Determination of Matrix Metalloproteinases in Human Radicular Dentin

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Abstract

Matrix metalloproteinases (MMPs) are present in sound coronal dentin and may play a role in collagen network degradation in bonded restorations. We investigated whether these enzymes can also be detected in root dentin. Crown and root sections of human teeth were powderized, and dentin proteins were extracted by using guanidine-HCl and EDTA. Extracts were analyzed by zymography and Western blotting for matrix metalloproteinases detection. Zymography revealed gelatinolytic activities in both crown and root dentin samples, corresponding to MMP-2 and MMP-9. MMP-2 was more evident in demineralized root dentin matrix, whereas MMP-9 was mostly extracted from the mineralized compartment of dentin and presented overall lower levels. Western blot analysis detected MMP-8 equally distributed in crown and root dentin. Because MMPs are also present in radicular dentin, their contribution to the degradation of resin-dentin bonds should be addressed in the development of restorative strategies for the root substrate. *(J Endod 2009;35:686–689)*

Key Words

Collagenase, crown, enzymes, gelatinase, human tooth, root

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Supported by grants from CAPES (# 3019/07-1 P.I. J Santos), CNPq (#300615/2007-8 and 473164/2007-8 P.I. M Carrilho), FAPESP (# 07/54618-4 P.I. M Carrilho), and Academy of Finland (#111724 P.I. L. Tjäderhane).

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0099-2399/$0 - see front matter

doi:10.1016/j.joen.2009.02.003

Current findings indicate that the loss of integrity of resin-dentin bonds with time is probably caused by a combined effect of hydrolytic deterioration of resinous components after water sorption (1, 2) and the degradation of denuded collagen fibrils exposed in incompletely infiltrated hybrid layers (3, 4). The latter is attributed to an endogenous proteolytic mechanism involving the activity of matrix metalloproteinases (5).

Matrix metalloproteinases (MMPs) form a structurally related but genetically distinct group of enzymes within the endopeptidase class and are mainly involved in the extracellular matrix degradation in both physiological and pathological conditions (6, 7). Collectively, these enzymes are mostly synthesized in latent zymogen forms, and they require the binding of a zinc ion in the catalytic site and the cleavage of a propeptide domain to become catalytically competent (6).

MMP-2, -8, and -9 have been detected in human crown dentin (8–10), and their release and activation may contribute to the organic matrix degradation during caries progression (11, 12) and along resin-dentin–bonded interfaces (13, 14). Although the presence of MMPs in coronal dentin has been confirmed, it is not well established yet whether they are synthesized and expressed similarly in radicular dentin. The immunohistochemical localization of MMP-2 in human dentin sections showed a much less intense immunoreactivity at the cementum-dentin junction when compared with dentin-enamel junction (15), and this was attributed to differences in the composition of crown and root dentin. The present study investigated whether MMPs can also be identified in root dentin. We hypothesized that the same MMPs previously observed in coronal dentin are also expressed in sound radicular dentin.

Materials and Methods

Human Dentin Samples

Forty sound human third molars with complete root formation were obtained from young patients (20-30 years) at the Oulu Health Care Centre under a protocol approved by the Ethical Committee of the Northern Ostrobothnia Hospital District. After organic debris/calculus removal, teeth were sectioned at the cementum-enamel junction. The pulp tissue was scraped off with scalers and endodontic files. Cementum and enamel were removed from the radicular and coronal teeth fragments with diamond burs operated in a high-speed handpiece under continuous water spray. Crown and root dentin fragments obtained from different teeth were pooled, cut into smaller sections (2 mm × 2 mm), frozen in liquid nitrogen, and pulverized into powder in a mixer mill (Model MM301; Retsch, Haan, Germany), with coronal and root dentin being separately powderized. Then, a 2-g aliquot was obtained from each pool of dentin powder (coronal and radicular) and stored at −20°C until further use.

Dentin Protein Extraction

The extraction of dentin proteins was performed by using the protocol described in detail by Martin-De Las Heras et al (8). All the reagents were purchased from Sigma (Sigma Aldrich Chemie GmbH, Steinheim, Germany) unless differently specified. Briefly, crown and root dentin powder (2 g each) was treated with 4 mol/L guanidine-HCl and centrifuged, and nonmineralized proteins were collected with supernatant (G1 extract). Dentin powder was then demineralized with 0.5

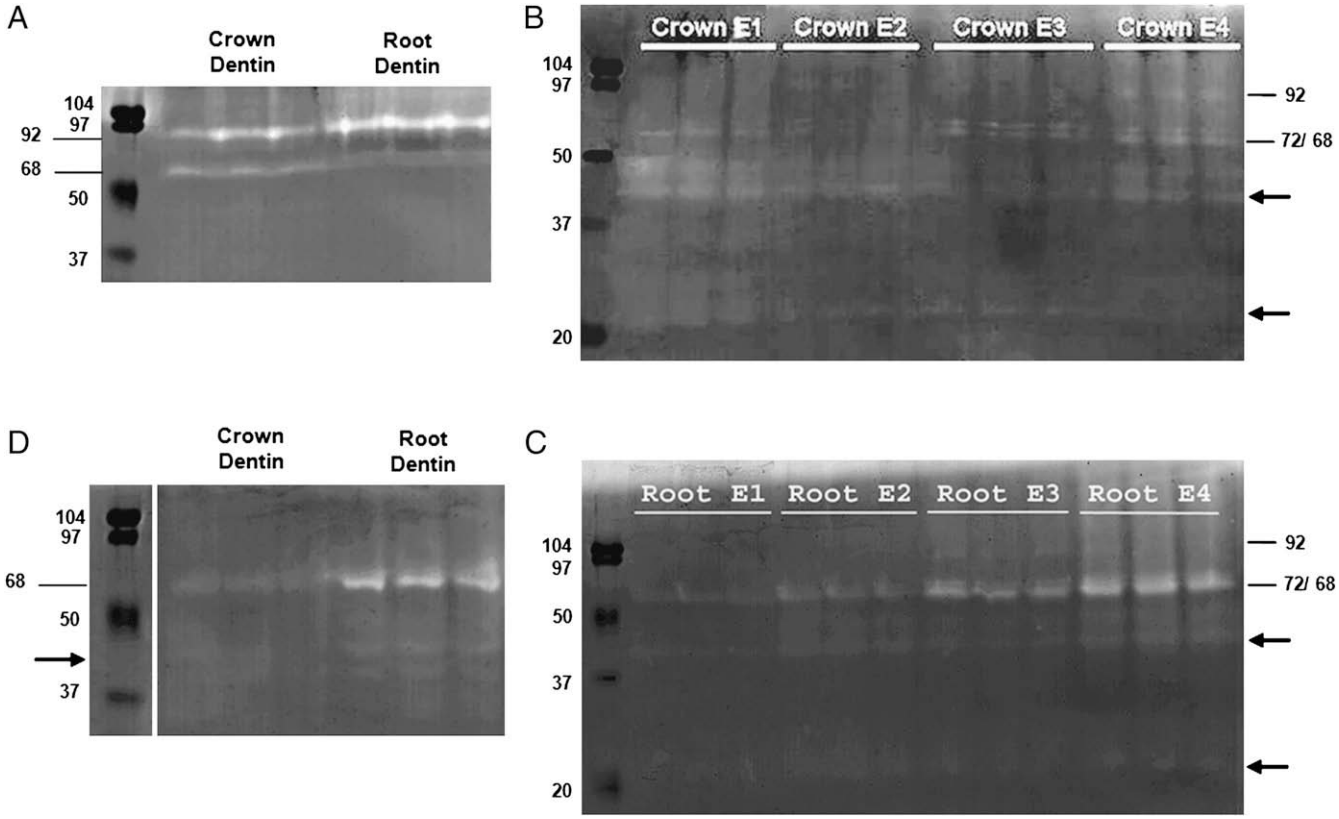

Figure 1. Gelatin zymograms of dentin proteins obtained from crown and root dentin. (*A*) Gelatinolytic activity detected in G1 extracts at approx. 92 and 68 kDa, corresponding to MMP-9 and MMP-2. (*B* and *C*) Crown and root dentin EDTA extracts (1–4) demonstrated 92 and 72/68 kDa bands, corresponding to MMP-9 and latent/active forms of MMP-2, respectively. (*B*) In coronal dentin, MMP-9 can be distinguished only in the last EDTA extract. (*C*) Root dentin proteins show increasing gelatinolytic activity with advancing demineralization steps, with MMP-9 apparent in the third and especially in the fourth extract. Low–molecular weight-bands (40-20 kDa) are also visible, most likely representing truncated forms of gelatinases (arrows). (*D*) Crown and root dentin samples obtained after the second guanidine extraction (G2 extracts). Stronger bands can be observed at the 68-kDa range in root dentin samples. Smaller molecular-weight bands presenting gelatinolytic activity can also be detected (arrow).

mol/L EDTA in four cycles to extract mineral-associated proteins (E1-E4 extracts). Finally, demineralized dentin underwent a second guanidine-HCl extraction (G2 extract). The total protein concentration of extracts obtained at each step of the protocol was measured by the Lowry protein assay (8), and 60-μg aliquots were obtained and lyophilized.

Gelatin Zymography

Dentin proteins aliquots were diluted in Laemmli sample buffer in a 2:1 ratio and electrophoresed under nonreducing conditions in 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 1 mg/mL fluorescently labeled gelatin (10). Prestained low-range molecular-weight SDS-PAGE standards (Bio-Rad, Hercules, CA) were used as molecular-weight markers. After electrophoresis, the gels were washed for 30 minutes in 50 mmol/L Tris-HCl, 2.5% Tween 80, and 0.02% (w/v) NaN_3 (pH 7.5) and then for 30 minutes in the same buffer supplemented with 5 mmol/L $CaCl_2$ and 1 μmol/L $ZnCl_2$ for the removal of SDS. Finally, the gels were incubated in activation solution (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, and 0.02% NaN_3, pH 7.5). Proteolytic activity was monitored under long-wave UV light until judged to be in linear range, and then the gels were stained in 0.2% Coomassie Brilliant Blue R-250 and destained in 10% acetic acid–10% methanol in H_2O. The zymography assay of dentin proteins was performed in triplicates and repeated three times.

Western Blot

The identity of dentin proteins was further assessed by immunoblotting. Protein aliquots (60 μg each) were mixed in Laemmli buffer with and without 0.5% β-mercaptoethanol and boiled for 5 minutes before electrophoresis in 11% SDS-PAGE gels. Separated proteins were transferred to nitrocellulose membranes (Protran; Whatman, Dassel, Germany) by means of a semidry apparatus (TE 77 PWR Semi-dry transfer unit; Amersham Biosciences, Piscataway, NJ). Nonspecific binding was blocked by tris buffered saline-Tween 20 (TBS-T) containing 5% nonfat dry milk. After sequential washes, the membranes were incubated with monoclonal antihuman MMP-2 (Oncogene, Boston, MA) and polyclonal antihuman MMP-8 (10) primary antibodies. After washes, peroxidase-linked antimouse and antirabbit secondary antibodies were added, and imunocomplexes were detected by a chemiluminescent method (ECL Western Blotting Analysis System; GE Healthcare, Buckinghamshire, UK). Western Blot analysis of crown and root dentin proteins was performed in duplicates and repeated twice.

Results

Zymography revealed gelatinolytic bands in both crown and root dentin samples. Proteins extracted in the first guanidine cycle (G1 extracts) yielded 92- and 68-kDa bands; the molecular weights corresponded to MMP-9 and MMP-2, respectively (Fig. 1*A*). Relative to crown dentin, root dentin presented stronger 92-kDa and weaker