

## Cell Surface Proteoglycan Expression by Human Periodontal Cells

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Cell surface proteoglycans are known to be involved in many functions including interactions with components of the extracellular microenvironment and serve to influence cell shape, adhesion, proliferation, and differentiation. They also can act as co-receptors, to help bind and modify the action of various growth factors and cytokines. Despite their strategic location and relevance to cell function, few studies have considered the nature of the cell surface proteoglycans associated with cells of the periodontium. Due to the structural complexity and multiplicity of cell types in the periodontium, we have selected three different cell lines (gingival connective tissue fibroblast, periodontal ligament fibroblast, and osteoblast) which each represent the unique functions within the periodontium to study the expression of cell surface proteoglycans. We hypothesized that a number of cell surface proteoglycans will be expressed by human periodontal cells and these may be related to the source and function of the cell. Western blotting and RT-PCR methods were used to study the expression of five cell surface proteoglycans (syndecan-1, -2, -4, glypican and betaglycan) in three cell lines of human periodontal cells *in vitro*. Our results demonstrated the expression of protein cores for syndecan-1 (43 kDa), syndecan-2 (48 kDa), syndecan-4 (35 kDa), glypican (64 kDa), and betaglycan (100–110 kDa). RT-PCR results confirmed that all of these cells produced mRNA for the cell surface proteoglycans under study, of which syndecan-2 showed a significant difference in expression between the periodontal ligament fibroblasts, gingival fibroblasts and osteoblasts. We conclude that the presence of specific cell surface proteoglycans on periodontal cells implies a likely role for these molecules in cell–cell, cell–matrix interactions involved in periodontal disease and/or regeneration of the periodontium, of which they may have distinctive functions related to the source and function of these cells.

**Keywords:** Periodontal, syndecan-1, syndecan-2, syndecan-4, betaglycan, glypican

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## INTRODUCTION

Cell surface proteoglycans are emerging as important molecules that mediate cell interactions with components of the extracellular microenvironment and serve to control cell shape, adhesion, proliferation, and differentiation.<sup>[1–4]</sup> With regard to their domain structure, cell surface proteoglycans are divided into two major groups. The first includes the syndecans (syndecan 1–4) and betaglycan, all of which are transmembrane proteins with an amino-terminal extracellular domain containing clustered sites for the potential attachment of GAG chains, a potential protease cleavage site near the single transmembrane domain, and a short cytoplasmic tail.<sup>[1,5–8]</sup> The other type of cell surface proteoglycan, glypican, is linked to the cell surface via a glycosyl phosphatidylinositol anchor.<sup>[9,10]</sup>

The periodontium is a dynamic supporting system that links teeth to the surrounding bone. It is composed of a number of discrete connective tissues (gingiva, periodontal ligament (PDL), cementum and alveolar bone), each with a unique architecture, composition and function. Destruction of these tissues during inflammatory periodontal disease involves loss of fibrous attachment, destruction of connective tissue matrix and resorption of alveolar bone and tooth roots, can lead to increased mobility of the tooth and may ultimately lead to tooth loss. Thus, the goal of periodontal therapy is to achieve regeneration of a healthy connective tissue new attachment, with PDL fibroblasts being suggested to play the most crucial role in these processes. The application of growth factors and chemotactic factors to PDL cells has suggested that the use of these factors for periodontal regeneration may be useful in periodontal therapy. In addition, PDL fibroblasts are a unique highly specialized connective tissue cell type with various characteristics important in periodontal regeneration, and different from gingival connective tissue fibroblasts (GF) and fibroblasts from other connective tissues.<sup>[11–14]</sup> The regeneration of damaged periodontal tissues involves a large array of cell types and several interdependent biological processes.

It has been proposed that the outcome of healing is determined to a large extent by the phenotypes of the cells that repopulate the healing site. However, phenotypic expression is also dependent on environmental factors such as matrix ligands that are known to regulate not only the intrinsic cellular program but also to modulate incoming signals.<sup>[15]</sup>

Since cell surface proteoglycans are well recognized for their ability to interact with a range of matrix proteins and growth factors, both as binding proteins and signalling mediators, we have hypothesized that a number of cell surface proteoglycans will be expressed by human periodontal cells and these may be related to the source and function of the cells. Indeed, despite their strategic location and relevance to cell function, to date, few studies have considered the nature of the cell surface proteoglycans associated with cells of the periodontium. Due to the structural complexity and multiplicity of cell types in the periodontium, we have selected three different cell lines (GF, PDL and osteoblast (OB)) which each represent the unique functions within the periodontium to study the expression of cell surface proteoglycans. The aim of the present study was to investigate the expression of five cell surface proteoglycans (syndecan-1, -2, -4, glypican and betaglycan) expressed by human periodontal cells and determine whether this may be related to the source and function of the cells.

## MATERIALS AND METHODS

### Cell Culture and Labelling

Normal human periodontal fibroblasts (GF, PDL and OB) were obtained from explant cultures of human gingiva, PDL and alveolar bone using methods described previously.<sup>[16,17]</sup> The cells were maintained in culture in 75 cm<sup>2</sup> flasks containing Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat inactivated fetal-calf serum, 1% penicillin/streptomycin and 1% non-essential amino acids. Subconfluent (80–90%)

monolayers between passages 5–10 were incubated for 48 h with medium containing 30  $\mu\text{Ci}$  of carrier-free  $^{35}\text{SO}_4/\text{ml}$  (Amersham, Life Science, England) for these studies.

### Proteoglycan Extraction and Purification

After labelling for 48 h, confluent fibroblast monolayer cells were rinsed twice with 10 ml of cold PBS and scraped with a rubber policeman in cold extraction buffer containing 0.5% (v/v) Triton X-100, 1 mM EDTA, 2.5 mM EGTA, 10 mM HEPES, 50 mM NaCl, 0.5 M sucrose, 0.05% (v/v) mercaptoethanol, 60 mM spermidine and a cocktail of protease inhibitors including, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1% aprotinin, 0.1% pepstatin A, and 0.05% leupeptin. The suspension was rapidly cooled to 4°C and was cleared by centrifugation (10,000  $\times g$ , 4°C for 30 min). The extract was further purified by adsorption onto a 15 ml DEAE-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 7 M urea, 0.1 M NaCl and 50 mM Tris-HCl (pH 7). Bound materials were eluted using a linear NaCl gradient (from 0.1 M NaCl) in urea buffer, at a flow rate of 1 ml/min at room temperature. Aliquots (100  $\mu\text{l}$ ) from each fraction were added to liquid scintillation cocktail (Beckman, USA) and assessed for  $^{35}\text{SO}_4$  activity in a scintillation counter (Beckman LS 6000 SC, USA). The fractions representing the peaks of  $^{35}\text{SO}_4$  peaks were pooled, dialyzed against deionized water and concentrated by using Ultrafree<sup>®</sup> Centrifugal Filter (10 kDa molecular weight cutoff, Millipore, USA using 2000  $\times g$  centrifugation). The  $^{35}\text{SO}_4$ -labelled molecules recovered at this stage represented more than 95% of the extracted radiolabelled material. The concentrated (500  $\mu\text{l}$ ) purified extract was then lyophilized.

### Enzymatic Deglycosylation

After solubilization in 50 mM HEPES pH 7, 100 mM NaCl, 1 mM  $\text{CaCl}_2$ , 0.1% Triton X-100, 50 mM 6-aminohexanoic acid, 1 mM PMSF, 0.1%

aprotinin, 0.1% pepstatin A, 0.05% leupeptin, the samples were digested with 1 mU/ml heparitinase (Seikagaku Kogyo Ltd, Japan) and/or 100 mU/ml chondroitinase ABC (Seikagaku Kogyo Ltd, Japan) overnight at 37°C. Controls consisted of undigested samples incubated in the same buffer without enzyme.

### SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

SDS-PAGE was carried out using a 7.5% or 10% resolving polyacrylamide gel<sup>[18]</sup> with a BioRad mini-Protean apparatus. Samples were boiled for 6 min either with or without 10% (v/v) 2-mercaptoethanol. After electrophoresis for 1.5 h at 100 V, the gels were electroblotted for 4 h at 60 V onto nitrocellulose membrane.<sup>[19]</sup> After electroblotting, membranes were stained with Ponceau S to ensure that each lane received equal amounts of protein. Each lane received cell extract obtained from half of a 75 cm<sup>2</sup> flask, however the quantity of  $^{35}\text{SO}_4$  present in each of these lanes differed slightly due to the different fraction of proteoglycans present in the three cell lines. Membranes were then subjected to Western blotting. The membranes were blocked in 5% non-fat dried milk in TBST (TBST; Tris-buffered saline with 0.1% Tween-20) for 1 h, then rinsed twice and washed with TBST. The membranes were then incubated with primary antibody diluted in 5% non-fat dried milk/TBST. Antibodies used were as follows: syndecan-1 (0.5  $\mu\text{g}/\text{ml}$  rat monoclonal anti-mouse clone 281-2, Seikagaku, Japan), mouse monoclonal antibodies 10H4 for syndecan-2, Ab8G3 for syndecan-4, and AbS1 for glypican were kindly provided by Dr G. David (Center for Human Genetics, University of Leuven, Belgium) and were used at a concentration of 1  $\mu\text{g}/\text{ml}$ . A polyclonal rabbit anti-rat betaglycan (Upstate Biotech Inc., USA) was used at a concentration of 1.2  $\mu\text{g}/\text{ml}$ . All incubations were performed at 4°C overnight. Membranes were then rinsed twice and washed as described above. Secondary antibodies were prepared in 5% non-fat milk/TBST as follows: 1:18,000 for syndecan-1

(sheep anti-rat, HRP-linked F(ab') fragment, Amersham Life Science, England), 1:15,000 for syndecan-2, -4 and glypican (sheep anti-mouse, HRP-linked F(ab') fragment, Amersham, Life Science, England), 1:20,000 for betaglycan (swine anti-rabbit Ig -HRP, Dako, USA). Membranes were incubated in secondary antibody for 1 h at room temperature followed by rinsing and washing as described above. Chemiluminescence (ECL, Amersham, Life Science, England) was used to detect the signals by exposing the membranes on Fuji Medical X-RAY films.

### Immunoprecipitation

Immunoprecipitation was performed to confirm the syndecan-1 results obtained using Western blotting. A monoclonal antibody for syndecan-1 (12.5 µg/ml) was incubated with Protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) in PBS containing 0.5% Triton X-100 (pH 8) for 4 h at room temperature. After washing with TBS, immunoprecipitation was performed by incubating the Protein G-Sepharose with cell extract at 4°C overnight. After washing and centrifugation, the pellet was suspended in Laemmli loading buffer and boiled for 6 min prior to electrophoresis using SDS-PAGE.<sup>[18]</sup> The proteins were then transferred onto nitrocellulose membrane as described above and examined by Western blotting.

### RT-PCR

Total RNA was isolated from confluent human periodontal fibroblasts using RNA Isolation reagent (Advanced Biotechnology, USA). The RNA concentration of all samples was determined spectrophotometrically and RNA quality was checked by agarose gel electrophoresis.

First strand cDNA was synthesized from 1 µg of total RNA using oligo-dT as primer and M-MLV reverse transcriptase (Promega, USA) in a reaction volume of 20 µl. PCR conditions for amplification of the different members of the syndecan family, glypican and betaglycan, were optimized using the PTC-100™ Programmable Thermal Controller (MJ Research Inc., USA). Separate PCR reactions of 2 µl of synthesized cDNA (except 3 µl for syndecan-2) in PCR buffer comprising 1.5 mM MgCl<sub>2</sub>, 400 nM mixed dNTP and 10 pmol of gene specific primers were carried out using 0.5 U of Red Hot Taq polymerase (Advanced Biotechnologies Ltd., UK) in a final volume of 20 µl. All primer pairs were from previously published PCR primers except for syndecan-4 which was designed using published cDNA sequence and confirmed by PCR product sequencing. The sequences for all the primers and the number of cycles performed are shown in Table I, the number of cycles being selected for the region where increase in product is linear with cycle number.

TABLE I Primer sequence and number of cycles used for PCR reactions

	Primers	bp	Cycles	References
$\alpha_2$ -Micro Globulin	<i>For</i> CCC CCA CTG AAA AAG ATG AG <i>Rev</i> TCA CTC AAT CCA AAT GCG GC	102	25	[37]
Syndecan-1	<i>For</i> AGC CAA GCT TAC CTT CAC AC <i>Rev</i> TAG AAT TCC TCC TGT TTG GTG G	416	25	[38]
Syndecan-2	<i>For</i> GGG AGC TGA TGA GGA TGT AG <i>Rev</i> CAC TGG ATG GTT TGC GTT CT	394	35	[39]
Syndecan-4	<i>For</i> ACC AGA CGA TGA GGA TGT AG <i>Rev</i> CTT CCT GAT CCT ACT GCT CA	380	25	See Methods
Glypican	<i>For</i> ATC ACC GAC AAG TTC TGG GGT A <i>Rev</i> CAT CTT CTC ACT GCA CAG TGT C	317	25	[39]
Betaglycan	<i>For</i> GAC AGT AGT GGT TGG CCA GA <i>Rev</i> AGC TGA CGC TGT GTA CGA AG	542	30	[40]

*For* = 5'–3' forward primer; *Rev* = 3'–5' reverse primer.

For PCR amplification, denaturation was carried out at 94°C for 1 min, annealing at 57°C (except syndecan-2 which required 49°C) for 30 s and extension at 72°C for 80 s. The PCR products were analyzed on a 2% (w/v) agarose gel and visualized by ethidium bromide staining. The relative intensity of each band was determined using the NIH Image analysis program (National Institutes of Health, USA). PCR for the housekeeping gene  $\beta_2$ -microglobulin ( $\beta_2$ M) was used as a control for equal loading of RNA into the RT-PCR reaction tubes. Obtaining bands of consistent intensity for  $\beta_2$ M allowed comparison of the amount of PCR products produced between different samples.

## RESULTS

### Isolation and Purification of Proteoglycans from Human Periodontal Fibroblasts

During the 48 h of metabolic labelling of the cells, the incorporation of  $^{35}\text{SO}_4$  into cell layer associated proteoglycan occurred rapidly during the first 8 h and continued to increase up to 30 h before a relatively steady state was reached by 48 h (results not shown). Proteoglycans were extracted using a Triton X-100 extraction buffer containing protease inhibitors. Following ion-exchange chromatography on DEAE-Sephacel, a single peak of  $^{35}\text{SO}_4$ -labelled material was eluted from the column at 0.6–0.65 M NaCl (Fig. 1).

### Expression of Syndecan-1, -2, -4, Glypican and Betaglycan Core Proteins by Human Periodontal Cells

To characterize the core protein moiety of the cell surface proteoglycans, samples obtained following ion-exchange chromatography were treated with chondroitinase ABC and/or heparitinase. Undigested (control) and treated samples were analyzed by SDS-PAGE and cell extracted proteoglycans were detected using specific antibodies against syndecan-1, -2, -4, glypican and betaglycan. All of

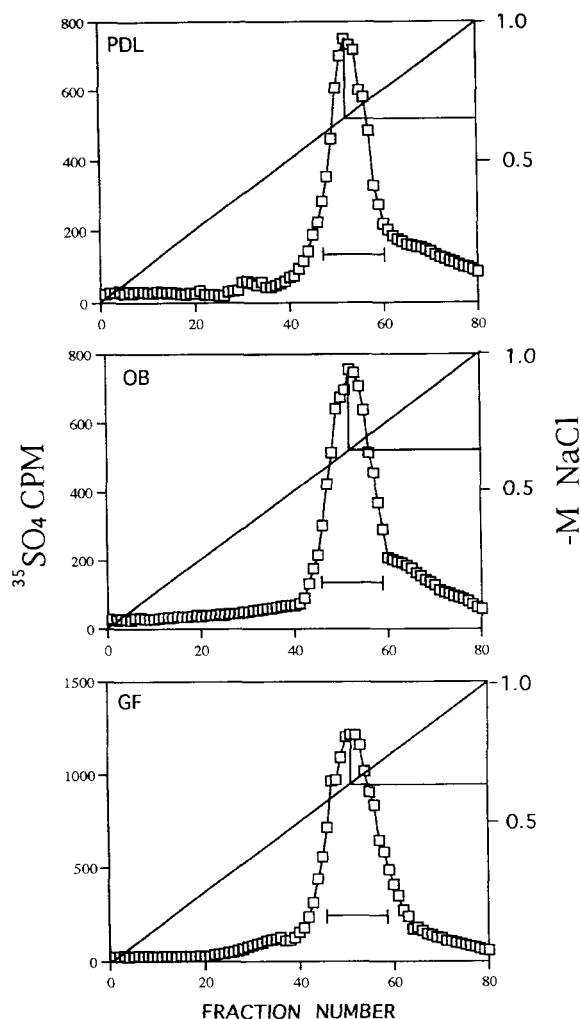
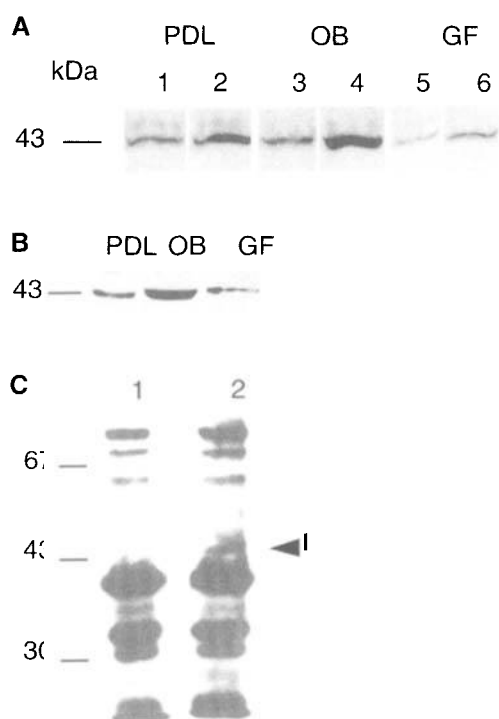


FIGURE 1 Purification of the cell surface proteoglycans by ion-exchange chromatography on DEAE-Sephacel. Bound material was eluted with a linear NaCl gradient from 0.1–1 M NaCl in urea buffer using a flow rate of 1 ml/min. After ion-exchange chromatography, the fractions containing cell extracted proteoglycans were pooled as indicated by the 15 ml bar (—): PDL = periodontal ligament fibroblasts; OB = osteoblasts; GF = gingival connective tissue fibroblasts.

these antibodies react with their respective core proteins and recognize both the glycanated and non-glycanated (precursor, processed or degraded) forms of the proteoglycans.<sup>[20]</sup> The results of such Western blots (Figs. 2–5) show specific bands at 43 kDa for syndecan-1, 48 kDa for syndecan-2, 35 kDa for syndecan-4, 64 kDa for glypican and



**FIGURE 2** Western blot analysis of the core proteins from human periodontal fibroblasts. Cell extracts from human periodontal fibroblasts were digested with chondroitinase ABC and/or heparitinase and electrophoresed in 7.5% or 10% polyacrylamide SDS-PAGE, transferred onto nitrocellulose membranes and incubated with specific antibodies. Panel A shows a 43 kDa syndecan-1 band obtained under reducing conditions (samples in lanes 1, 3 and 5 are undigested, while samples in lanes 2, 4 and 6 are digested with both enzymes). Panel B shows the same pattern of samples under non-reducing conditions and digestion with both enzymes. Panel C shows a 43 kDa band (lane 2) obtained using immunoprecipitation. The control lane (lane 1) contained only protein G and primary antibody but no cell extract. PDL = periodontal ligament fibroblasts; OB = osteoblasts; GF = gingival connective tissue fibroblasts.

100–110 kDa, for betaglycan. Syndecan-1 under reduced conditions and in control samples (primary antibody omitted, both undigested and digested with chondroitinase ABC and heparitinase) gave non-specific bands at high molecular weight (not shown). After treatment with 0.3% or 3%  $H_2O_2$ , the intensity of both the non-specific and specific bands was reduced in intensity. Furthermore enzyme digestion did not result in a change in the molecular weight of the detected bands, however the staining

intensity for each of the molecules under study was increased (Fig. 2A, lanes 2, 4, and 6). The molecular weight and staining intensity of syndecan-1 under non-reduced conditions was similar to that found in the reduced conditions (Fig. 2B, lanes 1, 2, and 3 in comparison with Fig. 2A, lanes 2, 4, and 6 respectively). Furthermore, immunoprecipitation demonstrated the presence of a 43 kDa syndecan-1 core protein (Fig. 2C, lane 2). Syndecan-2 core protein (Fig. 3) was detected as a protein band around 48 kDa in all cell extracts which did not change in molecular size following enzymatic digestion. A minor band was also noted at around 50 kDa for all samples. The syndecan-2 signal in OB and GF samples was very weak despite the amount of protein used for the detection being double that used for the other proteoglycans. Syndecan-4 and glypican showed 35 and 64 kDa bands respectively (Fig. 4) under reduced conditions. Control sample (primary antibody omitted, lane 1: undigested, lane 2: digested with chondroitinase ABC and heparitinase) showed two non-specific band doublets from secondary antibody around 50–60 kDa. Similar to syndecan-1, bands of similar molecular weight for syndecan-4 and glypican appeared in both undigested and digested samples (Fig. 4, lanes 3 and 4 for glypican and 5 and 6 for syndecan-4.) Betaglycan showed double bands around 100–110 kDa in all three cell lines, (Fig. 5A and B), however the intensity was lower in undigested samples (Fig. 5A, lane 3) compared to samples digested with both enzymes (Fig. 5A, lane 6, data shown only for OB). We also analyzed the culture medium for the presence of the proteoglycans and found at least two, syndecan-4 and betaglycan, were present. The molecular weight of syndecan-4 and betaglycan in the culture medium was slightly less than in the cell extracts, and also less intense (results not shown).

#### RT-PCR Analyses of Five Cell Surface Proteoglycan mRNAs

To complement the results of the Western blot analysis, RT-PCR was performed to determine the presence of mRNA for each of the proteoglycans

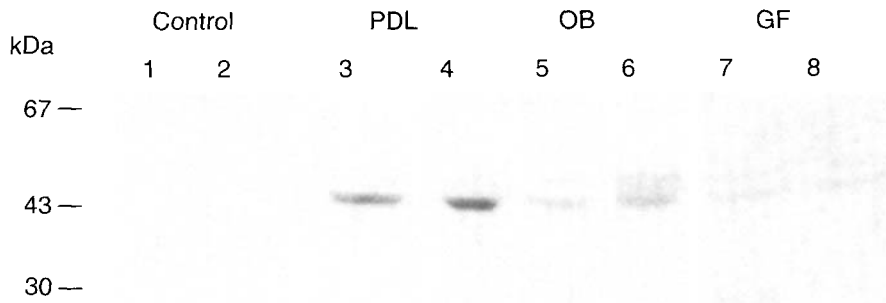


FIGURE 3 Syndecan-2 in PDL, OB and GF cell extracts. Samples in lanes 1, 3, 5 and 7 are undigested, while samples in lanes 2, 4, 6 and 8 have been digested with both chondroitinase ABC and heparitinase. Control samples (lanes 1 and 2) were not incubated with the primary antibody. A major protein band of around 48 kDa was present in the PDL samples (lanes 3 and 4) while staining for the OB and GF proteins was weaker (lanes 5–8). Proteins were detected as described in Fig. 2. PDL = periodontal ligament fibroblasts; OB = osteoblasts; GF = gingival connective tissue fibroblasts.

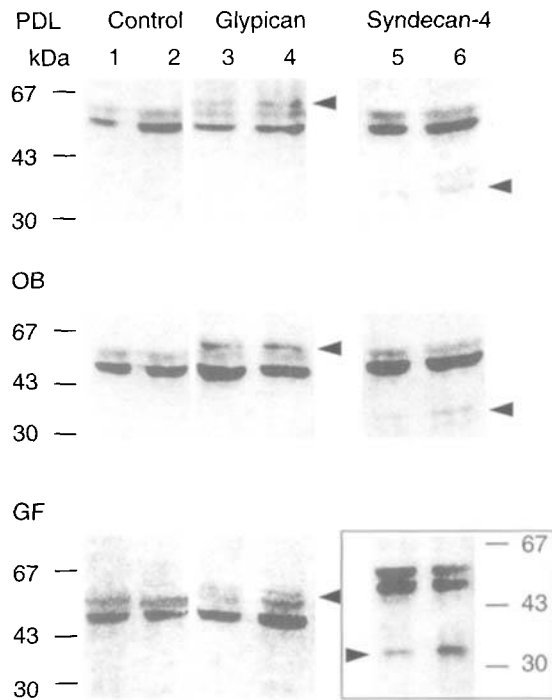


FIGURE 4 Syndecan-4 (35 kDa) and glypican (64 kDa) in the three cell lines. Lanes 1, 3, and 5 are undigested cell extracts while samples in lanes 2, 4 and 6 have been digested with both enzymes. Control samples (lanes 1 and 2) were not incubated with primary antibody. Proteins were analysed as described in Fig. 2. PDL = periodontal ligament fibroblasts; OB = osteoblasts; GF = gingival connective tissue fibroblasts.

each cell line was used to obtain three different preparations of RNA and three RT-PCR were performed for each RNA sample. The relative intensity of each PCR product was determined using the NIH Image analysis program and quantitative analyses between the three cell lines were carried out using statistical one way ANOVA (Fig. 6B). No statistically significant differences in the mRNA levels between the three cells lines were noted except for syndecan-2 which mRNA levels of PDL fibroblasts and OB had significant differences with GF (Fig. 6B,  $p < 0.05$ ).

DISCUSSION

To answer the question of whether the proteoglycans associated with the cell surfaces of human periodontal cells are related to known cell surface proteoglycans, Western blot analyses using a library of antibodies to five well characterized cell surface proteoglycans and RT-PCR were carried out. Our results on cultured human periodontal connective tissue cells demonstrate that they are able to synthesize, if not all, at least three members of the syndecan family (syndecan-1, -2, -4), as well as glypican, and betaglycan. To our knowledge this is the first report considering the synthesis of these five proteoglycans, in concert, by individual cell lines.

under investigation using specific primer pairs. All of the proteoglycans were detected in human periodontal fibroblasts using RT-PCR (Fig. 6A). Because RT-PCR is a very sensitive technique,

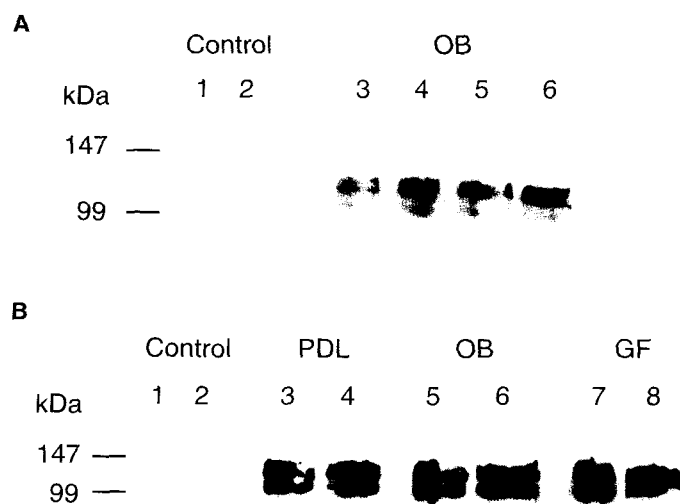


FIGURE 5 Panel A shows the double bands of betaglycan in osteoblast cell extracts. Samples in lanes 1 and 3 are undigested. Samples in lanes 2 and 6 are digested with both enzymes. Samples in lanes 4 and 5 are digested with either chondroitinase ABC or heparitinase respectively. Panel B shows undigested samples in lanes 1, 3, 5, and 7. All other lanes are digested with both enzymes. Control samples (lanes 1 and 2) were not incubated with primary antibody. Proteins were analysed as described in Fig. 2. PDL = periodontal ligament fibroblasts; OB = osteoblasts; GF = gingival connective tissue fibroblasts.

Syndecan-1 and -2 show a remarkable sequence similarity in their transmembrane and cytoplasmic domains of 52% and 66% respectively. Syndecan-1, previously shown to have a 53 kDa core protein in mouse mammary epithelial cells<sup>[21]</sup> and 82–88 kDa core proteins in human mammary epithelial and fibroblast cells,<sup>[22]</sup> appeared as a specific band around 43 kDa in our study both under reducing and non-reducing conditions. These data confirm previous statements that structural heterogeneity of syndecan varies according to the tissue of origin, and this variability seems to affect the potential functions of this proteoglycan.<sup>[8,23]</sup> Although syndecan-2, which has a 48/90 kDa core protein in human lung fibroblasts, has been reported to be characteristic for fibroblastic cells,<sup>[22,24]</sup> we found considerable variability in the expression of this proteoglycan in the various periodontal cell lines studied in this investigation. At the protein expression level, the PDL fibroblasts showed a strong expression of this proteoglycan. Gingival fibroblasts appeared to express only minor amounts of syndecan-2 core protein and OBs

had a moderate expression level which was not as great as the PDL fibroblasts. Consistent with this observation was the statistically significant stronger RT-PCR band obtained from PDL cells compared to the GF. In addition, a slightly modified PCR protocol was needed to identify syndecan-2 in our cells (more cDNA template and higher number of cycles). These findings suggest that there may be some variability in transcriptional and post-transcriptional regulation of syndecan-2 by human periodontal fibroblasts. The functional significance of these findings remains to be established.

Syndecan-4 and glypican, which have been reported in human lung fibroblasts to have 35 and 64 kDa core proteins<sup>[10,25]</sup> appeared at the expected size locations in our reduced samples. Identification of betaglycan by Western blot analysis demonstrated double bands around 100–110 kDa, close to those present in another cell lines. The OB appeared to express betaglycan more strongly than the other two periodontal cell lines both at the protein and mRNA levels. Such a ubiquitous distribution of betaglycan would be



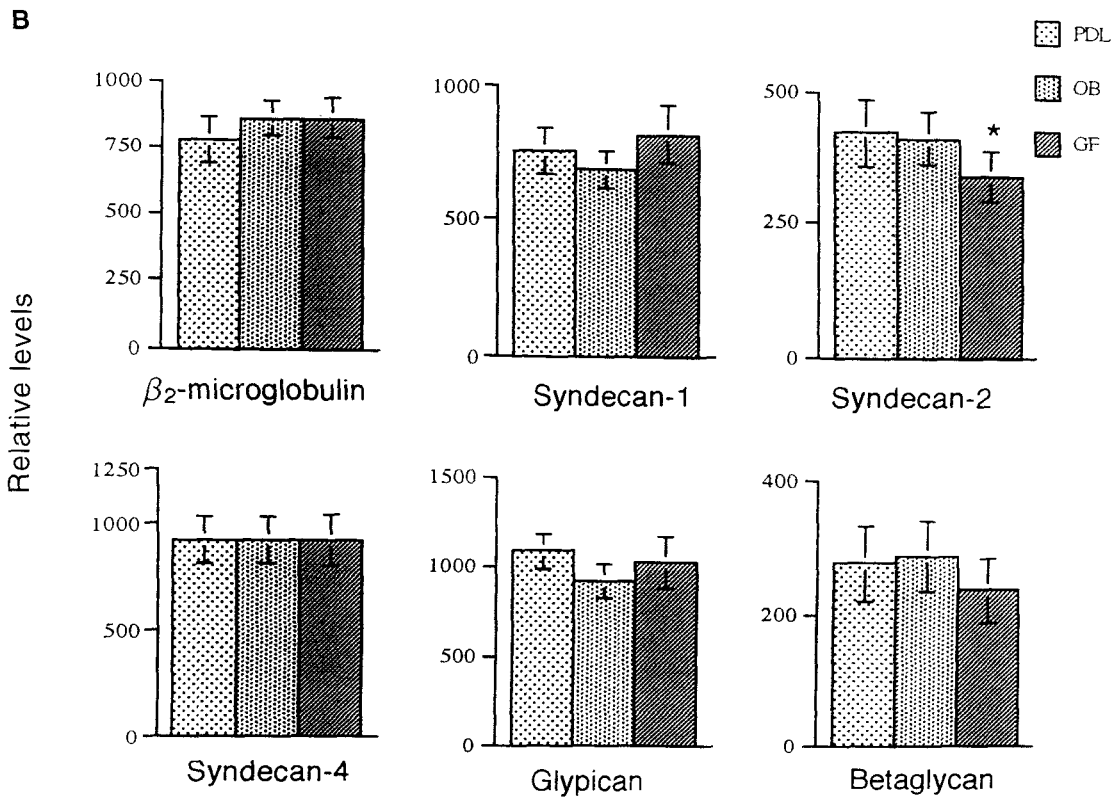


FIGURE 6B

FIGURE 6 Panel A: Expression of five cell surface proteoglycan mRNAs by human periodontal fibroblasts. RT-PCR analyses were performed using specific prime pairs for  $\beta_2$ M, syndecan-1, -2, -4, glypican and betaglycan. All PCR products were detected in these cell lines and all had the expected size and sequence as predicted from known cDNA sequences. Panel B: NIH Image analysis of each PCR product band intensity was normalized to the housekeeping gene  $\beta_2$ M mRNA levels. \* =  $p < 0.05$ . PDL = periodontal ligament fibroblasts; OB = osteoblasts; GF = gingival connective tissue fibroblasts.

heparitinase, or a related polypeptide. Such a finding is not inconsistent with the recognized "shedding" of these proteoglycans from cell surfaces and the need for glycosylation to occur only when the cells are in a functional relationship with their matrix. For example, syndecan can be lost from the cell surface by endocytosis, or by shedding of the extracellular domain, presumably by cleavage at the protease susceptible site near the plasma membrane.<sup>[32,33]</sup> These findings imply that some proteinase activity in our cell cultures may have been the cause of shedding of syndecan and, perhaps, of other proteoglycans as well. However, since all extractions were carried out in a well characterized

cocktail of protease inhibitors it is unlikely that proteolytic cleavage of the proteoglycans occurred during, or subsequent to, extraction. Furthermore, parallel preparation, under identical conditions, with other proteoglycans such as decorin and biglycan did not show any evidence of degradation (results not shown). The small amount, and uniform distribution of, syndecan-1 on cultured fibroblasts suggests that matrix interactions in these cells do not lead to its stabilization at the cell surface. Although fibroblasts do shed the extracellular domain of syndecan-1, some cycling of this proteoglycan between the cell surface and intracellular vesicles is likely.<sup>[1]</sup> For glypican, a rapid release from the cell

membrane into the pericellular matrix has also been shown<sup>[10]</sup> as well as a proteolytic cleavage of betaglycan by plasmin.<sup>[34]</sup> In addition to the results obtained for the cell surface proteoglycan extracts, additional evidence for cell surface shedding of these proteoglycans was obtained by the identification of at least two cell surface proteoglycans, syndecan-4 and betaglycan in the culture medium. Both were less intensely stained and slightly smaller in size than their counterparts retained with the cells and implies that further enzymatic degradation could have occurred once released into the culture medium.

Analysis of three cell lines derived from human periodontal connective tissues (GF, PDL, and OB) has revealed that these cells are able to synthesize at least five different cell surface proteoglycans. These cells are of cranial neural crest origin and undergo progressive specialization to eventually form tooth supporting tissues and have terminally different morphologies, tissue distribution and functions. Therefore we hypothesize that some differences may exist in terms of cell surface proteoglycan expression between these three cell lines. Of the five cell surface proteoglycans studied, only syndecan-2 showed any significant differences in expression. In general, the expression of syndecan-2 by the PDL fibroblasts and osteoblasts was higher than that found in the GF fibroblasts. This finding would seem to be in line with many of the characteristic differences between GF and PDL cells. For example, in terms of morphology, DNA content, proliferation, glycosaminoglycan synthesis, as well as cAMP production and alkaline phosphatase activity,<sup>[11,12]</sup> PDL cells have phenotypes more typical of OB than GF.<sup>[35]</sup> Since syndecan-2 may play a role in osteogenesis,<sup>[36]</sup> we propose that the presence of syndecan-2 on PDL and OB cells suggests a role for this molecule in the mineralization processes of hard connective tissues of the periodontium. This notion is further supported by our recent observation that syndecan-2 is specifically located within the cementum and alveolar bone matrices of the periodontium (manuscript submitted).

In conclusion, cell surface proteoglycans have been thought to play a role in cell-cell and cell-matrix interactions and may be involved in several different functions, such as binding to various growth factors as co-receptors, or as reservoirs of growth factors which enhance growth and matrix differentiation. Thus, given the important roles that these cell surface proteoglycans may be involved in the homeostasis in health, disease and repair or regeneration of the periodontal tissues. The clarification of specific roles for cell surface proteoglycans on periodontal cells requires further investigation.

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#### References

- [1] Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M.T., Spring, J., Gallo, R.L. and Lose, E.J. (1992). Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Ann. Rev. Cell Biol.*, **8**, 365-393.
- [2] Gallagher, J.T. (1989). The extended family of proteoglycans: social residents of the pericellular zone. *Curr. Opin. Cell Biol.*, **1**, 1201-1218.
- [3] Ruoslahti, E. and Yamaguchi, Y. (1991). Proteoglycans as modulators of growth factor activities. *Cell*, **64**, 867-869.
- [4] Woods, A. and Couchman, J.R. (1998). Syndecans: synergistic activators of cell adhesion. *Trends Cell Biol.*, **8**, 189-192.
- [5] Marynen, P., Zhang, J., Cassiman, J.J., Van den Berghe, H. and David, G. (1989). Partial primary structure of the 48- and 90-kilodalton core protein of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. *J. Biol. Chem.*, **264**, 7017-7024.
- [6] Carey, D.J., Evans, D.M., Stahl, R.C., Asundi, V.K., Conner, K.J., Garbes, P. and Cizmeci-Smith, G. (1992). Molecular cloning and characterization of N-syndecan, a novel transmembrane heparan sulfate proteoglycan. *J. Cell Biol.*, **117**, 191-201.
- [7] Gould, S.E., Upholt, W.B. and Kosher, R.A. (1992). Syndecan-3: a new member of the syndecan family of membrane-intercalated proteoglycans that is expressed in high amounts at the onset of chick limb cartilage differentiation. *Proc. Natl. Acad. Sci. USA*, **89**, 3271-3275.

- [8] David, G. (1992). Structural and Functional diversity of the heparan sulfate proteoglycans. *Adv. Exp. Med. Biol.*, **313**, 69–78.
- [9] Carey, D.J. and Evans, D.M. (1989). Membrane anchoring of heparan sulfate proteoglycans by phosphatidylinositol and kinetics of synthesis of peripheral and detergent-solubilized proteoglycans in Schwann cells. *J. Cell Biol.*, **108**, 1891–1897.
- [10] David, G., Lories, V., Decock, B., Marynen, P., Cassiman, J.J. and Van den Berghe, H. (1990). Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. *J. Cell Biol.*, **111**, 3165–3176.
- [11] Ogata, Y., Niisato, N., Sakurai, T., Furuyama, S. and Sugiya, H. (1995). Comparison of the characteristics of human gingival fibroblasts and periodontal ligament cells. *J. Periodontol.*, **66**, 1025–1031.
- [12] Mariotti, A. and Cochran, D.L. (1990). Characterization of fibroblasts derived from human periodontal ligament and gingiva. *J. Periodontol.*, **61**, 103–111.
- [13] Giannobile, W.V. (1996). Periodontal tissue engineering by growth factors. *Bone*, **19**, 235–375.
- [14] Matsuda, N., Lin, W.L., Kumar, N.M., Cho, M.I. and Genco, R.J. (1992). Mitogenic, chemotactic, and synthetic responses of rat periodontal ligament fibroblastic cells to polypeptide growth factors *in vitro*. *J. Periodontol.*, **63**, 515–525.
- [15] McCulloch, C. (1995). Origins and functions of cells essential for periodontal repair: the role of fibroblasts in tissue homeostasis. *Oral Diseases*, **1**, 271–278.
- [16] Bartold, P.M. and Page, R.C. (1987). Isolation and characterization of proteoglycans synthesized by adult human gingival fibroblasts *in vitro*. *Arch. Biochem. Biophys.*, **253**, 399–412.
- [17] Sodek, J. and Berckman, F.A. (1987). Bone cell cultures. *Methods Enzymol.*, **145**, 303–324.
- [18] Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- [19] Towbin, H., Staehelin, T. and Gordon, J. (1979). Electro-phoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
- [20] Roskams, T., Rosenbaum, J., De Vos, R., David, G. and Desmet, V. (1996). Heparan sulfate proteoglycan expression in chronic cholestatic human liver diseases. *Hepatology*, **24**, 524–532.
- [21] Rapraeger, A., Jalkanen, M., Endo, E., Koda, J. and Bernfield, M. (1985). The cell surface proteoglycan from mouse mammary epithelial cells bears chondroitin sulfate and heparan sulfate glycosaminoglycans. *J. Biol. Chem.*, **260**, 11046–11052.
- [22] Lories, V., Cassiman, J.J., Van den Berghe, H. and David, G. (1992). Differential expression of cell surface heparan sulfate proteoglycans in human mammary epithelial cells and lung fibroblasts. *J. Biol. Chem.*, **267**, 1116–1192.
- [23] Salmivirta, M., Elenius, K., Vainio, S., Hofer, U., Chiquet-Ehrismann, R., Thesleff, I., Jalkanen, M. (1991). Syndecan from embryonic tooth mesenchyme binds Tenascin. *J. Biol. Chem.*, **266**, 7733–7739.
- [24] Lories, V., De Boeck, H., David, G., Cassiman, J.J. and Van Den Berghe, H. (1987). Heparan sulfate proteoglycans of human lung fibroblasts. *J. Biol. Chem.*, **262**, 854–859.
- [25] David, G., Van der Schueren, B., Marynen, P., Cassiman, J.J. and Van den Berghe, H. (1992). Molecular cloning of Amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. *J. Cell Biol.*, **118**, 961–969.
- [26] Cheifetz, S. and Massague, J. (1989). Transforming growth factor beta (TGF-beta) receptor proteoglycan. Cell surface expression and ligand binding in the absence of glycosaminoglycan chains. *J. Biol. Chem.*, **264**, 12025–12028.
- [27] Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J.L., Lane, W.S. and Massague, J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell*, **67**, 785–795.
- [28] Kjellen, L. and Lindahl, U. (1991). Proteoglycans: structures and interactions. *Ann. Rev. Biochem.*, **60**, 443–475.
- [29] Lopez-Casillas, F., Wrana, J.L. and Massague, J. (1993). Betaglycan presents ligand to the TGF-beta signaling receptor. *Cell*, **73**, 1435–1444.
- [30] Lawrence, D.A. (1996). Transforming growth factor-beta: a general review. *Eur. Cytokine Netw.*, **7**, 363–374.
- [31] Woods, A., Couchman, J.R. (1994). Syndecan-4 heparan sulfate proteoglycan is a selectively enriched and widespread focal adhesion component. *Mol. Biol. Cell.*, **5**, 183–192.
- [32] Jalkanen, M., Rapraeger, A., Saunders, S. and Bernfield, M. (1987). Cell surface proteoglycan of mouse mammary epithelial cells is shed by cleavage of its matrix-binding ectodomain from its membrane-associated domain. *J. Cell Biol.*, **105**, 3087–3096.
- [33] Subramanian, S.V., Fitzgerald, M.L. and Bernfield, M. (1997). Regulated shedding of syndecan-1 and -4 ectodomains by thrombin and growth factor receptor activation. *J. Biol. Chem.*, **272**, 14713–14720.
- [34] Lamarre, J., Vasudevan, J. and Gonias, S.L. (1994). Plasmin cleaves betaglycan and release a 60kDa transforming growth factor-beta complex from the cell surface. *Biochem. J.*, **302**, 199–205.
- [35] Nojima, N., Kobayashi, M., Shionome, M., Takahashi, N., Suda, T., Hasegawa, K. (1990). Fibroblastic cells derived from bovine periodontal ligaments have the phenotypes of osteoblasts. *J. Periodont. Res.*, **25**, 179–185.
- [36] David, G., Xiao, M.B., Van der Schueren, B., Marynen, P., Cassiman, J.J. and Van den Berghe, H. (1993). Spatial and temporal changes in the expression of fibroglycan (syndecan-2) during mouse embryonic development. *Development*, **119**, 841–854.
- [37] Matthers, T., Werner-Favre, C., Tang, H., Zhang, X., Kindler, V. and Zubler, R.H. (1993). Cytokine mRNA expression during an *in vitro* response of human B lymphocytes: kinetics of B cell tumor necrosis factor alpha, interleukin (IL) 6, IL-10, and transforming growth factor beta-1 mRNAs. *J. Exp. Med.*, **178**, 521–528.
- [38] Engelmann, S., Ebeling, O., Schwartz-Albiez, R. (1995). Modulated glycosylation of proteoglycans during differentiation of human B-lymphocytes. *Biochim. Biophys. Acta*, **1267**, 6–14.
- [39] Grover, J. and Roughley, P.J. (1995). Expression of cell surface proteoglycan mRNA by human articular chondrocytes. *Biochem. J.*, **309**, 963–968.
- [40] Schick, B.P. and Jacoby, J.A. (1995). Serglycin and betaglycan proteoglycans are expressed in the megakaryocytic cell line CHRF 288-11 and normal human megakaryocytes. *J. Cell. Physiol.*, **165**, 96–106.