Dentin proteoglycans: An immunocytochemical FEISEM study

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Abstract: Dentin proteoglycans are fundamental constituents of the dentin matrix and are distributed ubiquitously both in dentin and cement. They have several important functional properties; in particular, they have a fundamental role in the maintenance and the correct stabilization of collagen fibers. The use of phosphoric acid on dentin, as proposed in most common dental adhesive systems to establish a reliable bond, may affect the molecular structure of proteoglycans. The aim of this study was to evaluate, after the application of EDTA or phosphoric acid on dentin, the dentin proteoglycans with an immunocytochemical approach with high resolution SEM. For this purpose, dentin disks obtained from recently extracted human molars were etched with a 35% water solution of phosphoric acid for 15 s, 30 s, and 60 s. Control specimens were conditioned with EDTA. Specimens were immunolabeled with a monoclonal antibody antichondroitin sulfate and visualized with a gold-conjugated secondary antibody. Conditioning dentin with EDTA resulted in a distinct labeling of the proteoglycans, as visualized on branching fibrillar structures in the order of 10–20 nm. The use of 35% phosphoric acid on dentin revealed a coagulation of proteoglycans after etching for 15 s while a very low labeling signal was detectable after 30 s. No labeling was obtained after etching dentin with phosphoric acid for 60 s. These results suggest that the use of 35% phosphoric acid on dentin is able to produce significant structural modifications of the dentin proteoglycans even after short application times. Additionally, when applied on the dentin surface for more than 30 s, phosphoric acid produces a dramatic decrease in proteoglycans' antigenicity, probably due to structural modifications of the three-dimensional conformation of these molecules. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 61: 40–46, 2002.

Key words: dentin; phosphoric acid; proteoglycans; immunocytochemistry; electron microscopy

INTRODUCTION

Most modern dental adhesive techniques require the use of a preliminary etching step, usually performed clinically with 35% phosphoric acid (PA) on the instrumented dentin and enamel surfaces.[1,2] On dentin, the acid opens the dentin tubules by removing the smear plugs, and results in a superficial porous demineralized area on the intertubular dentin through which the fluid adhesive agents penetrate.[3] This process creates a complex hybrid structure formed by debris of instrumentation, residual mineral crystals, collagen fibrils (CF), and noncollagenous proteins that can be embedded by the polymerized adhesive monomer, producing a hybrid layer.[4,5] Several studies have demonstrated that the use of PA as a dentin etchant greatly improves the bonding capability, but on the other hand it may affect the organic substrate of dentin, inducing structural modifications of the CF[6–8] and of the noncollagenous proteins that constitute the dentin matrix.[9]

Proteoglycans (PG) and phosphoproteins represent the main constituents of the dentin noncollagenous proteins.[1,8,9] PG are carbohydrate polyanions with a high molecular weight (from 11,000 up to 220,000)[10,11] constituted mainly of a polypeptide core to which is attached one or more glycosaminoglycan (GAG) chains.[12–14] The GAG chains are repeating disaccharide units with sulfate ester groups linked at positions 4 or 6, and they usually are identified as hyaluronan,

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heparin sulfate, keratan sulfate (KS), dermatan sulfate, and chondroitin sulfate (CS). The presence of KS, CS-4, and CS-6 is very well described on predentin, dentin, and cement.[15,16] These GAGs regulate the biophysical properties of the dentin PG as well as their three-dimensional appearance. In fact, these molecules have the ability to fill space, bind and organize water molecules, and repel negatively charged molecules. Their high affinity to water molecules and their tight relationship with the collagen fibril network[17–21] give the PG a fundamental role in determining the properties of the dentin demineralized matrix, which represents the substrate of the dental adhesive technique.[2]

Several histochemical techniques have been employed to visualize PG, in particular, the use of periodic acid-Schiff (PAS),[22] alcian blue,[23] azure A, thionine, toluidine and cuprolinic blue, and ruthenium red.[24] Additionally, autoradiographic methods have been used.[25] All of these techniques have been applied to describe the morphologic aspects of dentin PG.[26–29] Recently an immunocytochemical approach has been proposed at the light microscopy level.[9]

The aim of this research project was to identify the human dentin PG following different application times of a solution of 35% PA (and, as control specimen, after conditioning with EDTA at pH 7.0) by means of an immunocytochemical technique with high-resolution SEM. For this purpose we utilized a monoclonal antibody anti-CS followed by a secondary antibody conjugated with 15 nm of gold particles. The possibility of obtaining morphologic details at a high level of magnification also allowed an investigation of the relationships between PG and the dentin CF.

The null hypothesis tested was that the application of PA on dentin would not modify the dentin PG antigenicity nor would it affect their structural physical properties.

MATERIALS AND METHODS

Dentin preparation

Ten dentin disks were obtained from five impacted human third molars immediately after extraction. The patient signed an informed consent. Disk preparation was performed, in order to expose middle/deep dentin, using a low-speed diamond saw (Remet, Franco Cicerchia, Casalecchio di Reno, Italy) under intense water-cooling. The dentin specimens then were ground with 600-grit wet abrasive paper to reduce the thickness and a standard smear layer on the surface, and small dentin pieces of 2 mm wide × 2 mm long and 1 mm thick were obtained.

Dentin then was etched with (1) EDTA (0.5*M*) at pH 7.0 for 30 min; (2) 35% PA solution for 15 s; (3) 35% PA solution for 30 s; and (4) 35% PA solution for 30 s followed by rinsing in saline solution and a re-application of 35% PA for 30 s. All the specimens then were rinsed with distilled water for 30 s.

Immunohistochemistry

Specimens were immersed in a 0.05-*M* Tris HCl buffer solution (TBS) at pH 7.6, with 0.15*M* of NaCl and 0.1% bovine serum albumin, and pre-incubated for 30 min in normal goat serum (NGS, British BioCell International, Cardiff; United Kingdom) in TBS (0.05*M*) at pH 7.6. Overnight incubation was performed using the primary antibody, a monoclonal antibody anti-chondroitin sulfate (mouse monoclonal, Clone CS-56, Sigma Chemical Co., St. Louis, MO) at 4°C. Gold labeling was performed using a secondary antibody, a goat anti-mouse IgG conjugated with 15 nm of colloidal gold particles (British BioCell International, Cardiff; United Kingdom) in 0.02*M* of TBS at pH 8.2 (0.02*M* of Tris HCl buffered at pH 8.2 with 0.15*M* of NaCl and 0.1% BSA) for 90 min at room temperature. Specimens then were rinsed in TBS (0.02*M*) at pH 8.2, washed in water, fixed in 2.5% glutaraldehyde in 0.1*M* of Sorensen's phosphate buffer (PB) at pH 7.2 for 4 h, and rinsed in 0.15*M* of PB. After dehydration in an ascending ethanol series, specimens were submitted to hexamethyldisilazane (HMDS) drying.[30]

FEISEM analysis

Specimens were mounted on the microscope stubs and coated with a 5-nm thick layer of evaporated carbon using a Balzers Med 010 Multicoating System (Bal-Tec AG, Liechtenstein). Observations were performed under a field emission in-lens SEM (FEISEM) JEOL JSM 890 (JEOL, Tokyo, Japan) at 7 kV accelerating voltage and 1×10^{-11} A probe current. Final images were obtained mixing both backscattered (BSI) and secondary (SEI) electron signals and by optimization of gain and brightness control levels.[31,32]

Two different observers made the observations, and images were taken from the areas of the specimens observed more frequently and considered as representative for the sample. All measurements of structures of interest were performed utilizing the image-analysis software of the microscope (JSMSCS, JEOL Italia SpA, Milan, Italy).

Controls

Controls consisted of dentin specimens processed, as previously described, and incubated overnight in TBS (0.05*M*) at pH 7.6 without the primary antibody.

RESULTS

FEISEM observations showed the CS distribution as well as the dentin morphology after the different times of application of PA. The images are the result of a mixing of the BSI, which reveals the gold nanoparticles as spheric white spots approximately 15 nm in diameter, with the SEI, which shows the dentin features at a high level of resolution.

EDTA-conditioned specimens [Fig 1(A–D)] revealed residual particles of smear layer on the intertubular dentin (ITD) surface while smear plugs appeared to be removed by the dentin tubule orifices. Labeling was

more evident on the peritubular matrix than it was on the ITD [Fig. 1(A,B)]. Higher magnification images of the peritubular and intratubular structures revealed an intense labeling of minor fibrillar structures branching from major CF [Fig. 1(C)]. A detail of these small fibrillar structures, identified by the gold nanoparticles and their relationships with the CF, is shown in Figure 1(D).

Specimens etched with the 35% PA solution revealed the typical morphologic features of PA etching (Figs. 2 and 3), such as smear-layer removal, funneling, and collar formation around the tubular orifices, regardless of the etching time. Differences from the EDTA-conditioned samples and among the different times of application were clearly evident from observations of the gold-labeling that identifies the dentin CS.

After the 15-s application of PA, the colloidal gold particles were clustered in spots [Fig. 2(A,B)]. The clusters of gold particles mostly were localized in the peritubular dentin, with a few spots evident on the ITD. Details of the clusters revealed a preferential localization of the gold particles on globular structures intimately joined to the dentin CF [Fig. 2(C)]. The globules showed as spheric electron-reflective material, with diameters ranging from 20 to 50 nm. Gold particles also were detected inside the open tubules, labeling lateral globular structures of the collagen ra-

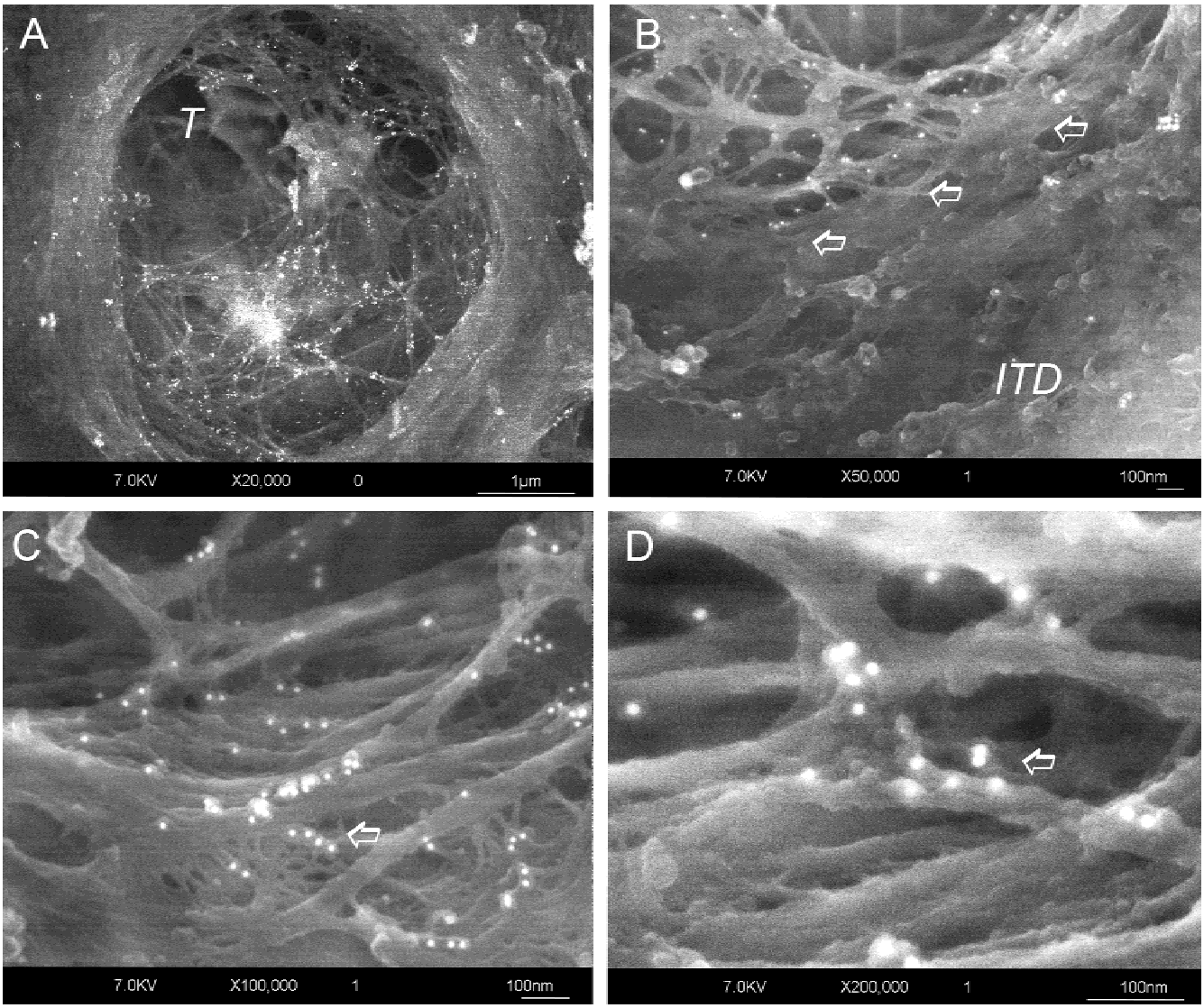

Figure 1. FEISEM micrographs of the dentin surface conditioned with EDTA for 30 min. (A) The odontoblastic tubule (T) appears partially open, with only small remnants of the tubular plugs. The *lamina limitans* is shown as an intricate network of CF. An intense labeling is detectable on minor fibrils. (B) Labeling of the PG is particularly evident inside the tubule and along the peritubular dentin matrix (arrows). Minor labeling can be detected on the intertubular dentin (ITD). (C) Image revealing the fibrils network of the peritubular dentin. Labeling of the PG (arrows) is particularly evident on minor branching fibrils that connect major CF. (D) High magnification image revealing the PG (arrows) on the peritubular dentin matrix.

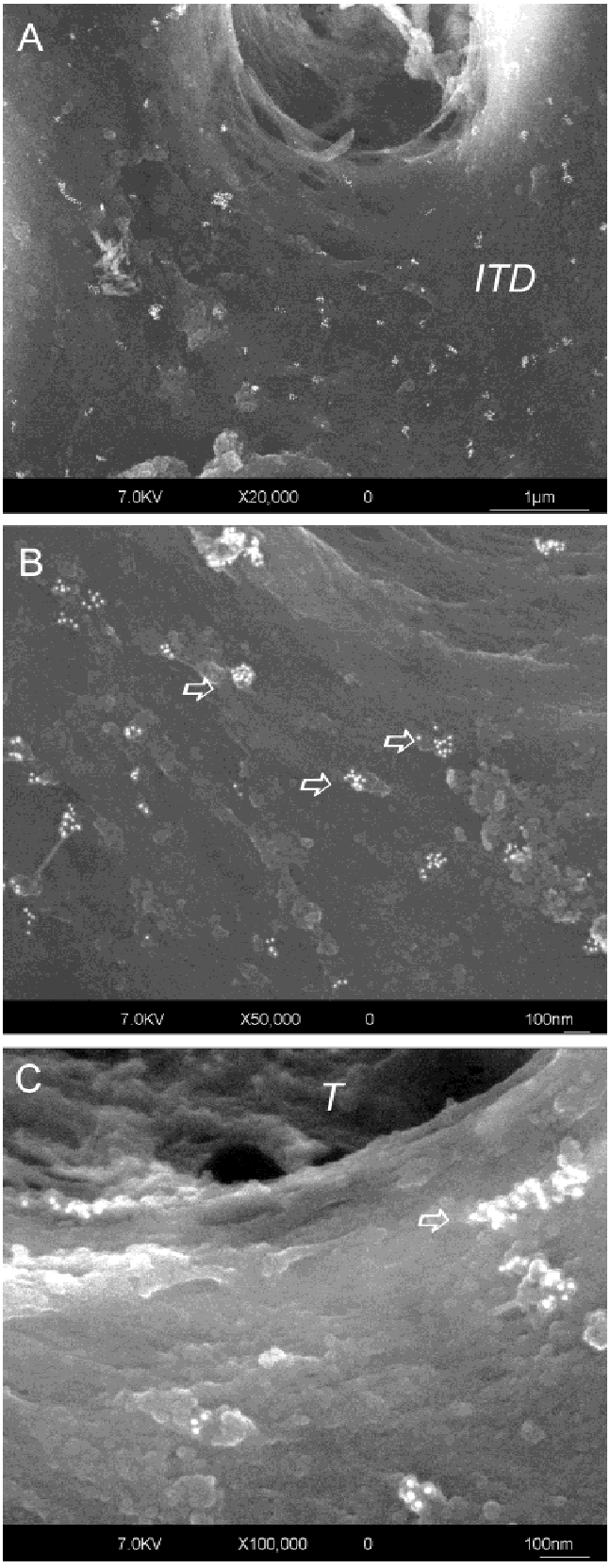

Figure 2. FEISEM micrographs of the dentin surface etched with PA for 15 s. (A) The intertubular dentin (ITD) surface reveals a non-uniform labeling of the PG as they appear as clustered in spots. (B) The PG are coagulated in globules (arrows) on a substrate of CF. (C) The clusters of gold-labeling particles also were evident on the peritubular dentin (T = odontoblastic tubule).

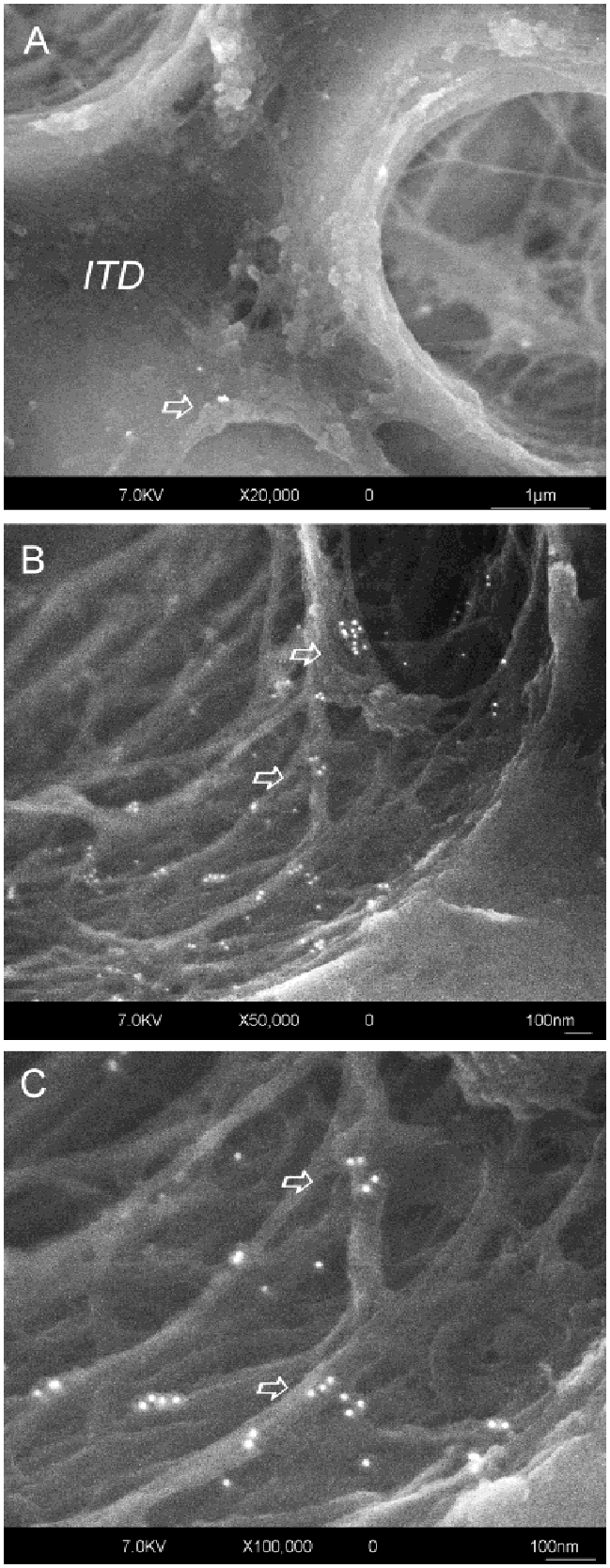

Figure 3. FEISEM micrographs of the dentin surface etched with PA for 30 s. (A) Only few scattered clusters of gold particles were still detectable on the dentin surface (arrow). (B) Inside the tubule the labeling (arrows) was still evident, revealing a minor effect of the acid on the PG of the inner surface of the open tubules. (C) High magnification image revealing detail of fibrils of different diameters, with the gold particles identifying the PG (arrows).

dial fibers that suspend the *lamina limitans* to the tubule lateral walls.

PA application longer than 15 s resulted in a dramatic decrease of the labeling, even if the clustering of the gold particles (compared to the EDTA-conditioned specimens) was confirmed. In particular, specimens etched for 30 s revealed few scattered clusters of gold nanoparticles in the intertubular dentin. High magnification of the clusters revealed features similar to the ones observed after a 15-s application, even if a smaller number of particles per cluster were identified [Fig. 3(A–C)].

Etching with 35% PA for 60 s resulted in an extremely rare cluster of labeling scattered throughout the demineralized dentin surface (image not shown).

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

DISCUSSION

Proteoglycans (PG) are fundamental components of the extracellular matrix and are distributed ubiquitously throughout human connective tissues. PG are large molecules characterized by GAG side chains that give to the complex an extremely high-fixed negative charge density, which, in turn, creates structures with high osmotic pressure. These structures have a high capacity to imbibe large amounts of water, and this peculiarity is transferred to nonmineralized connective tissues as a strong resistance to a load force that would decrease the amount of water in the PG domain.[33] On the other hand, the presence of the CF in the connective tissue matrices typically gives high resistance to traction forces. Thus the CF and the PG interact, creating a complex network of fibrillar structures that act as scaffolding in the extracellular matrix of both mineralized and nonmineralized tissues.[13,14,17,19,33]

The dentin matrix is exposed by an etching procedure that routinely is performed on the dentin surface following clinical instrumental procedures. The superficial demineralization of dentin is carried out in order to increase dentin permeability by removing the smear plugs and thus opening the tubule orifices and making the presence of a superficial demineralized layer of dentin available for adhesive infiltration.[2,34] The result is the formation of a hybrid tissue of dentin matrix and polymerized acrylic monomer commonly known as hybrid layer.[4,5] The presence of this layer has been demonstrated to be fundamental for several dental adhesives to achieve acceptable clinical and laboratory bonding properties.[35,36] Thus the etching procedure represents an essential prerequisite to creating the most suitable substrate for bonding to dentin.[37]

Questions relative to the most useful type of acid, the most adequate pH and concentration of the solution, the correct mode (undisturbed or under continuous brushing), and the proper length of time for the application of the etching have been investigated extensively in order to achieve a satisfactory bonding of the dental adhesive system to the dentin substrate.[3] Nevertheless, very scarce information is available regarding the modifications of the dentin matrix molecule after the application of these acids. Previous studies have provided evidence that CF can be affected by the use of PA and that long-time etching may induce structural modifications to the molecular integrity of the CF.[6–8]

Several studies previously have investigated the nature and the distribution of PG in predentin, dentin, and cement. The existence of PG in dentin tissue was first chemically identified many years ago,[38] but today's highly specific techniques have revealed several details as to their distribution and functional properties in the dentin tissue.

It has been demonstrated that CS-4 and CS-6 are not homogeneously distributed in the dentin matrix[39] as strong staining is evident in the dentin tubules (surrounding the odontoblastic processes),while a lower labeling has been detected on the intertubular dentin matrix. Moreover, dermatan sulfate PG was localized in the odontoblastic layer.[39] The presence of PG also was investigated in the predentin tissue,[40] revealing mostly the presence of CS-4 and CS-6.[41] CS also was found in rat[42] and human[43] cement. And with an immunolabeling technique, it was specifically localized on bovine cement in small pericellular spaces close to the lacunae,[44] with lumican and fibromodulin[45] also present in small amounts. Lumican also recently has been identified in human predentin, revealing a possible fundamental role in the mineralization process of the dentin matrix,[46] and in human cement at the border of inserted periodontal ligament fibrils.[47]

A recent study also suggests that PG play an important role in repairing the cement lines, mediating the attachment between new and old mineralized tissue,[48] and that the adhesion of PG seems to be more important than the intermingling of dentinal and cement CF in determining cemento-dentinal attachment.[49]

The results of the present study demonstrate the three-dimensional organization of dentin PG at the FEISEM level and reveal how the use of 35% PA solution on dentin affects PG structural organization as well as their morphologic appearance. In fact, major differences were observed when comparing dentin specimens obtained after demineralization with neutral EDTA and after etching with PA.

The use of neutral EDTA for 30 min was chosen as

the control specimen of the study because of the mild activity of the acid on the dentin surface.[31] We chose it also because of the previously demonstrated possibility of EDTA's preserving the antigenic binding sites of the protein compartment of the dentin matrix.[32] EDTA-treated specimens revealed a gold immunolabeling of minor fibrillar structures branching and connecting the CF in an intricate network that constitutes the dentin matrix.

These observations confirm the hypothesis on the PG nature of minor fibrils formulated in previous high-resolution SEM studies[3,8,31,50] on human dentin. In fact, the FEISEM images obtained in this study reveal an intense labeling of these structures of 10–30 nm in diameter, demonstrating the existence of an intricate network of PG fibrils that connect major CF. These morphologic data confirm the functional interaction between the PG and the CF previously shown by other studies. In fact, it is well known that PG electrostatically interact with the type I CF, regulating the fibrillogenesis and the fibrillostasis,[16] and that the GAG are not incorporated into the CF but interact with them, determining the CF morphology and functional properties.[17,51–54]

Previously a hypothesis was made as to the presence of two different classes of PG on human dentin. One localized the predentin in the spaces between the CF and was extremely susceptible to degradation,[15,27,40] being constituted of globules and filaments in close association with the CF.[17,27] The second class of PG has been shown to be more resistant to fixative and etching solutions.[27,54] Our data confirm that PG in close relationship with CF are resistant to ETDA conditioning; they reveal a very well preserved and repetitive morphology with a selective immunolabeling. On the other hand, PA shows a time-dependent effect on PG.

Morphologic and quantitative differences in the labeling intensity easily could be observed when comparing samples treated with EDTA (Fig. 1) with samples etched with PA for 15 s (Fig. 2) or 30 s (Fig. 3). The gold labeling appears not only to be greatly reduced by the PA etching, but a massive coagulation of the gold particles is shown, thus suggesting a shrinkage or collapse of the dentin PG. These modifications induced by the acid may be the result of structural changes caused in the GAG, modifying their negative charge that regulates the capability of binding water molecules.

The use of a liquid PA instead of a gel formulation (mostly used in clinical conditions) was preferred in this study in order to avoid interference due to the different viscosity and thickening agents of the PA gels. In fact, it previously was demonstrated that the different PA gel formulations available on the market are able to produce different morphologic features on a dentin surface, depending on their thickening agents and pH.[3] Additionally, a previous immunohistochemical study demonstrated that in a comparison of liquid versus gel formulation, dentin PG are affected differently by PA, showing a lower sensitivity to the gel formulation.[9]

Unetched specimens were not considered as no labeling could be revealed when the smear layer was still present on the dentin surface (data not shown).

CONCLUSIONS

The possibility of achieving a distinct and selective labeling of dentin PG using a monoclonal antibody allowed a selective labeling of the substrate and revealed several morphologic differences in the PG antigenicity relative to the different times of application of PA tested in the study.

The higher sensitivity[55,56] of the immunolabeling technique due to the use of a monoclonal antibody suggests that the application of a 35% solution of PA on the dentin surface for periods of time longer than 15 s results in a dramatic decrease of PG antigenicity, probably due to structural modifications occurring to the dentin PG during the etching process. These modifications also may affect dentin CF, as an intimate relationship between dentin PG and type I CF was observed.

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References

1. Marshall GW Jr, Marshall SJ, Kinney JH, Balooch M. The dentine substrate: Structure and properties related to bonding. J Dent 1997;25:441–458.
2. Perdigão J, Lopes M. Dentin Bonding—Questions for the new millennium. J Adhes Dent 1999;1:191–209.
3. Perdigão J, Lambrechts P, Van Meerbeek B, Tomé AR, Vanherle G, Lopes AB. Morphological field emission–SEM study of the effect of six phosphoric acid etching agents on human dentin. Dent Mater 1996;12:262–271.
4. Nakabayashi N, Kojima K, Masuhara E. The promotion of adhesion by the infiltration of monomers into tooth substrates. J Biomed Mater Res 1982;16:265–273.
5. Van Meerbeek B, Dhem A, Goret-Nicaise M, Braem M, Lambrechts P, Vanherle G. Comparative SEM and TEM examination of the ultrastructure of the resin–dentin interdiffusion zone. J Dent Res 1993;72:495–501.
6. Scott PG, Leaver AG. The degradation of human collagen by trypsin. Connect Tissue Res 1974;2:299–307.
7. Okamoto Y, Heeley JD, Dogon IL, Shintani H. Effects of phosphoric acid and tannic acid on dentine collagen. J Oral Rehab 1991;18:507–512.
8. Breschi L, Lopes M, Gobbi P, Mazzotti G, Perdigão J. Immunocytochemical identification of Type I collagen on etched human dentin. [Abstract 1706]. J Dent Res 2001;80:249.
9. Oyarzun A, Rathkamp H, Dreyer E. Immunohistochemical and ultrastructural evaluation of the effects of phosphoric acid etching on dentin proteoglycans. Eur J Oral Sci 2000;108:546–554.
10. Bourdon MA, Oldberg A, Perschbacher M, Ruoslahti E. Molecular cloning and sequence analysis of a chondroitin sulfate proteoglycan cDNA. Proc Natl Acad Sci USA 1985;82:1321–1325.

11. Doege K, Sasaki M, Horigan E, Hassell JR, Yamada Y. Complete primary structure of the rat cartilage proteoglycan core protein deducted from cDNA clones. J Biol Chem 1987;262: 17757–17767.
12. Okazaki J, Embery G, Hall RC, Hughes Wassell DT, Waddington RJ, Kamada A. Adsorption of glycosaminoglycans onto hydroxyapatite using chromatography. Biomaterials 1999;20: 309–314.
13. Evered D, Wheln J, editors. Functions of proteoglycans. Ciba Foundation Symposium 124. Chichester: John Wiley & Sons; 1986.
14. Wight TN, Mecham RP, editors. Biology of proteoglycans. New York: Academic Press; 1987.
15. Goldberg M, Takagi M. Dentine proteoglycans: Composition, ultrastructure and functions. Histochem J 1993;25:781–806.
16. Cheng H, Caterson B, Yamauchi M. Identification and immunolocalization of chondroitin sulfate proteoglycans in tooth cementum. Connect Tissue Res 1999;40:37–47.
17. Scott JE. Proteoglycan–fibrillar collagen interactions. Biochem J 1988;252:313–323.
18. Vogel KG, Paulsson M, Heinegård D. Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. Biochem J 1984;223:587–597.
19. Hedbom E, Heinegård D. Binding of fibromodulin and decorin to separate sites on fibrillar collagens. J Biol Chem 1993;268: 27307–27312.
20. Kobe B, Deisenhofer J. The leucine rich repeat: A versatile binding motif. Trends Biochem Sci 1994;19;415–421.
21. Schonherr E, Hausser H, Beavan L, Kresse H. Decorin-type I collagen interaction. Presence of separate core protein-binding domains. J Biol Chem 1995;270:2776–2783.
22. Mac Manus JFA, Cason JE. Carbohydrate histochemistry studied acetylation techniques. I. Periodic acid methods. J Exp Med 1950;91:651–658.
23. Scott JE. Alcian blue. Now you see it, now you don't. Eur J Oral Sci 1996:104:2–9.
24. Ruggieri A, Dell'Orbo C, Quacci D. Electron microscopic visualization of proteoglycans with ruthenium red. Histochem J 1977;9:249–252.
25. Odeblad E, Bostrom H. An autoradiographic study of the incorporation of ^{35}S-labeled sodium sulphate in different organs of adult rats and rabbits. Acta Pathol Microbiol Scand 1952;31: 339–343.
26. Jones JL, Leaver AG. Glycosaminoglycans of human dentine. Calcif Tissue Res 1974;16:37–44.
27. Goldberg M, Septier D. Differential staining of glycosaminoglycans in the predentine and dentine of rat incisor using cuprolinic blue at various magnesium chloride concentrations. Histochem J 1992;24:648–654.
28. Nagai N, Takuma S, Goto Y Ogiwara H. Electron microscopy of dentine and predentine of developing rat molars stained with Ruthenium red. J Biol Buccal 1974;2:73–83.
29. Embery G. The isolation of chondroitin 4 (^{35}S) sulphate from the molar teeth of young rats receiving sodium (^{35}S) sulphate. Calcif Tissue Res 1974;14:59–65.
30. Perdigão J, Lambrechts P, Van Meerbeek B, Vanherle G, Lopes ALB. A field emission SEM comparison of four post-fixation drying techniques for human dentin. J Biomed Mater Res 1995; 29:1111–1120.
31. Breschi L, Gobbi P, Mazzotti G, Ellis TH, Sacher E, Stangel I. A field emission in-lens SEM study of enamel and dentin. J Biomed Mater Res 1999;46:315–323.
32. Lin C, Douglas WH, Erlandsen SL. Scanning electron microscopy of type I collagen at the dentin–enamel junction of human teeth. J Histochem Cytochem 1993; 41:381–388.
33. Ruoslahti E. Structure and biology of proteoglycans. Ann Rev Cell Biol 1988;4:229–255.
34. Pashley DH, Carvalho RM. Dentine permeability and dentine adhesion. J Dent 1997;25:355–372.
35. Perdigão J, Lopes M, Geraldeli S, Lopes GC, Garcia-Godoy F. Effect of a sodium hypochlorite gel on dentin bonding. Dent Mater 2000; 16:311–323.
36. Prati C, Chersoni S, Pashley DH. Effect of removal of surface collagen fibrils on resin–dentin bonding. Dent Mater 1999;15: 323–331.
37. Haller B. Recent developments in dentin bonding. Am J Dent 2000;13:44–50.
38. Pincus P. Sulphated mucopolysaccharides in human dentine. Nature 1950;166:187.
39. Takagi M, Hishikawa H, Hosokawa Y, Kagami A, Rahemtulla F. Immunohistochemical localization of glycosaminoglycans in predentin and dentin in rat incisors. J Histochem Cytochem 1990;38:319–324.
40. Hall R, Septier D, Embery G, Goldberg M. Stromelysin-1 (MMP-3) in forming enamel and predentine in rat incisor-coordinated distribution with proteoglycans suggests a functional role. Histochem J 1999;31:761–770.
41. Goldberg M, Septier D. Electron microscopic visualization of proteoglycans in rat incisor predentine with cuprolinic blue. Arch Oral Biol 1983;28:79–83.
42. Vidal BC, Mello MLS, Valdrighi L. Histochemical and isotopical aspects of the rat cementum. Acta Anat 1974;89:546–551.
43. Bartold PM, Miki Y, McAllister B, Narayanan AS, Page RC. Glycosaminoglycans of human cementum. J Periodont Res 1988;23:13–17.
44. Bartold PM, Reinboth B, Nakae H, Narayanan AS, Page RC. Proteoglycans of bovine cementum: isolation and characterization. Matrix 1990;10:10–19.
45. Cheng H, Caterson B, Neame PJ, Lesters GE, Yamauchi M. Differential distribution of lumicans and fibromodulin in tooth cementum. Connect Tissue Res 1996;34:87–96.
46. Hall RC, Embery G, Lloyd D. Immunochemical localization of the small leucine-rich proteoglycan lumican in human predentine and dentine. Arch Oral Biol 1997;42:783–786.
47. Ababneh KT, Hall RC, Embery G. The proteoglycans of human cementum: Immunohistochemical localization in healthy, periodontally involved and ageing teeth. J Periodontal Res 1999; 34:87–96.
48. Yamamoto T, Domon T, Takahashi S, Suzuki R, Islam MN. The fibrillar structure of cement lines on resorbed root surfaces of human teeth. J Periodont Res 2000;35:208–213.
49. Yamamoto T, Domon T, Takahashi S, Islam N, Suzuki R, Wakita M. The structure and function of the cemento–dentinal junction human teeth. J Periodont Res 1999;34:261–268.
50. Perdigão J. An ultramorphological study of human dentine exposed to adhesive systems [PhD thesis]. The Netherlands: Catholic University of Leuven; 1995.
51. Scott JE. Proteoglycan–collagen interactions and subfibrillar structure in collagen fibrils. Implications in the development and ageing of connective tissues. J Anat 1990;169:23–35.
52. Goldberg M, Spetier D. Effect of dissociative extraction with 1.5M calcium chloride on proteoglycans in rat-incisor predentine visualized with cuprolinic blue. Arch Oral Biol 1984;29: 131–135.
53. Kneene DR, San Antonio JD, Mayne R, McQuillan DJ, Sarris G, Santoro SA, Iozzo RV. Decorin binds near the C terminus of type I collagen. J Biol Chem 2000;275:21801–21804.
54. Scott JE, Haigh M. Proteoglycan–type I collagen fibril interactions in bone and non-calcifying connective tissues. Biosci Rep 1985:5;71–81.
55. Willingham MC. Conditional epitopes: Is your antibody always specific? J Histochem Cytochem 1999;47:1233–1235.
56. Hall RC, Embery G. The use of immunohistochemistry in understanding the structure and function of the extracellular matrix of dental tissues. Adv Dent Res 1997;11:478–486.