Immunohistochemical expression of biglycan and decorin in the pulp tissue of human primary teeth during resorption

Monique Saveriano De Benedetto[a]
Filipe Modolo Siqueira[b]
Marcelo Betti Mascaro[c]
Vera Cavalcanti Araújo[d]
Marcelo José Strazzeri Bönecker[e]

[a] Department of Pediatric Dentistry, São Leopoldo Mandic Dental Research Institute - SLMandic, Campinas, SP, Brazil.

[b] Department of Pathology, Health Sciences Center, Universidade Federal de Santa Catarina - UFSC, Florianópolis, SC, Brazil.

[c] Department of Anatomy, Universidade Nove de Julho - UNINOVE, São Paulo, SP, Brazil.

[d] Department of Oral Pathology, São Leopoldo Mandic Institute and Research Center - SLMandic, Campinas, SP, Brazil.

[e] Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Universidade de São Paulo - USP, São Paulo, SP, Brazil.

Abstract: Primary teeth are interesting models that can be used to study physiological and pathological processes involving cells and extracellular matrices in hard and soft tissues. This study investigated the expression and distribution of biglycan and decorin—the non-collagenous components of the extracellular matrix—in primary teeth tissue, during physiological root resorption. Thirty healthy human primary teeth were grouped together according to root length: Group I - two-thirds root length, Group II - one-third root length, and Group III - teeth with no root. The streptavidin-biotin-peroxidase immunohistochemical method was used with antibodies against the previously named antigens. The proteoglycans studied were found in the pulp and dentin extracellular matrix in all groups without any differences in the proteins, among the groups. Biglycan was observed mainly in predentin and in pulp connective tissue in the resorption area. In addition, decorin was observed mainly in pulp connective tissue, but near the resorption area. Biglycan and decorin were distributed differentially in the dental tissues. The present immunohistocytochemical data, combined with previously reported data, suggest that these proteoglycans could be involved in regulating the physiological resorption process in healthy primary teeth.

Descriptors: Immunohistochemistry; Extracellular Matrix; Proteoglycans; Endodontics; Tooth, Deciduous.

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Corresponding Author:
Monique Saveriano De Benedetto
E-mail: *niquesdb@usp.br*

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Introduction

Root resorption and pulp inflammation are physiological events that occur in the lifespan of primary teeth. These events have prompted studies addressing expression and interaction among different cells and chemical substances.[1] Moreover, it is still unclear as to how the connective tissue of the pulp responds to inflammatory processes triggered during root resorption.

Dental pulp is a loose connective tissue composed of cells and the extracellular matrix (ECM). The ECM of pulp tissue comprises a variety of proteins and polysaccharides secreted locally, forming a discrete network. Matrix macromolecules include collagenous proteins, non-collagenous proteins, proteoglycans and phospholipids.[2]

Proteoglycans are large, complex macromolecules distributed ubiq-

Table 1- Clone, title and incubation period of primary antibodies anti-biglycan (BGC) and anti-decorin (DEC) and enzymatic treatment. Antibodies were donated by Prof. Larry W. Fischer, Bethesda, MD, USA.

Antibody	Clone	Title	Incubation period (min)	Enzymatic treatment
BGC[a]	Polyclonal	1:750	40	Chondroitinase ABC, 37°, 60 min
DEC[a]	Polyclonal	1:750	40	Chondroitinase ABC, 37°, 60 min

uitously in the human body, and are found in matrices of mineralizing and non-mineralizing tissues. They consist of glycosaminoglycan chains, comprising repeating disaccharide units, linked covalently to a core protein.[3] Biglycan and decorin belong to the family of small leucine-rich proteoglycans (SLRPs). Several studies have shown that biglycan and decorin are important components of dental tissues. Moreover, these proteoglycans participate in the formation of mineralized tissues, such as dentine.[3] Research involving animal dental tissues in initial stages of development has been conducted to determine the role of biglycan and decorin in dentinogenesis.[4]

Recently, the histological conditions of the primary teeth pulp in physiological root resorption were analyzed, and the expression of proteins in the ECM of pulp tissue was described.[5] Nevertheless, there are currently no studies on the immunoexpression of biglycan and decorin in primary teeth.

This study investigated the expression and distribution of biglycan and decorin, in primary teeth dental tissues during physiological root resorption.

Methodology

This study was approved by the Human Ethics Research Committee of the School of Dentistry (protocol number 177/04), *Universidade de São Paulo*, São Paulo, Brazil. Children were treated only when parental consent was obtained.

Thirty healthy human primary teeth, extracted for either occlusal or orthodontic reasons, were distributed into three sample groups according to their root lengths, according to the stage of root resorption:

- Group I - two-thirds root length,
- Group II - one-third root length, and
- Group III - teeth with no root.

All specimens were fixed in neutral-buffered formalin (10%), decalcified in ethylenediaminetetraacetic acid (EDTA; Invitrogen, Carlsbad, USA)[4-7] under irradiation by a PELCO 3440 laboratory microwave oven (Ted Pella, Redding, USA) for 35 h.[5] The specimens were placed in a beaker containing 25 mL of EDTA, which was then placed in a larger glass container filled with ice. A temperature probe was submersed in the EDTA, and the specimens were immediately irradiated at a medium setting for 15 min periods with the temperature programmed to a maximum of 37°C. The decalcifying solution was changed every 30 min, 3 hours a day, until completing 35 h. The teeth were then embedded in paraffin.

The blocks were cut, stained by hematoxylin-eosin (Sigma-Aldrich, St. Louis, USA) and histologically prepared to be viewed under optical microscopy (Leica DMR, Wetzlar, Germany). Eighteen specimens (6 teeth from each group) were selected for immunohistochemical study. Briefly, the 33-µm sections from paraffin-embedded materials were deparaffinized in xylene and then rehydrated in alcohol and distilled water. The sections were washed in methanol with 6% hydrogen peroxide (H_2O_2 1:1) twice for 20 min, and washed with Tris-HCL (pH 7.4) twice for 5 min. The sections were then incubated for 1 hour at 37°C in a 20 mM Tris-HCL buffer, pH 8.0, containing 0.2 U/mL of chondroitinase ABC (Seikagaku, Tokyo, Japan).

Nonspecific staining was blocked by incubating the sections for 60 min with normal goat serum diluted at 1:1 in PBS–10% bovine serum albumin. Immunohistochemistry was performed in the Dako-Autostainer (Dako Corporation, Carpinteria, USA). The sections were then incubated with the primary antibodies (Table 1), diluted in a solution of TRIS (pH = 7.4), and 1% of bovine serum albumin (BSA, Biotest S/A, São Paulo, Brazil). In the negative controls, the primary antibodies were replaced by bo-

vine serum albumin in Tris-HCL, pH 7.4, instead of the primary antibody. Slides of normal kidneys were the positive controls.[8,9]

Next, the sections were washed thoroughly and exposed to the secondary antibodies. This was followed by application of the streptavidin-biotin kit (Dako Corporation, Carpinteria, USA). Sections were then incubated in 0.3% diaminobenzidine (DAB) solution (Sigma-Aldrich, St. Louis, USA), for 3 min, and counterstained with Mayer's hematoxylin, dehydrated and mounted with glass coverslips and mounting media. Median sections for the 18 specimens (6 teeth from each group) selected for immunohistochemical study were evaluated using a light microscope, by 2 calibrated examiners blinded to the group being analyzed. Five regions of each tooth were scored according to a previously published[5] system for scoring immunoexpression intensity:

- no (0),
- weak (1),
- moderate (2) and
- intense expression (3).

It was possible to compare all cases of each proteoglycan studied, because all the specimens were treated equally.[10] Comparisons of the scores for each proteoglycan, among the groups, were analyzed using the Kruskal-Wallis test at a significance level of $p < 0.05$.

Results

Morphologic features

The dental pulp revealed four distinct zones:

- an odontoblastic zone,
- a cell-free zone,
- a cell-rich zone, and
- the pulp core (Figure 1A).

Descriptive analysis of microscopic sections taken from primary teeth in the process of root resorption, stained by hematoxylin-eosin, showed a loose connective tissue with a variable number of fibroblast-like cells, odontoblasts and undifferentiated mesenchymal cells dispersed among collagen fibers, blood vessels and nerve fibers in the coronary part of the teeth in Groups I and II. The odontoblastic layer was preserved mainly in coronary areas (Groups I and II). In apical areas of Groups I and II, inflammatory alterations of the pulp were observed, such as the presence of mononuclear inflammatory cells, odontoclasts in resorption lacunae on the inner surface (Figure 1B) and variations in the odontoblast layer that shows disorganization. In Group III, the remaining pulp was permeated by an inflammatory cell infiltration, and the odontoblast layer was discontinuous.

Immunohistochemical features

Tissue staining specificity was confirmed by lack of immunostaining in the negative controls.

Biglycan and decorin (semi-quantitative analysis)

Both biglycan and decorin were expressed in the ECM of the pulp tissue of the human primary teeth at different stages of root resorption, without differences in the proteins, among the groups.

Biglycan

In the three groups, this proteoglycan had weak expression in dentin, in the odontoblastic layer and in the adjacent resorption zone. However, the expression was intense in predentin and in the tissues in the resorption process.

Decorin

Decorin was not expressed in the odontoblastic layer. The expression was weak in dentin, in predentin and in tissues in the process of resorption, in all the groups. Only in the adjacent resorption zone was the expression intense.

Results are summarized in Figure 1 and Table 2.

Discussion

Biglycan and decorin have previously been reported to be involved in angiogenesis[11] and renal tissues,[12] and have been studied mainly in bone ECM.[13,14] In addition, these proteoglycans have been studied in inflamed human periodontium[15] and in odontogenic tumors.[9]

Biglycan and decorin are SLRPs that contain two

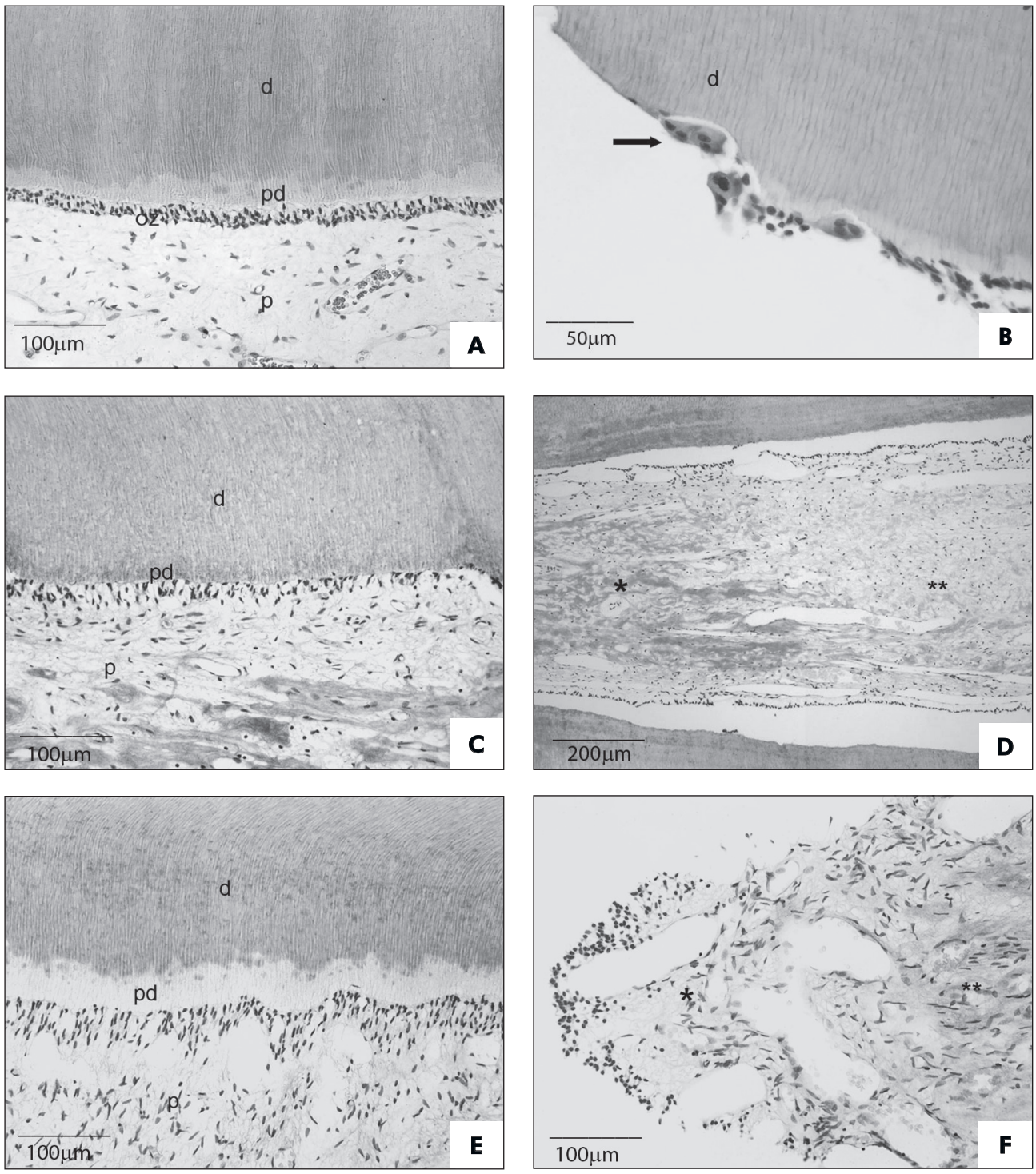

Figure 1 - Hematoxylin-eosin staining of the dentin and pulp of deciduous teeth and immunohistochemical expression of biglycan and decorin. **A:** Coronary morphology showing dentin (**d**), predentin (**pd**), odontoblastic zone (**oz**) and pulp (**p**). **B:** Apical areas of teeth of Group II showing disorganization in odontoblastic layer and odontoclasts in resorption lacunae (**arrow**). **C:** and **D:** Group I of biglycan. **C:** Weak expression in dentin (**d**), intense in predentin (**pd**) and moderate in pulp (**p**). **D:** Moderate expression in tissues in resorption process, apical areas (*) and weak expression in resorption adjacent zone (**). **E** and **F:** Group II of decorin. **E:** Weak expression in dentin (**d**) and predentin (**pd**) and moderate in pulp (**p**). **F:** Weak expression in tissues in resorption process, apical areas (*) and moderate in resorption adjacent zone (**).

Table 2- Scores of immunoexpression intensity (median) of each protein according to the stage of resorption of primary teeth and studied locations.

	Biglycan			Decorin		
	Group I	Group II	Group III	Group I	Group II	Group III
Dentin	1 ^{Aa}	1 ^{Aa}	1 ^{Aa}	1 ^{Aa}	1 ^{Aa}	1 ^{Aa}
Predentin	3 ^{Aa}	3 ^{Aa}	3 ^{Aa}	1 ^{Ab}	1 ^{Ab}	1 ^{Ab}
Odontoblasts	1 ^{Aa}	1 ^{Aa}	1 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}
Adjacent resorption zone	1 ^{Aa}	1 ^{Aa}	1 ^{Aa}	3 ^{Ab}	3 ^{Ab}	3 ^{Ab}
Tissues in resorption	3 ^{Aa}	3 ^{Aa}	3 ^{Aa}	1 ^{Ab}	1 ^{Ab}	1 ^{Ab}

Values with different letters have statistically different results ($p < 0.05$) analyzed in rows, based on Kruskal-Wallis test. Scores 1 (weak), 2 (moderate) and 3 (intense). Capital letters for comparisons between the groups of the same protein. Small letters for comparisons between different proteins of the same group.

and one chondroitin sulfate chain, respectively, on small core proteins near the NH_2 terminal sequences, where they have clear differences. The presence of these proteoglycans was detected immunohistochemically in human and animal pulp, and in dental germs under development.[4,6,16,17] The specificities of these antibodies were previously described[18] and the use of chondroitinase ABC was imperative to remove the chondroitin sulfate chains and expose the epitopes for antibodies linkage.

Biglycan

In the literature, biglycan seems to be associated with more specialized cell types.[13] Therefore, biglycan-deficient mice develop an osteoporosis-like phenotype, because the biglycan influences genes associated with proliferation and differentiation of osteoblast progenitors.[19,20] Moreover, biglycan mRNA was intensely expressed in rat tooth germs, including presecretory odontoblasts. This suggests that biglycan was involved in odontoblast differentiation,[16] but that its expression decreases in mature odontoblasts.[4] Consistent with these observations, our results demonstrate a weak expression of this proteoglycan in the odontoblastic layer.

In this study, biglycan was intensely stained in predentin, in all three groups studied (Figure 1C). These results were in agreement with the literature, which reports an intense presence of biglycan in predentin dental germs of newborn rats[7] at 7 and 11 days of age.[4] Moreover, biglycan was observed in human tooth predentin associated with collagen fibers.[6] Additionally, the mean diameter of collagen fibrils in the biglycan knockout mice was smaller in the proximal predentin, but larger in the central and distal predentin.[17] Furthermore, biglycan was able to increase the coalescence of calcospherites[21] and apatite formation.[22] Therefore, these data suggest that biglycan can affect predentin mineralization.

In this study biglycan was weakly stained in dentin (Figure 1 C). A similar distribution pattern was observed in both permanent human[6] and adult rat teeth.[7]

In the pulp tissue, biglycan was expressed in this study in the resorption area (Figure 1D). This finding is in agreement with the literature describing biglycan as a proinflammatory proteoglycan that acts on macrophages, increasing IL-1β and stimulating the expression of TNF-alpha.[23] Stimulation of fibroblasts with IL-1β has a long-lasting effect, leading to significantly increased osteoclastogenesis;[24] moreover, fibroblasts, endothelial cells and macrophages are capable of responding to TNF-alpha and release enzymes from specific granule storage sites called metalloproteinases. These proteolytic enzymes mediate ECM degradation.[25] TNF-alpha can act in osteoclastogenesis via receptors on stromal or osteoblastic cells to enhance the receptor activator of the nuclear factor kappa B ligand (RANKL) expression.[26] The RANKL/Osteoprotegerin system is known to regulate osteoclast differentiation and maturation. The RANKL stimulates osteoclast formation and osteoprotegerin inhibits it. Moreover, the RANKL/Osteoprotegerin system has been shown to be related to odontoclast activity.[27] Our data suggest the important role of biglycan not just in tooth development, but also in all phases of the biological cycle of the primary teeth. Biglycan also participates in the pre-

dentin mineralization processes and in the inflammatory pulp connective tissue stimulus.

Decorin

Decorin plays a vital role in many important cellular processes in several tissues. It interacts with various growth factors to regulate processes like collagen fibrillogenesis, ECM compilation and cell-cycle progression.[28] Unlike biglycan, the bone mass in decorin-deficient mice remains normal.[20]

In this study, decorin was expressed uniformly in all the groups studied. Its expression was weak in dentin and predentin (Figure 1E). These results were partly consistent with the literature, which describes a weak labeling for decorin in dentin and a moderate expression in the predentin of rat and human permanent dental tissue.[4,6,7] Comparison of different species and dental cycle stages could explain these dissonant results. Moreover, in human predentin, decorin is associated with collagen fibers, but compared to biglycan, this association seems to be linked to block initiation of mineralization.[29] According to the literature, the expression of decorin in the pulp of young rats may contribute to the undifferentiated state of some cells, and to the continued unmineralized state of pulp tissue.[4] In this study, although primary teeth under resorption were used, decorin was also expressed in pulp tissue, even in Group 3, suggesting a possible role as a mineralization inhibitor, as previously reported.

In the resorption area, a weak expression of decorin was observed; curiously, a stronger expression was verified in the adjacent resorption area (Figure 1F). In Group III, the expression was strong and uniform in pulp. Decorin induces apoptosis in tumor cells acting in the cell cycle, increasing the production of p21 (WAF). Moreover, it decreases the TGF beta 1 expression.[30] *In vivo,* an increase in TGF beta 1 results in rat lung fibrosis; in addition, overexpression of decorin can decrease this fibrosis by influencing collagen fiber production. In addition, decorin was also associated with angiogenesis, mainly in the inflammatory process.[11] By combining this information, it may be inferred that the strong expression of decorin near the resorption area could demonstrate how this area prepares for resorption, including an alteration in collagen fiber structure, an increase in vascularization and an induction of cell apoptosis.

Both proteoglycans participate in events that occur in the primary tooth lifespan. Biglycan seems to be linked to odontoblast differentiation, dentin mineralization and pulp inflammation processes. On the other hand, decorin seems to be associated to the blocking of dentin-pulp complex mineralization and to pulp resorption preparation.

Conclusion

Biglycan and decorin were found differentially in hard and soft primary dental tissue in resorption. Moreover, this distribution is common in the different phases of resorption. Biglycan was expressed mainly in the predentin and in the pulp resorption area; on the other hand, decorin was expressed mainly near the pulp resorption area.

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