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In situ study of the gelatinase activity in demineralized dentin from rat molar teeth

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ABSTRACT

Matrix metalloproteinases (MMPs) in dentin are believed to participate in various physiological and pathological events in coronal dentin, but their exact source and location is not clear. The purpose of this study was to evaluate the activity of gelatinases in decalcified rat molars crowns by *in situ* zymography. Hemi-mandibles of five male Wistar rats were fixed in paraformaldehyde, decalcified in EDTA and glycerol solution and embedded in paraffin. Sections from the region of molar teeth were incubated with or without DQ gelatin in 50 mM Tris–$CaCl_2$ at 37 °C for 2 h and observed by means of confocal microscopy. Gelatinolytic activity was observed throughout the coronal dentin with varying intensities in different locations. High gelatinase activity was observed in the dentinal tubules, dentin–enamel junction (DEJ) and predentin, and it was weaker and less uniform in the intertubular dentin. This study shows that the location of gelatinase and relative activity can be detected by means of *in situ* zymography and confocal microcopy, and this methodology may provide a useful tool in studies on the role of gelatinases in tooth development, maturation and in pathological conditions.

Introduction

Matrix metalloproteinases (MMPs) are a family of structurally related, but genetically distinct, enzymes that can degrade components of the extracellular matrix (ECM) and basement membrane (BM). This group of enzymes is classified into collagenases, gelatinases, stromelysins, membrane-associated MMPs and other MMPs, depending mainly on the substrate specificity and molecular structure (Uitto et al., 2003; Sorsa et al., 2004). Metalloproteinases require calcium and zinc ions to maintain their proper tertiary structure and functionally active sites, respectively (Tezvergil-Mutluay et al., 2010).

Type I collagen constitutes about 90% of the organic matrix of dentin. In general, collagens can be degraded by the interstitial collagenases, which include MMP-1, MMP-8, and MMP-13, resulting in the release of 3/4- to 1/4-length peptides. These peptides lose the triple-helical conformation and can then be further degraded by the gelatinases MMP-2 and MMP-9 (Sorsa et al., 2004). The fibronectin-like domain uniquely present in MMP-2 and 9 is required for gelatin binding and makes gelatin the preferred substrate for gelatinases (Hannas et al., 2007). In addition, MMP-2 is also able to cleave soluble triple helical type I collagen at the typical Gly-Ile/Leu sites, producing the 3/4 and 1/4 fragments (Aimes and Quigley, 1995), albeit at much slower rate than collagenases (Craig et al., 1991).

Several MMPs, especially gelatinases MMP-2 and MMP-9 (Martin-De Las Heras et al., 2000; Mazzoni et al., 2007; Sulkala et al., 2007; Boushell et al., 2008), collagenase-2 (MMP-8) (Sulkala et al., 2007), stromelysin-1 (MMP-3) (Mazzoni et al., 2007), MMP-2 activator MT1-MMP (Caron et al., 1998; Palosaari et al., 2002) and enamelysin (MMP-20) (Palosaari et al., 2002; Sulkala et al., 2002), have all been identified in either odontoblasts or in the predentin/dentin compartments of fully formed teeth. In addition to MMPs, healthy and carious dentin also contain other collagenolytic enzymes, cysteine cathepsins (Tersariol et al., 2010; Nascimento et al., 2011), the presence correlating significantly with MMPs (Tersariol et al., 2010).

Three different methods have mostly been used to assess gelatinases in dentin: substrate zymography (using SDS Page), Western blot and immunohistochemistry (Sulkala et al., 2007; Boushell et al., 2008; Niu et al., 2011). Substrate zymography and Western blot require dentin crushing, followed by protein extraction (Sulkala

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et al., 2007). These techniques allow evaluation of the presence or activity of some MMPs by molecular weight and specific substrate (zymography) or by molecular weight and conjugation with antibody (Western blot), but it is not possible to know the exact localization of the proteases extracted from dentin. Immunohistochemical localization presents the specific location of different proteases in dentin, but does not discriminate between zymogens and active enzymes, and no information about the activity can be inferred. In addition, the results depend to a large degree on the specificity of the antibody and in case of dentin potential bonding to organic or mineral components potentially masking the epitopes, with the possibility of both over- and underestimation of the protein (Boushell et al., 2011a,b).

In situ zymography (ISZ) is a technique that uses an enzymatic substrate-based support or overlay to detect and localize specific protease activities in tissue sections (Kupai et al., 2010). In this assay, an enzymatic substrate for a specific protease(s) is deposited on the surface of a tissue section. During the incubation period, the substrate is cleaved in a time- and dose-dependent manner by the appropriate active enzymes in their native location. After the incubation period, the lyses of the labeled substrate can be detected by light or fluorescence microscopy depending on the substrate type, permitting the localization of the specific protease activity in tissue sections (Kupai et al., 2010). The location of active gelatinases by ISZ has been demonstrated in different tissues, including bone and dentin after decalcification (Porto et al., 2009).

Although studies have already demonstrated the presence of MMPs in dentin, the presence, activity and role of the collagenases and gelatinases in teeth have not been totally elucidated. In addition, there are no reports in the literature concerning the localization of MMP activity in histological sections from fully formed decalcified teeth, but only in developing teeth, and in particular in rodent incisors (continuously growth teeth) (Sakuraba et al., 2006; Porto et al., 2009). Therefore, the aim of this preliminary study was to evaluate, using *in situ* zymography, the location and origin of the gelatinolytic activity in decalcified dentin of rat molars, and also to map the gelatinolytic activity in the distinct regions of this tissue.

Materials and methods

Animals

Five male Wistar rats (8 weeks old, mean weight of 234 ± 18 g), obtained from the Animal Facility Center of the State University of Campinas were maintained in a room with 12-h day/night cycles with food and drinking water *ad libitum*. Experimental procedures were approved by the Institutional Animal Research Committee of the State University of Campinas (São Paulo, Brazil).

Tissue preparation

The rats were euthanized by cervical dislocation and their hemimandibles were retrieved for histological evaluation, using the protocol adapted from Porto et al. (2009). For tissue fixation, the right hemi-mandibles of all animals were kept in 4% paraformaldehyde (EMS, Electron Microscopy Sciences, Washington, PA, USA), pH 7.4, at 4 °C for 12 h. After tissue fixation, the hemi-mandibles were decalcified using a protocol described by Begum et al. (2010). The hemi-mandibles were washed 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, Brazil), 0.01 M PBS containing 10% glycerol, and 0.01 M PBS containing 15% glycerol. The specimens were then decalcified in ethylenediaminetetraacetic acid (EDTA)/glycerol (EDTA-G) solution 14.5 g EDTA (Merck AG, Darmstadt, Germany), 1.25 g NaOH (Merck), and 15% glycerol in 100 ml distilled pH 7.3 at 4 °C. The EDTA-G solution was replaced every 2 days. After decalcification (~40 days), the specimens were washed at 4 °C for 12 h in successive washes 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. The region of the first and second molars was selected and included in a low melting point paraffin wax (EMS). Longitudinal sections (5-μm thick) in the middle of the crown molar teeth were cut on a microtome (Leica, Nussloch, Germany) and place on silanized microscope slides. Some sections were stained with hematoxylin and eosin (HE).

In situ zymography

In order to detect gelatinase activity in rat molar dentin, a protocol based on Porto et al. (2009) was used. For deparaffinization, the following washes were performed: 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. The excess water surrounding the sample area was wiped with absorbent paper. After partially drying the slices, 80 mL of DQ-gelatin (1:10) (DQ-gelatin, E12055; Molecular Probes, Eugene, OR, USA) in 50 mM Tris–$CaCl_2$ (Definir, Tris–$CaCl_2$, ex: 50 nm Tris–HCl, pH 7.4 and 5 mM $CaCl_2$) was applied on top of the tissue sections, and incubated at 37 °C for 2 h in a dark humid chamber. As the degradation of fluorescent-labeled gelatin exhibits fluorescence, the gelatinolytic activity was observed as green fluorescence (absorption maxima, ~495 nm; fluorescence emission maxima, ~515 nm) by confocal fluorescence microscopy (Leica TCS SP5, Leica Microsystems, Heidelberg, Germany). Negative control sections were incubated with 50 mM Tris–$CaCl_2$ as described above, but without DQ-gelatin. To confirm that the observed activity is due to MMP enzymes, some sections were incubated with a metalloproteinase inhibitor, 1,10-phenanthroline (Phe) (Sigma–Aldrich, St. Louis, MO, USA) at 2 mM in 50 mM Tris–$CaCl_2$. All photomicrographs were obtained using the same confocal microscope calibration.

Results

In the sections incubated with DQ-gelatin, the gelatinolytic activity was observed by the fluorescence throughout the dentin (Figs. 1–3). Negative controls (sections without DQ-gelatin) showed only faint dentin auto-fluorescence, which was easily discriminated from the fluorescence caused by the degradation of labeled gelatin. Sections incubated with DQ-gelatin + MMPs inhibitor (Fig. 1D) had a loss of fluorescence, even when examined at higher magnification (data not shown) and were comparable with negative controls (Fig. 1B). Although gelatinolytic activity was present also in the intertubular dentin, the highest fluorescence was found in the dentinal tubules (Figs. 2B, 2D, 3B and 3C); in the predentin (Fig. 2C and D) and in the dentin–enamel junction (D, E, J) (Fig. 3B and C). Especially close to the dentin–pulp border, the activity appeared in granular form in dentinal tubules (Fig. 2C), while the presence of these granular structures was much less apparent close to the DEJ (Fig. 3C).

Discussion

Since dentin is a calcified tissue, studies that involve activity assays of proteins, such as zymography, are not easily performed. The main is that the MMPs associated to collagen in mineralized intertubular dentin are masked by mineral crystals, and the demineralization process to assess organic matrix may cause loss