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ABSTRACT

This study determined if dentin proteases are denatured by phosphoric acid (PA) used in etch-and-rinse dentin adhesives. Dentin beams were completely demineralized with EDTA for 30 days. We “acid-etched” experimental groups by exposing the demineralized dentin beams to 1, 10, or 37 mass% PA for 15 sec or 15 min. Control beams were not exposed to PA but were incubated in simulated body fluid for 3 days to assay their total endogenous telopeptidase activity, by their ability to solubilize C-terminal crosslinked telopeptides ICTP and CTX from insoluble dentin collagen. Control beams released 6.1 ± 0.8 ng ICTP and 0.6 ± 0.1 ng CTX/mg dry-wt/3 days. Positive control beams pre-incubated in p-aminophenyl-mercuric acetate, a compound known to activate proMMPs, released about the same amount of ICTP peptides, but released significantly less CTX. Beams immersed in 1, 10, or 37 mass% PA for 15 sec or 15 min released amounts of ICTP and CTX similar to that released by the controls ($p > 0.05$). Beams incubated in galardin, an MMP inhibitor, or E-64, a cathepsin inhibitor, blocked most of the release of ICTP and CTX, respectively. It is concluded that PA does not denature endogenous MMP and cathepsin activities of dentin matrices.

KEY WORDS: demineralized, MMPs, cathepsins, collagen, bonding.

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Effect of Phosphoric Acid on the Degradation of Human Dentin Matrix

INTRODUCTION

Matrix metalloproteinases (MMPs) have been implicated in various physiological and pathological processes of the pulpodentin complex (Tjäderhane *et al.*, 2001; Bourd-Boittin *et al.*, 2005; Lehmann *et al.*, 2009). Detection of MMPs in mineralized dentin (Martin-De Las Heras *et al.*, 2000; Sulkala *et al.*, 2004, 2007; Mazzoni *et al.*, 2007, 2011) and carious dentin (Tjäderhane *et al.*, 1998; Chaussain-Miller *et al.*, 2006) indicates that MMPs trapped within peripheral dentin may be activated once they are exposed by acid demineralization. The recent discovery of cysteine cathepsins in normal and carious dentin (Nascimento *et al.*, 2011; Tersariol *et al.*, 2010; Scaffa *et al.*, 2012) increases the list of potential endogenous proteases in dentin matrices. Demineralization of the sound, unaffected collagen matrix exposes endogenous dentin proteases, although most of them remain bound to the collagen (Martin-De Las Heras *et al.*, 2000).

Dentin demineralization is also involved in dentin bonding. In etch-and-rinse bonding procedures, 32 to 37 mass% phosphoric acid (PA) is used to expose the collagen fibril meshwork for micromechanical retention of adhesive resins (Nakabayashi and Pashley, 1998). Although a small fraction of these proteases may be extracted by acids (Mazzoni *et al.*, 2007; DeMunck *et al.*, 2009), most remain bound to the matrix in their active forms, where they can slowly hydrolyze the collagen matrix (Pashley *et al.*, 2004).

Traditionally, investigators have extracted proteases from the dentin matrix for identification by Western blotting and evaluation of their functional activity by zymography (Tjäderhane *et al.*, 1998; Martin-De Las Heras *et al.*, 2000; Sulkala *et al.*, 2004, 2007; Mazzoni *et al.*, 2007; Breschi *et al.*, 2010). In those studies, it was assumed that soluble proteases functioned similarly to those that were matrix-bound, and that all proteases were equally extractable. An alternative approach is to study the functional activity of matrix-bound MMPs *in situ*. This approach measures the end results of protease activity by

quantifying the rate of solubilization of hydroxyproline-containing peptide fragments (Garnero *et al.*, 1998), or the rate of release of collagen C-terminal telopeptides by cysteine cathepsins and MMPs (CTX and ICTP, respectively), as these proteases slowly degrade the dentin matrix (Garnero *et al.*, 2003; Fedarko *et al.*, 2004; Osorio *et al.*, 2011). The advantage of this indirect approach is that one may assay the total protease activities of the dentin matrix in their natural bound state, and their responses to activators and inhibitors.

Clinical acid-etching of dentin with 37% PA has been reported to decrease MMP activity (Pashley *et al.*, 2004; Mazzoni *et al.*, 2006; Nishitani *et al.*, 2006). It is not clear if this is due to denaturation of MMPs or the creation of CaHPO₄ precipitates over the enzymes. To differentiate between these 2 possibilities, we demineralized dentin beams completely in EDTA to remove all mineral. This prevented any calcium precipitates from forming when such dentin was “acid-etched” with PA. Thus, the conditions used in this experiment were far removed from clinical conditions.

If one completely demineralizes dentin with ethylenediaminetetraacetic acid (EDTA), most of the matrix-bound proteases remain on the dentin matrix (Martin-De Las Heras *et al.*, 2000). One may then expose the matrix-bound MMPs and cathepsins to various concentrations of PA without the buffering effects of apatite mineral. If matrix-bound MMPs and cathepsins of EDTA-demineralized dentin are already active, and if they are susceptible to denaturation by PA, then acid-etching EDTA-demineralized dentin matrix with PA should denature MMPs and cathepsins and reduce their activities.

Thus, the purpose of this work was to measure the total functional MMP and cathepsin enzyme activities of EDTA-demineralized dentin matrices before and after exposure to various concentrations of PA. The null hypotheses were that EDTA-demineralization of human dentin does not activate protease enzymes, and that further exposure of EDTA-demineralized dentin to PA does not inactivate dentin MMP or cathepsin activity.

MATERIALS & METHODS

Measurement of Matrix-bound Endogenous Proteases in Demineralized Dentin

Seventy extracted human third molars were obtained from 18- to 21-year-old patients under a protocol approved by the Georgia Health Sciences University. The teeth were stored frozen until required. After they were thawed, the enamel and superficial dentin were removed by means of an Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) under water cooling. Dentin beams with dimensions 6 x 2 x 1 mm were sectioned from the mid-coronal dentin (70 beams, 1 *per* tooth).

The beams were completely demineralized in 0.5 M EDTA (pH 7.4) for 30 days at 4°C with constant stirring. Demineralization was followed by serial measurements of beam stiffness by 3-point loading. Mineralized dentin has a modulus of elasticity between 16,000 and 19,000 MPa. Dentin beams completely demineralized in EDTA have a modulus of elasticity of 4 to 6 MPa (Carrilho *et al.*, 2009). Ten beams were assigned to each of 7 groups. Group 1 was the negative control, which was not treated with any acid.

Group 2 medium contained the same medium as group 1 plus 2 mM p-aminophenylmercuric acetate (APMA), an agent known to activate pre-forms of MMPs. After 2 hrs of incubation in APMA, the beams were transferred to APMA-free incubation buffer. Groups 3, 4, and 6 were treated with 1, 10, or 37% PA, respectively, for 15 min to determine if increasingly higher PA concentrations would cause more denaturation. Group 5 specimens were treated with 37% PA for 15 sec to simulate normal clinical etching to serve as controls for the prolonged etching for 15 min used in groups 3, 4, and 6. Group 7 beams were incubated in the same buffer as controls but contained 200 μM galardin, an MMP inhibitor, plus 50 μM E-64, a cathepsin inhibitor. All beams exposed to PA or APMA were then dropped into 50 mL of buffered medium (pH 7) for 5 min to dilute the reagents and buffer the specimens. The pH values of 1, 10, and 37% PA were 1.42, 0.51, and -0.37, respectively. Each beam was then immersed in 0.5 mL of a buffered medium composed of 5 mM HEPES, 2.5 mM CaCl₂·H₂O, 0.05 mM ZnCl₂, and 120 mM NaCl adjusted to pH 7.4. The sealed tubes were incubated in a shaker-water bath at 37°C for 3 days. The entire 0.5 mL of medium was removed after 3 days. From 10- to 20-μL aliquots of the incubation medium were used to measure solubilized ICTP and CTX collagen fragments.

Solubilized Telopeptides

We determined matrix degradation by MMPs by measuring the quantity of solubilized type I collagen C-terminal cross-linked telopeptides (ICTP) (Garnero *et al.*, 2003; Osorio *et al.*, 2011) over the 3-day incubation periods, using the ICTP ELISA kit (TSZ ELISA, Framingham, MA, USA; Cat. #HU9655). The only source of ICTP telopeptide fragments in mineralized matrices is attributed to the telopeptidase activity of MMPs (Garnero *et al.*, 1998, 2003; Osorio *et al.*, 2011). We determined matrix degradation by cysteine cathepsins by measuring the amount of solubilized C-terminal peptide, CTX, in the incubation medium using the Serum CrossLaps ELISA (Immunodiagnostic System, Scottsdale, AZ, USA).

Statistical Analyses

The ICTP and CTX release rates (in ng telopeptide/mg dry dentin *per* 3 days) from all groups were compared for normality (Kolmogorov-Smirnov test) and homoscedasticity (modified Levine test). Since the normality and equality variance assumptions of the data were valid, they were analyzed by 2 different one-way analyses of variance (ANOVAs) (one for ICTP and the other for CTX), with dentin treatment (APMA PA, etc.) as the single factor. *Post hoc* multiple comparisons were performed with the Tukey test using SigmaStat 3.11 (Systat Software, San Jose, CA, USA). Statistical significance was pre-set at $\alpha = 0.05$.

RESULTS

For the negative control group that was never exposed to PA (incubated in SBF only), the EDTA-demineralized dentin beams released 6.1 ± 0.8 ng ICTP and 0.6 ± 0.1 ng CTX *per* mg dentin dry-weight/3 days (Fig.). For the positive control group that was

incubated in 2 mM APMA to activate MMP proforms, the ICTP release rate remain unchanged compared with controls, but APMA caused a large, significant ($p < 0.05$) decrease in CTX release (Fig.). The rate of release of ICTP from EDTA-demineralized beams did not change significantly as the PA concentration or exposure time increased. Group 7 specimens were incubated in a buffered medium containing 200 μ M galardin, an MMP inhibitor, and E-64, a cathepsin inhibitor. The presence of both inhibitors significantly reduced ($p < 0.05$) the rate of release of ICTP and CTX to near zero, indicating that ICTP and CTX are released from dentin collagen by MMPs and cathepsins. Other experiments in which demineralized dentin beams were incubated in only galardin or only E-64 revealed that their action was limited to MMPs or cathepsins, respectively (data not shown). The rate of release of CTX from EDTA-demineralized dentin was unchanged as a function of PA concentration or exposure time. These values were not significantly different from those of the corresponding negative controls (Fig.). Treatment of dentin with APMA or galardin/E-64 was the only variable that significantly ($p < 0.05$) lowered CTX values below those of the control.

DISCUSSION

Osorio *et al.* (2011) were the first to demonstrate that MMP-2 has telopeptidase activity in dentin, although they did not use that term. Using an ICTP-specific ELISA assay, they incubated completely demineralized dentin beams in a medium for 1, 7, or 21 days. The control media contained 0.87 ± 0.11 μ g/L ICTP per 24 hrs. When they added 10 μ g of exogenous active recombinant human MMP-2, the ICTP concentration increased to 210 ± 11 μ g/L in the media. This indicated that MMP-2 attacked the C-terminal telopeptides in demineralized dentin, to release cross-linked ICTP fragments. The results of the present study confirm that endogenous dentin MMPs-2, -8, and -9, known to exist in the dentin matrix (Mazzoni *et al.*, 2007; Sulkala *et al.*, 2007), do indeed release ICTP from dentin matrix. In addition, endogenous cathepsins also attack C-terminal telopeptides in dentin matrix to release the smaller CTX peptide fragments.

The total MMP activity of EDTA-demineralized dentin, measured as the release of ICTP telopeptide fragments, is an order of magnitude larger than the total cysteine cathepsin activity of demineralized dentin, measured as the release of CTX telopeptide fragments. By measuring the relative amounts of ICTP and CTX telopeptide fragments in the incubation medium, one can follow the relative amounts of MMPs vs. cathepsin protease activity. Thus, the dentin matrix contains 2 classes of endogenous proteases that can act as telopeptidases for demineralized collagen matrix degradation. Both ICTP and CTX release were inhibited by galardin and E-64, respectively. Future work will identify their relative collagenolytic activity.

The results of this work require rejection of the first null hypothesis, that EDTA-demineralization of human dentin does not activate protease enzymes. That is, EDTA-demineralized control dentin exhibited significant MMP and cathepsin activity without the use of acids or APMA to activate proforms of dentin MMPs, confirming previous findings (Carrilho *et al.*, 2009).

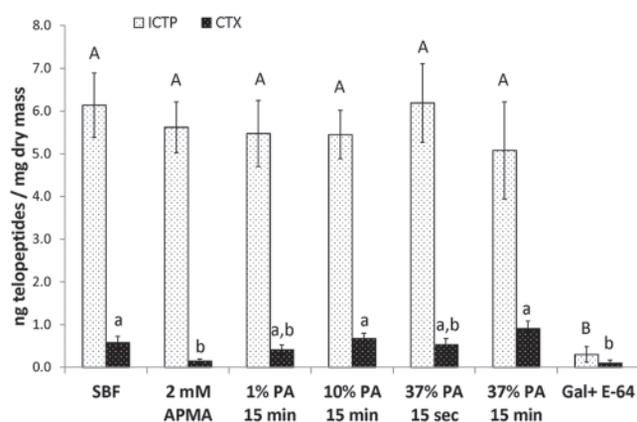


Figure. Bar chart of C-terminal telopeptide (ICTP and CTX) release from EDTA-demineralized dentin beams after various treatments with increasing concentrations of phosphoric acid. Values are ng telopeptide/mg dentin dry-wt/3 days. Bar heights are mean values ($n = 10$); brackets indicate \pm SD. Bars identified by different letters are significantly different. APMA = 2 mM p-aminophenylmercuric acetate pre-treatment for 2 hrs at 37°C. PA = phosphoric acid. SBF = simulated body fluid. Bars identified with similar upper-case letters are not significantly different ($p > 0.05$) by one-way ANOVA for effects of treatments on ICTP release rate. Bars identified by different lower-case letters are significantly different ($p < 0.05$) by one-way ANOVA followed by Tukey's test for effects of treatments on CTX release rate. Gal/E-64 refers to group 7, where the incubation medium contained 200 μ M galardin, a potent MMP inhibitor, and E-64, a cathepsin inhibitor.

Treatment of the demineralized dentin with 2 mM APMA for 1 hr at 37°C did not activate matrix-bound MMPs. Rather, APMA treatment significantly lowered the total cathepsin activity of dentin because it reduced the release of CTX peptide fragments, indicating that some cathepsins were already active. Organic mercurials are known to oxidize with sulfuryl groups in proteins, causing their inactivation (Means and Feeny, 1971; Pasternak *et al.*, 1975).

It is possible that the MMP proforms are activated by members of the small integrin-binding ligand N-linked glycoproteins (SIBLINGs) in dentin. Bone sialoprotein activates proMMP-2; dentin matrix protein-1 activates MMP-9 (Fedarko *et al.*, 2004). MMP-2 is known to activate other proforms of MMPs (Nagase, 1997). Cathepsins are also known to activate MMPs (Tersariol *et al.*, 2010).

Our previous work indicated that acid-etching mineralized dentin powder with 37% PA for 15 sec caused a large (65%) reduction in the collagenolytic activity of that powder (Pashley *et al.*, 2004). Similar results were obtained by Nishitani *et al.* (2006). Mazzoni *et al.* (2006) acid-etched dentin powder with 10% PA for 15 sec, resulting in 98.1% reduction in collagenolytic activity compared with mineralized dentin powder control. These results were interpreted as being due to very rapid exposure of the collagen matrix and its matrix-bound MMPs by PA-demineralization, which was followed, almost immediately, by denaturation of the MMPs by the very low pH of these solutions. However, a recent experiment on the effects of self-etching adhesives provides an alternative

explanation. Iwasa *et al.* (2011) examined the buffering capacity of dentin powder exposed to single-step, self-etching adhesives. The baseline pHs of the self-etching adhesives before etching ranged from 0.97 to 2.83. After the adhesives were mixed with dentin powder for 3 min, the pH values rose to 6.3-7.11. Scanning electron microscopy examination of the etched dentin powder revealed that the surfaces were covered with a “dense, insoluble precipitate” that obscured the fibrillar nature of the underlying collagen matrix. We believe that such precipitates are reaction products of the interaction of acids with dentin matrix apatite that mask the collagen fibrils from matrix proteases.

The present paper investigated the question from a different perspective. By completely demineralizing dentin powder with 0.5 M EDTA, and then exposing the demineralized powder to PA, there is no possibility of a layer of CaHPO₄ forming over the demineralized collagen fibrils, because all of the calcium was removed by EDTA for 30 days. “Acid-etching” of the EDTA-demineralized beams with all concentrations of PA tested for up to 15 min had no effect on the total MMP activity of dentin (*i.e.*, on the release of ICTP fragments). The prolonged exposure of EDTA-demineralized dentin was used to show that even under extreme conditions that are far from clinical practice, PA does not denature matrix-bound proteases. That is, there was no large decrease in MMP or cathepsin activity following exposure to PA. These results argue against the idea that 37% PA can chemically denature matrix-bound proteases. Thus, these results require acceptance of the second test null hypothesis, that exposure of EDTA-demineralized dentin to PA does not inactivate dentin MMP activity.

Within the limits of the present study, it may be concluded that treatment of dentin with 37% PA does not denature the endogenous proteases of the dentin matrix. We speculate that matrix-bound endogenous proteases are far more stable to thermal or chemical denaturation than are those same proteases in soluble form (Fernandez *et al.*, 2002; Berberich *et al.*, 2005; Roessl *et al.*, 2010). The closeness of the active site of MMPs and cathepsins to their collagen-binding sites (Nagase *et al.*, 2006; Brömme and Wilson, 2011) may stabilize collagen-bound MMPs compared with soluble forms.

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