over 0-5 days, as per manufacturer's instructions. Triplicate assays were performed for each supernatant on three separate occasions.

Influence of bacterial supernatant on collagen synthesis by PDL cells

PDL cells were seeded on to round glass coverslips in 6-well culture dishes at 10⁴ cells/cm², and cultured for 24 h, after which media was replaced with media supplemented with 0 ng/ml or 20 ng/ml of SAG supernatants (day 0) which was

renewed every 24 h. At 5-13 days, cells were fixed in 2% formaldehyde for 30 min and then washed in PBS. Cells were blocked with 1% normal horse serum in PBS (Sigma Aldrich), for 10 min and then incubated with mouse monoclonal anti-collagen type I (Sigma Aldrich; 1:2,000 dilution in 0.5% horse serum, PBS) for 1 h. Immunoreactivity was detected with rabbit anti-mouse IgG FITC conjugate secondary antibody (Sigma Aldrich; 1:1000) at for 30 min, and viewed under a fluorescent microscope ($\times 40$ objective).

Influence of bacterial supernatant on endothelial cell migration

A migration assay was performed using endothelial cells cultured as spheroids [17]. Briefly, single uniform spheroids were prepared from 1000 EA.hy926 cells per well of a 96-well round bottom hydrophobic plate, suspended in Waymouth MAB 87/3 medium supplemented with 5% heat-inactivated foetal calf serum and 30% methyl cellulose (Sigma Aldrich) for 48 h, 37°C, 5% CO₂. Spheroids were transferred, one per well of a flat bottomed 96-well hydrophilic plate, pre-coated with 0.125 mg of type 1 collagen (extracted from rat tail [17]) and cultured in 100 μ l Waymouth medium supplemented with 0.5% heat-inactivated foetal calf serum, and 0 ng/ml or 100 ng/ml of SAG supernatant, 37°C, 5% CO₂ for 0-72 h. Cells migrating away from the spheroid bodies, eventually form a ring of cells resembling sprout-like extensions associated with angiogenesis. For spheroids located close to the centre of the well, cell migration was recorded as digital images after 6 h and then every 24 h over 3 days. Spheroid width was measured using Adobe Photoshop (Adobe Systems Incorporated) and the ruler tool, and means with standard deviations calculated for each time-point from a minimum of 12 wells for each supernatant.

Influence of bacterial supernatant on mRNA expression of decorin

PDL cells were seeded at 10⁴ cells/cm² in 6-well plates for 24 h, after which the culture medium was supplemented with or without SAG supernatant, and changed every 24 h. At days 10 and 13, cells were washed with PBS and the total RNA extracted using a Qiagen RNeasy Mini kit (Qiagen Ltd, UK). cDNA was synthesised from 500 ng of total RNA, after which PCRs were performed using primer sequences to decorin (sense 5'-CAATAGCATCACCGTTGTGG-3'; anti-sense 5'-CCGGACAGGGTTGCTATAAA-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense 5'-TCCCTCAAGATTGTGACAA-3'; anti-sense 5'-AGATCCACAA CGGATACATT-3'). PCR cycling parameters used were 94°C, 3 min, 35 cycles at 94°C, 1 min, 58°C, 1 min and 72°C, 5 min. RT-PCR products were examined on 2% agarose gels containing ethidium bromide and viewed under UV light, using a BioRad image analysis system (BioRad Laboratories Ltd.). Band intensities were determined by normalisation to GAPDH using the BioRad Quantity One calibration programme, to provide a descriptive analysis of

temporal trends and gross changes in the expression patterns. Experiments were performed on three separate occasions. The amplification of GAPDH was also performed for 15, 25, 35 and 45 cycles, using the same starting concentrations of 500ng total RNA, 1mM dNTPs, 25 μ g/ μ l random hexamers and 4 U/ μ l reverse transcriptase. RT-PCR products were readily seen following 35 cycles, which increased in intensity after 45 cycles, confirming that the reaction had been stopped in the exponential phase and had not reached the plateau phase (Figure 2).

Influence of bacterial supernatant on decorin synthesis

PDL cells were seeded at 10⁴ cells/cm² with or without SAG supernatant as described above. Media was collected at days 10 and 13 and dialysed against double distilled water. Protein concentrations were determined using the BCA assay according to the manufacturer's instructions and then adjusted to 20 mg/ml. Samples (20 μ l containing 400 μ g protein per well) were mixed with an equal volume of 0.2 M tris-HCl buffer, 0.06M sodium acetate, pH 8 containing 2 units/ml chondroitinase ABC (protease free, Seikagku Corporation, Tokyo) and incubated at 37°C for 1-2 h. The resultant core proteins were then mixed with an equal volume of 0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2- β -mercaptoethanol, 0.004% bromophenol blue and examined by SDS-PAGE using a Phastsystem (GE Healthcare) followed by Western blot analysis for detection of immuno-reactivity with antibodies rabbit anti-mouse decorin (LF-113) as previously described by [29].

Influence of PDL derived extracellular matrix on bacterial protein synthesis

PDL cells were cultured in DMEM culture media (as above) at 37°C, 5% CO₂, to 95% confluency. Media was then changed for media with 0.2% FBS and cultured for a further 48 h. Collected media was exhaustively dialysed against double distilled water, lyophilised and extracellular matrix (ECM) protein concentration determined using the BCA assay (Thermo, Fisher Scientific Inc, IL, USA). SAG bacteria (duplicate samples for each isolate) were inoculated into BHI broth to give OD_{550nm} = 0.2 and cultured anaerobically for 18 h. One ml of BHI was transferred to 9 ml of fresh BHI broth and cultured until mid-log phase (approximately 4 h). Bacteria were then cultured for a further 4 h, anaerobically, with or without 250 μ g/ml of protein collected from the PDL cells. This higher concentration of ECM proteins (compared with the 20 or 100 ng/ml concentrations of bacterial supernatants utilised in the above assays) was selected to reflect the higher concentration of matrix components compared to bacterial cells *in vivo* and did not affect the growth of the bacteria in culture. One-ml aliquots of bacterial suspensions were collected for subsequent analysis.

Bacterial aliquots were lyophilised and 10 μ g of each bacterial suspension was dissolved in sample buffer (0.062M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2- β -mercapthoethanol,

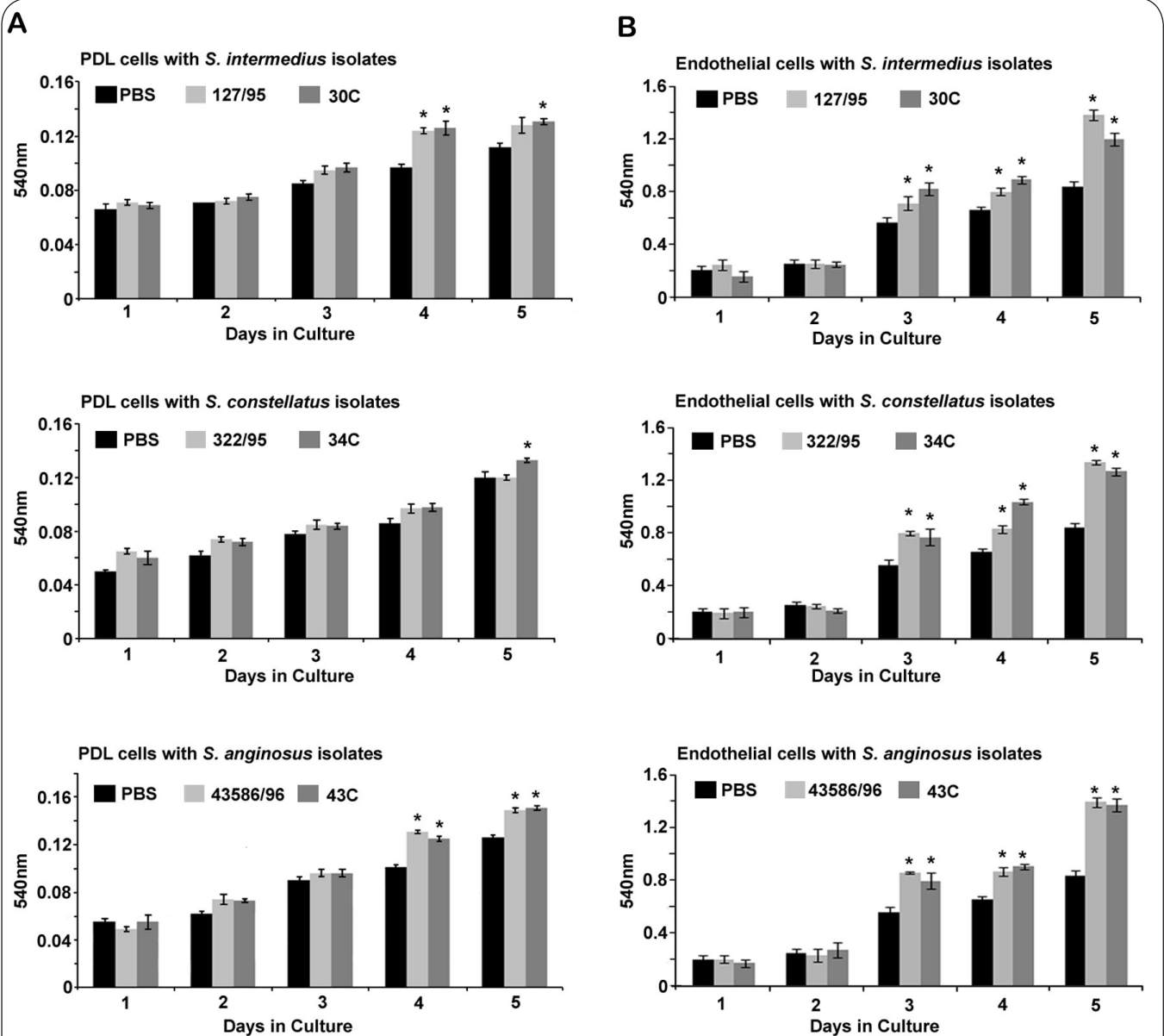


Figure 1. The influence of non-lethal concentrations of supernatant proteins derived from SAG bacterial cultures on (A) PDL and (B) endothelial cell numbers over 5 days of culture. Cells were cultured for 24 h following reseeding at 10^4 cells/cm² before supplementation of media with either 0 or 20ng/ml (PDL cells) or 0 or 100ng/ml (endothelial cells) of the SAG supernatants. Cell growth was determined using the MTT assay with cell numbers proportional to absorbance at 540nm. * p<0.05 indicates statistically significant difference when compared to the non supplemented control at the same day of culture.

0.002% bromophenol blue) and separated on 4-15% gradient SDS-PAGE gels using the Phast System (GE Healthcare) as previously described [29]. Gels were stained with 0.05% Coomassie brilliant blue, 50% methanol, 10% acetic acid for 1 h and destained with 50% methanol, 10% acetic acid.

Influence of PDL derived extracellular matrix on bacterial enzyme activity

One ml of bacterial suspension, cultured with or without 250 µg/ml ECM proteins, was diluted with distilled water to equate to a turbidity of 5-6 on the McFarland Scale and

analysed using API ZYM (bioMerieux, Marcy-l'Etoile, France) for the semi-quantitative micro-identification of enzymatic activities. Based on colour development and comparing to manufacturer's reading table, activity was graded from 0-5. Due to subjectivity, two individual readers were used.

Chondroitinase and hyaluronidase activity was measured using a spectroscopic assay monitoring the formation of unsaturated bond resulting from the digestion of chondroitin 4-sulphate and hyaluronan [30]. Duplicate 1 ml bacterial suspension samples, taken at mid-log stage were added to 4 ml BHI media containing 400 µg/ml of either hyaluronan

or chondroitin 4-sulphate (Sigma Aldrich), with or without supplementation of 250 µg/ml ECM proteins and incubated anaerobically for 7 days. Bacterial samples were centrifuged at 3000 *g*, 10 min, 20 µl of supernatant was mixed with 4 ml of distilled water and the absorbance 232_{nm} recorded. Non-inoculated broth served as a negative control for background subtraction.

Influence of PDL derived extracellular matrix on intermedilysin (ILY) production by *S. intermedius*

Total RNA was extracted from 1 ml samples of *S. intermedius* isolates using a Qiagen RNeasy Mini kit (Qiagen Ltd, West Sussex, UK) and cDNA was generated. PCR of the ILY gene was performed according to previous methods [31] using ILY forward primer (5'-AACACCTACCAAACAAAAGCAGC-3'), ILY reverse primer (5'-ACTGTGGATGAAGGGTTGTCC-3'), 1.5 mM MgCl₂ and the cycling parameters of 95°C, 5 min, followed by 30 cycles at 95°C, 1 min, 55°C for 1 min, followed by a final 72°C, 2min extension step. To quantify ILY expression, the 16S rRNA of *S. intermedius* isolates was also amplified from bacterial cDNA using the primer pair 27F (5'-GTGCTGCAGAGAGTTTGCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTACGACTT-3'). PCR was performed for 1 cycle of 94°C, 6 min, 65°C, 1 min, 72°C, 2 min followed by 11 cycles where denaturation was performed for 1 min and the annealing temperature was decreased by 2°C each cycle, after which 25 cycles were carried out under the same conditions. The final cycle, extension was performed for 12 min.

Results

Influence of SAG supernatants on PDL cell behaviour

Culture of PDL cells in the presence of supernatants derived from SAG isolates appeared to result in stimulation in cell expansion (Figure 1A). For cells exposed to supernatants derived from *S. intermedius* and *S. anginosus*, a significantly higher number of cells were observed by day 4 ($p < 0.05$) and this was consistent for isolates derived from clinically healthy and pathologically infected sites. For PDL cells exposed to supernatant derived from *S. constellatus*, cell expansion appeared to be greater compared to the non supplemented control, but this was observed to be statistically significant for cells exposed to *S. constellatus* 34C (isolate from clinically healthy site) at day 5. All isolates appeared to reduce the ability of the PDL cells to synthesise matrix components (Figure 2). Decorin mRNA expression appeared to be affected with all isolates, demonstrating a trend towards an approximate 20% to 43% reduction in levels compared to the non supplemented control (Figure 2A). These results were supported by Western blot analysis which demonstrated a very large reduction in protein levels of decorin in the media collected from PDL cells grown in the presence of SAG isolates compared to the non supplemented control (Figure 2B). Also observed was an increase in low molecular

weight decorin degradation products in the molecular weight range 20-12kDa. Immunofluorescence labelling of cell cultures for type 1 collagen demonstrated reduced levels at day 7 and a complete absence at days 10 and 13 following exposure to SAG supernatants (Figure 2C). These observations for collagen and decorin synthesis were consistent for all isolates studied.

Influence of SAG supernatants on endothelial cell behaviour

Exposure of endothelial cells to SAG supernatants stimulated cell expansion (Figure 1B), with significantly greater cell numbers observed by day 3 in supplemented cultures compared with non supplemented cultures ($p < 0.05$ for all comparison). No significant differences were observed in comparing effects of supernatants for isolates obtained from either clinically healthy or pathogenic sites ($p > 0.05$). The effect of SAG soluble components on the migration of endothelial cells was also examined by measuring radial outward migration from a pre-formed spheroid of endothelial cells (Figure 3) which was intended to mimic endothelial migration during sprout formation from pre-existing vessels [17]. By 6 h, in the absence of SAG supernatants, cells demonstrated a statistically significant higher migration ability (2- to 3-fold higher) compared to cells cultured in the presence of the SAG supernatants ($p < 0.05$). For *S. intermedius* 127/95, *S. constellatus* 322/95 and *S. anginosus* 43586/96 and 43C, the rate of migration of the cells over the subsequent 6-72 h appeared similar and cells maintained a 30-60% reduction in the overall distance of cell migration compared with migratory distances achieved by cells cultured in the absence of bacterial supernatant ($p < 0.05$ for all comparisons). Migration rates for cells in the presence of supernatant from isolates 30C (*S. intermedius*) and 34C (*S. constellatus*) appeared slightly greater compared with other isolate supernatants, but this was shown not to be significant. The distance of migration of cells in the presence of these bacterial supernatants was also significantly decreased compared with non supplemented controls.

Protein expression by SAG in the presence and absence of PDL extracellular matrix

SDS PAGE analysis of the bacterial supernatants derived from the SAG isolates revealed a series of faint bands ranging in molecular weight from 188-22kDa (Figure 4). In the presence of ECM proteins synthesised by PDL cells, a strongly staining band was apparent for all isolates, with variable molecular weight ranging between 59 and 69 kDa. In addition, for each isolate, a different profile of lower molecular weight staining protein bands (55-49kDa). For all SAG, a protein band with molecular weight of approximately 72kDa was also observed in the presence of the ECM components, which was absent in SAG with no ECM. Separation of the bacterial culture medium and the ECM components alone failed to produce any distinguishable bands, indicating that all bands observed

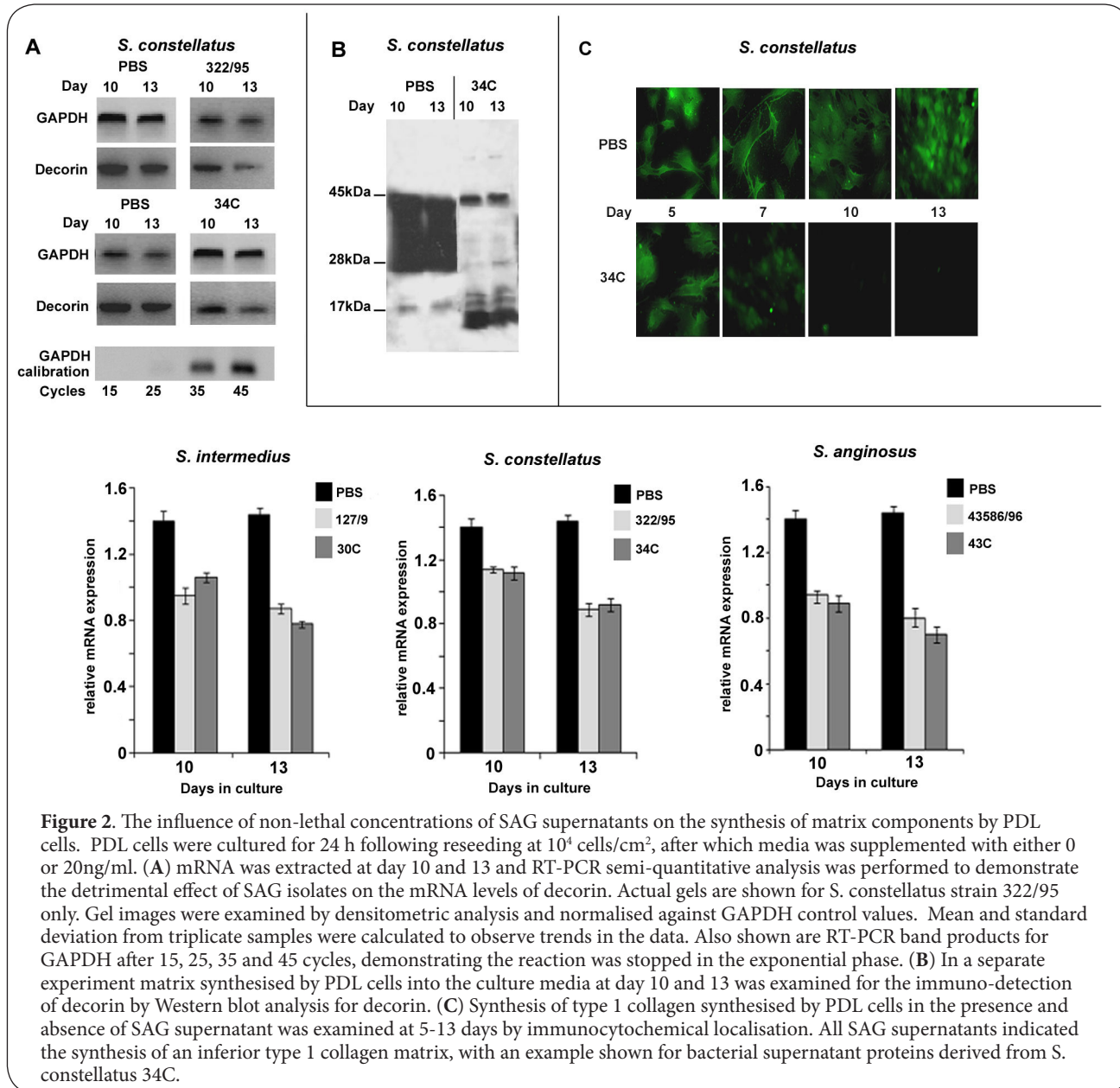


Figure 2. The influence of non-lethal concentrations of SAG supernatants on the synthesis of matrix components by PDL cells. PDL cells were cultured for 24 h following reseeding at 10^4 cells/cm², after which media was supplemented with either 0 or 20ng/ml. (A) mRNA was extracted at day 10 and 13 and RT-PCR semi-quantitative analysis was performed to demonstrate the detrimental effect of SAG isolates on the mRNA levels of decorin. Actual gels are shown for *S. constellatus* strain 322/95 only. Gel images were examined by densitometric analysis and normalised against GAPDH control values. Mean and standard deviation from triplicate samples were calculated to observe trends in the data. Also shown are RT-PCR band products for GAPDH after 15, 25, 35 and 45 cycles, demonstrating the reaction was stopped in the exponential phase. (B) In a separate experiment matrix synthesised by PDL cells into the culture media at day 10 and 13 was examined for the immuno-detection of decorin by Western blot analysis for decorin. (C) Synthesis of type 1 collagen synthesised by PDL cells in the presence and absence of SAG supernatant was examined at 5-13 days by immunocytochemical localisation. All SAG supernatants indicated the synthesis of an inferior type 1 collagen matrix, with an example shown for bacterial supernatant proteins derived from *S. constellatus* 34C.

in the presence of the SAG were bacterially derived. There was no similarity seen in the profile of protein bands on comparing isolates from clinically healthy and pathogenic sites or in comparing species within the SAG group.

Assessment of the enzyme profile produced by the SAG isolates is shown in **Table 1**. The three species within the SAG differed in their ability to produce an array of enzymes. There was negligible activity for esterase and no activity of esterase lipase, lipase or β -glucouronidase for any isolate (data not shown). Of note, the API ZYM assay demonstrated an increase in alkaline phosphatase, phosphohydrolase and α -glucuronidase, except where levels were already graded as relatively high (scoring 4 or 5). Trypsin-like protease activity

increased in the presence of ECM components for commensal isolates of *S. constellatus* and *S. intermedius*, to levels similar to those present with *S. constellatus* and *S. intermedius* isolated from pathogenic sites. Trypsin-like protease levels were low for pathogenic isolates of *S. anginosus* and absent from commensal isolates. Activity of the glycosaminoglycan depolymerising enzymes chondroitinase and hyaluronidase was detectable in *S. constellatus* and *S. intermedius* isolates and was not seen to increase in the presence of the ECM proteins.

Detected only in the *S. intermedius* isolates, mRNA expression of ILY, normalised against 16SrRNA levels, was assessed in several isolates assigned to this stain (**Figure 5**). For isolates collected from clinically healthy sites, ILY levels

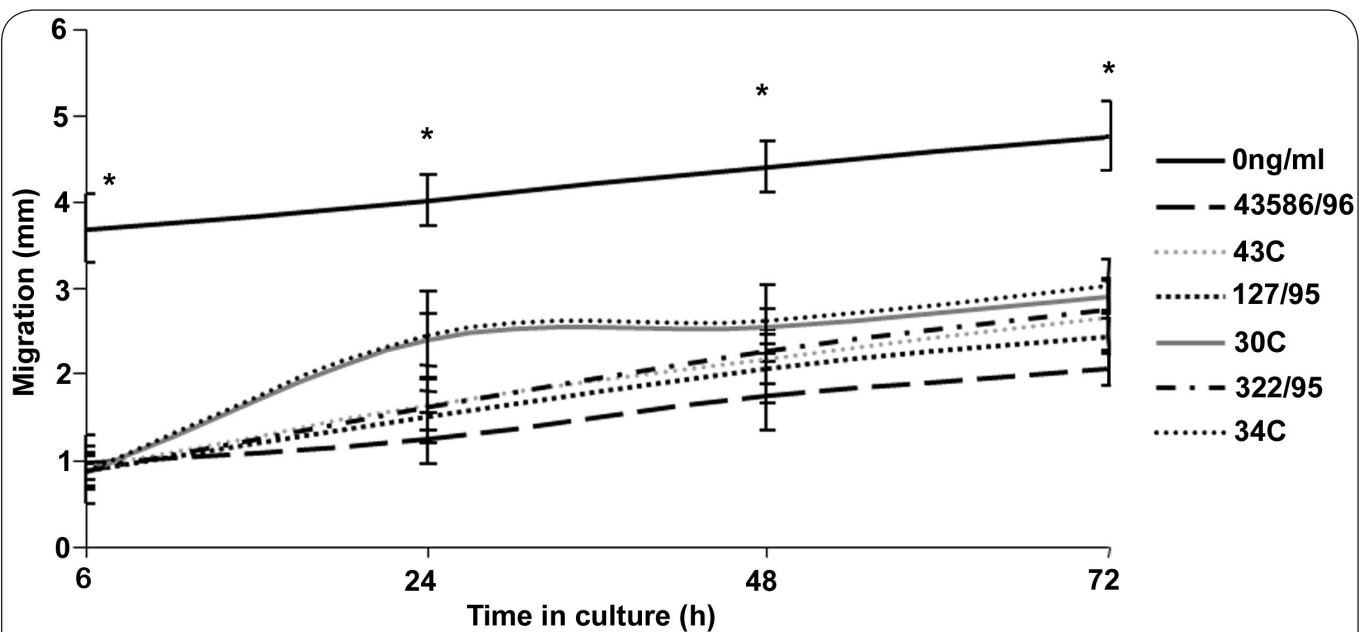


Figure 3. The influence of bacterial supernatants on the migration of endothelial cells. Spheroids containing 1000 endothelial cells were prepared and plated onto a hydrophilic plate and cultured for up to 3 days. Photographic images of the cells migrating away from the spheroid bodies were taken and the distance of migration was calculated using the ruler tool in Adobe Photoshop. * $p < 0.05$ indicates statistically significant difference in cell migration compared with the non supplemented control.

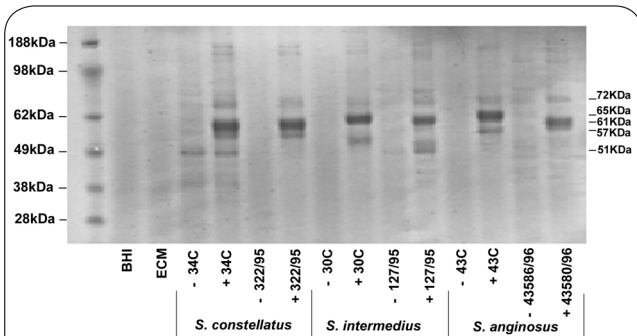


Figure 4. Molecular weight profile of proteins produced into the culture supernatant of SAG isolates in the absence and presence of ECM proteins synthesised by PDL cells. ECM was prepared as conditioned media following the culture of confluent PDL cells for 48 h. SAG were grown from the mid-log phase in broth supplemented in the ECM proteins for 4 h and the culture supernatant was examined by SDS PAGE. Also shown is a separation of the brain heart infusion broth alone (BHI) and the ECM proteins in the culture broth in the absence of bacteria (ECM).

were observed to increase in the presence of ECM proteins to levels similar to those identified in strains collected from pathogenic sites. There was no further increase in ILY for these pathogenic strains in the presence of ECM proteins.

Discussion

This study has demonstrated that protein components released from SAG isolates were all similarly capable of disrupting the proliferation of and matrix synthesis by PDL cells, and

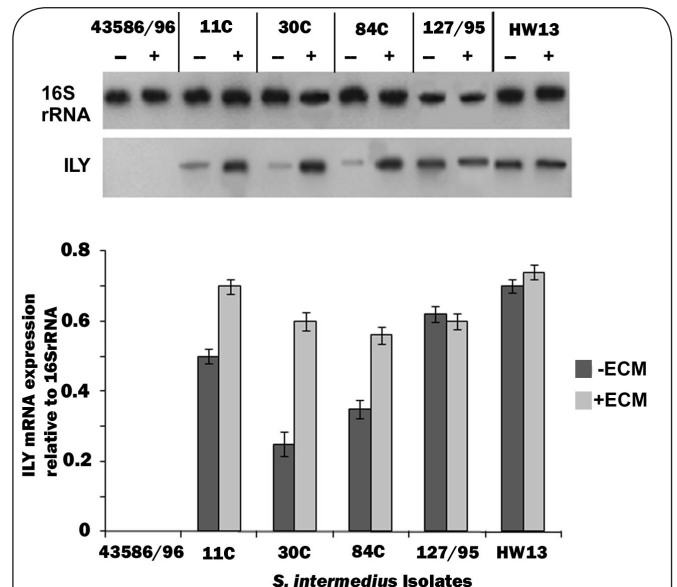


Figure 5. Analysis of intermedilysin (ILY) production by *S. intermedius* in the absence and presence of ECM proteins in conditioned media of PDL cells. Total RNA was isolated from five *S. intermedius* strains and expression of the ILY gene was quantified by normalisation of the PCR product against the PCR product for 16S rRNA. Also included was the *S. anginosus* strain 43586/96 demonstrating the specificity of ILY to *S. intermedius*.

the proliferation and migration of endothelial cells. This would have an obvious impact on tissue repair processes required in the maintenance of the structural integrity of

Table 1. Enzyme activity identified in SAG isolates in the presence and absence of extracellular matrix (ECM) proteins synthesised by PDL cells, as determined by the API ZYM and glycosaminoglycan depolymerise assays. ▲ indicates an increase in activity of greater than 2 scores, as judged by the API ZYM assay, compared to the control in the absence of ECM proteins.

		Alkaline Phosphatase	Esterase (C4)	Leucine arylamidase	Valine arylamidase	Cysteine arylamidase	Trypsin-like protease	α -chymotrypsin	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	α -galactosidase	α -glucosidase	β -glucosidase	N-acetyl- β -glucosaminidase	α -mannosidase	Chondroitinase	Hyaluronidase
ECM only		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
322/95 (<i>S. constellatus</i>)	No ECM	4	1	5	2	3	2	1	5	3	-	2	2	-	-	+	+
	+ ECM	4	1	5	2	2	2	3	5	5	-	4	3	-	-	+	+
34C (<i>S. constellatus</i>)	No ECM	5	-	5	3	2	0	3	5	2	-	2	4	-	-	+	+
	+ ECM	5	-	5	3	3	3	3	5	5	-	4	5	-	-	+	+
127/95(<i>S. intermedius</i>)	No ECM	5	-	5	3	3	2	3	5	3	-	2	2	2	2	+	+
	+ ECM	5	-	5	3	4	2	3	5	4	-	4	3	5	4	+	+
30C (<i>S. intermedius</i>)	No ECM	1	-	2	2	1	0	0	2	2	-	0	1	1	3	+	+
	+ ECM	▲	-	▲			▲	▲	▲	▲	-	▲	▲	▲	4	+	+
43586/96 (<i>S. anginosus</i>)	No ECM	3	-	5	4	4	1	2	5	2	1	1	1	-	0	-	-
	+ ECM	▲	-	5	4	4	2	4	4	4	3	4	3	-	3	-	-
43C(<i>S. anginosus</i>)	No ECM	1	-	4	-	-	-	1	1	3	-	2	-	0	-	-	-
	+ ECM	▲	-	4	-	-	-	-	2	4	1	4	-	3	-	-	-

PDL and represent the first crucial steps in the establishment of infection and tissue destruction. In addition, this study also demonstrated that extracellular components synthesised by PDL cells were capable of increasing the hydrolytic enzyme activity of SAG, thus providing the potential to exacerbate tissue damage. Significantly, the results indicate similar responses for *S. intermedius*, *S. constellatus* and *S. anginosus* isolates, irrespective of whether they were commensal isolates, collected from healthy body sites or pathogenic isolates from clinical infection.

The cell population examined in this study would be heterogeneous in nature, but would contain a high proportion of mesenchymal progenitor cells capable of proliferation and subsequent differentiation into collagen synthesising cells. The results of this study have shown that soluble components released by SAG isolates at concentrations as low as 20 ng/ml increased the active proliferation of the PDL progenitor cell population. Our results demonstrated that soluble components of SAG isolates severely disrupted collagen levels within the extracellular matrix and organisation of the collagen fibrils, with a near absence of collagen in cultures after 10 days. The latter observation may be due to the hydrolytic enzyme activity in the SAG supernatant, which was identified in this

study (API ZYM results demonstrate enzyme activity in the absence of ECM components synthesised by the PDL cells) or by SAG components stimulating matrix degradation activity by the PDL cells [14]. However, this study also demonstrated that in the presence of SAG soluble components, the mRNA expression and protein synthesis of decorin was severely reduced, which may be additionally reduced due to host degradative activity or bacterial hydrolytic enzyme activity. In considering decorin's role in collagen fibrillogenesis [12,13], the reduction in levels of a functional decorin would be expected to lead to an inferior collagen matrix, making it highly susceptible to hydrolytic enzymes released by the SAG isolates.

Decorin has also been ascribed both direct and indirect roles in regulating cell signalling and proliferation. Decorin is proposed to sequester growth factors to the matrix such as TGF β [32], Wnt-I-induced secreted protein I (WISP-I) [33], TNF α [34] and BMP2 [35], protecting it from proteolysis but at the same time regulating its release to the target cell, indirectly regulating its activity. Of note, TGF β 1 has been shown to increase proliferation and chemotaxis of PDL cells [36-38], thus the reduction in decorin available to bind TGF β may indirectly account for the increased cell growth seen

in this study. Likewise the reduction of decorin may release free TNF α propagating its pro-inflammatory cellular responses. Decorin has also been shown to directly signal to PDL cells [14] and squamous carcinoma cells [39, 40] arresting cell proliferation. Although the signal transduction pathway has not been identified in PDL cells [14], the reduced synthesis of decorin in the presence of SAG components may further account for the increased proliferation of these mesenchymal progenitor cells.

Within this study, soluble components from all SAG isolates stimulated endothelial cell expansion, however, migration of endothelial cells across a collagen matrix was reduced, suggesting that within a clinical SAG infection site angiogenesis would be impaired. Neither mRNA expression nor protein synthesis of decorin were detected for the endothelial cells in monolayer culture (results not shown). This concurs with previous data which indicate that decorin is expressed by sprouting endothelial cells contributing to angiogenesis during inflammatory conditions, but not quiescent endothelial cells [41]. Indeed, *in vitro* studies have indicated that within a collagen matrix, decorin activates the insulin-like growth factor-1 receptor and interacts with the $\alpha 2\beta 1$ integrin leading to reorganisation of the cytoskeleton and focal adhesion to promotes endothelial adhesion and motility [17]. The reduction in decorin synthesis by PDL like cells, observed in our study, could therefore also impact on angiogenesis within the periodontal tissue at sites of SAG infections. These would be additional to the direct effects of soluble components released by SAG on the endothelial cell motility.

SDS PAGE demonstrated that the profile of soluble components released by the various SAG isolates was variable, with some potential for common components, but at differing concentrations. In particular, there were large variations in the protein profiles of isolates designated commensal, derived from clinically healthy, or pathogenic, from infected sites, yet identified as the same species. All SAG isolates were able to influence the biological activity of both PDL cells and endothelial cells towards phenotypes which do not favour tissue repair. In considering the reciprocal process, the results of the present study also demonstrated that components within the ECM were able to increase the production of specific hydrolytic enzymes produced by the SAG isolates. In particular, ECM components were shown to increase naphthol-AS-B1-phosphohydrolase activity, which along with alkaline phosphatase, esterase lipase, lipase, leucine arylamidase and acid phosphatase, has been proposed as a bacterial 'housekeeping' enzyme required for survival [42]. *Streptococcus constellatus* and *S. intermedius* isolates produced enzymes which are capable of modifying the carbohydrate moieties of various proteoglycans or glycoproteins which are utilised by the bacteria as a carbon source. These included chondroitinase and hyaluronidase which were at a relatively low level and did not increase in the presence of the ECM components. Enzymes such as α -galactosidase, β -glucosidase,

N-acetyl- β -glucuronidase and α -mannose demonstrated a varied and often low expression across the SAG isolates. However, in the presence of ECM components a 2 to 4 fold rise in the levels of α -glucuronidase was seen for all isolates. For proteolytic enzymes, trypsin-like protease was observed to rise for commensal isolates of *S. constellatus* and *S. intermedius*, but levels in the *S. anginosus* isolates were relatively low. The ECM synthesised by PDL cells contains a diverse array of protein or glycoconjugates and it has not been possible within this study to identify if specific proteins are responsible for triggering the bacterial expression of the hydrolytic enzymes. This response may equally be unspecific, with bacteria responding to a changing protein or carbohydrate nutrient supply. For *S. intermedius* isolates, ECM components induced higher levels of ILY, whilst high levels of ILY were apparent in pathogenic strains before exposure to ECM components. ILY is a toxin produced only in *S. intermedius* and is proposed to interfere with phagocytosis of PMNs and macrophages and, in conjunction with cholesterol, appears to be cytotoxic to these immune cells [43]. Although produced by *S. intermedius* isolates in this study, ILY in the SAG supernatant at the sub-toxic concentrations utilised, was probably of insufficient level to be cytotoxic to either PDL cells or endothelial cells, as evidenced by an increase in cell proliferation in the presence of the SAG soluble components. However, it has previously been 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 [44].

Collectively, the results of this study would suggest that the ECM of PDL plays a prominent role in the establishment and propagation of SAG infection. Our previous studies have demonstrated that these SAG isolates favour binding to the dermatan sulphate conjugated decorin and biglycan and fibronectin, which are all predominant in soft tissues of PDL [22], lending these tissues in particular to bacterial biofilm formation. Whilst this study focussed on periodontal ligament, similar components are present in other soft connective tissues of the body and thus the ECM of these tissues could equally play a prominent role in the establishment of lung, brain and liver abscesses. Soluble components from the SAG isolates appeared to produce a deleterious effect on the biological activity of PDL cells and endothelial cells regardless of whether the SAG were designate as commensal or pathogenic bacteria, possibly suggesting that the ECM components drive the development of a pathogenic phenotype within the SAG. The ECM components have the potential to increase hydrolytic enzyme factors with a consequential influence on the biological activity of the host mesenchymal and immune cells. Whilst not leading to direct gross tissue destruction, degradation of carbohydrate moieties on glycoconjugates such a fibronectin, may lend them susceptible to proteolytic degradation, by either bacterial or mammalian enzymes. The bacterial expression of these enzymes presents the potential for altering the glycosaminoglycan chains of cell membrane

heparin sulphate proteoglycans, the structure of which appears to be important in fulfilling a signalling role [45,46]. Of further consideration, research in the literature already suggests that binding cell surface heparin sulphate proteoglycans with various microbial surface cell recognition adhesion matrix molecules (MSCRAMMs) released from the bacterial surface may stimulate intracellular signalling, influencing host cell survival, promotion of antimicrobial peptide synthesis and the initiation of the inflammatory response [26,47-49]. It is therefore apparent that whilst this study has elucidated a novel mechanism by which SAG change cellular behaviour via changes in the expression of decorin, this only represents part of the pathological mechanism, which remains on the whole unclear, probably because it is multifactorial in nature.

Competing interests

The Authors declare that they have no competing interests.

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