doi:10.1111/j.1365-2591.2007.01263.x

Immunohistochemical identification of type I and type III collagen and chondroitin sulphate in human pre-dentine: a correlative FEI-SEM/TEM study

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Abstract

Orsini G, Ruggeri A Jr, Mazzoni A, Papa V, Piccirilli M, Falconi M, Di Lenarda R, Breschi L. Immunohistochemical identification of type I and type III collagen and chondroitin sulphate in human pre-dentine: a correlative FEI-SEM/TEM study. *International Endodontic Journal*, **40**, 669–678, 2007.

Aim To identify type I- (I-CF) and type III-collagen fibrils (III-CF) and chondroitin 4/6 sulphate (CS) within human pre-dentine by means of a correlative analysis under field emission in-lens-scanning electron microscopy (FEI-SEM) and transmission electron microscopy (TEM).

Methodology Human-extracted teeth were obtained and submitted to either a pre-embedding or a post-embedding immunolabelling procedure using monoclonal primary antibodies anti-I-CF, anti-III-CF and anti-CS. Gold-conjugated secondary antibodies were coupled to primary antibodies to visualize labelling under the electron beam. Correlative labelling patterns were obtained for I-CF and CS under both FEI-SEM and TEM.

Results Field emission in lens-SEM analysis revealed an intricate three-dimensional network of I-CF and CS clarifying the intimate relationship between the two main components of the pre-dentine organic matrix. TEM analysis revealed odontoblasts exhibiting intracellular labelling for CS, which became more intense and diffuse over the pre-dentine organic matrix. The same diffuse immunoreaction was revealed for I-CF, whereas a weak immunolocalization of III-CF was found scattered throughout the pre-dentine layer and over the collagen fibrils.

Conclusions Both the pre- and post-embedding immunohistochemical approaches have led to the visualization of CF- and CS-labelling distribution within the pre-dentine layer, adding further knowledge on the elucidation of collagen–proteoglycans interaction in the organic matrix of human dental roots.

Keywords: chondroitin sulphate, electron microscopy, immunohistochemistry, pre-dentine, type I collagen, type III collagen.

Received 15 November 2006; accepted 8 February 2007

Introduction

Dentine is first deposited by the odontoblasts as a layer of non-mineralized organic matrix called pre-dentine that lines the innermost pulpal portion and that varies in thickness; however, it remains constant during ageing because the amount that calcifies is balanced by addition of newly-secreted matrix (Nanci 2003). The first sign of pre-dentine formation is the appearance of large-diameter collagen fibrils, called von Korff fibres, that appear at least during radicular circumpulpal dentine formation and mainly consist of type III-collagen fibrils (III-CF) (Ohsaki & Nagata 1994, Kitasako *et al.* 2002). Although their existence

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remained controversial (Ten Cate 1978), ultrathin sections of demineralized cat teeth revealed frequent bundles of III-CF passing from between the odontoblasts into the pulp (Bishop *et al.* 1991). Then, as the odontoblasts continue to increase in size, they produce type I-collagen fibrils (I-CF) extending their processes into the forming extracellular matrix and budding off a number of membrane-bound matrix vescicles that are considered as the first sites of early mineral deposition. In addition to collagen, proteoglycans (PGs) and their constituent glycosaminoglycans (GAGs) are critical components of the pre-dentine and dentine extracellular organic matrix that regulate mineral deposition, acting as promoters or inhibitors, being their roles indicated by their distribution (Goldberg *et al.* 2003, Nanci 2003).

Biochemical, histochemical and autoradiographic investigations demonstrated that small leucine-rich PGs, including chondroitin 4/6 sulphate (CS)-rich decorin and biglycan, have some important functional implications in collagen assembly of pre-dentine, where nascent collagen fibrils are secreted from the proximal to the distal area before undergoing mineralization (Goldberg & Takagi 1993, Hall *et al.* 1999). The assembly of the collagenous organic matrix prior to mineralization is a key step in the formation of dental hard tissues; however, conflicting views have been expressed on the role of PGs in dentinogenesis and mineral formation (Goldberg & Takagi 1993). One approach towards elucidating the morphological features of mineralized tissue components is to take advantage of the resolving power of the electron microscope and to reveal their localization by an *in situ* identification carried out by means of immunohistochemistry (McKee & Nanci 1993, Mesgouez *et al.* 2006).

Numerous experiments have investigated pre-dentine and dentine PGs in continuously forming rat incisors (Septier *et al.* 1998, 2001, Goldberg *et al.* 2003), and in bovine teeth (Waddington *et al.* 2003, Milan *et al.* 2005). Garcia *et al.* (2003) have immunolocalized by immunohistochemistry and immunofluorescence presence of type I collagen, osteonectin and bone sialoprotein in pre-dentine and odontoblasts of dental human pulps. However, no studies using the high resolution power of the electron microscopy have currently mapped either presence or distribution patterns of both collagen and PGs throughout pre-dentine of human teeth, because of the difficulty of exposing the radicular circumpulpal pre-dentine without causing structural alterations. Indeed, there appear to be no data currently available on the appearance of circumpulpal human radicular pre-dentine and on the final distribution rate of I-CF, III-CF and CS. This information is of outmost importance as the pattern of expression and the distribution of matrix constituents is pertinent to describe the mechanisms involved in human dentinogenesis. The aim of the present study was to investigate the distribution of labelling patterns of I-CF, III-CF and CS-rich PGs within the pre-dentine of human dental roots, using a correlative morphological approach (Breschi *et al.* 2003a), i.e. a pre- and post-embedding immunohistochemical techniques were analysed under field emission in-lens-SEM (FEI-SEM) and transmission electron microscopy (TEM).

Materials and methods

Ten human teeth (incisors and canines) scheduled for extraction for periodontal reasons in subjects with a mean age of 56.9 years were selected. Informed consent was obtained under a protocol that has been approved by the Ethics Committee of the University of Bologna, Italy. Crowns were removed with a low speed diamond saw (Remet, Casalecchio di Reno, Italy) under water irrigation. Each tooth was then transversally sectioned to obtain 2-mm thick dentine discs in the middle third of the root. Dentine discs were randomly assigned to the following groups: FEI-SEM group, in which discs were fractured to expose the inner surface of the canal lumen and underwent a pre-embedding immunohistochemical procedure. In TEM group, discs were left intact, immediately fixed, decalcified and processed for post-embedding immunohistochemistry (specific sections will follow).

Pre-embedding technique and FEI-SEM tissue processing

Un-fixed specimens of the FEI-SEM group were processed for a double pre-embedding immunolabelling procedure (Breschi *et al.* 2003b). After carefully removing the pulp, and exposing the inner surface of the canal lumen to two monoclonal primary antibodies were used: an IgG anti-I-CF and an IgM anti-CS (mouse monoclonal, Sigma Chemical Co., St Louis, MO, USA), to detect simultaneously the distribution of antigenically intact I-CF and CS-containing PGs. Specimens were immersed in 0.05 mol L^{-1} Tris–HCl buffered solutions with 0.15 mol L^{-1} NaCl and 0.1% bovine serum albumin (TBS; pH 7.6), and then pre-incubated for 30 min in TBS 0.05 mol L^{-1} normal goat serum

(British BioCell International, Cardiff, UK) at pH 7.6 to obtain preliminary antigen saturation. Incubation was subsequently performed by using both primary antibodies at 37 °C for 90 min, specimens were then rinsed with 0.05 mol L^{-1} TBS at pH 7.6, then with 0.02M TBS at pH 8.2 and placed again in TBS 0.05 mol L^{-1} normal goat serum for 30 min. Gold labelling was performed by using two secondary antibodies conjugated with gold nanoparticles of different sizes, i.e. an IgG goat anti-mouse IgG conjugated with 30-nm gold particles (British BioCell International) for type I collagen identification, and an IgG goat anti-mouse-IgM conjugated with 15-nm colloidal gold for chondroitin 4/6 sulphate identification (British BioCell International). Reaction with secondary antibodies was performed in 0.02-mol L^{-1} TBS at pH 8.2 for 90 min at room temperature. The specimens were then rinsed in 0.02-mol L^{-1} TBS at pH 8.2 and in distilled water. Specimens were fixed in 2.5% glutaraldehyde in 0.1-mol L^{-1} cacodylate buffer at pH 7.2 for 4 h at room temperature, rinsed in 0.15-mol L^{-1} cacodylate buffer and in distilled water. Specimens were then dehydrated in ascending ethanol series (25%, 50%, 75%, 90%, 95% and 100%) and dried with hexamethyldisilazane (Sigma Chemical Co.) (Perdigão *et al.* 1995. Specimens were mounted on the microscope stubs and coated with a 1-nm thick layer of evaporated carbon using a Balzers Med 010 Multicoating System (Bal-Tec AG, Liechtenstein).

Additional specimens were prepared with a single immunohistochemical pre-embedding technique and incubated with a monoclonal primary antibody anti-III-CF (Sigma Chemical Co., Breschi *et al.* 2003a).

Observations were performed under a field emission in lens SEM (FEI-SEM) JEOL JSM 890 (JEOL, Tokyo, Japan) at 7-KV accelerating voltage and 1×10^{-12} Amp probe current. Final images were obtained with both back scattered and secondary electron signals and were performed by utilizing the proprietary IMAGE-ANALYSIS software of the microscope (JSMSCSI, JEOL Italia SpA, Milan, Italy).

Control specimens were performed as previously described by Breschi *et al.* (2003b).

Tissue processing for TEM group and post-embedding technique

Discs assigned to the TEM group were carefully rinsed in saline solution and immediately fixed in 0.4% paraformaldehyde–0.1% glutaraldehyde in 0.1-mol L^{-1} cacodylate buffer at pH 7.2, overnight at 4 °C. Specimens were then washed for 1 h in cacodylate buffer and decalcified using 4.13% EDTA (Warshawsky & Moore 1967) for 90 days followed by extensive rinsing in 0.1-mol L^{-1} cacodylate buffer at pH 7.2. Samples were dehydrated in graded concentrations of ethanol and embedded in LR White resin (London Resin, London, UK). Semi-thin sections (1 μm) were cut with glass knives on a Reichert Jung Ultracut E ultramicrotome and stained with toluidine blue. Selected areas of the 1-μm thick sections were trimmed for ultrathin sectioning (80 nm) using a diamond knife and mounted on 200-mesh formvar carbon-coated nickel grids.

Then, grid-mounted tissue thin sections were processed for immunohistochemical labelling. Three incubations were carried out following the same protocol as previously described in the pre-embedding technique using primary antibodies anti-I-CF, III-CF and anti-CS. After incubation with the primary antibodies, samples were rinsed and gold labelling was performed by using an IgG goat antimouse-IgG conjugated with 15-nm colloidal gold for I-CF and III-CF identification and an IgG goat anti-mouse-IgM conjugated with 15-nm colloidal gold for CS identification. Grids were then stained with 4% uranyl acetate and lead citrate for examination in a Jeol 1010 TEM operated at 60 kV (JEOL Ltd, Tokyo, Japan). The TEM was connected with a Digital Camera MegaView III equipped with the Analysis Imaging System GmbH (Munster, Germany).

Controls consisted of sections incubated with secondary antibodies only.

Results

Low-magnification FEI-SEM micrographs revealed an intricate network of unmineralized collagen fibrils. No crystallites or other debris were visible. Tubular lumen was not visible but collagen network appeared to be circularly arranged in the peritubular area. This configuration seems to create a sketch of a tubular structure in this unmineralized tissue.

The use of FEI-SEM allowed visualization of type I collagen and PGs labelling distribution in the predentine tissue (Fig. 1a–d) by mixing back scattered and secondary electron signal. Low magnifications images showed an homogenous distribution of the 30-nm gold particles anchored to collagen fibrillar network (Fig. 1a,b). High-magnification images revealed the 15-nm gold particles specific for chondroitin 4/6 sulphate of PGs (Fig. 1c,d). CS were mainly imaged as globular aggregates labelled by a cluster of 2–3 gold nanoparticles along the branching points of the collagen

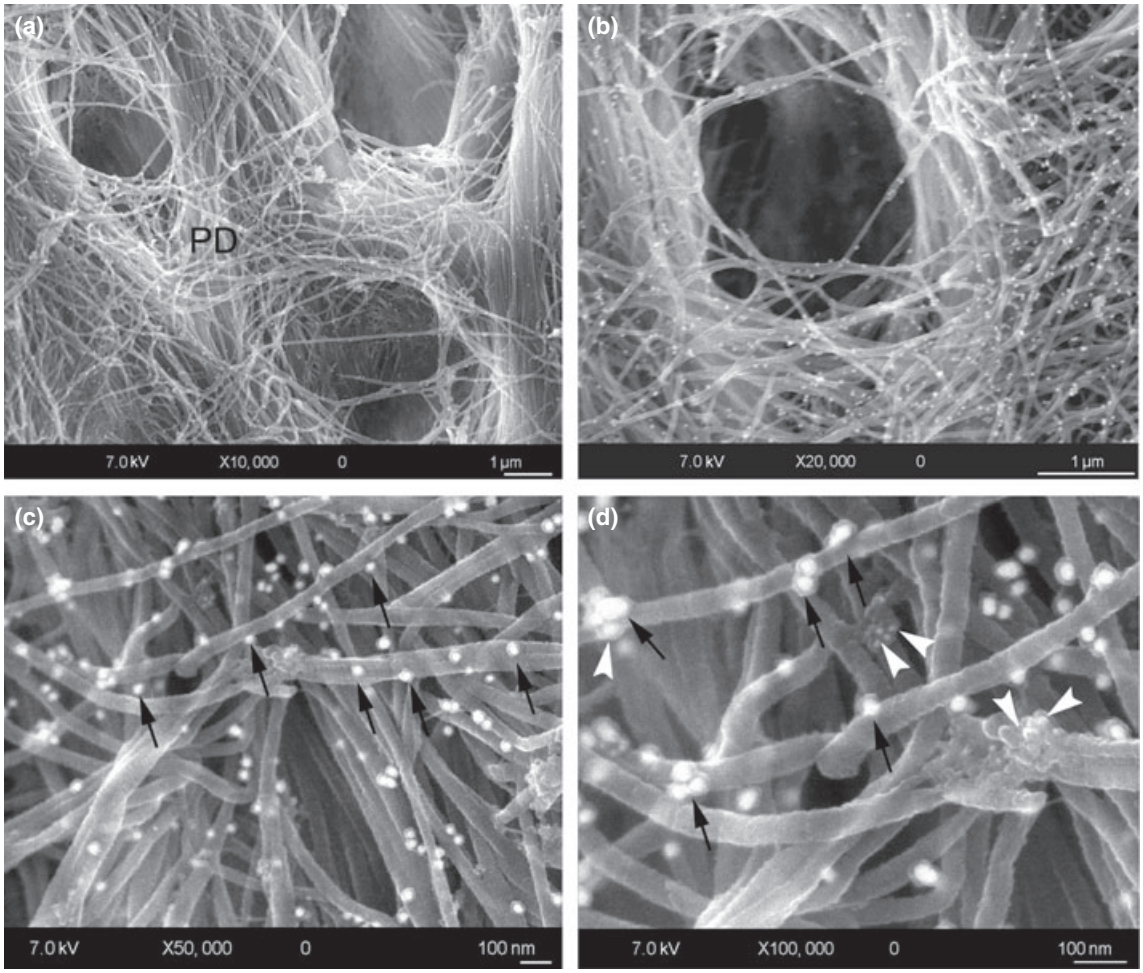

Figure 1 Field emission in-lens-scanning electron microscopy (FEI-SEM) micrographs showing chondroitin 4/6 sulphate (CS) and type I-collagen fibril (I-CF) labelling in human pre-dentine after pre-embedding technique: (a) low-magnification image of pre-dentine (PD) showing an intricate network of not-mineralized fibrils lining the innermost pulpal portion of the pulp chamber. (b) Tubule orifice and peritubular dentine matrix revealing the gold nanoparticles of 30-nm diameter (identifying I-CF) as white reflective spots along the fibrils. (c) High-magnification image of the intertubular dentine matrix revealing a porous network of I-CF (70–80-nm diameter) showing the typical banding and a repetitive-positive labelling for I-CF (arrows). (d) High-magnification image of the peritubular dentine network revealing the three-dimensional arrangement of both I-CF and CS. The FEI-SEM image reveals major fibrils positively labelled for I-CF (arrows) and small branching fibrils (30–40-nm diameter) and globule-like structures (20- to 30-nm diameter) linked to the I-CF clearly labelled with 15-nm diameter gold nanoparticles thus identifying CS (white arrowheads).

fibrils (Fig. 1d). CF cross-banding was sometimes identified as an A D-periodic repetition of elevations and depressions of CF surface (Fig. 1d).

By transmission electron microscopy, an intact layer of odontoblasts lining the dental pulp was observed and a thin distinct layer of organic matrix, the pre-dentine, could be discerned between the odontoblasts and the mineralization front initially constituted by mantle dentine, and followed by mature dentine, characterized by numerous dentinal tubules hosting the odontoblastic processes (Fig. 2a). Ultrastructural analysis showed that pre-dentine presented many thin collagen fibrils (Fig. 2b), mainly dispersed in the organic extracellular matrix. Near the odontoblasts, von Korff-like fibres running in bundles were identified, mainly consisting of two portions of fibrils connected to each other at an angle and localized in proximal pre-dentine (Fig. 2c). Although the embedded tissue resulted in good overall preservation, few ultrastructural data were evident on some intracellular compartments of odontoblasts, as

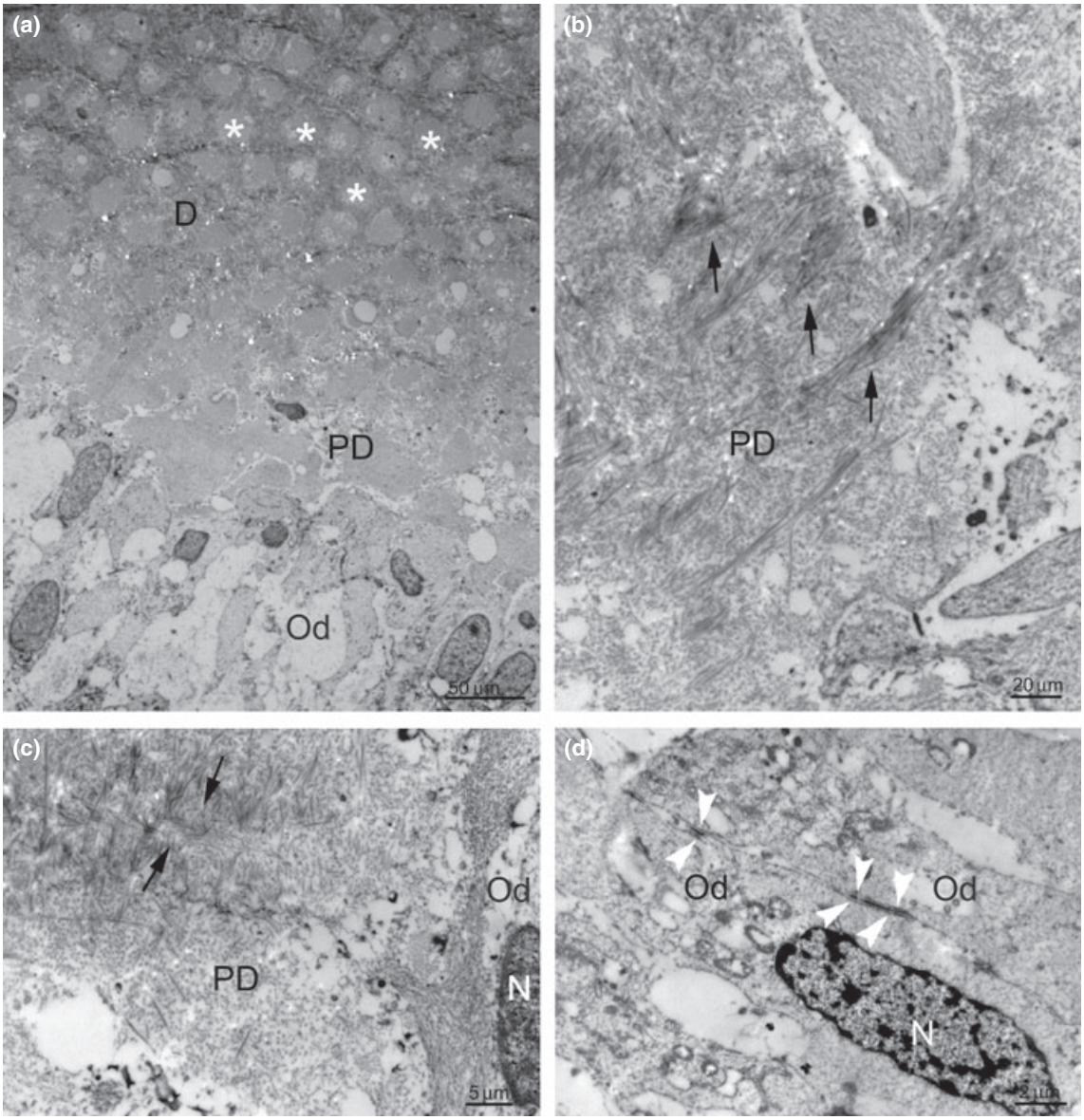

Figure 2 Transmission electron microscopy (TEM) micrographs illustrating the morphology of the pre-dentine. (a) Low-magnification image of the TEM prepared specimens showing the odontoblastic layer (Od). A thin layer of unmineralized organic matrix (PD, pre-dentine) is interposed between the Od and the mineralized matrix (D, dentine) which presents numerous dentinal tubules (asterisks). Magnification bar: 50 µm. (b) The pre-dentine (PD) is characterized by bundles of collagen fibres (arrows). Magnification bar: 20 µm. (c) In close proximity with an odontoblast (Od), the so called 'von-Korff-like fibres' can be visualized (arrows); N, nucleus. Magnification bar: 5 µm. (d) Odontoblasts (Od) are polarized cells typically lining the pulp, in contact with each other by means of junctional complexes (white arrowheads); N, nucleus. Magnification bar: 2 µm.

protein-synthesizing organelles and secretory granules were poorly defined. Furthermore, the cross-sectional method of obtaining dentine discs may have resulted in lack of detail. Junctional complexes were found between the odontoblastic cells (Fig. 2d).

Transmission electron microscopy analysis revealed CS labelling over the entire pre-dentine layer (Fig. 3a). Some CS labelling was present over the odontoblasts cytoplasm (Fig. 3b), but the endoplasmic reticulum and the classical collagen-containing cylindrical secretory

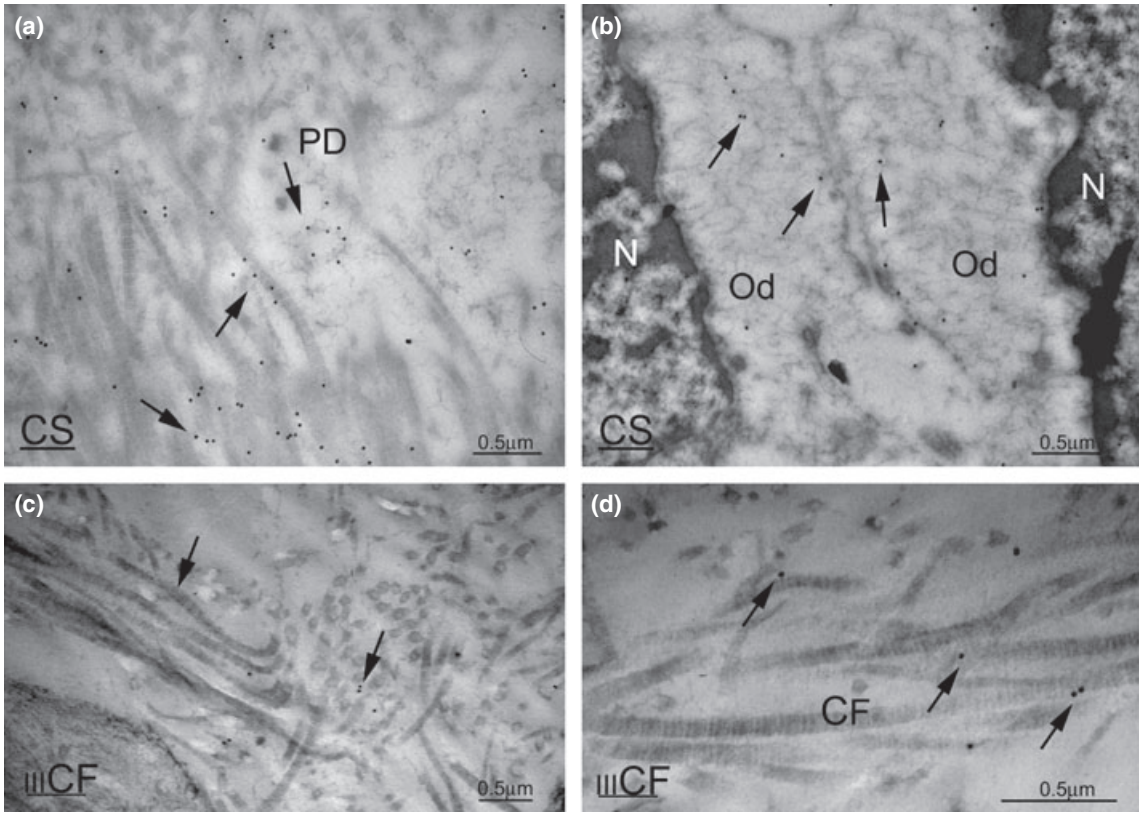

Figure 3 Transmission electron microscopy micrographs showing immunoreactivity for chondroitin 4/6 sulphate (CS) and III-CF by a post-embedding procedure. (a) CS labelling is diffuse (arrows) over the pre-dentine organic matrix. (b) Two odontoblasts (Od) showing initial immunoreaction for CS into the cytoplasms (arrows). Few gold particles were present over the nuclei (N). (c) III-CF immunoreaction was very weak over the longitudinal and cross-sectional collagen fibrils forming the von Korff-like fibres (arrows). (d) High magnification of collagen fibrils (CF), showing typical collagen fibrillar periodicity. There are very few gold particles (arrows) that seem to be scarcely specific for the visualized CF. Magnification Bar: 0.5 μm.

granules were generally poorly preserved and it was difficult to associate any labelling with them. Few gold particles were present over the nuclei. In mantle dentine, where mineralization occurs focally, the labelling was seen throughout and over the electron-dense mineralization foci (data not shown). No reduction of CS antigenicity occurred after EDTA treatment of the samples.

Labelling for type I-CF basically followed the same pattern encountered for CS immunoreaction (data not shown). On the other hand, Type III-CF within the interodontoblastic collagen fibres and over the thin CF dispersed into pre-dentine showed a weak labelling (Fig. 3c). In electron micrographs that showed longitudinal CF, collagen banding was evident (Fig. 3d).

Control specimens (incubated without the primary antibody) showed no labelling, thus confirming no cross-reaction between the secondary antibody and the pre-dentine substrate.

Discussion

Precise composition and distribution of specific molecules may be obtained by immunohistochemical techniques with colloidal gold probes, thus fulfilling a fundamental need to relate form to function in the mineralized tissues (McKee & Nanci 1993). Interpretation of immunohistochemical data derived from studies on mineralized tissues requires an appreciation of the nature of matrix–mineral relationships and how they are affected by the various tissue-processing protocols used to prepare the specimens for ultrastructural examination (McKee & Nanci 1995).

High-resolution FEI-SEM and TEM correlative assay allowed the assessment of the three-dimensional conformation of the pre-dentine organic matrix and revealed positive immuno-reactivity of human radicular pre-dentine for I-CF, III-CF and CS.

A distinct and intense immuno-reaction for I-CF and CS was seen under FEI-SEM over the three-dimensional network of fibrillar structures constituting the pre-dentine organic matrix, thus confirming a good preservation of collagenous and noncollagenous components, and showing aspects similar to the ones previously reported for intact sound dentine (Breschi *et al.* 2003a, Suppa *et al.* 2006). As highly specific monoclonal antibodies are used, the use of a pre-embedding double immunolabelling technique on unfixed calcified root dentine results in a selective binding with the target substrate (Breschi *et al.* 2002, 2003a,b). In previous investigations, it has been demonstrated that reaction occurs only in small epitopes because of the high specificity of the immuno-reaction, thus leading to a weak labelling, when protein epitopes are not perfectly preserved from denaturation and fixation (Scott 1990, Willingham 1999). Moreover, the presence of noncollagenous protein over the CF surface may strongly reduce the labelling (Dahl *et al.* 1998). The difficulty in identifying and immunolabelling the pre-dentine of human teeth is due to its low accessibility. This might be especially true for FEI-SEM evaluation, as, even if allowing a visualization with high resolution the morphological features of dentine, it may have some limitation in distinguishing the thin pre-dentine layer that is interposed between the odontoblasts and the mineralization front. The thickness of pre-dentine could be regarded as reflecting the time required for extracellular processing and organization of newly-secreted collagen into an insoluble meshwork of I-CF required to support mineral phase; however, this layer has been reported to be approximately in a range of 10–50 μm (Nanci 2003). III-CF-incubated specimens showed no distinct labelling under FEI-SEM. Indeed, TEM analysis may help render more distinguishable organic matrix of pre-dentine, thus correlating the initial gross findings obtained by FEI-SEM with the more fine structural details allowed by TEM observation.

As previous results have demonstrated a nonrepetitive-positive labelling of samples treated with a pre-embedding approach at TEM, because of technical problems during sample preparation, a post-embedding technique was performed for studying the EDTA decalcified TEM group (Breschi *et al.* 2003a). The findings of the present study demonstrated that decalcified specimens undergoing post-embedding technique revealed similar antigenicity, testified by a positive labelling for I-CF and CS over the pre-dentine, i.e. extended EDTA demineralization does not seem to alter the native structural or biochemical properties of pre-dentine, thus confirming the FEI-SEM data obtained on undecalcified unfixed samples. This can be considered true especially for the major components of a hard tissue, whilst, in some cases, when a minor secretory product should be identified, EDTA might partially extract or denature the protein, resulting in a slight reduction of the immunohistochemical labelling (Camarda *et al.* 1987).

It has been reported, at least in rodents, that radicular pre-dentine and mantle dentine appear as an unorganized bundle of collagen fibrils that tend to become more ordered and parallel to the long axis of the tooth (Ten Cate 1978, McKee *et al.* 1996). However, Bosshardt & Schroeder (1996) and Bosshardt *et al.* (1998) have reviewed that species particularities may account for different fibril organization and dentine rate formation. In fact, in the rat molar, the speed of root elongation is initially extremely fast and slow down considerably as root formation progresses. On the other hand, in human teeth, root elongation and dentine mineralization progress very slowly and at quite a constant rate (Bosshardt & Schroeder 1996). TEM and SEM studies showed the existence of inter-odontoblastic collagen fibrils in human dentine (Sogaard-Pedersen *et al.* 1990), in the root of cat dentine and pre-dentine (Bishop *et al.* 1991) and in mechanically exposed monkey dental pulp (Kitasako *et al.* 2000, Goldberg *et al.* 2003). Indeed, because of their frequent appearance, they could be an important source of collagen for dentine substrate (Goldberg *et al.* 2003).

Classic von Korff fibres were postulated to help guide the odontoblasts in their pulpward migration during dentinogenesis and to bind the soft tissue pulp and odontoblasts to the dentine (Bishop *et al.* 1991, Kitasako *et al.* 2002). The findings of the present study, in particular the TEM images, revealed the presence of von Korff-like fibres and a generally not well-organized accumulation of thin fibrils in the proximal part of pre-dentine, near the odontoblasts. The results confirmed recent reports in which expression of I-CF was identified in pre-dentine of dental germs and erupted teeth, and in cultured odontoblasts (Garcia *et al.* 2003, Mesgouez *et al.* 2006). Furthermore, TEM micrographs revealed very weak positive labelling for III-CF of the forming

collagen fibrils throughout the entire pre-dentine layer, until the mineralization front. This is only partially in accordance with the studies of Ohsaki & Nagata (1994) that showed a strong staining for III-CF in interodontoblastic fibres of rat molars. In human developing teeth, Lukinmaa *et al.* (1993) have reported that odontoblasts produce type III collagen. The expression of type III proalpha collagen mRNA was examined by *in situ* hybridization and the protein product by immunostaining. Type III collagen immunoreactivity was observed in early pre-dentine and again in pre-dentine towards the completion of dentinogenesis, when mRNA was no longer detected. The study of Palosaari *et al.* (2001) confirmed these findings in cultures of mature human odontoblasts and suggested that absence of type III collagen in physiological dentine may be due to protein degradation or removal from pre-dentine during the organization of the matrix.

It is believed that the presence of PGs in the prematrix of collagen-based hard tissues, such as bone and dentine, may serve at least two functions: to help in the maturation of collagen fibrils and to inhibit mineral from forming prematurely until the collagen is ready (Boskey *et al.* 1997). Regarding the localization of the PGs covalently attached to CS-GAGs, many studies have been carried out in dentine and, in particular, the presence of PGs was recently investigated in predentine extract of bovine teeth using Western blot, electrophoresis and chromatographic analyses, revealing mostly the presence of dermatan sulphate (DS)-rich PGs, with CS-4 and CS-6 additionally identified as major constituents (Goldberg & Septier 1983, Hall & Embery 1997, Hall *et al.* 1999, Waddington *et al.* 2003, Milan *et al.* 2005). On the other hand, pre-dentine–dentine interface showed an increased proportion of CS, with corresponding decrease of DS. Immunolabelling data on rat incisors have shown that a sharp increase of the CS-rich decorin was observed in distal pre-dentine, being biglycan concentration apparently constant (Goldberg *et al.* 2003). In addition, unglycosylated forms of PGs and metabolic fragments were also identified in this pre-mineralized tissue, supporting the studies that have immunohistochemically demonstrated gradients of CS and/or DS decreasing from the pulpal aspect towards the mineralization front (Septier *et al.* 1998, Hall *et al.* 1999). Furthermore, specific metalloproteinase (MMP) gradients, identified predominantly in pre-dentine, may be the candidate proteases providing dynamic modification and PGs turnover processes occurring during mineralization (Hall *et al.* 1999, Embery *et al.* 2001). Hall *et al.* (1999) suggested that, in rat incisors, MMP-3 (stromelysin-1) may have a functional role in the degradation of CS/DS containing PGs, consequently allowing the increase of keratan sulphate PGs concentration near the mineralization front. In contrast, in mature human dentine pulp complex, Palosaari *et al.* (2003) did not detect MMP-3, but a copious expression of MMP-10 (stromelysin-2), the other member of the stromelysin group, sharing an 82% sequence homology. Therefore, it has been postulated that different stromelysins may be involved in the regulation of dentine mineralization in different species, which could be explained by the diversities in the nature of dentinogenesis in rat incisors and human teeth (Palosaari *et al.* 2003).

Conclusions

Findings of the present study revealed high percentage of CS labelling in pre-dentine of human dental roots, confirming that this GAG chains attached to predentine PGs have a pivotal role in fibril formation and promotion of the initial collagen aggregation (Embery *et al.* 2001). However, by means of the tested immunohistochemical methods, no evident CS gradient, with an expected decrease in proximity of the distal predentine, was present, because of the extreme thinness of the pre-dentine layer examined.

Finally, the use of monoclonal antibodies by means of a pre- and a post-embedding technique may represent a reproducible method to investigate collagen and PGs distribution in human pre-dentine organic matrix. A future study focusing on the difference of PGs composition in human pre-dentine and dentine will be carried out, using immunohistochemical techniques supported by biochemical assays. Moreover, specific evaluation of the distribution of small leucine-rich PGs such as decorin and biglycan will be needed to understand their postulated different roles also in human pre-dentine.

Acknowledgements

The authors wish to thank Mr Aurelio Valmori for extensive technical and photographical assistance. The investigation was supported with grants ex-60% MIUR, Italy.

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