

Expression and distribution of cell-surface proteoglycans in the normal Lewis rat molar periodontium

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Cell-surface proteoglycans participate in several biological functions such as cell–cell and cell–matrix interactions, cell adhesion, the binding to various growth factors as co-receptors and repair. To understand better the expression and distribution of cell-surface proteoglycans in the periodontal tissues, an immunohistochemical evaluation of the normal Lewis rat molar periodontium using panels of antibodies for syndecan-1, -2, -4, glypican and betaglycan was carried out. Our results demonstrated the expression and distribution of all proteoglycans in the suprabasal gingival epithelium, soft and hard connective tissues. Both cellular and matrix localization was evident within the various periodontal compartments. The presence of these cell-surface proteoglycans indicates the potential for roles in the process of tissue homeostasis, repair or regeneration in periodontium of which each function requires further study.

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Proteoglycans associated with cell membranes form a distinct class of structurally and functionally related molecules whose expression may vary with differentiation and tissue origin (1). Proteoglycans may be associated with cell membranes via 3 separate mechanisms (i) as an integral membrane protein spanning the lipid bilayer, (ii) partial insertion into the lipid bilayer of a phosphatidyl inositol component of the proteoglycan, or (iii) binding of a glycosaminoglycan side chain to specific plasma membrane receptors (2). Given their strategic position upon cell surfaces these proteoglycans have been ascribed varied functions, most of which relate to cell–cell and cell–matrix interactions.

Cell-surface proteoglycans are divided into two broad groups based on their domain structures. The first includes the transmembrane syndecan family (SLIPS: the syndecan-like integral membrane proteoglycans), of which 4 types have been identified which all have similar cytoplasmic and transmembrane domains but differ in their extracellular domains and structure (3–6). Betaglycan is

also a transmembrane proteoglycan. Although its core protein amino acid sequence is unrelated to the sequences of other intercalated membrane proteoglycans (7, 8), the overall domain structure of betaglycan is similar. Another cell-surface proteoglycan, glypican, is unique in that it is linked to the cell surface via a glycosyl phosphatidylinositol anchor (9–15).

From many studies it is now clear that the above cell-surface proteoglycans are important molecules which mediate cell interactions with components of the extracellular microenvironments and serve to control cell shape, adhesion, proliferation and differentiation (2, 3, 16, 17). Although many of the above cell surface proteoglycans have been studied in a variety of cell culture systems, apart from syndecan-1 and CD-44 (18–20), there has been no comprehensive study of the distribution and expression of cell surface proteoglycans by cells of the periodontium.

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 cell. The aim of this project was to study the expression of the cell-surface proteoglycans within the various periodontal compartments of the mature rat molar periodontium with view to determining whether site-specific variations in expression are evident.

The significance of this project lies in gaining an important understanding of the biological properties of the principal cells which reside in the soft tissues of the periodontium. Cell-surface proteoglycans are extremely important with respect to their role in cell-matrix interactions. In particular, many cell-surface proteoglycans are involved in binding growth factors to the cell in the initial phases of cellular interactions with growth factors. With the current interest in periodontics in the use of growth factors to stimulate periodontal regeneration an understanding of how the cells react to these agents is crucial to the development of rational treatment strategies.

Material and methods

Antibodies

Panels of primary antibody were used, as follows: syndecan-1 (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). A polyclonal rabbit anti-rat antibody was obtained for betaglycan (Upstate Biotech Inc., USA). All primary antibodies were diluted in PBS containing 0.1% BSA at concentrations previously determined to give optimal staining (1:20–1:50 dilution). All incubations were performed overnight at 4°C. Negative control tissues were stained by the omission of the primary antibody. Secondary antibodies used to detect the above antibodies were: sheep anti-rat for syndecan-1 (Amersham Life Science, UK), swine anti-mouse for syndecan-2, syndecan-4 and glypican (Dako, USA) and swine anti-rabbit for betaglycan (Dako, USA).

Tissue preparation of normal Lewis rat periodontium

Three, normal, 10-wk-old male Lewis rats were used for this study following ethical approval by the University of Queensland animal ethics committee. The animals were perfused intracardially with 50 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.2, under ketamine anaesthesia. Mandibular molars, together with the surrounding with alveolar bone and covering gingival tissues, were dissected out and

post-fixed in the perfusate for 4 h at 4°C. Tissues were then decalcified with 4% EDTA, pH 7.3, 4°C for 21–28 d with multiple changes. The specimens were then embedded in paraffin using standard histological procedures. Longitudinal sections (5 µm thick) were cut in the bucco-lingual plane of the tooth. The sections were then immunostained with each of the antibodies for the selected cell-surface proteoglycans.

Immunohistochemistry

After dewaxing, rehydration, the sections were incubated in PBS containing 3% H₂O₂ to block endogenous peroxidase activity. No enzymatic treatments of the tissues to expose epitopes were necessary. After 3 washes in PBS, the tissues were blocked by incubation with 10% normal swine serum (or 10% normal goat serum for the syndecan-1 antibody) followed by primary antibody incubations which were performed overnight at 4°C. After 2 washes in 0.1% Triton-X/PBS and once in PBS, the specimens were incubated with the appropriate biotinylated secondary antibody followed by alternatively incubating in conjugated streptavidin-peroxidase (Dako, USA) or avidin-biotin complex (Vectastain[®], Vector Laboratories Inc., USA). Specific immunostaining was visualized with 3,3'-diaminobenzidine tetrahydrochloride containing 0.015% H₂O₂. The sections were counterstained with Mayer's haematoxylin, dehydrated and mounted with synthetic mounting medium. An assessment of the intensity of staining was made by three observers in agreement (W.W., H.L., W.G.Y.) using an Olympus BX 50 microscope (Tokyo, Japan) for the qualitative assessments as + (weakly positive); ++ (moderately positive); +++ (strongly positive); – (no positive staining); ? (no cells present in the section). Comparisons were made between cells active in maintenance of the integrity of the periodontium with cells in tissues in an inactive state. Since the purpose of this study was qualitative only, no attempt was made to quantitate the level of immunostaining within the different tissue compartments.

Results

Localization of cell-surface proteoglycans in gingival epithelium and gingival connective tissue

Immunohistochemical staining using antibodies against syndecan-1, syndecan-2, syndecan-4, glypican and betaglycan showed specific distributions of these cell-surface proteoglycans within the gingival epithelium and gingival connective tissue (Table 1). In the oral aspect of the gingival

Table 1. Summary of the immunoreactivity in gingival epithelia and connective tissue for 5 cell-surface proteoglycans

	Syndecan-1	Syndecan-2	Syndecan-4	Glypican	Betaglycan
Basal cells	-	-	-	+?	-
Keratinized suprabasal cells	++	++	++	++	++
Non-keratinocyte cells (Melanocyte cells, Langerhans cells, Merkel cells)	++	+	+	+	+
Basement membrane	-	-	-	-	-
Gingival fibroblasts	++	++	+	++	+++
Gingival CNT matrix	++	++	+	++	+++

+ = Weakly positive staining; ++ = moderately positive staining; +++ = strongly positive staining; +? = some area positive staining; ? = no presenting cells in the section; - = no positive staining.

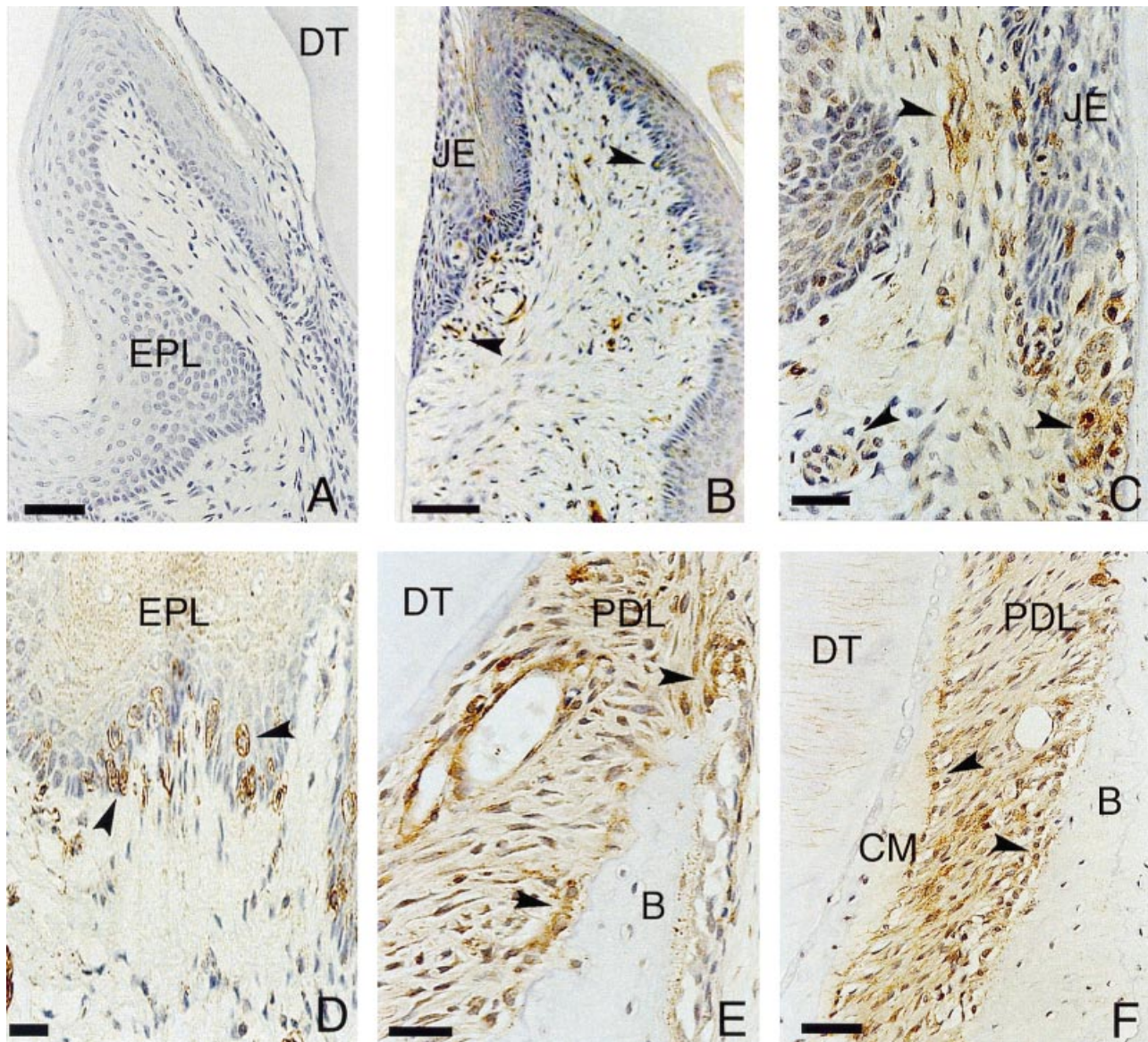


Fig. 1. Distribution of syndecan-1 in rat periodontium. A. Control section (primary antibody omitted). B. The affinity of this antibody for keratinizing epithelial cells but not basal epithelial cells or basement membrane (right arrow) and gingival fibroblasts below the JE (bottom arrow) is evident. C. Cells stained positively are gingival fibroblasts (top and right arrows) and epithelial cell rests of Malassez (bottom arrow). D. Cell surface staining of dendritic non-keratinocytes in epithelium (arrow). Note the basement membrane is not stained. E. Staining of active osteoblasts at the crestal bone (top arrow) and along the inner lining of alveolar bone. F. Staining of the cementoid and weak staining of the osteoid is noted. Staining of PDL fibroblasts and matrix in panels D and E is evident and appears to be stronger than that noted in the gingival connective tissue matrix staining as shown in panel B. EPL = oral epithelium, DT = dentine, JE = junctional epithelium, PDL = periodontal ligament, B = alveolar bone, CM = cementoid. Scale bar = 50 μ m for A and B, 25 μ m for B-E.

epithelium, which is keratinized or parakeratinized, the suprabasal layers exhibited moderate to strong staining in the cytoplasm with all antibodies (Fig. 1, panels A–C; Figs 2–5, panel A). In the basal cell

layer, basal keratinocytes and the basement membrane did not stain for any of the proteoglycans studied except glypican, which localized to some, but not all, basal cells. However, strong positive

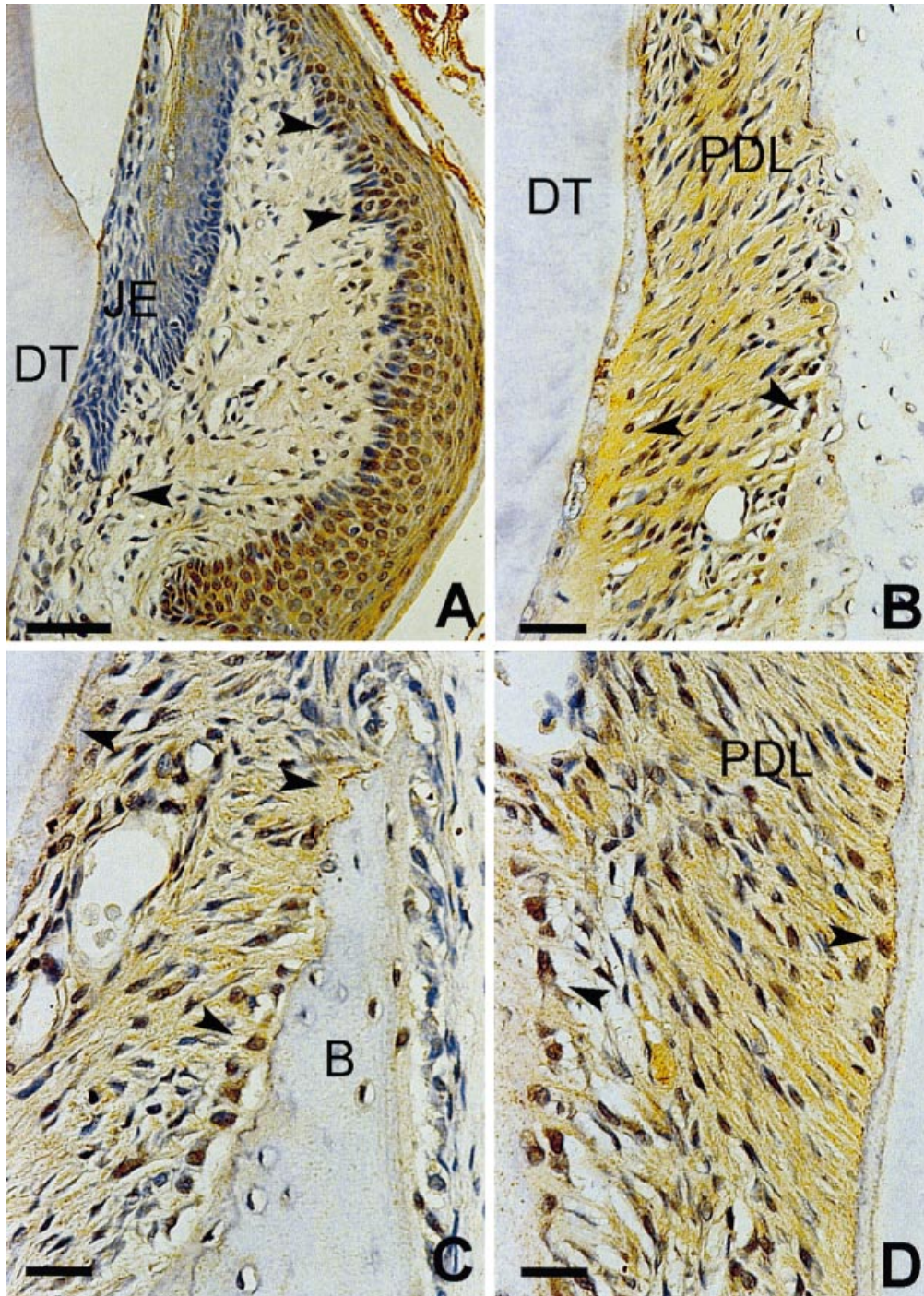


Fig. 2. Distribution of syndecan-2 in rat periodontium. A. The affinity of this antibody for non-keratinizing cells even in basal cells of oral gingival epithelium is evident (top arrows) and gingival fibroblasts below the JE (bottom arrow). B. Arrowheads show staining in cementoblasts lining the root surface (left) and osteoblasts lining the inner surface of the alveolar bone proper (right). Staining is markedly present in the cementoid and osteoid. C. Arrowhead at top left shows staining in the acellular cementum. Positive staining is also seen in the crestal bone region and in the osteoblasts lining the inner surface of the alveolar bone proper (right). D. Arrowhead on right shows staining in PDL fibroblasts and positively stained acellular cementum. The left arrowhead shows staining in osteoblasts. Staining is also present in PDL fibroblasts and matrix in panels B–D, and this appears to be stronger than that seen in the gingival connective tissue matrix staining in panel A. Scale bars = 50 μ m for A, 25 μ m for B–D. DT = dentine, JE = junctional epithelium, PDL = periodontal ligament, B = alveolar bone.

staining was noted on the cell membranes and dendritic processes of non-keratinocytes, such as melanocytes, Langerhans cells and Merkel cells (Fig. 1, panel D). The non-keratinized cells of the sulcular epithelium, stained weakly for all of the cell-surface proteoglycans studied. The stratified squamous keratinized layers, which are continuous between the junctional epithelium and the sulcular epithelium, were weakly stained intracellularly by all antibodies. Interestingly, intense staining for all of the cell surface proteoglycans studied (except syndecan-1) was found in the lining cells of the junctional epithelium which interfaced with the tooth surface (Figs 2, 4, panel A; Figs 3, 5, panels A and B). In summary, gingival basement membrane and basal keratinocytes were not positive for the cell-surface proteoglycans studied. Dendritic intra-epithelial cells showed membrane staining. Squamous cells undergoing keratinization showed intracellular staining as was also found in epithelial cell rests of Malassez.

In general, the gingival connective tissue demonstrated mild to moderate staining patterns for syndecan-1, -2, and glypican for both the gingival fibroblasts and the gingival connective tissue matrix (Fig. 1, panels B–D; Figs 2, 4, panel A). Betaglycan was moderately to strongly expressed in the gingival fibroblasts and gingival connective tissue matrix (Fig. 5, panel A). However, in contrast, syndecan-4, stained weakly in both the gingival fibroblasts and gingival connective tissue matrix (Fig. 3, panel A). In the area underlying the junctional epithelium, gingival fibroblasts showed intense and extensive staining with all antibodies (Fig. 1, panels B and C; Figs 2–5, panel A).

Localization of cell-surface proteoglycans in the periodontal ligament, cementum and bone

A summary of the distribution of cell-surface proteoglycans in the periodontal ligament is given in Table 2. Syndecan expression by the periodontal ligament fibroblasts, especially those close to, or in contact with, the cementum and bone surfaces was found to be cell associated. Moderate to strong positive staining of the periodontal ligament matrix was generally observed (Fig. 1, panels E and F; Fig. 2, panels B and C; Fig. 3, panel E). A very strong immunoreactive staining pattern was found

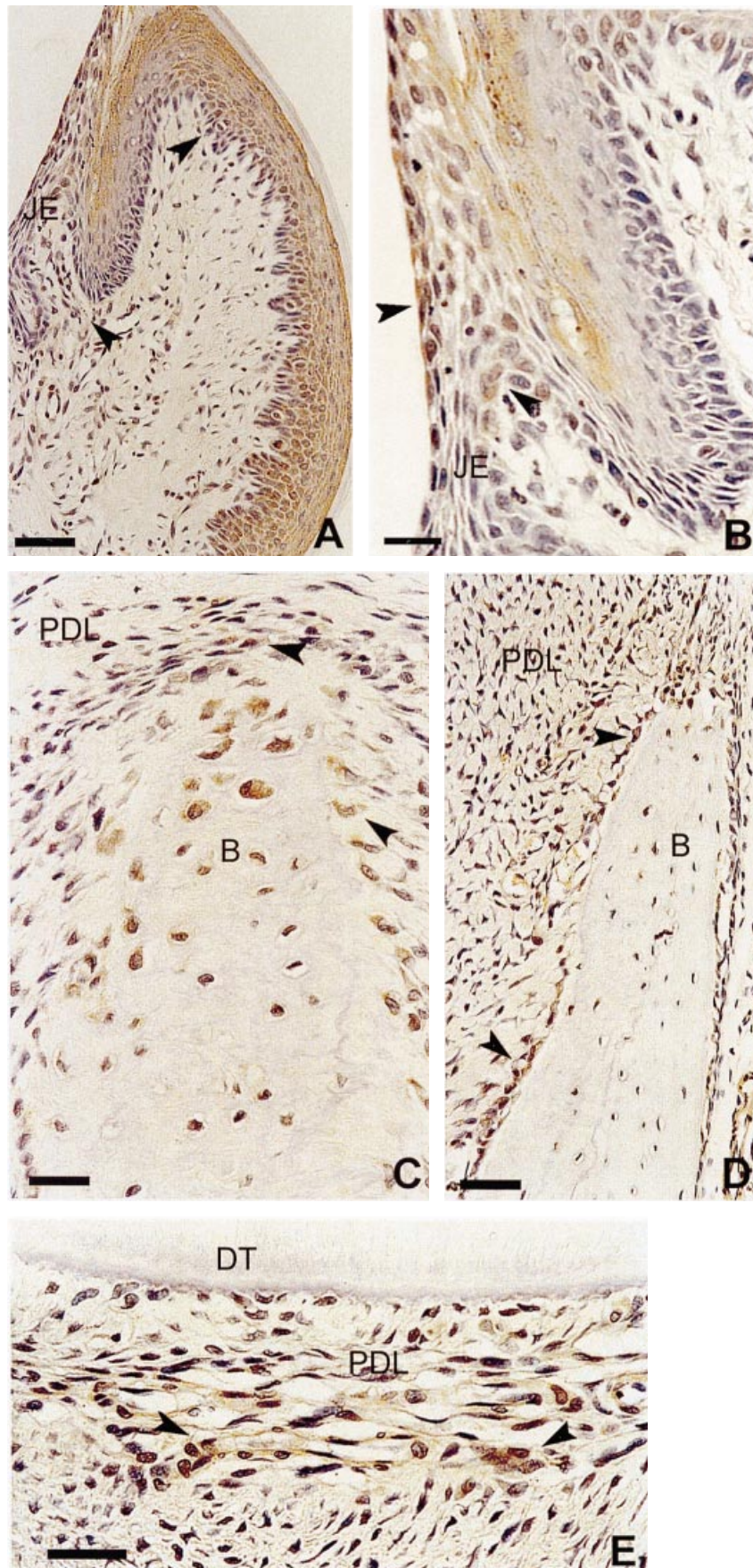
for glypican and betaglycan throughout the entire periodontal ligament in both the fibroblast cells and their surrounding matrix (Fig. 4, panels B and C; Fig. 5, panel C). In addition, strong staining was noted in cells adjacent to the crestal bone (Figs 4, 5, panels D and E).

All the syndecans were expressed in a similar pattern in the alveolar bone to that found in the periodontal ligament fibroblasts and their surrounding matrix. In particular, strong intracellular staining of osteoblasts was noted at the crestal bone, and on the inner surface of the alveolar bone proper. Strong positive staining was noted in cementoblasts along the root surface. Maximum immunoreactivity was noted in the cytoplasm of active osteoblasts and cementoblasts (Fig. 1, panel F; Fig. 2, panel B; Fig. 3, panels C and D). Marked expression of syndecan-2 was noted in the acellular cementum (Fig. 2, panels C and D). Within the cementoid and osteoid tissues, syndecan-2, syndecan-1 and syndecan-4 all stained positively (Fig. 1, panel F; Fig. 2, panel B; Fig. 3, panel D). Expression of both glypican and betaglycan were moderately to strongly localized in osteoblasts on the inner surface of the alveolar bone proper, as well as in cementoblasts of cellular cementum (Fig. 4, panels B, C and D; Fig. 5, panels C and D). It should be noted that the positive staining seen in the cementoid for glypican and betaglycan was not noted in osteoid (Fig. 4, panel B; Fig. 5, panel C).

General findings

Table 3 shows that other tissues showed variable expression of the cell-surface proteoglycans studied. In the teeth, odontoblasts and their product, predentine, showed positive immunoreactivity for all antibodies. In the periodontal ligament, the epithelial cell rests of Malassez (remnants of Hertwig's epithelial root sheath) were noted to have moderately strong staining for all antibodies, especially syndecan-1. Furthermore, endothelial cells appeared to stain strongly for syndecan-1 and glypican but not for betaglycan. The subendothelial/or smooth muscle cells of blood vessels showed moderate staining with all antibodies, except for syndecan-4. Megakaryocytes were positive for all antibodies while the red and white blood cells stained

Fig. 3. Distribution of syndecan-4 in rat periodontium. A. This affinity of this antibody for keratinizing epithelial cells but not basal cells or basement membrane is clearly shown (arrows) B. The junctional epithelial cells attached to the tooth (left arrow) are positive suggesting a role for this proteoglycan in epithelial attachment to enamel surfaces. Strong staining is evident in the keratinizing cells between the junctional epithelium and sulcular epithelium (right arrow). Basal cells and basement membrane of the sulcular epithelium are not stained. C. Positively stained osteoblasts and some osteocytes are evident in the crestal bone. D. Osteoblasts lining the inner surface of the alveolar bone are positive. Weak staining of osteoid is observed. E. Staining is present in some PDL fibroblasts (both arrow heads) and throughout the PDL matrix. Scale bar = 50 μ m for A and D, 25 μ m for B, C and E. DT = dentine, JE = junctional epithelium, PDL = periodontal ligament, B = alveolar bone.



positively for syndecan-1, syndecan-2 and betaglycan but negatively for syndecan-4 and glypican. In general, striated muscle cells were positive for all antibodies.

Discussion

Our previous *in vitro* studies have demonstrated that at least five cell-surface proteoglycans are

expressed by human periodontal cell lines (gingival fibroblasts, periodontal ligament fibroblasts and osteoblasts) (21). Therefore, it was of interest to establish where these cell-surface proteoglycans were distributed *in vivo*.

Using immunostaining of Lewis rat molar periodontal tissues with antibodies against a variety of cell-surface proteoglycans we have shown cell and tissue specific differences in the expression of these

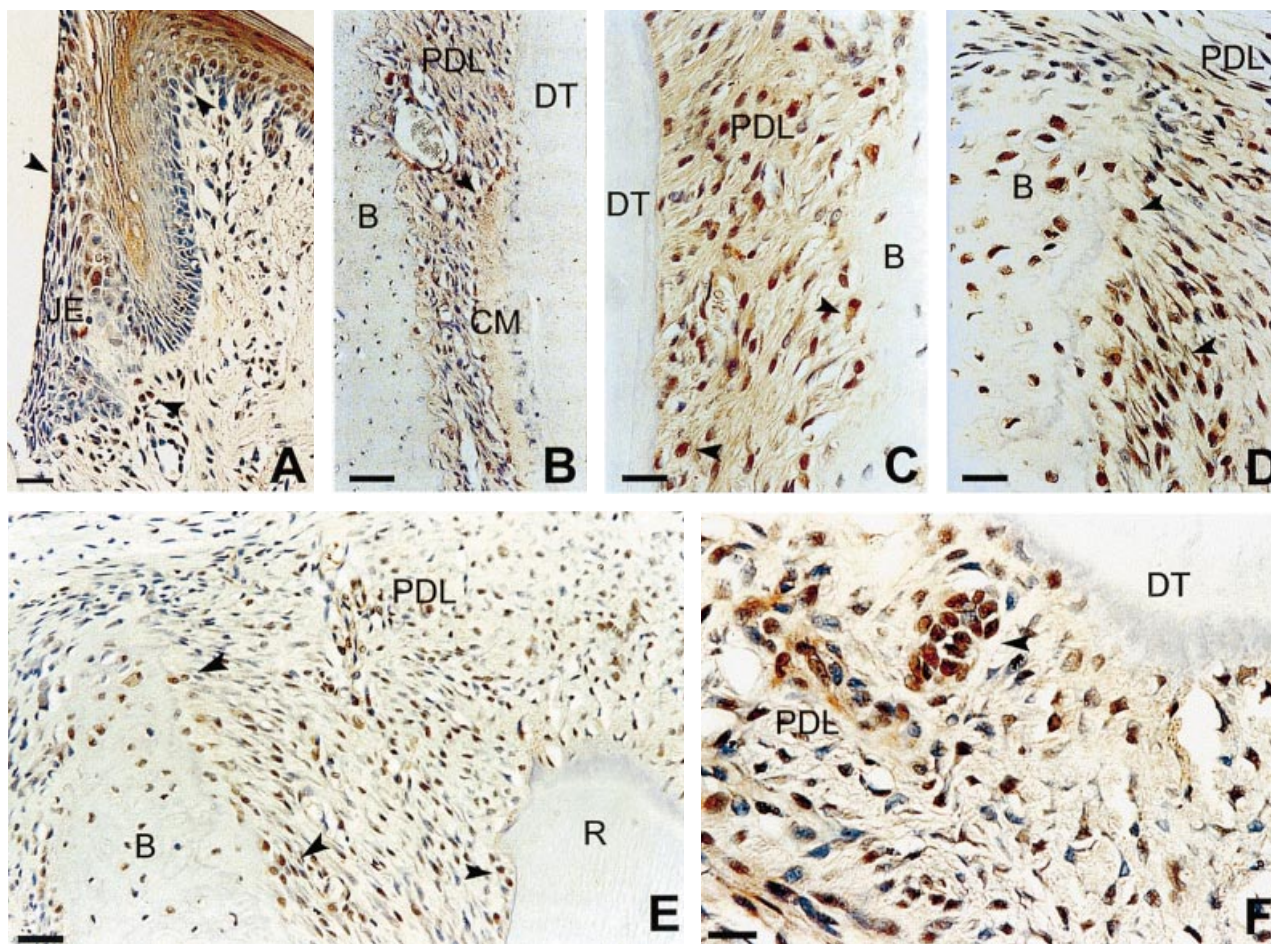


Fig. 4. Distribution of glypican in rat periodontium. A. The epithelia stain as for syndecans 1, 2, and 4, positive staining is present at the lining JE attached to the tooth surface (left arrow) and in dendritic non-keratinocytes (top arrow). Staining is also present in the gingival fibroblasts below the JE (bottom arrow). B. Positive staining is pronounced in the cementoblasts and cementoid matrix. C. Staining is present in the osteoblasts (right arrow) and PDL fibroblasts (left arrow). Staining is also present throughout the PDL matrix. D. Staining is present in the osteoblasts and some of osteocytes in the crestal bone area (top arrow) and in PDL fibroblasts adjacent the alveolar bone (right arrow). E. Staining is present in the osteoblasts lining along the alveolar bone (left arrows) and in PDL fibroblasts adjacent to the root surface (right arrow). F. The epithelial cells rests of Malassez showed intracellular staining (arrow). Staining is also present in the surrounding PDL fibroblasts and their matrix. Scale bars = 50 μm for A, B and E and 25 μm for C, D and F. DT = dentine, JE = junctional epithelium, PDL = periodontal ligament, B = alveolar bone, CM = cementoid, R = root.

Fig. 5. Distribution of betaglycan in rat periodontium. A. Expression of betaglycan in the keratinizing oral gingival epithelia (top arrow) and gingival fibroblasts below the JE (bottom arrow). B. The lining JE attached to the tooth surface is positive (left arrow). Staining is also present in the gingival fibroblasts below the JE (bottom arrow). C. Staining in cementoblasts and the cementoid is positive (left arrow). The right arrow shows stained osteoblasts. No osteoid staining was observed. Staining is also present in PDL fibroblasts and matrix and is stronger than that seen in the gingival connective tissue. D. Osteoblasts and some osteocytes at the crestal bone area are positive. E. The periodontal ligament fibroblasts (left arrow) and the osteoblasts lining the alveolar bone (right arrow) are positive staining. Scale bars = 50 μm for A, C and D and 25 μm for B and E. DT = dentine, JE = junctional epithelium, PDL = periodontal ligament, B = alveolar bone, CM = cementoid, CT = cementum, R = root.

proteoglycans. Within the epithelia, the expression of most proteoglycans was associated with intracellular changes of the keratinized pattern of the rat gingival epithelium which, unlike human, shows

keratinization of the sulcular epithelium as well as the oral aspect. Interestingly, basal keratinocytes did not express the cell-surface proteoglycans studied, nor did these proteoglycans appear to be

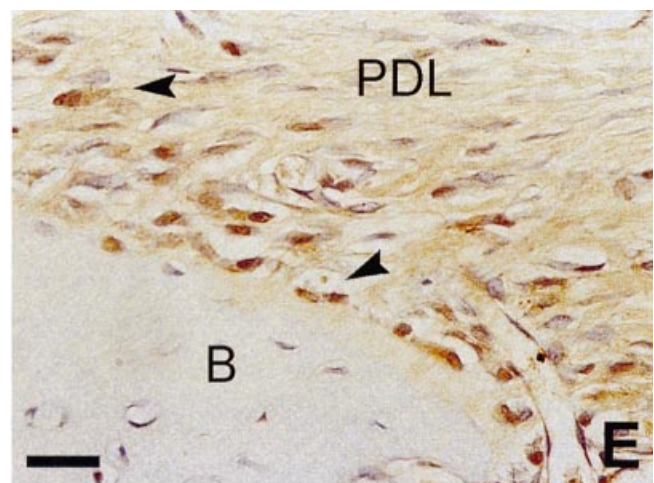
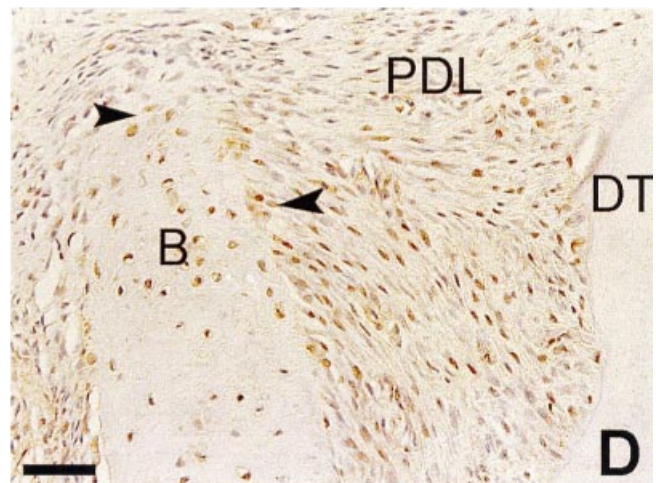
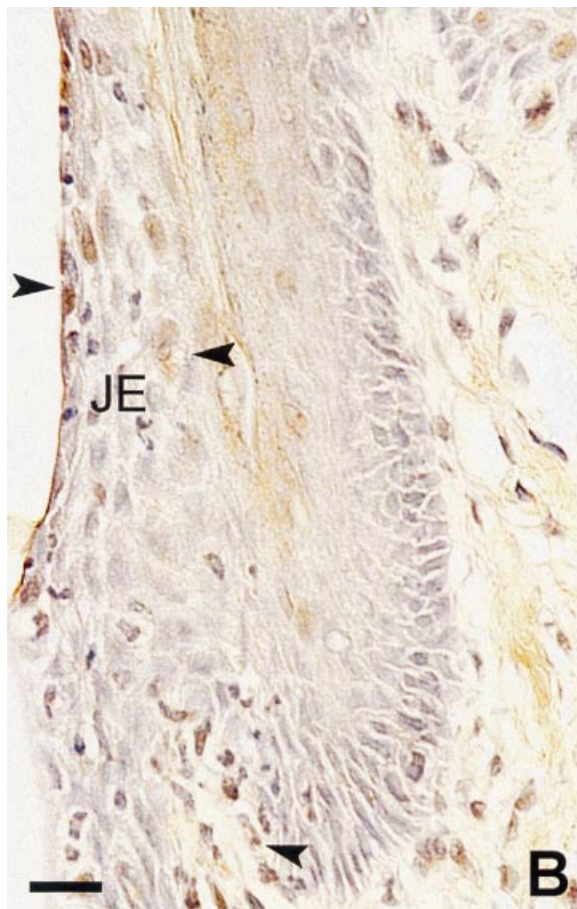
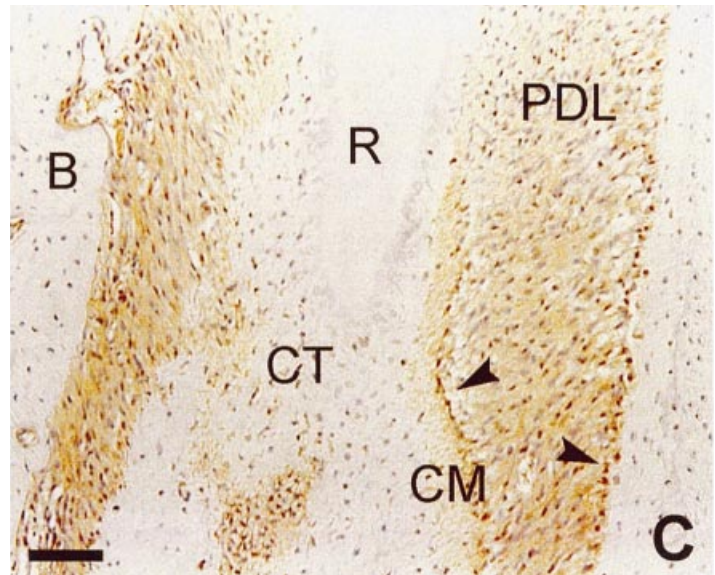
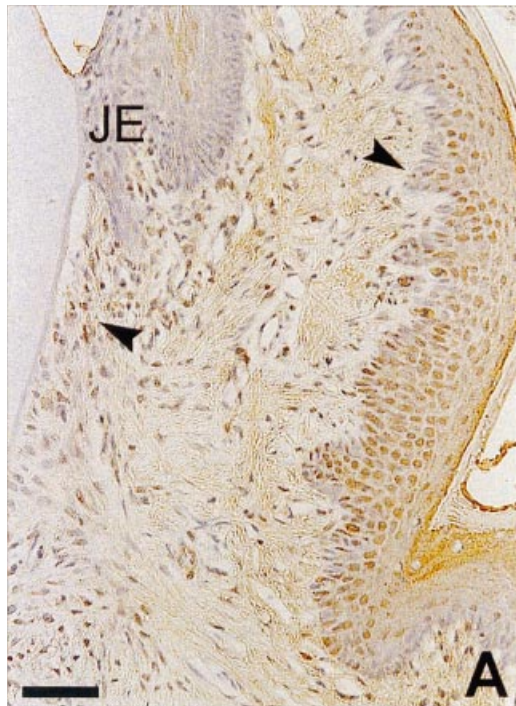


Table 2. Summary of immunoreactivity of components in the periodontium for 5 cell-surface proteoglycans

	Syndecan-1	Syndecan-2	Syndecan-4	Glypican	Betaglycan
PDL fibroblasts	++	++	++	+++	+++
PDL matrix	++	++	++	+++	+++
Cementoblasts	++	++	+	++	++
Cementocytes	-	++	-	-	+
Cementoid	+	++	+	+	+
Acellular cementum	-	++	-	-	-
Osteoblasts	++	++	++	++	++
Osteocytes	-	++	++	++	++
Osteoclasts	+++	?	?	?	?
Osteoid	+	++	+	-	-

Table 3. Summary of immunoreactivity in other tissues for 5 cell-surface proteoglycans

General findings	Syndecan-1	Syndecan-2	Syndecan-4	Glypican	Betaglycan
Odontoblasts	++	++	++	++	++
Predentine	++	++	++	++	++
Endothelial cells	+++	++	++	++	-
Subendothelial/smooth muscle of blood vessels	+++	++	+	++	++
Megakaryocytes	+	+	+	+	+
Blood element (RBC/WBC)	+	+	-	-	+
Striated muscle	+++	+++	+	++	++
Epithelial cell rests of Malassez	+++	++	++	++	++

involved in the structure of the basal lamina, since the basement membranes were not positive for any of these proteoglycans. In contrast, the dendritic cells within the gingival epithelia were positive for all of the cell-surface proteoglycans studied. Since these cells do not generally affect cell functions with the surrounding keratinocytes, the proteoglycans on these cells may serve non-attachment functions. While this is an interesting observation, no attempt was made to characterize these dendritic cells as Langerhans cells, melanocytes or Merkel cells. Positive staining in junctional epithelial cells adjacent to enamel and in the epithelial cell rests of Malassez further indicate a predilection for expression of cell-surface proteoglycans by differentiated epithelial cells of the periodontium.

The positive staining of the junctional epithelial cells for all proteoglycans studied except syndecan-1 is consistent with previous reports (18, 19) and indicates that these proteoglycans may play a role in adhesion or attachment functions of these cells. This attachment site, termed the "internal basal lamina", is a product of the epithelial cells, and consists of nonfibrillar type IV collagen, laminin, nidogen and perlecan (22). An earlier report has shown that syndecan-1 binds to several ECM proteins but not to collagen type IV or laminin under physiological ionic conditions (23) and this may explain, in part, the absence of this proteoglycan in the oral epithelial basement membranes.

Whether or not the various syndecans have somewhat different cell-matrix interactions leading to distinct functions remains to be established (24).

The underlying gingival connective tissue fibroblasts and matrix showed generalized moderate staining and distribution. However, it was notable that the staining of gingival connective tissue fibroblasts was most intense in the area below the junctional epithelium and close to or attached to the tooth surface. The roles of fibroblasts in tissue homeostasis such as differentiation, migration and contractility are becoming better understood. For example, some cell-surface proteoglycans on mesenchymal cells have been shown to be involved in cell adhesion, movement and differentiation both in soft and hard connective tissues (17, 24). Our results demonstrate strong immunostaining for all the cell-surface proteoglycans studied in both the gingival and periodontal ligament fibroblasts and their matrix and indicate that these proteoglycans may play major roles in cell-cell and cell-matrix interactions in these tissues.

Of interest in this context was the finding that the lamina propria adjacent to the junctional epithelium and the crestal bone (areas of particular cellular, metabolic and synthetic significance with regard to tissue regeneration) showed positive reactivity towards syndecans-1, -2, -4, glypican and betaglycan in both the resident cells and adjacent matrix. Positive staining of osteocytes

was also observed for syndecan-4, glypican and betaglycan in the crestal bone. Furthermore, we found that osteoblasts, active on the inner surface of the alveolar bone proper, showed positive staining for all the cell-surface proteoglycans studied. However, there appeared to be some differences. For example, syndecan-2 showed positive staining in osteoblasts and also in new bone matrix (osteoid). On the other hand, both glypican and betaglycan demonstrated intense positive intracellular staining in all osteoblasts along the entire alveolar bone surface, but no positive staining in osteoid was observed. These findings appear to be in accordance with others which have shown that glypican-1 is expressed by developing and mature osteoblasts found in periosteum and trabeculae during both intramembranous and endochondral ossification (10). The presence of betaglycan in the cells of the mineralized tissues was not unexpected, since this molecule is recognized as the type III receptor for TGF- β and thus serves an important function in the responsiveness of cells to TGF- β stored within the mineralized matrix.

In conclusion, this study has demonstrated the presence of a number of cell-surface proteoglycans on cells of the periodontium. Given their diverse structures and functions we propose that these cell-surface proteoglycans may function in cell-cell, cell-matrix and cell-growth factor interactions. Thus, they are likely to play key roles in tissue homeostasis, repair or regeneration of the periodontium. The precise roles of each cell-surface proteoglycan await further investigation. In particular, the temporal and spatial distribution of these molecules during development and regeneration requires investigation to determine their role in these two critical processes.

Acknowledgements

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