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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 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.

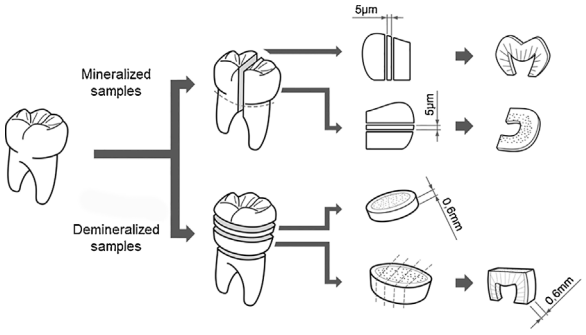

Fig. 1. Schematic illustration of sample preparation: transversal and longitudinal sections of mineralized and demineralized human molars.

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, Carpinteria, CA, USA) for 30 min, washed with PBS and then they were incubated with diaminobenzidine solution (DAB