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In situ analysis of gelatinolytic activity in human dentin

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ABSTRACT

Matrix metalloproteinases (MMPs) such as gelatinases are differentially expressed in human tissues. These enzymes cleave specific substrates involved in cell signaling, tissue development and remodeling and tissue breakdown. Recent evidences show that gelatinases are crucial for normal dentin development and their activity is maintained throughout the entire tooth function in the oral cavity. Due to the lack of information about the exact location and activity of gelatinases in mature human dentin, the present study was designed to examine gelatinolytic levels in sound dentin. *In situ* zymography using confocal microscopy was performed on both mineralized and demineralized dentin samples. Sites presenting gelatinase activity were identified throughout the entire biological tissue pursuing different gelatinolytic levels for distinct areas: predentin and dentinal tubule regions presented higher gelatinolytic activity compared to intertubular dentin. Dentin regions with higher gelatinolytic activity immunohistochemically were partially correlated with MMP-2 expression. The maintenance of gelatinolytic activity in mature dentin may have biological implications related to biomineralization of predentin and tubular/peritubular dentinal regions, as well as regulation of defensive mechanisms of the dentin-pulp complex.

1. Introduction

Hard biological tissues, such as dentin, are complex hierarchical composite substrates (Kinney et al., 2003; Ryou et al., 2012) as result of the intricate arrangements of organic matrix and apatite nanocrystals, and their interaction. Odontoblasts are specialized cells responsible for the production of predentin matrix, containing type I collagen matrix, non-collagenous proteins, proteases and proteoglycans (Bleicher, 2014), which participate in the mineralization process. The arrangement of collagen matrix macromolecules determines the shape and the structure of the mineralized component. Mineral phase-interactive acidic matrix proteins, such as Small Integrin Binding LIgand N-linked Glycoproteins (SIBLINGs) intermediate collagen and mineral interactions by regulating the site of initial crystal deposition and the type of mineral crystal deposited (Addadi and Weiner, 1985). Furthermore, mineralization of hard tissues is a complex biological process that relies on specific proteinases and other enzymes to degrade or modify the acidic matrix proteins to allow mineralization (Charadram et al., 2012).

Human dentin contains several enzymes capable to degrade dentin collagen matrix proteins, including type I collagen. At present, these enzymes may belong to the calcium/zinc-dependent endopeptidases class know as matrix metalloproteinases (MMPs) or cysteine cathepsins (Tjäderhane et al., 2013). Some MMPs, including gelatinases MMP-2 and MMP-9 have been identified in odontoblasts and in predentin/dentin compartments of fully formed and mineralized teeth (Boushell et al., 2008; Mazzoni et al., 2007; Sulkala et al., 2007). The main function attributed to gelatinases is the degradation of the extracellular matrix (ECM) (Visse and Nagase, 2003). However, it is now widely acknowledged that MMPs play an important role in cell signaling by generating peptides with specific biological activities (Chaussain et al., 2009; Page-McCaw et al., 2007). Although active gelatinases have been identified in mature dentin, their location and distribution are not

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exactly known. Localization and quantitation of dentinal MMPs with immunohistochemistry and with different biochemical techniques at different locations in dentin have produced conflicting results (Boushell et al., 2011, 2008; Niu et al., 2011). Since part of dentinal enzymes may reside in or be closely associated with the mineral compartment of dentin (Santos et al., 2009; Sulkala et al., 2007), demineralization required for traditional sample preparation methods may lead to substantial loss of such enzymes. Immunofluorescence techniques combined with confocal laser scanning microscopy have been used to detect collagenolytic enzymes in intact and carious dentin without demineralization (Vidal et al., 2014), but only to limited regions adjacent to the dentin-pulp border. *In situ* zymography with confocal microscopy is accepted as a viable technique to identify gelatinolytic activity on specific sites of biological tissues. To date, it has been mostly used to map enzymatic activity only in rat teeth (Pessoa et al., 2013; Porto et al., 2009); studies employing human dentin have been mostly limited to the dentin-composite interface (Mazzoni et al., 2013, 2012a, 2012b). Therefore, the aim of this study was to investigate the specific location of active gelatinase sites in both mineralized and demineralized human dentin, by means of an *in situ* zymography assay, and immunohistochemically examine the co-localization of MMP-2 in the corresponding areas presenting gelatinolytic activity. The tested null hypothesis was that there would be no differences in enzymatic activity levels for distinct regions in sound dentin.

2. Materials and methods

Twenty-four erupted sound human third molars with complete root formation were extracted for surgical reasons with patients' (age 18–23 years) informed consent after approval by the local Ethical Committee (#095/2012). Twelve teeth were cleaned and stored at 4 °C in saline solution with 0.2% sodium azide (Sigma-Aldrich, St. Louis, MO, USA) for one week. The remaining twelve teeth were cleaned and immediately fixed in 4% paraformaldehyde (EMS, Electron Microscopy Sciences, Washington, PA, USA) pH 7.4, at 4 °C for 12 h.

2.1. Demineralized sample preparation

After fixation, teeth were demineralized using a protocol described by Pessoa et al. (Pessoa et al., 2013). Briefly, teeth were immersed for 12 h at 4 °C in each of the following series of solutions: 0.01 M phosphate buffered saline (PBS) (Cultilab, Campinas, SP, Brazil) containing 5% glycerol (Synth, São Paulo, SP, Brazil), 0.01 M PBS containing 10% glycerol, and 0.01 M PBS containing 15% glycerol. Specimens were then demineralized in ethylenediaminetetraacetic acid (EDTA)/glycerol (EDTA-G) solution with 14.5 g EDTA (Merck AG, Darmstadt, Germany), 1.25 g NaOH (Merck), and 15% glycerol in 100 ml distilled water, pH 7.3 at 4 °C. The EDTA-G solution was replaced every two days. After dentin demineralization (approximately 120 days), the specimens were immersed for 12 h at 4 °C in successive solutions of 15% sucrose and 15% glycerol in PBS, 20% sucrose and 10% glycerol in PBS, 20% sucrose and 5% glycerol in PBS, 20% sucrose in PBS, 10% sucrose in PBS, 5% sucrose in PBS, and 100% PBS. Demineralized teeth were then longitudinally sectioned (Fig. 1), dehydrated in ethanol and embedded in a low melting point paraffin wax (EMS). Longitudinal and transversal sections (5 μm thick) from the crown were cut with a microtome (Leica, Nussloch, Germany) and placed on silanized microscope slides. Immediately prior to the *in situ* zymography assay, dentin sections were deparaffinized with the following: Xylene (Synth) 2 × 1 min; Xylene 1:1 with 100% ethanol (Synth) for 3 min; 100% ethanol for 2 × 3 min; 95% ethanol, 3 min; 70% ethanol, 3 min; 50% ethanol − 3 min; 100% distilled water.

2.2. Mineralized sample preparation

The occlusal enamel surface was removed using a low speed

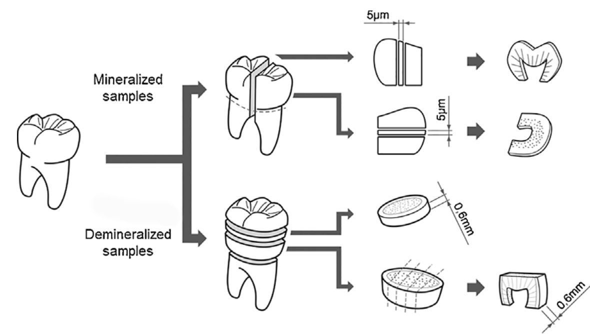

Fig. 1. Schematic illustration of sample preparation: transversal and longitudinal sections of mineralized and demineralized human molars.

diamond disc (Isomet 1000 Precision Saw, Buehler, Lake Bluff, IL, USA) under water cooling. A transversal 0.6-mm dentin slab was first sectioned. The remaining sectioned tooth, containing the pulp chamber, was then cut longitudinally into 0.6-mm sequential slabs (Fig. 1). Dentin slab surfaces were wet-polished with 600, 1000, 1200, 2000 grit SiC paper (BuehlerMet, Buehler, Lake Bluff, IL, USA) and 6, 3, and 1 μm water-based diamond polishing pastes (MetaDi Diamond Suspension, Buehler). The samples were ultrasonically cleaned for 5 min after each step and kept in distilled water until tested to avoid dehydration.

2.3. In situ zymography

In order to detect gelatinase activity in dentin, a protocol based on Pessoa et al. (Pessoa et al., 2013) and Porto et al. (Porto et al., 2009) was used. Excess water from both mineralized and demineralized samples was removed with sterilized absorbent paper followed by incubation in a solution containing DQ-gelatin (DQ-gelatin E-12055, catalog No. D12054, Molecular Probes, Eugene, OR, USA) diluted (1:10) in 50 mM Tris-$CaCl_2$ (Tris-$CaCl_2$, ex: 50 nm Tris-HCl, pH 7.4 and 5 mM $CaCl_2$). The incubation period was 2 h for the demineralized samples and 24 h for the mineralized samples. All specimens were kept in a dark humid chamber at 37 °C during the incubation period, and then washed in distilled water for 5 min prior to analyses with a multiphoton confocal microscopy (Leica TCS SP5, Leica Microsystems, Heidelberg, Germany) equipped with 63x/1.4NA oil immersion lens using a 488 nm argon laser (490–540 nm band pass filter). As the degradation of fluorescent-labeled gelatin produces fluorescence, the gelatinolytic activity in specific sites was observed as green fluorescence (absorption maximum ∼495 nm; emission maximum ∼515 nm). The z-stack scans (0.5 μm) were compiled into single projections until 70 μm final volume with image resolution of 1024 × 1024 pixels. Images were qualitatively analyzed by two experienced blinded examiners. Negative control sections were incubated with 50 mM Tris-$CaCl_2$ as described above, but without DQ-gelatin. All photomicrographs were obtained using the same confocal microscope calibration.

2.4. Immunohistochemistry

Longitudinal and transversal sections (∼5 μm thick) from the demineralized teeth were subjected to immunohistochemistry reaction for MMP-2 detection. Endogenous peroxidase was inhibited by 2 × 5 min H_2O_2 treatment. Enzymatic digestion was performed with PBS (Sigma) for 30 min. The antibody non-specific binding was prevented by 3% BSA for 30 min, and the sections were incubated overnight at 40 °C with monoclonal anti-human MMP-2 antibody (Clone 42-5D11, catalog No. IM33, mouse mAb, EMD Milipore Corporation, Temecula, CA, USA) diluted in PBS (pH 7.4) 1:50. Sections were incubated with secondary antibody (LSAB-Link + Labeled streptavid-Biotin, catalog No. K1015, Dako Corporation, Carpinterıa, CA, USA) for 30 min, washed with PBS and then they were incubated with diaminobenzidine solution (DAB

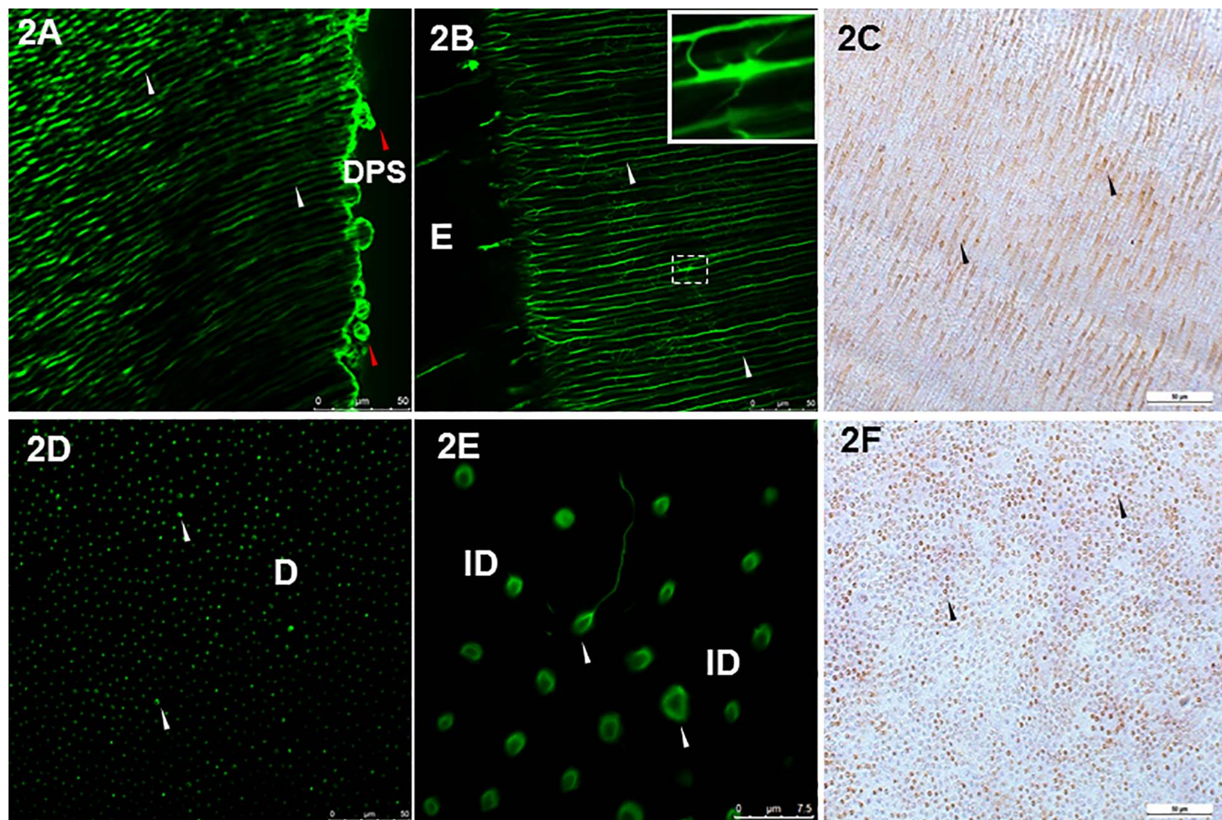

Fig. 2. Representative photomicrographs obtained from mineralized human molar-sections by confocal *in situ* zymography (2A, 2B, 2D and 2E) and immunohistochemistry (2C and 2F). The sections were oriented transversally (2A, 2 B and 2C) or longitudinally (2D, 2E and 2F) to dentinal tubules. 2A, 2B, 2D and 2E show the *in situ* zymography, in which green fluorescence indicates sites with gelatinolytic activity. White arrowheads indicate fluorescence in dentinal tubules. Red arrowheads indicate high fluorescence in globular structures (calcospherites) at the dentin-pulp border. In 2B, a high magnification photomicrography of the square-shaped dotted figure is presented. Note that high gelatinolytic activity was observed exclusively inside tubular branches and anastomoses indicating that enzymes are not intracellular. 2C and 2F show the immunohistochemistry for MMP-2 in dentinal tubules (black arrowheads), which correlates with correspondent sites presenting high gelatinolytic activity in the *in situ* zymography. D: dentin; ID: intertubular dentin; DPS: region corresponding to dental pulp space; E: enamel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Substrate Kit®, catalog No. K3468, Dako Corporation, Carpinterıa, CA, USA). Mayer's hematoxylin was used to counterstain and the sections were observed under a light microscope (Leica DMR, Nussloch, Germany) to localize MMP-2 in dentin. Negative controls were performed by omitting the primary antibody.

3. Results

3.1. In situ zymography

High gelatinolytic activity was observed at the dentin-pulp border. In mineralized samples, where odontoblasts and predentin were mostly lost during sample processing, high gelatinolytic activity was observed at the mineralization front, following the shape of calcospherites (Fig. 2A, red arrows). In demineralized samples, the presence of a continuous-intense fluorescence indicated high activity throughout the width of predentin, while odontoblast bodies were negative and only very faint activity was observed in pulp tissue (Fig. 3B and C).

Intense gelatinolytic activity was also present in dentinal tubules in both mineralized (Fig. 2) and demineralized (Fig. 3) samples. In mineralized samples, tubular activity remained relatively unchanged from the dentin-pulp border to dentin-enamel junction (DEJ) and was readily observable also in the lateral tubular branches especially closer to the DEJ (Fig. 2B). In DEJ itself, activity was inconsistently seen in only a few samples (demineralized/mineralized) (data not shown). Transversal sections following dentinal tubules orientation demonstrated gelatinolytic activity in all tubules, but complete absence of gelatinolytic activity in mineralized intertubular dentin (Fig. 2D and E). However, in demineralized sections, fainter but distinct activity was present also in intertubular dentin (Fig. 3A–C).

3.2. Immunohistochemistry

The immunohistochemistry analysis showed a co-localization of MMP-2 in dentin areas (dentin tubules and predentin) presenting high gelatinolytic activity in the *in situ* zymography for both mineralized (Fig. 2C and F) and demineralized samples (Fig. 3D). However, the number of tubules with no MMP-2 detection was relatively large (Fig. 2F) compared to *in situ* zymography (Fig. 2D and E).

4. Discussion

Distinct distribution of gelatinolytic active sites throughout the fully-formed human dentin led to rejection of the null hypothesis. Moreover, immunohistochemistry evaluation showed that such active sites were correlated with dentin regions containing MMP-2. These findings corroborate previously published data (Pessoa et al., 2013) obtained from rat dentin. The high gelatinolytic activity observed in dentinal tubules, but not in the intertubular dentin in mineralized teeth, confirms previous studies showing that human dentin contains gelatinases not only within mineralized intertubular dentin, but also in non-mineralized compartments (Santos et al., 2009; Sulkala et al., 2007). Intratubular activity observed in mineralized samples may represent gelatinases in dentinal fluid, complexed with intratubular proteins or intracellular within odontoblast processes. Even though the length of odontoblast processes in human dentin is still not completely agreed

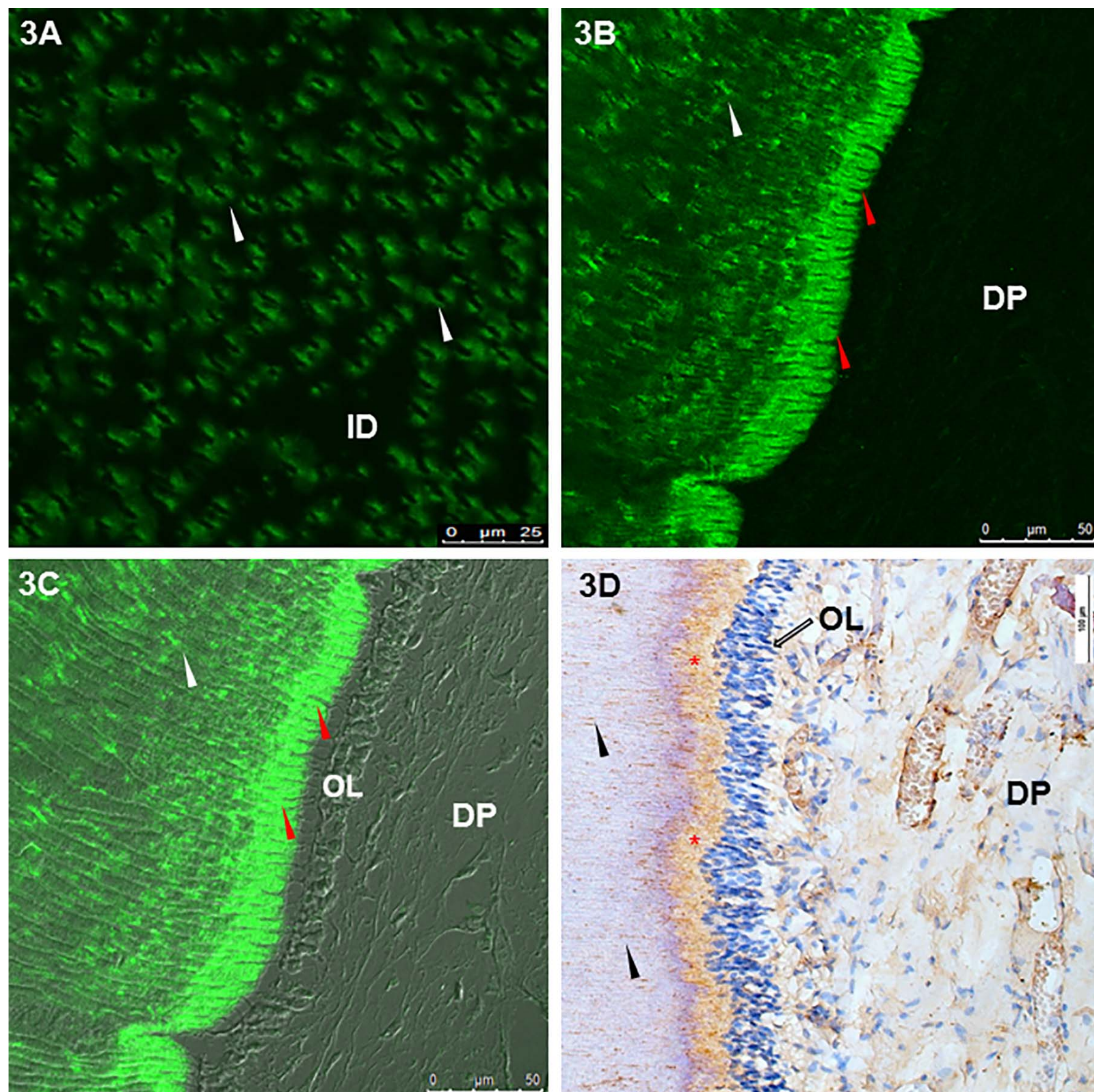

Fig. 3. Representative photomicrographs from demineralized human molar-sections obtained by confocal in situ zymography (3A, 3B, and 3C) and immunohistochemistry (3D). The sections were oriented transversally (3A) or longitudinally (3B and 3C) to dentinal tubules (white arrowheads). In 3A, 3B, and 3C, green fluorescence indicates sites with gelatinolytic activity. Red arrowheads (3B and 3C) show high gelatinolytic activity in predentin. In 3C, a merged photomicrograph of the in situ zymography (3B) with transmitted light shows sites with gelatinolytic activity and nearby cellular structures, notice the odontoblast layer (OL) adjacent to a high gelatinolytic active site. 3D shows a representative immunohistochemistry photomicrograph to MMP-2, where asterisks indicate MMP-2 at the predentin area and black arrowheads indicate their presence in dentinal tubules. ID: intertubular dentin region. DP: dental pulp. OL: odontoblast layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

upon (Tjäderhane and Haapasalo, 2009), it may be unlikely that the processes would reach all the way to the DEJ in a uniform and consistent manner observed in Fig. 2B. In addition, gelatinolytic activity was observed exclusively inside tubular branches and anastomoses, which indicates that the enzymes are not intracellular. These branches have been classified as major, fine, and microbranches (Mjör and Nordahl, 1996). Even the major branches (0.5–1.0 µm diameter), let alone fine branches (300–700 nm diameter), would be too small for odontoblast cellular processes. Still, gelatinolytic activity was observed in the fine network of fine microbranches especially on the outer dentin. Therefore, dentinal fluid or MMP-protein complexes constitute a more plausible explanation. In dentinal tubules, MMPs may in principle form complexes with either collagen or non-collagenous proteins. Dentinal tubules close to dentin-pulp border contain collagen fibrils which gradually disappear with increasing proximity towards DEJ (Dai et al., 1991). This disappearance has been suggested to be caused by collagenolytic enzymes in dentinal fluid (Hannas et al., 2007).

The high gelatinolytic activity in the dentin-pulp border, predentin and peritubular regions, observed in the *in situ* zymography analysis correlated with immunohistochemical MMP-2 detection. Although MMP-2 is not the only proteolytic enzyme in dentin matrix, it is probably the most abundant gelatinolytic MMP present in mineralized dentin (Mazzoni et al., 2012a, 2012b). Since the activity of MMPs in general seems to be predominant over the activity of cathepsins (Scaffa et al., 2017), it seemed only plausible to select MMP-2 as a starting point for the immunohistochemistry analysis. Nevertheless, the presence of gelatinolytic enzymes in dentin may explain the observed disparity between the number of tubules with no MMP-2 detection by immunohistochemistry and the relatively large gelatinolytic activity identified by the non-specific *in situ* zymography assay. Further studies should explore the co-distribution and activity of additional MMPs and even cysteine proteases in sound mature dentin.

MMP-2 regulates the processing and function of non-collagenous proteins that participate in peritubular dentin formation and

mineralization, such as dentin sialophosphoprotein (DSPP) (Yamakoshi et al., 2006), dentin matrix protein-1 (DMP-1) (Chaussain et al., 2009) or fetuin (Ray et al., 2003). An important non-collagenous protein in dentin mineralization is dentin sialophosphoprotein (DSPP), or rather its proteolytically processed products, N-terminal dentin sialoprotein (DSP) and C-terminal dentin phosphoprotein (DPP, also called phosphophoryn, PP) (Butler, 2008). Dentin sialoprotein also occurs in a proteoglycan form (Huang et al., 2008; Qin et al., 2003; Yamakoshi et al., 2005) called DSP-PG (Huang et al., 2008). During dentin mineralization, DSPP first seems to be cleaved by bone morphogenetic protein-1/Tolloid-like proteinase (BMP1/Tolloid-like proteinases) into fully active DSP and DPP. DSP and DSP-PG are further processed and possibly inactivated by MMP-2, MMP-20 and other proteinases (Prasad et al., 2010; Yamakoshi et al., 2006). DPP is believed to be directly involved in controlling the nucleation and growth of hydroxyapatite crystals during dentin mineralization. DSP, on the other hand, is believed to act as a weak mineral nucleator (Boskey et al., 2000; Boskey, 2003), possibly involved in the initiation of dentin mineralization, but not in tissue maturation (Suzuki et al., 2009). The presence of DSP-PG in predentin, but not in mineralized dentin, indicates that a major portion of the proteoglycan form of DSP is removed prior to the mineralization of collagen (Huang et al., 2008).

The interaction between DMP1 and collagen fibrils may be essential for matrix-mediated mineralization (He et al., 2003a, 2003b). Gajjeraman and co-workers (Gajjeraman et al., 2007) demonstrated the *in vitro* mineralization of type I collagen with DMP1, with great resemblance to dentin in both ultrastructural (with calcospherite-type mineralization) and mechanical properties (*i.e.* elastic modulus). The fundamental role of DMP1 in collagen matrix mineralization is further indicated by the finding that the N-terminal domain of DMP1 stabilizes amorphous calcium phosphate during the growth phase and inhibits hydroxyapatite formation, while the C-terminal domain is required for crystal formation. DMP1 is present mainly in peritubular dentin and at the mineralization front (Maciejewska et al., 2008; Massa et al., 2005; Orsini et al., 2008), while DMP1 N-terminal fragment, called DMP1-PG (Qin et al., 2006), is abundant in predentin (Maciejewska et al., 2008). MMP-2 has been reported to split the C-terminal peptide portion of DMP1 (Chaussain et al., 2009) subsequently promoting mineralization (Gericke et al., 2010).

Fetuin-A (a-2-Heremans Schmid-glycoprotein (a-2-HSglycoprotein, AHSG) is the most abundant serum protein in dentin (Lee et al., 2009; Thomas and Leaver, 1975). Fetuin-A inhibits undesirable ectopic calcification allowing bone or dentin mineralization. In contrast to other ectopic calcification inhibitors, Fetuin-A acts in all extracellular fluids and is not locally restricted, indicating that Fetuin-A may act as a unique systemic inhibitor of ectopic calcification (Jahnen-Dechent et al., 2008). Both gelatinases MMP-2 and MMP-9, have high binding affinity to Fetuin-A. In this context, Fetuin-A may not only activate and control the gelatinase activity (Ochieng et al., 1995), but also protect the gelatinases, especially MMP-9, from autolytic degradation (Ray et al., 2003). Since extracellular fluids are the main function sites for Fetuin-A, mostly located in peritubular dentin (Takagi et al., 1990), it is highly possible that Fetuin-A/MMP interactions in dentinal fluid and peritubular dentin surfaces regulate peritubular dentin formation.

The present study demonstrated that *in situ* zymography is a viable method to be used with both mineralized and demineralized dentin samples. Since gelatinases do not participate only in dentin formation and/or mineralization, but also in caries progression, defense, erosion and degradation of dentin-restoration interface (Mazzoni et al., 2015, 2013; Tjäderhane et al., 2015), *in situ* zymography is a powerful tool to investigate the real role of proteolytic enzymes in these conditions. Indeed, recent studies have demonstrated the intense gelatinase activity at the bottom of the hybrid layer (Mazzoni et al., 2013, 2012a, 2012b), where partially demineralized collagen fibrils are not enveloped by the adhesive resin and thus become highly prone to degradation by endogenous enzymes. Reduction of such enzymatic activity by using specific MMP inhibitors was detected by *in situ* zymography and confirmed with a zymography assay (Mazzoni et al., 2013), which demonstrates the usefulness of this technique.

5. Conclusion

This study presents compelling evidence that active gelatinases are present not only in demineralized, but also in mineralized human dentin. Different activity levels were identified for distinct areas showing higher gelatinolytic activity in predentin and dentinal tubule regions compared to intertubular dentin. *In situ* zymography proved to be a valuable method to investigate the existence of active gelatinases in mineralized dentin. The immunohistochemistry analysis revealed that areas with corresponding high gelatinolytic activity observed in the *in situ* zymography were partially correlated with MMP-2 expression.

Acknowledgments

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