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ABSTRACT

This study analyzed the amounts of solubilized telopeptides cross-linked carboxyterminal telopeptide of type I collagen (ICTP) and C-terminal crosslinked telopeptide of type I collagen (CTX) derived from matrix-metalloproteinases (MMPs) and cysteine cathepsins (CTPs) subsequent to application of a filler-free (Res.A) or an ion-releasing resin (Res.B) to ethylenediaminetetraacetic acid (EDTA)-demineralized dentin with or without zoledronate-containing primer (Zol-primer) pre-treatment. The chemical modification induced following treatments and artificial saliva (AS) storage was also analyzed through attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). Totally EDTA-demineralized specimens were infiltrated with Res.A or Res.B with or without Zol-primer pre-treatment, light-cured, and immersed in AS for up to 4 wk. ICTP release was reduced following infiltration with Res.B and further reduced when Res.B was used with Zol-primer; remarkable phosphate mineral uptake was attained after AS storage. CTX release was increased in Res.A- and Res.B-treated dentin. However, when Zol-primer was used with Res.A, the CTX release fell significantly compared to the other tested resin-infiltration methods. In conclusion, zoledronate offers an additional inhibitory effect to the ion-releasing resins in MMP-mediated collagen degradation. However, Zol-primer induces a modest reduction in CTX release only when used with resin-based systems containing no ion-releasing fillers.

KEY WORDS: proteolysis, phosphorylation, matrix metalloproteinase, cathepsins, calcium phosphates, bisphosphonates.

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Zoledronate and Ion-releasing Resins Impair Dentin Collagen Degradation

INTRODUCTION

Matrix metalloproteinases (MMPs) are proteolytic enzymes existing within mineralized dentin in active or pro-enzyme forms (Palosaari *et al.*, 2003). These MMPs are exposed and activated when phosphoric acid or self-etching primers (Mazzoni *et al.*, 2006; Lehmann *et al.*, 2009) are used to create micromechanical retention of adhesive resins. Although a small fraction of these proteases may be extracted by acids (De Munck *et al.*, 2009), most remain matrix-bound in their active forms and hydrolyze collagen (Pashley *et al.*, 2004). Remineralization of demineralized dentin requires inhibition of matrix proteases. Bioactive silicate-doped resin co-monomer blends reduce MMP-mediated matrix degradation processes due to apatite precipitation that remineralizes MMPs and the collagen matrix to which they are bound (Osorio *et al.*, 2012). A poly(vinylphosphonic acid) such as zoledronate may provide additional MMP inhibition due to its chelation ability (Tezvergil-Mutluay *et al.*, 2010). The combination of these 2 agents might represent a further strategy for the inhibition of endogenous proteases within the resin-dentin interface. The rationale is that the calcium and phosphate ions released by bioactive fillers may be biomimetically deposited onto MMP-rich dentin collagen fibrils (Tjäderhane *et al.*, 2013).

Cysteine cathepsins (CTPs) represent another class of endogenous proteases that may be activated during demineralization of sound and carious dentin (Nascimento *et al.*, 2011). It is unclear whether zoledronate and/or bioactive silicate-doped resins can also interfere with the proteolytic activity of cathepsins.

Thus, the objectives of the present work were to assess the enzymatic activities of functional MMPs and CTPs and to identify the chemical modification of completely ethylenediaminetetraacetic acid (EDTA)-demineralized dentin when a filler-free or an ion-releasing filled resin is used with or without zoledronate-containing primer (Zol-primer) pre-treatment. The 2 null hypotheses were that the use of the Zol-primer applied along with the ion-releasing filled or unfilled resin: (i) has no significant effect on the degradation activity

of dentin endogenous proteases (MMPs and CTPs); and (ii) induces no change in EDTA-demineralized dentin matrices.

MATERIALS & METHODS

Preparation of Experimental Adhesives

A resin co-monomer blend was formulated and designated as Res.A. A microfiller ($< 30 \mu\text{m}$) was created as described by Sauro *et al.* (2013) by the mixing of 20 wt% beta-tricalcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$; β -TCP; Sigma-Aldrich, St. Louis, MO, USA] and 80 wt% type I Portland cement (Italcementi Group, Cesena, Italy) in deionized water (ratio, 2:1) and incubation of the mixture at 40°C for 12 hr after paste was allowed to set ($24 \text{ hr}/21^\circ\text{C}$). It was incorporated into Res.A (filler/resin-ratio: 40/60 wt%) to create an ion-releasing resin (Res.B). The experimental Zol-primer was created by solvating Res.A (30 wt%) with a water/ethanol solution (70 wt%); this latter was previously formulated with deionized water (50 vol%) saturated (7 mg/mL) with zoledronate [1-hydroxy-2-(1H-imidazol-1-yl) ethane-1,1-diyl]bis-phosphonic acid (MW 290; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and absolute ethanol (50 vol%) (pH adjusted to 6.8 with 0.1 M NaOH).

Dentin Specimen Preparation and Resin Infiltration

Ten unerupted human third molars were used in accordance with the ethical guidelines (Research Ethics Committee) and stored in 0.5 wt% chloramine-T/water solution at 4°C for no longer than 1 month. Six dentin beams ($5 \times 1 \times 1 \text{ mm}$) were sectioned from the mid-coronal dentin of each tooth (total = 60). The beams were randomly distributed into different groups to reduce the tooth-effect variability: (1) mineralized control; (2) EDTA-demineralized control (no resin infiltration); (3) EDTA-demineralized dentin infiltrated by Res.A; (4) EDTA-demineralized dentin infiltrated by Zol-primer and Res.A (Zol-Pr/Res.A); (5) EDTA-demineralized dentin infiltrated by Res.B; and (6) EDTA-demineralized dentin infiltrated by Zol-primer and Res.B (Zol-Pr/Res.B). Complete dentin demineralization was attained with 0.5 M ethylenediamine tetra-acetic acid (EDTA; pH = 7.4) for 7 days at 25°C (Osorio *et al.*, 2012). The demineralization of the specimens was checked by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) (Spectrum-One, Perkin-Elmer, London, UK). Primer treatment was performed for 1 hr, while resin infiltration was performed for 12 hr by constant agitation at 25°C in the dark. Excess resin was removed by strong air-blowing of the specimens for 3 sec on each side. Light-curing was performed for 40 sec ($600 \text{ mW}/\text{cm}^2$; Optilux VLC, Demetron Kerr, Orange, CA, USA). A further group with only zoledronate (no resin infiltration) was not included, since this would make an additional step when a 3-step etch-and-rinse adhesive is used, and the optimal time for application of such a zoledronate solution is still under investigation.

Solubilized Telopeptides

The specimens were immersed in 0.5 mL artificial saliva (AS) containing 5 mM HEPES, 2.5 mM CaCl_2 , 0.05 mM ZnCl_2 , and 120 mM NaCl (pH 7.4) at 37°C for 4 wk. The sealed tubes were aged in a shaker-water bath at 37°C before the removal of 20- μL

aliquots from the AS incubation medium; these were analyzed for solubilized cross-linked carboxyterminal telopeptide of type I collagen (ICTP) and C-terminal crosslinked telopeptide of type I collagen (CTX) fragments.

MMP degradation was analyzed with the ICTP ELISA kit (UniQ EIA, Orion Diagnostica, Helsinki, Finland) (Garnero *et al.*, 2003). Cathepsin-mediated degradation of collagen (Karsdal *et al.* 2011) was followed by measurement of the amount of solubilized C-terminal peptide (CTX) by Serum Cross Laps ELISA (Immunodiagnostic System, Scottsdale, AZ, USA).

The pH of the AS was evaluated by means of a pH electrode (Mettler-Toledo, Leicester, UK), while the dry mass of each dentin specimen was measured by means of a digital microbalance. The ICTP and CTX release was calculated as pg-telopeptide/mg dry dentin and was statistically analyzed by the Komolgorov-Smirnov normality test. The results were then analyzed by 2 different one-way analyses of variance (ANOVA) (for ICTP and CTX), with dentin treatment (Zol-primer and/or Res.A/B) as the single factor and *post hoc* multiple comparisons Tukey's test ($\alpha = 0.05$) (SigmaPlot 11.5, Systat Inc., Chicago, IL, USA).

ATR-FTIR Vibrational Analysis

Middle-dentin specimens ($n = 3$ each group) were prepared ($2 \times 2 \times 1.5 \text{ mm}$), treated as previously described, and subjected to qualitative ATR-FTIR analysis (0- and 4-week AS storage). Three different positions of each specimen were analyzed by ATR-FTIR spectrometry (Spectrum One, Perkin-Elmer, London, UK) in the region of 650 to $4,000 \text{ cm}^{-1}$ with a resolution of 4 cm^{-1} and 12 scans *per* spectrum. A moderate pressure was applied (5 psi) to establish good contact between the specimen and the ATR surface. All spectra were subjected to normalization, baseline correction, and smoothing processes and were averaged into a single spectrum by the FTIR software.

RESULTS

Mineralized dentin showed almost no loss of dry mass after incubation in AS (4 wk), but a -54.0% loss of dry mass in demineralized, un-infiltrated matrices (Fig. 1). Beams infiltrated with Res.A and Zol-Pr/Res.A lost 32.6% and 23.6% of their dry mass, respectively. The dry mass of the specimens infiltrated with Res.B fell significantly to -18.8% ($p < .05$), while those treated with Zol-Pr/Res.B produced a slight gain in dry mass (+5.5%); only these latter 2 groups showed alkalinity of the incubation medium (Fig. 1).

The release of ICTP and CTX telopeptide fragments after 4 wk of incubation is shown in Fig. 2. One-way ANOVA showed significance at a 95% confidence level. Mineralized dentin beams released very little ICTP (34.3 pg/mg dry dentin), while the demineralized controls released the most ICTP (1,100 pg/mg dry dentin) (Fig. 2A). The demineralized beams infiltrated with Res.A with or without the Zol-primer showed no significant reduction in ICTP release. Beams infiltrated with Res.B showed a significant reduction in ICTP release ($p < .05$), which was reduced further by the use of Res.B along with Zol-primer ($p < .05$).

Mineralized beams released very little CTX ($2.5 \pm 0.1 \text{ pg/mg}$ dry dentin), while after EDTA demineralization, the CTX release

increased significantly ($p < .05$) to 16.5 ± 1.2 pg/mg dry dentin (Fig. 2B). Demineralized beams infiltrated with Res.A significantly increased the CTX release to 52.7 ± 1.9 pg/mg ($p < .05$). The CTX release from beams infiltrated with the Zol-Pr/Res.A fell significantly ($p < .05$) to 34.7 ± 2.7 pg/mg compared with Res.A. Demineralized dentin infiltrated with Res.B or Zol-Pr/Res.B showed the highest CTX release (Fig. 2).

The results of ATR-FTIR analysis showed collagen ($1,240$ cm^{-1}) and water ($1,219$ cm^{-1}) in demineralized dentin before and after incubation (Fig. 3). Calcium-phosphate complexes were observed after Zol-Pr/Res.A treatment ($1,270$ to 970 cm^{-1}), while the presence of zoledronate was identified at 945 cm^{-1} and $1,168$ cm^{-1} ; these latter peaks disappeared after four-week storage. Res.B and Zol-Pr/Res.B induced formation of orthophosphates and mineral precipitation after four-week AS aging. Calcium-phosphate complex ($1,270$ to 970 cm^{-1}), P-O(H) stretching (878 cm^{-1}), pyrophosphate stretching (746 cm^{-1}), and carbonate stretching vibrations ($1,150$ and $1,110$ cm^{-1}) were also identified in these two groups (Res.B and Zol-Pr/Res.B).

DISCUSSION

The loss of dry mass was less than 1% in mineralized dentin (Fig. 1) because its endogenous proteases (MMPs and cathepsin K) were inactive. In completely demineralized dentin matrices, the loss of dry mass was -54% due to replacement of the mineral phase of the matrix with water and due to the activation of pro-forms of matrix endopeptidases by SIBLING proteins (Fedarko *et al.*, 2004). Once activated, dentin proteases cleave helical and telopeptide segments of the collagen matrix that slowly solubilize. Infiltration of Res.A or Zol-primer/Res.A may have encapsulated the collagen or the proteases or both, and lowered their hydrolytic activity. Infiltration of Zol-primer plus the ion-releasing fillers in Res.B not only prevented loss of dry mass but also produced a slight dry mass gain of +5.5%. This could be due to the release of silica, calcium, and phosphates which may have covered the endogenous proteases with apatite-like crystallites, making the proteases inactive (Osorio *et al.*, 2012).

EDTA-demineralized dentin matrices (DDM) released more ICTP and CTX telopeptides fragments than the mineralized controls. This study confirmed the results of Tezvergil-Mutluay *et al.* (2013), who showed that the release of ICTP in DDM is remarkably higher (67-fold) than that of CTX (Figs. 2A, 2B) by use of the same measurement method and incubation at pH 7.4. We speculate that this discrepancy between the amounts of ICTP and CTX released into the incubation medium is due to the differences in the amount and optimum pH activity of MMPs and cathepsin K.

The optimum pH for matrix metalloproteinase activity is about 7.4 (Holman *et al.*, 1999), while that for cathepsin K is 5.0 (Kometani *et al.*, 2010). MMPs can release ICTP-fragments at near maximum rates at pH 7.4, while for cathepsin K this is 2 pH units above its optimum pH of 5.0. Although it can still produce CTX, it does so at a much lower rate than that of MMPs. That is why the CTX release was so much lower than the ICTP release.

Figure 1. Dry mass loss and alkalinizing activity. **(A)** Percentage of the dry mass loss of each treatment and no-treatment group after 4 wk of storage in artificial saliva. Same letters indicate no significant differences between groups ($p > .05$). **(B)** Alkalinizing activity/pH values of the materials tested in this study at different storage periods in artificial saliva evaluated by means of a professional pH electrode (Mettler-Toledo, Leicester, UK).

The neutral pH of EDTA, along with its specific chelation ability, allows for activation of MMPs only when dentin is completely demineralized. Conversely, the low pH of phosphoric acid can induce a severe release of the calcium and phosphates necessary for MMP and CTP activation. Phosphoric acid would have been less appropriate for this study, since it would degrade the collagen matrix earlier, thus interfering with the standardization of the experiment (Osorio *et al.*, 2012).

ICTP release was significantly ($p < .05$) reduced 4 wk after the matrix was infiltrated with Res.B. The inhibition of MMPs induced by Res.B may be due to the Ca^{2+} and $(\text{PO}_4)^{3-}$ release from tricalcium phosphate and calcium silicate fillers, which react and form amorphous calcium phosphate (Hench and Wilson, 1993). If this amorphous calcium phosphate hydrolyzed to apatite crystallites, they could immobilize the proteases and inactivate some of them. Osorio *et al.* (2012) showed Ca/PO uptake by DDM after 4 wk of AS aging, treated with silicate-doped resins. They also suggested the phosphorylation (Bertassoni *et al.*, 2011) and remineralization of dentin. Likewise, our ATR-FTIR results showed, after AS aging, the presence of calcium-phosphate complex in DDM ($1,038$ cm^{-1} and $1,083$ cm^{-1}), carbonates, and the presence of P-O(H) groups along with pyrophosphates (P-O-P) (Fig. 3).

Moreover, Ca/PO precipitation can bind MMPs to form complexes [Ca/PO-MMP] with a high molecular weight that restricts their collagenase activity (Makowski and Ramsby, 2004). Alkaline environments may favor the conversion of calcium phosphates into apatite (Hoppe *et al.*, 2011). When zoledronate primer was used with Res.B, the release of ICTP fell to its lowest value. It is likely that zoledronate initially chelated calcium and zinc from MMPs to decrease their enzyme activity. Later mineralization of MMPs and collagen may have inactivated more MMPs. Osorio *et al.* (2012) also remarked that ICTP was

Figure 2. The resin-based blends used in this study were formulated with urethane dimethacrylate (30 wt%), ethoxylated bisphenol-A dimethacrylate (5 wt%), triethyleneglycol dimethacrylate (20 wt%) (Esstech, Essington, PA, USA), hydroxyethyl methacrylate (15 wt%), ethanol (28 wt%), camphorquinone (1.0 wt%), and ethyl-4-dimethylaminobenzoate (1.0 wt%) (Sigma-Aldrich). **(A)** Means and standard deviations of ICTP released from the resin-bonded dentin at the final storage period (4 wk) in artificial saliva. Means designated with the same letters indicate no significant differences ($p > .05$). **(B)** Means and standard deviations of CTX released from various groups after a four-week storage period in artificial saliva. Same letters indicate no significant differences ($p > .05$).

released from DDM treated with similar resins. A biological interaction between demineralized collagen and β TCP has been reported to occur, creating nucleation sites to adsorb calcium/phosphates (Gamal, 2011); β TCP was present in the microfiller of the Res.B.

A possible biomimetic effect induced by the Zol-primer in the remineralization of DDM infiltrated with Zol-Pr/Res.B may have occurred. Indeed, analogs of non-collagenous proteins such as polyvinylphosphonic acid (PVPA) are used both to inhibit MMPs (Tezvergil-Mutluay *et al.*, 2010) and to remineralize collagen fibrils (Tay and Pashley, 2008) and/or fossilize MMPs (Tjäderhane *et al.*, 2013). Polyvinylphosphonic acid can diffuse and bind to specific sites along the surfaces of the collagen fibrils and along the tropocollagen molecules, thereby guiding mineral deposition within the gap regions of the collagen fibrils (Weiner, 2008). Our ATR-FTIR results showed the presence of zoledronate (945 and 1,168 cm^{-1}) in demineralized dentin only subsequent to its application, but these peaks disappeared after AS storage (four-week), since zoledronate was probably incorporated into mineral clusters due to creation of chemical bonds with ions released by the Res.B ($\text{Ca}^{2+}/\text{PO}_4^{3-}$) and/or those of the storage media (AS). Indeed, the specimens treated with Zol-primer-Res.A showed the presence of calcium-phosphate complexes in DDM that were not detected in the Res.A-treated specimens (Fig. 3). Thus, the first null hypothesis requires rejection, since important changes were identified in the degradation of dentin collagen, in particular with specimens treated with Zol-primer in combination with Res.B and aged for 4 wk. Moreover, since the results of this study showed that the combination of Zol-primer along with Res.B induced important mineral deposition which increased the dentin dry mass (Fig. 1), the second null hypothesis must also be rejected.

We speculate that the main mechanism by which zoledronate reduces MMP-mediated collagen degradation when used in combination with Res.B may be attributed to the presence of this bisphosphonate within the DDM, which likely chelated calcium from MMPs. This also enhanced calcium phosphate

precipitation within the demineralized collagen matrix that, in turn, inactivates MMPs, as suggested by Osorio *et al.* (2012). The significant reduction in the loss of dry mass of the resin matrix provided indirect evidence that zoledronate and Res.B inhibited endogenous matrix proteases. However, Zol-primer used in combination with Res.A (unfilled) had no significant effect on the release of ICTP, but did decrease ICTP release when combined with Res.B compared with the control demineralized dentin. Further quantitative and ultra-morphology studies are already ongoing to confirm these outcomes.

Zoledronates have the highest potency to inhibit bone resorption *via* cellular mechanisms (Widler *et al.*, 2002) without affecting MMP synthesis (Heikkilä *et al.*, 2002). Systemic intravenous administration of zoledronate has been associated with bisphosphonate-associated osteonecrosis of the jaw (BRONJ) (Grbic *et al.*, 2008). However, because in this study zoledronate was applied locally to dentin at a low concentration and subsequently polymerized within the resin, we believe the risk of BRONJ to be very low.

Nevertheless, our results indicated an increase of CTX release subsequent to Res.A or B-infiltration (Fig. 2B) compared with the control DDM. How can infiltration of demineralized dentin with resin-based materials increase CTX release? Resin infiltration should decrease telopeptide release either directly, by inactivating matrix proteases, or indirectly, by encapsulating the collagen matrix. We hypothesize that the resin systems used in this study increased the release of methacrylic acid by the hydrolysis of HEMA and other methacrylate esters. Methacrylic acids created by hydrolysis of ester bonds of the methacrylates in Res.A and Res.B may have reduced the local pH (within the interface), thereby facilitating cathepsin activity by bringing the enzyme closer to its optimum pH of 5.5. It has been advocated that cathepsin K is active mainly in an acidic pH (Tjäderhane *et al.*, 2013).

However, the treatment with Zol-primer produced a significant reduction in CTX release when used with Res.A, but not with Res.B (Fig. 2B). This cathepsin K-inhibition effect may

Figure 3. FTIR spectra ($1,270\text{--}970\text{ cm}^{-1}$) of control dentin matrix or dentin matrices immediately after treatment with the 2 experimental resin-based materials with or without pre-application of the zoledronate-containing primer (Zol-primer). The spectra were acquired in 3 different positions at time 0 and after 4 wk of AS immersion. These images represented the average of 3 single scanning analyses which were then processed by smoothing, baseline correction, and normalized to the amide I peak. **(A)** FTIR spectra showed the absorption band of collagen (amide III; $1,250\text{--}1,200\text{ cm}^{-1}$) and O–H in-plane bending ($1,219\text{ cm}^{-1}$). Orthophosphate optical absorption band ($1,038\text{ cm}^{-1}$) and absorption bands at $1,083\text{ cm}^{-1}$ (orthophosphate stretching vibrations— ν_3) are particularly visible after application of the Res.B [Ca/PO-silicate fillers] and Zol-Pr/Res.B. Zoledronic acid was identified at 945 and $1,168\text{ cm}^{-1}$ (C–H rocking CH in ZOL). **(B)** FTIR spectra attained after 4 wk of artificial AS storage, which show mineral precipitation, especially in the dentin treated with Res.B alone and Zol-Pr/Res. The Zol-primer also induced some mineral precipitation when combined with Res.A. These modifications suggest the formation of calcium-phosphate-ester complexes. In the specimens treated with Res.B, it was possible to observe carbonate stretching vibrations between $1,150$ and $1,110\text{ cm}^{-1}$. The specimens treated with Res.B and Zol-Pr/Res.B presented P–O(H) stretching at 878 cm^{-1} , H_2O oscillating motion at 791 cm^{-1} , and OPO stretching of pyrophosphates at 746 cm^{-1} .

have been due to zoledronate release from the matrix subsequent to resin matrix degradation, which produced a prolonged and more effective inhibitory activity on cathepsin K; this is why zoledronate had less available calcium and phosphates to bind and precipitate on collagen compared with Res.B. It is well-known that zoledronate is a broad-spectrum inhibitor, since this involves a cation-chelation effect (Widler *et al.*, 2002); thus, if a possible release of zoledronate occurred, this could rapidly chelate Ca^{2+} and Zn^{2+} , decreasing the activity of MMP active-site Zn^{2+} . Further studies are required to confirm the release of zoledronate from our resin-based materials. Moreover, Heikkilä *et al.* (2002) showed that zoledronate needs to be in intimate contact to create covalent bonds to inhibit them. It is possible that higher zoledronate concentrations might offer further inhibition of CTX release. Unfortunately, our pilot study indicated (results not shown) that higher concentrations of zoledronate interfere with the polymerization of the resin systems used in this study.

Although cathepsin K exhibits maximum catalytic (91%) activity at pH 5.5 (Marini *et al.*, 2000), there may still be some catalytic activity, albeit at low rates at higher pH (6–10). Indeed, Kometani *et al.* (2010) showed that human cathepsins K exhibit 11% of their activity at pH 7.5; these findings support the observed activity of cathepsins found in the present investigation when Res.B was used. Furthermore, the percentage of protonation (enzymatic properties) of MMPs also plays an important role in enzymatic activity (Marini *et al.*, 2000).

In conclusion, the use of the zoledronate-containing primer along with ion-releasing filler materials induces mineral formation within demineralized dentin, which impairs the MMP-mediated degradation activity. However, this primer presents anti-cathepsin K activity only when used with unfilled resins. The future challenge remains in the regulation of ion-releasing kinetics from the Ca/PO silicate-doped resins as well as the incorporation of particles with slow controlled-release of zoledronate within the resin-dentin interface.

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