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2017-02


http://hdl.handle.net/10138/233340
https://doi.org/10.1016/j.archoralbio.2016.11.011

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

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ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form 26 October 2016
Accepted 20 November 2016

Keywords:
Cysteine cathepsins
Matrix metalloproteases
Proteolytic activity
Dentin
Immunohistochemistry

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-immunolabelling technique was used to identify, at once, the occurrence of those enzymes in dentin. Activities of CTs and MMPs in dentin extracts were evaluated spectrophotometrically. 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 (Fonovic & 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 physiopathological processes occurring in dentin matrix (Dickinson 2002; Hannaset et al., 2007). The co-expression of two distinct
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-immunolabeling 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, CaCl₂ 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, Cambridge, 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 proteolytic 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., 2006). 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 (carbohydrazoxy-phenylalanine-arginine-7-amido-4-methyl coumarin). For the assay, 10 μl of the dentin extract was added to 50 mM sodium
phosphate buffer (pH 5.7) containing 10 μM of the substrate with 1 mM DTT and incubated at 37 °C for 24 h. Excitation and emission wavelengths were set at 380 and 460 nm, respectively. The total collagenolytic/gelatinolytic MMP activity in dentin extract was measured with the MMP-specific internally quenched fluorescent peptide substrate Abz-GPQLAGQ-EDDnp [ortho-aminobenzoic acid– Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-N-(2,4-dinitrophenyl)- ethylenediamine]. For MMPs, 10 μL of the dentin extract was added to 50 mM HEPES buffer (pH 7.5) containing 10 μM of the substrate and incubated at 37 °C for 24 h. The excitation and emission wavelengths were 320 and 420 nm. The substrate’s hydrolysis were checked using 2 mmol/L 1,10 phenanthroline (specific MMP inhibitor) or 10 μM E-64 (specific CT inhibitor). The assays were performed in duplicate, in a 96-well plate by a fluorometer (Tecan GENios Pro Multifunction Microplate Reader; Tecan, Mannedorf, Switzerland). The specific enzymatic activity, for both CTs and MMPs was calculated as the difference between final and initial fluorescence readings (delta fluorescence) and expressed in arbitrary units of fluorescence per microgram of protein in the extract (AUf/μg).

3. Results

Positive immunolabeling patterns for CT-B and CT-K were observed in partially demineralized dentin surfaces under FEI-SEM examination as indicated by the presence of the gold nanoparticles, which appear as spherical spots of 15 nm in diameter (Fig. 1). Both CT-B and CT-K were localized along the collagen fibrils. High-magnification images show globular aggregates by a cluster of 2–3 gold nanoparticles along the branching points on collagen (Fig. 1B).

TEM images of demineralized dentin revealed different distribution of the CT-B and CT-K labeling (Fig. 2). The intensity of immunoreaction for CT-B was scattered and higher than CT-K in dentin matrix. Labeling for CT-K showed weak distribution over the collagen fibrils (Fig. 2). Similar labeling patterns were obtained for CT-B and CT-K under both FEI-SEM and TEM observations. The double-labeling of CT-B and MMP-2 was performed with two sizes of colloidal gold particles (black spots) conjugated with the antibodies, in which CT-B is represented by the larger 30 nm and MMP-2 by the smaller 15 nm particles. TEM images revealed the co-occurrence of CT-B and MMP-2 in dentin matrix, but with different distribution (Fig. 2E,F). Labeling for MMP-2 was randomly distributed over the collagen matrix, while CT-B tended to localize in aggregates (Fig. 2E,F).

The in situ zymography revealed the relative effects of different families of enzymes on the total enzymatic activity of dentin. For the controls, an intense green fluorescence indicating gelatinolytic activity was observed in mineralized dentin, located especially in dentinal tubules (Fig. 3B,C). The treatment with E-64 and 1,10-phenanthroline inhibited the gelatinolytic activity, seen as decrease in green fluorescence (Fig. 3E,F,H,I) with different rates of specificity. Phenanthroline inhibition of MMPs resulted in

Fig. 1. FEI-SEM micrographs of partially demineralized dentin after a pre-embedding immunolabeling procedure. Labeling can be identified as electron-dense white spots under the electron beam (arrows). (a,c) Low magnification view (>30,000) of the partially demineralized dentin surface showing a mild CT-B and CT-K labeling signal. (b) A higher magnification view (>50,000) of the partially demineralized surface: positive immunohistochemical staining identifying CT-B located along the collagen fibrils. (d) High magnification FEI-SEM micrographs (>100,000) revealing a moderate labeling for CT-K staining.
stronger reduction in fluorescence (Fig. 3) indicating that intrinsic MMPs gelatinolytic activity was higher than that of CTs.

Proteolytic activity in G2-dentin extract was detected using CT- and MMP-specific fluorogenic substrates (Fig. 4). The activity analysis for CT-substrate demonstrated strong inhibition by E-64 (Fig. 4A) and also a decrease in the rate of hydrolysis of the MMP-substrate in the presence of MMP inhibitor 1,10-phenanthroline (Fig. 4B). Results showed, therefore, the presence of both active MMPs and CTs in dentin.

4. Discussion

The proteolytic enzymes potentially involved in the collagen metabolism can be examined by immunocytochemistry, a powerful imaging technique for correlating composition with structure (Nanci, 1999). To the best of our knowledge, this is the first study that evaluates the in situ co-occurrence/distribution of CTs and MMPs in human dentin matrix by immunogold labeling correlative FEI-SEM and TEM microscopies.

FEI-SEM demonstrated the presence of CT-B and CT-K in dentin surface after brief demineralization with EDTA. Calcium chelation by EDTA provides a mild process of tissue demineralization, preserving the antigenic binding sites better than strong acids do (Breschi et al., 2003) and it has been therefore used to reveal the presence of various proteins with FEI-SEM, including MMP-2, -3 and -9 (Mazzoni et al., 2009, 2011). Even though FEI-SEM images indicated the localization of CTs apparently onto collagen fibrils, it must be taken in consideration that SEM images represent, at maximum, a bidimensional depiction of surface topography, hence the actual interaction between the enzymes and dentin organic matrix cannot be fully confirmed through this method. Therefore, TEM analyses with completely demineralized dentin were performed, confirming CT-B and CT-K binding along collagen fibrils. The binding might occur directly to collagen or collagen-
bound proteoglycans (PGs), since it has been already demonstrated that CTs readily form complexes with PG glycosaminoglycan (GAG) side chains (Fonović & Turk, 2014; Li et al., 2004). GAGs may have a significant effect on CT activities, as they differentially regulate their activities (Fonović & Turk, 2014; Li et al., 2004). This is especially true for CT-K, which is highly dependent on forming a complex with chondroitin-4-sulfate GAG for its collagenolytic activity (Li et al., 2004), but not against non-helical substrates such as gelatin (Selent et al., 2007).

The proteolytic activity of proteases can be verified in different ways (Martin-De Las Heras et al., 2000; Mazzoni et al., 2012; Tersariol et al., 2010). In this study, the collagenolytic/gelatinolytic activities were demonstrated by substrate hydrolysis at the presence/absence of enzymes inhibitors. In addition, the gelatinolytic activity of enzymes was further assessed by in situ zymography. Both MMPs and CTs activities were observed spectrofluorometrically using fluorogenic substrates. Using this technique, once the peptide is enzymatically hydrolyzed, an increase in the fluorescence can be detected. Although these enzymes may be involved in enamel and dentin formation, their fate after mineralization is not known. The G2-extract represents the proteolytic activity of enzymes that were extracted with EDTA.

Fig. 3. Dentin slices pre-incubated without inhibitors (control) or with E-64 and 1,10-phenanthroline and incubated for 24 h with quenched fluorescein-labeled gelatin; (A,D, G): Differential interference contrast (DIC), showing the optical density of the dentin surface; (B,E,H): Acquired image at green-channel, showing fluorescence in mineralized dentin; (C,F,I): Merged images. (A-C) control group indicating that the fluorescein conjugated gelatin was strongly hydrolyzed; (D-F) dentin slice incubated with E-64 inhibitor, showing partial inhibition of gelatinolytic activity in dentin; (G-I) dentin slice incubated with 1,10-phenanthroline inhibitor, showing stronger inhibition of gelatinolytic activity; (J) Specific fluorescence emission intensity in control and cathepsin-inhibited (with E-64) or MMP-inhibited (with 1,10-phenanthroline) samples.
while were found originally deeply embedded in mineral (Martín-De Las Heras et al., 2000). The proteolytic activity in the G2 extract suggests that these enzymes were deposited in the dentin matrix during mineralization and were most probably tightly bound to the collagen. Dentin enzyme activity analysis against enzyme-specific synthetic substrates demonstrated that both CTs and MMPs are active in dentin matrix and fully capable to degrade their target substrates.

To better determine the role of each class of enzymes in proteolytic degradation of collagen, we performed in situ zymography using specific enzymes inhibitors. Gelatin was used herein as a substrate, as commonly reported in literature for dental and craniofacial tissues (DeVito-Moraes et al., 2016; Mazzoni et al., 2012; Pessoa et al., 2013). Although other dye-quenched fluorogenic substrates such as collagen and casein might result in different patterns of proteolytic/gelatinolytic activity (Sakuraba et al., 2006), the dramatic reduction in fluorescence by addition of E-64 and 1,10-Phenanthroline support our results. Indeed, the in situ zymography demonstrated more intrinsic MMP activity, indicating stronger role in dentin collagenous matrix degradation in pathologies. To the best of our knowledge, this is the first study to directly compare dentinal CT- and MMP-activities. Previously, Tezvergil-Mutluay et al. (2013) demonstrated 90% lower CT-dependent release of CTX (C-terminal crosslinked telopeptide of type I collagen) compared to MMP-dependent ICTP (C-terminal crosslinked telopeptide of type I collagen). Even though these telopeptidase activities may not directly reflect the total amounts of different enzyme groups in dentin, together with our immunolabeling results, they suggest that MMPs may be present in markedly higher quantities in dentin.

The reason for the higher MMPs than CTs activity in dentin is not well understood, but may be related to the role of theses enzymes in collagen metabolism processes. These two families of proteases play different coordinated roles in matrix degradation and bone resorption. CT-K plays a unique role among the various protease, since its collagenolytic activity does not depend on destabilization of the triple helix in contrast to the other enzymes (Garnero et al., 1998). Thus, once collagen is cleaved, the triple helix would unwind and become available for degradation by other gelatinases (Delaisé et al., 2003).

In other tissues, MMPs and CTs are known to be able to regulate the activities of each other, and possible CT-MMP interactions have also been suggested to occur in dentin (Nascimento et al., 2011; Tjäderhane et al., 2013). In principle, this would require a close proximity with each other and the substrate, i.e. collagen. Although double-immunolabeling with MMP-2 and CT-B antibodies showed co-occurrence of the enzymes, which is suggestive of an interaction, it could not be confirmed by this method. Some degree of joint action in enzyme activities is supported by the in situ zymography findings. CT-specific inhibition with E-64 and MMP-specific inhibition with 1,10-phenanthroline maintained approximately 50% and 20% of gelatinolysis compared to untreated controls, respectively. Therefore, it appears that the total activity is not only additive, but inhibition of one enzyme family has some inhibition on other, too. Thus, even though the results do not support the hypothesis of CT-MMP interplay in dentin matrix degradation, the possibility of interactions of other enzymes still remains. Further studies with inhibitors that specifically inhibit certain members of MMP- or CT-families are needed to examine this possibility.

The latent forms of CTs and MMPs enzymes can be activated in acidic conditions. CTs are auto-activated in low pH, and most are considered unstable and inactive in neutral pH (Turk, Turk, & Turk, 2000). Conversely MMPs are neutral proteinases, but latent forms can be activated with acidic pH followed by neutralization (Tjäderhane et al., 1998a). CT-B enhances the activity of the MMPs by shifting the balance between MMPs and TIMPs through inactivation of the MMP-specific tissue inhibitors TIMP-1 and TIMP-2 (Nagase 1997; Kostoulas et al., 1999). Therefore, CTs activated at the acidic conditions may further activate dentin-bound MMPs, which in turn would become functional after pH neutralization, resulting in dentin matrix enzymatic degradation. This suggests the potential pH-related interactions and synergistic effects of CTs and MMPs in collagen degradation.

In conclusion, MMPs and CTs are co-distributed within sound dentin matrix, which supports the interaction of both families of enzymes for the proteolytic degradation of dentin in pathological conditions. While active enzymes were extracted from dentin, MMPs activity is predominant over CTs. Further investigations on proteolytic activity associated with a proteomic approach may show the involvement and interplay between the main proteolytic enzymes in dentin. In this way, the actual role of the different classes of enzymes participating in the mechanisms of collagen degradation in physiological and pathological conditions could be established.

Conflict of interests
None.

Funding
FAPESP (2007/54618-4 P.I. Carrilho and 2009/14005-9 P.I. Scaffa) and CNPq (312768/2013-3 P.I. Carrilho), Brazil.

Fig. 4. Enzymes activity in G2 extract over the enzyme-specific substrates with and without (control) respective specific inhibitors. (A) Activity against cathepsin-specific ZFR-MCA peptide. E-64 resulted with 93% inhibition compared to untreated control. (B) Activity against MMP-specific Abz-GPQGLAGQ-EDDnp peptide. MMP-specific inhibitor 1,10-phenanthroline inhibited 76% of the activity.
Ethical approval

The manuscript was approved by the Ethics Committee in Research of the Faculty of Dentistry at Piracicaba - University of Campinas (117/2009).

Acknowledgments

The authors thank the funding agencies FAPESP (2016/07996-2 – PI: M.R. Carrilho) and CNPq (312768/2013-3 – PI: M.R. Carrilho), Brazil.

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