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Zinc reduces collagen degradation in demineralized human dentin explants

R Osorio^{1,*}, M Yamauti¹, E Osorio¹, ME Ruiz-Requena², DH Pashley³, FR Tay^{3,4}, and M. Toledano¹

¹Department of Dental Materials. School of Dentistry. Campus de Cartuja s/n. University of Granada. E- 18071 Granada. Spain

²Department of Biochemistry and Molecular Biology. School of Medicine. Av. de Madrid s/n. University of Granada. E- 18071 Granada. Spain

³Department of Oral Biology, School of Dentistry, Medical College of Georgia, 1120 15th Street, Augusta, GA, USA

⁴Department of Endodontics, School of Dentistry, Medical College of Georgia, 1120 15th Street, Augusta, GA, USA

INTRODUCTION

Zinc ions are frequently found in biological systems. Protein-protein interactions are controlled by the metal and dictated by the ligand preferences of the zinc ion¹. A Zn-specific inhibition of osteoclast mediated bone resorption provides evidence of an important role for Zn in the formation/resorption of mineralized tissues².

Metzincins are multi-domain proteins with endopeptidase activity that are dependent on zinc ions in their catalytic domain for proteolysis. Matrix metalloproteinases (MMPs) are expressed as inactive pro-enzymes, where the catalytic domain is shielded by a pro-domain that interacts with the zinc ion in the catalytic center via a cysteine residue. The catalytic site becomes accessible to substrates through proteolytic removal of the pro-peptide. The endopeptidase activity is mediated by a zinc ion coordinated between three histidine residues that are present in the catalytic center. The exact mechanism of proteolysis involves polarization of an active-site-bound water molecule to function as a nucleophile that attacks the polarized carbonyl group of the scissile peptide bond³. Catalytic zinc sites provide convenient targets for anti-MMP drugs because a wide range of functional groups (i.e. sulfonamides or hydroxamates) can coordinate directly with the zinc ion, displacing the zinc-water in the active site and inhibiting the enzyme⁴.

MMP-9 and MMP-2 substrate specificities include type I collagen² and are responsible for the degradation of extracellular matrix (ECM) components. Once MMPs are released or anchored to the ECM and activated, they are regulated by endogenous tissue inhibitors (TIMPs), co-secreted or ECM-anchored⁵.

MMP-8, -2, -9, -20 are present in dentin and contribute to dentin matrix organization and mineralization. They play a role in dentin matrix modulation during caries progression^{6,7}. MMPs also contribute to collagen degradation at the dentin-resin bonded interfaces,

*corresponding author: Raquel Osorio. Facultad de Odontología. Campus de Cartuja s/n. University of Granada., E-18071 Granada. Spain. Telph.: +34 958 243788; Fax: +34 958 240908, toledano@ugr.es.

jeopardizing the longevity of bonded restorations^{8–10}. It may be that zinc concentration is important in the formation/resorption process of this mineralized tissue.

Zinc is widely used in dentistry and is a component of tooth pastes, denture retention adhesives, oral rinsing solutions, cementing media, dental amalgams, cavity liners, temporary filling and root canal filling materials. Large amounts of soluble zinc ions are constantly released from zinc oxide cements that usually have a high solubility in humid environments¹¹. Zinc ions may also be released from amalgam by electrochemical reactions¹². These ions are released into the saliva and oral fluids, but products of biodegradation of dental restorations are also transported into the underlying tooth structure, pulp and soft tissues. It has been speculated that some proteinases may be inhibited by zinc or other divalent metals¹³.

The objective of the present study was to determine if the activity of MMPs on mineralized and demineralized dentin may be modulated by an excess of Zn^{2+} or zinc chelators (i.e. chlorhexidine digluconate, doxycycline). The null hypothesis tested was that zinc in excess or zinc-chelators do not reduce the activity of bound dentin MMPs or exogenous active MMP-2 on human dentin explants.

MATERIALS AND METHODS

A human dentin explant degradation assay was performed. Eight extracted non-carious human third molars were obtained with informed consent from two donors, under a protocol approved by the Institution review board. The teeth were stored in 0.1% (w/v) thymol solution at 4°C and used within one week after extraction. Dentin disks (0.75 mm $\pm$ 0.08 mm thick) were obtained from the mid-coronal portion of each tooth using a diamond saw under water cooling. Four dentin beams (0.75 mm $\times$ 0.75 mm $\times$ 5.0 mm) were obtained from each dentin disk as described by Carvalho *et al.*, 1996¹⁴.

Half of the dentin beams from each donor were demineralized by immersion in 10% phosphoric acid (pH 1.0) for 12 h at 25°C (PA-demineralized), as described by Carrilho *et al.*, 2009⁸. Demineralized dentin beams were rinsed in deionized water under constant stirring at 4°C for 72 h. Dentin beams were dried over anhydrous calcium sulphate (8 h). The dry mass of each dentin beam was measured with a microbalance. Specimens were rehydrated in 0.9% NaCl containing 10 U/ml of penicillin G and 300 μ g/ml of streptomycin for 24 h (pH 7.0).

Mineralized dentin (10mg) or demineralized dentin (2 mg) (two dentin beams, one from each donor) was placed in each Eppendorf tube. Four incubation media were established: 1) artificial saliva (AS) 50 mM HEPES, 5 mM $CaCl_2 \cdot 2H_2O$, 0.001 mM $ZnCl_2$, 150 mM NaCl and 100 U/ml penicillin, 1000 μ g/mL streptomycin (pH 7.2); 2) 40 mM chlorhexidine digluconate in AS (pH 7.4); 3) doxycycline (1:1) was added to the AS. Final concentration of doxycycline was 5 mg/ml (pH 7.4); 4) 3.33 mg/ml of zinc chloride was added to the AS (pH 7.0). Active MMP-2 was added at a dilution rate of 1:10 in buffer solution for MMP-2 (50 mM borate, 5 mM $CaCl_2$, 20% glycerol, 0.005% Brij 35) to half of the specimens in each group (pH 7.5). The final concentration of active MMP-2 was 0.01 μ g/ μ l. Chemicals are described in Table 1.

Specimens were incubated in 500 μ l of media at 37°C for 24 h, 1 week and 3 weeks. Supernatants (100 μ l) of the conditioning media after each storage period were withdrawn after agitation and analyzed for the collagen degradation product liberation (C-terminal telopeptide of type I collagen - ICTP determination) using a radioimmunoassay kit (ICTP-RIA Cat. No. 68601, Orion Diagnostica Oy, FI-02101 Espoo, Finland). A standard curve

was constructed with ICTP ranging from 0.01 to 250 µg/l. Each measurement was performed in triplicate. The amount of liberated ICTP from the PA-demineralized dentin in the absence of any inhibitor, and in the presence of active MMP-2 after 3 weeks was set as 100%, and the collagen degradation in the presence of inhibitors was calculated in each single experiment as a percent of this value¹⁵.

Mean ICTP concentration values were analyzed by ANOVA and Student-Newman-Keuls multiple comparisons. Differences between storage times were analyzed by Friedman's and Wilcoxon pair-wise comparisons tests. ($P < 0.05$).

RESULTS

Mean ICTP values were affected by the conditioning media ($F = 186.74$; $P < 0.001$), dentin demineralization ($F = 1034.16$; $P < 0.001$) and storage time ($F = 27.31$; $P < 0.001$). Interactions were also significant ($P < 0.001$). Power of the ANOVA was 0.81.

The total amount of liberated ICTP is displayed in Table II and the percent of collagen degradation in the different incubation media and time in the absence or presence of active MMP-2 are presented in Figures 1–2.

Mineralized dentin explants released only negligible amounts of ICTP: 0.87 µg/L (24 h) to 4.04 µg/L (3 weeks), 0.39 to 1.8% of collagen degradation respectively. When MMP-2 was added to the media, ICTP values increased significantly and ranged from 4.46 µg/L (24 h) to 9.02 µg/L (3 weeks), 1.98 to 4.01% of collagen degradation.

The amount of collagen degradation was significantly higher in PA-demineralized dentin ($p < 0.001$). The total amounts of ICTP liberated after incubation of PA-demineralized dentin in artificial saliva range from 70 µg/l (24 h) to 178 µg/l (3 weeks), these values increased significantly overtime, and correspond to 31 and 79% of collagen degradation, respectively. When MMP-2 was added, ICTP values ranged from 210 µg/l – 93% collagen degradation after 24 h to 225 µg/l – 100% collagen degradation after 3 weeks. No significant differences were found between obtained values in the three storage periods.

Addition of active MMP-2 significantly increased collagen degradation at 24 h evaluation (except for those groups in which doxycycline was present). When exogenous MMP-2 was added, the amounts of collagen degradation were similar at 24 h, 1 week or 3 weeks within each experimental group. When only dentin MMPs were considered, ICTP values were lower at 24 h, and a significant increase in collagen degradation was produced after 1 week of incubation. These values were maintained significantly similar at the three weeks evaluation period.

Doxycycline almost completely inhibited collagen degradation in mineralized and demineralized dentin. Collagen degradation ranged from 0.005% to a maximum of 2.88% after 21 days of incubation of the demineralized dentin, in the presence of active MMP-2. This inhibitory effect was maintained over the entire study period.

Chlorhexidine digluconate significantly reduced collagen degradation in demineralized dentin from 18 to 26% in the absence and presence of active MMP-2, respectively, but this inhibition was only evident at the 24 hours incubation period. Collagen degradation increased after 3 weeks, with values similar to those of the control artificial saliva groups.

When zinc was added, collagen degradation in demineralized dentin was reduced to 24 % at 24 h, 53 % after 1 week, and 60 % after 3 weeks. Liberated ICTP from PA-demineralized dentin in the zinc-containing solution was significantly lower than that of the artificial saliva

and higher than the attained value in the Doxycycline groups at any storage time. ICTP concentration in zinc-rich solutions was significantly similar to those of chlorhexidine groups, only at 24 hours evaluation. When active MMP-2 was incorporated under the same experimental conditions, collagen degradation was 65 to 70 % reduced, and this effect was maintained over the three tested periods.

DISCUSSION

The ICTP is the carboxyterminal telopeptide of type I collagen, joined via trivalent cross-links and liberated during collagen degradation. The telopeptide is produced through the action of matrix metalloproteinases, and it is considered an index of MMP-driven collagenolysis¹⁶. Determination of ICTP is one of the most reliable techniques to quantify enzymatic activity on type I collagen^{15–17}. Active MMP-2 was added to one-half of the specimens in each group because using complex biological samples, may produce very low concentrations of ICTP, that reduces the sensitivity in identifying the potentially less effective inhibitors. Active MMP addition may also help to overcome the problem of individual variability (variations in MMP concentration in dentin beams derived from different donors). Previously employed enzyme assays utilized synthetic peptides as substrates, but it is important to confirm the activity of MMP-inhibitors against the native substrate and in the presence of TIMPs¹⁸.

In dentin substrates, MMPs activity has been assessed by gelatine zymography^{12,19,20}, but profiling is limited and quantification with densitometry is not very accurate³. Zymography does not discriminate between free and TIMP-inhibited enzymes. In the present study, monitoring substrate conversion was employed. An endogenous protein substrate (dentin) of the proteinase of interest is added to the sample, and degradation is measured by ICTP determination. Availability of an appropriate substrate was guaranteed. The technique is only limited by the overlapping substrate specificity of metzincin subfamilies, so quantification of individual proteinases activity is not possible³.

Doxycycline completely inhibited collagen degradation. It is a broad spectrum MMP inhibitor⁵. Chlorhexidine digluconate reduced collagen degradation (up to 85% and up to 25% in the absence and presence of exogenous MMP-2, respectively). Zinc ions directly participate in the bond-breaking step in the catalytic domain of MMPs⁴. The zinc-bound water is a critical component for the catalytic zinc site, because the zinc ion serves as a powerful electrophilic catalyst by providing all or a combination of: 1) an activated water molecule for nucleophilic attack, 2) polarization of the carbonyl of the scissile bond, and 3) stabilization of the negative charge in the transition state⁴. Doxycycline and chlorhexidine digluconate have been previously identified as zinc chelators^{4,21}. These compounds, with zinc-binding groups, may have potent effects but lacked selectivity because of the strong homology between catalytic sites of MMPs³.

The inhibitory effect of chlorhexidine on dentin MMPs was previously shown^{8–10}, but our data demonstrate that this effect may be limited to 24 h. Chlorhexidine in solution may produce digluconate anions which may result in rapid precipitation in the presence of other mono- and divalent cations derived body fluids²². Previous enzymatic assays using chlorhexidine as dentin MMPs inhibitor were performed, but only at 24 h incubation periods^{8,10}.

Chlorhexidine digluconate has been shown to produce stabilization of the adhesive resin-dentin interface over time^{9,10}, even though the relationships between the collagenolytic activity of dentin, and the role of MMPs in hybrid layer degradation has been established^{8–10}, the exact inhibitory mechanism of chlorhexidine on MMPs requires further clarification. Chlorhexidine only binds electrostatically to demineralized dentin collagen²³.

It may slowly diffuse out of a collagen matrix via a competitive desorption mechanism in the presence of other cations. The inhibitory effect of chlorhexidine on ICTP liberation has been shown to be long-lasting in resin infiltrated dentin, if compared to non-infiltrated demineralized dentin²⁴. Chlorhexidine salts in solution may bind to hydrophilic polymers; carboxyl groups provide anionic binding sites for the cationic chlorhexidine molecules during their sorption from solution²⁵. Resin polymers at the adhesive interface may act as chlorhexidine delivery materials²⁶.

High zinc concentration strongly reduced collagen degradation. The mechanism of enzyme inhibition by zinc was previously hypothesized^{11,12} but not completely explained. Larsen and Auld²⁷ showed that zinc inhibits carboxypeptidase A by the formation of zinc monohydroxide that bridges the catalytic zinc ion to a side chain in the active side of the enzyme. Lysil and prolyl hydroxylation of collagen side-chains were also inhibited by excess Zn^{2+} in various cell types¹².

Zinc may have a structural role in proteins. In structural zinc sites, the zinc ion mainly stabilizes the tertiary structure of the protein in a manner analogous to disulfide bonds and is usually coordinated by four amino acid side chains, in tetrahedral geometry. High stability constants have been reported for these tetradentate zinc complexes⁴. Thus, zinc is important not only for its role in enzyme catalysis, but also in protein folding/stability²⁸. Four defined zinc-binding sites were found in collagen and procollagen molecules. Two of these sites are located close to each end and two at 126 and 206 nm from the C-terminal²⁹, near the fibronectin binding domain of MMPs, amino acids 757–776³⁰, same location than the collagenase cleavage sites²⁹. We hypothesize that subtle conformational changes occur following the zinc binding and lead to the protection of sensitive cleavage sites of metalloproteinases. N-terminal or C-terminal modification by zinc-binding in other proteins has also been shown to induce improvements in thermostability and in proteolytic resistance³¹.

Zinc has been shown to promote binding between collagen and other oligomeric matrix proteins; other divalent cations do not induce binding (Ca^{2+} , Mg^{2+} or Mn^{2+})²⁹. Protein-protein interactions are controlled by the presence of metallic cations and probably dictated by the zinc ion as the preferential ligand¹. Binding models that predict the existence of a single binding site on a protein molecule indicate preferential high affinity for a specific cationic species, whereas the existence of multiple binding sites can accommodate different cations with different affinities for the protein molecule¹. The amino acid side chains serving as zinc ligands in these structures often create hydrogen bonds contacts with other residues to three-dimensionally orientate the binding site configuration and lower the entropy for binding of the metallic ion⁴. Zinc-bound water is a hydrogen bond donor⁴, and phosphoric acid-etched collagen may act as a hydrogen bond acceptor. However, there is no predilection of a particular binding model based on current data; the data simply suggests that different divalent cations have different affinity constants to collagen.

Zinc may be considered as a competitive inhibitor of MMPs, exerting a protector effect through binding at the collagen sensitive cleavage sites of metalloproteinases. Selectivity for a specific MMP may be a function of differences in Zn/Ca ratios^{4,32}. Thus, the MMP inhibition effect of excess zinc ions in the present study may be due to the presence Ca^{2+} in the incubation media, as Ca^{2+} may inhibit Zn^{2+} binding depending on the relative abundance of these divalent ions^{29,32}. In an articular cartilage explant degradation model, local variations in the mass ratio between Ca^{2+} and Zn^{2+} altered MMP-collagen interactions²⁹. These different mass ratios can alter the differential hydration free-energy of Zn^{2+} , as the latter is dependent on its co-ordination geometry of zinc-water. We speculate that zinc-water/protein interactions may have decreased the hydration free-energy of Zn^{2+} relative to

the free energies of other divalent cations such as calcium²⁸. This differential metallic ion binding affinity hypothesis is also indirectly supported by data available from other proteins such as metallothioneins³³.

This is the first time that zinc has been shown to be a potent competitive inhibitor of MMPs in models of dentin explants. Research on metallic ion binding sites on demineralized dentin, particularly on factors governing their affinities, is desired in order to create metal-activated switches for increasing dentin collagen stability.

Presented results may explain, in part, the high clinical success of zinc containing/releasing materials that has been widely employed in restorative dentistry (silver amalgam, zinc oxide cements or setting calcium hydroxide). New adhesive/primers formulations including zinc in their composition should be tested, as they may exert a protective effect on MMPs-mediated collagen degradation at the resin-dentin hybrid layer.

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Table I

Chemicals used in the experiment.

Solutions and chemicals	Batch number (Manufacturer)
Ortho-Phosphoric acid	0000124351 (Panreac, Barcelona, Spain)
Deionized water	0000201888 (Panreac)
Anhydrous calcium sulphate, Drierite, 6 mesh	14796TJ (Sigma Aldrich, St. Louis, MO)
0.9% Sodium chloride	5211B1 (Braun Medical SA, Barcelona, Spain)
20 % Chlorhexidine digluconate	9418600021 (Guinma, Valencia, Spain)
HEPES buffer, Sterile, pH 8.0 (1 M)	8D007674 (Applichem GmbH, Darmstadt, Germany)
Sodium chloride 2-hydrate powder	0000159010 (Sigma-Aldrich)
Zinc chloride	0000144046 (Sigma-Aldrich)
MMP-2 active, Recombinant CHO cells (0.1 mg/mL)	D00065439 (Calbiochem, Gibbstown, NJ)
Borate (di-Sodium tetra-borate anhydrous)	0000173320 (Panreac)
Calcium chloride anhydrous	0000208286 (Panreac)
Glycerol	0000200971 (Panreac)
Brij® 35	0000173320 (Panreac)
Doxycycline	C03 (Invicta Farma SA, Madrid, Spain)
Streptomycin sulphate salt	026K8901 (Sigma-Aldrich)
Penicillin G potassium salt	1386789 (Sigma-Aldrich)

Table 2

Mean and standard deviation of ICTP concentration ($\mu\text{g/l}$) in conditioning media after human dentin explants incubation in the different experimental groups.

Dentin state and medium / incubation time	24 hours	1 week	3 weeks
Mineralized +AS	0.87 (0.11) 1a	4.08 (1.06) 2 a	4.04 (0.97) 2a
Mineralized +AS+ Chlorhexidine digluconate	0.80 (0.05) 1a	2.34 (0.8) 1,2 b	3.78 (1.59) 2a
Mineralized +AS+ Doxycycline	<0.01 (0) 1a	<0.01 (0) 1c	<0.01 (0) 1b
Mineralized +AS+ ZnCl_2	0.13 (0.11) 1a	0.23 (0.19) 1c	0.53 (0.15) 1b
Mineralized +MMP-2+AS	4.46 (0.77) 1b	6.74 (0.56) 1d	9.02 (1.63) 2c
Mineralized +MMP-2+AS+Chlorhexidine digluconate	2.33 (0.59) 1c	4.55 (0.73) 2a	8.01 (1.49) 3c
Mineralized +MMP-2+AS+ Doxycycline	<0.01 (0) 1a	<0.01 (0) 1c	<0.01 (0) 1b
Mineralized +MMP-2+AS+ ZnCl_2	0.23 (0.12) 1a	0.97 (0.24) 2c	1.81 (0.48) 3b
PA-demineralized +AS	70 (16.67) 1A	160 (20.95) 2A	178 (22.51) 2A
PA-demineralized +AS+ Chlorhexidine digluconate	30.82 (8.29) 1B	203 (19) 2A,B	200 (16.55) 2A,B
PA- demineralized +AS+Doxycycline	<0.01 (0) 1C	<0.01 (0) 1C	<0.01 (0) 1C
PA- demineralized +AS+ ZnCl_2	16.32 (5.84) 1B,C	42.14 (5.86) 2D	45.66 (5.98) 2D
PA-demineralized +MMP-2+AS	210 (11.40) 1D	220 (16.46) 1B	225 (13.52) 1E
PA-demineralized +MMP-2+AS+Chlorhexidine digluconate	152 (27) 1E	195 (21.07) 2B	205 (12.60) 2B
PA-demineralized +MMP-2+AS+ Doxycycline	0.1 (0.09) 1C	<0.01 (0) 1C	0.04 (0.05) 1C
PA-demineralized +MMP-2+AS+ ZnCl_2	58.26 (8.57) 1A	47.6 (9.69) 1D	56.05 (7.13) 1D

For each horizontal row: values with identical numbers indicate no significant difference ($p>0.05$).

For each vertical column: values with identical letters indicate no significant difference ($p>0.05$).

Mineralized and PA-demineralized dentin was analyzed separately.