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Co-distribution of cysteine cathepsins and matrix metalloproteases in human dentin

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ABSTRACT

It has been hypothesized that cysteine cathepsins (CTs) along with matrix metalloproteases (MMPs) may work in conjunction in the proteolysis of mature dentin matrix. The aim of this study was to verify simultaneously the distribution and presence of cathepsins B (CT-B) and K (CT-K) in partially demineralized dentin; and further to evaluate the activity of CTs and MMPs in the same tissue. The distribution of CT-B and CT-K in sound human dentin was assessed by immunohistochemistry. A double-immunolabeling technique was used to identify, at once, the occurrence of those enzymes in dentin. Activities of CTs and MMPs in dentin extracts were evaluated spectrofluorometrically. In addition, *in situ* gelatinolytic activity of dentin was assayed by zymography. The results revealed the distribution of CT-B and CT-K along the dentin organic matrix and also indicated co-occurrence of MMPs and CTs in that tissue. The enzyme kinetics studies showed proteolytic activity in dentin extracts for both classes of proteases. Furthermore, it was observed that, at least for sound human dentin matrices, the activity of MMPs seems to be predominant over the CTs one.

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1. Introduction

Dentin matrix has endogenous proteolytic activity, which has been attributed to the collagenolytic/gelatinolytic function of enzymes such as matrix metalloproteases (MMPs) (Martin-De Las Heras et al., 2000; Mazzoni et al., 2007; Sulkala et al., 2007) and cysteine cathepsins (CTs) (Nascimento et al., 2011; Tersariol et al., 2010). More recently, we have also identified in dentin the presence of cysteine cathepsin K (CT-K) (Vidal et al., 2014), the most potent mammalian collagenase (Fonović & Turk, 2014).

The gene expressions of MMPs (Palosaari et al., 2003) and CTs (Tersariol et al., 2010) in mature human odontoblasts suggest that these enzymes could share multiple functions in physio-pathological processes occurring in dentin matrix (Dickinson 2002; Hannaset al., 2007). The co-expression of two distinct

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families of extracellular matrix–degrading enzymes in a specific tissue is infrequent, particularly because the cells in question (*i.e.* odontoblasts) are not thought to be very much active after tissue (*i.e.* dentin) maturation and under physiologic conditions. However, in pathological conditions, the collagen degrading activity of these enzymes has been widely recognized (Hannas et al., 2007; Tjäderhane et al., 2013). Our previous data suggested that CTs might be responsible for activating dentin-bound or salivary MMPs, establishing a synergy between these two classes of enzymes acting in different stages in caries progression (Nascimento et al., 2011). Even though the involvement of MMPs in dentin pathologies was suggested already about fifteen years ago (Tjäderhane et al., 1998a, 1998b), CTs were only detected in dentin recently (Nascimento et al., 2011; Tersariol et al., 2010; Vidal et al., 2014). Therefore, no detailed information about the individual or synergistic roles of these enzymes on dentin matrix remodeling/proteolysis processes is available (Tjäderhane et al., 2013).

We hypothesize that, to work synergistically, these two groups of enzymes should be localized very close together and in the vicinities of their target substrates. This is supported somehow by our previous studies wherein we found the existence of at least some MMPs and CTs members distributed in the same space occupied by collagen, both in sound and carious teeth (Vidal et al., 2014), which in turn indicates a possible interplay between these different classes of proteases as they can be found active when extracted from dentin (Nascimento et al., 2011; Tersariol et al., 2010). The present study investigated the distribution of CT-B and K in dentin matrix and evaluated whether co-occurrence of CTs and MMPs can be observed in this tissue. Additionally, it was tested whether the proteolytic activity of dentin would be predominantly due to activity of either CTs or MMPs.

2. Material & methods

2.1. Material

Fifteen freshly extracted human molars, obtained with patient's informed consent under a protocol approved by IRB Committee of Piracicaba School of Dentistry/UNICAMP were used in this study. Reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise specified.

2.2. Pre-embedding technique: FEI-SEM analysis

Specimens were processed for a pre-immunolabelling procedure as described by Mazzoni et al., 2009. Briefly, cryofractured dentin fragments were partially demineralized in 0.5 M ethylene diamine tetracetic acid (EDTA; pH 7.4) for 30 min, immersed in a 0.05 M Tris HCl buffer solution (TBS) at pH 7.6, with 0.15 M NaCl and 0.1% bovine serum albumin and then pre-incubated in normal goat serum in 0.05 M TBS at pH 7.6 for 30 min. Specimens were then incubated overnight with one of the primary antibodies: rabbit IgG anti-human cathepsin B (Calbiochem, Billerica, MA, USA) or mouse IgG anti-human cathepsin K (Biovendor, Brno, Czech Republic) (dilution 1:100 in 0.05 M TBS, pH 7.6, 37 °C). Gold labeling was performed using a secondary antibody, a goat anti-mouse or anti-rabbit IgG conjugated with 15 nm colloidal gold particles (British BioCell International, Cardiff, UK, dilution 1:20) in 0.02 M TBS (pH 8.2) for 90 min.

All specimens were fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer pH 7.2 for 4 h, rinsed and dehydrated in graded concentrations of ethanol. The samples were critically point dried and coated with carbon using a Balzers Med 010 Multicoating System (Bal-Tec AG, Liechtenstein). Observations were performed using FEI-SEM (JSM 890, JEOL, Tokyo, Japan) at 7 kV and 1×10^{-12} A. Images were obtained using a combination of backscattered and secondary electron detectors (Breschi et al., 2003).

2.3. Post-embedding technique: TEM analysis

Dentin fragments submitted to the post-embedding technique were immediately fixed in 4% paraformaldehyde–0.1% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.2, for 4 h) and demineralized in 0.5 M EDTA for 3 months. Then, the specimens were dehydrated in graded concentrations of ethanol and embedded in LR White resin (London Resin, Berkshire, UK). Ultrathin sections (80 nm thick) were obtained, mounted on formvar nickel grids and processed for immunohistochemical labelling (Breschi et al., 2003).

Immunolabeling was performed as described above using the same primary anti-CT-B, anti-CT-K and secondary antibodies. Additionally, a double-labeling technique was performed to identify, simultaneously, CT-B and MMP-2 (mouse IgG anti-human MMP-2, Abcam, Cambridge, UK) on dentin matrix. After incubation with the primary antibodies, samples were rinsed and incubated with two colloidal gold-conjugated secondary antibodies, a goat anti-rabbit IgG conjugated with 30-nm colloidal gold particles for the identification of CT-B and a goat anti-mouse IgG conjugated with 15-nm colloidal gold particles for MMP-2 identification. Grids were then stained with 3% uranyl acetate and lead citrate for examination in a ZEISS 109 TEM operated at 60 kV (Zeiss, EM 109, Carl Zeiss, Oberkochen, Germany). Controls consisted of sections incubated with secondary antibodies only.

2.4. In situ *zymography*

Two freshly extracted third molars were used to obtain longitudinal slices of 200 μm thickness (Isomet – Buehler Ltda, Lake Bluff, IL, USA). To verify the specificity of enzymes activity, the slices were previously incubated in deionized water (control), E-64 (specific inhibitor of CTs) or 1,10-Phenanthroline (MMPs inhibitor) for 30 min at room temperature. *In situ* zymography was performed using a quenched fluorescein-conjugated gelatin as substrate (E-12055, Molecular Probes, Eugene, OR, USA), as described by Mazzoni et al., 2012. The gelatin stock solution was diluted 1:8 in a buffer (NaCl 150 mM, $CaCl_2$ 5 mM, Tris-HCl 50 mM, pH 8.0) with 10 μL of anti-fading agent (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK). A 80-μL of this mixture was placed on top of each slab and incubated in a dark humid chamber at 37 °C for 24 h.

The hydrolysis of quenched fluorescein-conjugated gelatin substrate was assessed by a multi-photon confocal microscope, excitation: 488 nm and emission: 530 nm (Zeiss, LSM 780, Carl Zeiss, Oberkochen, Germany). Optical sections of 85 μm thick were acquired and the stacked images were analysed, quantified, and processed with ZEN 2010 software (Carl Zeiss).

2.5. Monitoring of the protelotytic activity in dentin extract

Dentin powder of 10 freshly extracted human molars was obtained as described previously (Scaffa et al., 2012). Aliquot of 1.2 g was used for enzyme extraction (Martin-De Las Heras et al., 2000). Briefly, dentin powder was treated with 4 M guanidine-HCl; then demineralized with 0.5 M EDTA and treated again with 4 M guanidine-HCl (G2-extract). The amount of protein in G2-extract was determined according to Lowry et al. (1951), and specific activities were calculated with reference to protein concentration.

The total CTs activity was monitored spectrofluorometrically by using the CT-specific fluorogenic substrate Z-FR-MCA (carbobenzoxy-phenylalanine-arginine-7-amido-4-methyl coumarin). For the assay, 10 μL of the dentin extract was added to 50 mM sodium

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