RESEARCH REPORTS
Biological

F.D. Nascimento[1], C.L. Minciotti[2], S. Geraldeli[3], M.R. Carrilho[1], D.H. Pashley[4], F.R. Tay[4], H.B. Nader[5], T. Salo[6,7], L. Tjäderhane[6,7]*, and I.L.S. Tersariol[2,5]*

[1]Grupo de Estudos em Odontologia, Universidade Bandeirante de São Paulo (UNIBAN), Brazil; [2]Centro Interdisciplinar de Investigação Bioquímica, Universidade de Mogi das Cruzes, São Paulo, Brazil; [3]The University of Iowa College of Dentistry, Iowa City, IA, USA; [4]School of Dentistry, Medical College of Georgia, Augusta, GA, USA; [5]Universidade Federal de São Paulo (UNIFESP), Disciplina de Biologia Molecular, São Paulo, Brazil; [6]Institute of Dentistry, PO Box 5281, 90014 University of Oulu, Finland; and [7]Oulu University Hospital, Oulu, Finland; *corresponding authors, leo.tjaderhane@oulu.fi, ivarne@umc.br

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Cysteine Cathepsins in Human Carious Dentin

ABSTRACT

Matrix metalloproteinases (MMPs) are important in dentinal caries, and analysis of recent data demonstrates the presence of other collagen-degrading enzymes, cysteine cathepsins, in human dentin. This study aimed to examine the presence, source, and activity of cysteine cathepsins in human caries. Cathepsin B was detected with immunostaining. Saliva and dentin cysteine cathepsin and MMP activities on caries lesions were analyzed spectrofluorometrically. Immunostaining demonstrated stronger cathepsins B in carious than in healthy dentin. In carious dentin, cysteine cathepsin activity increased with increasing depth and age in chronic lesions, but decreased with age in active lesions. MMP activity decreased with age in both active and chronic lesions. Salivary MMP activities were higher in patients with active than chronic lesions and with increasing lesion depth, while cysteine cathepsin activities showed no differences. The results indicate that, along with MMPs, cysteine cathepsins are important, especially in active and deep caries.

KEY WORDS: MMP, enzyme, cathepsin, human, tooth, dentin.

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INTRODUCTION

Several physiological states, such as bone remodeling, angiogenesis, and organ development, require controlled collagen degradation (Benjamin, 2001; Lecaille *et al*., 2002; Ortega *et al*., 2003). In contrast, various pathological conditions such as osteoporosis (Yamashita and Dodds, 2000), arthritis (Buttle, 1994), tumor invasion (Seiki, 2003), and dentinal caries (Chaussain-Miller *et al*., 2006) are linked with excessive collagen degradation. The mammalian enzymes with collagenolytic activities include members of the matrix metalloproteinase (MMP) family, such as MMP-1, -2, -8, -13, and -14 (Lauer-Fields *et al*., 2002), human neutrophil elastase (Kafienah *et al*., 1998), and cysteine proteinases (Lecaille *et al*., 2002).

Lysosomal cysteine proteinases of the papain enzyme family are traditionally believed to degrade proteins that have entered the lysosomal system (Kirschke *et al*., 1995; Mort and Buttle, 1997). Nevertheless, the role of cysteine cathepsins is not limited to protein degradation within lysosomes. Moreover, cysteine cathepsins can degrade type I collagen (Burleigh *et al*., 1974), laminin, fibronectin (Buck *et al*., 1992), and proteoglycans (Nguyen *et al*., 1990) extracellularly, and they are involved in several diseases related to extracellular matrix degradation, *e.g*., arthritic diseases (Hashimoto *et al*., 2001; Pozgan *et al*., 2010). Extracellular cathepsin B is also related to the collagenous matrix degradation in gingivitis and periodontitis (Kennett *et al*., 1997) and during tumor invasion and progression (Mohamed and Sloane, 2006). Cathepsins B and L cleave in the non-helical telopeptide extensions of collagens (Kirschke *et al*., 1982), and cathepsin K cleaves the collagen at the triple helical region (Brömme *et al*., 1996). Cysteine proteinases can also activate the tartrate-resistant acid phosphatase (Ljusberg *et al*., 2005), an important enzyme involved in dentin resorption (Okamura *et al*., 1993).

Recently, we demonstrated the expression of cysteine cathepsins in human pulp tissue and odontoblasts and their presence in intact dentin (Tersariol *et al*., 2010). Even though cysteine cathepsins have been indicated to participate in various physiological and pathological conditions in the oral cavity (Dickinson, 2002), their role in dental caries has not been examined. Since MMPs have been indicated to participate in dentinal caries development and progression (Tjäderhane *et al*., 1998; Sulkala *et al*., 2001), and cathepsins are

present in intact dentin (Tersariol *et al*., 2010), the aim of this study was to examine the involvement of cysteine cathepsins in dentinal caries lesions. The hypothesis was that cysteine cathepsin activity is detectable in carious dentin.

MATERIALS & METHODS

Cathepsin B Immunostaining

The study protocol was approved by the Committee on Human Subjects, University of Mogi das Cruzes, São Paulo, Brazil, and samples were collected with patients' informed consent. The samples consisted of 8 teeth, with active caries lesions, that were extracted for therapeutic reasons from individuals with ages ranging from 20 to 30 yrs. The extracted teeth exhibited coronal deep lesions (n = 4) or cervical deep lesions (n = 4) in dentin, with a depth varying between 3 and 5 mm. Lesion activity was diagnosed according to color and texture (Fejerskov *et al*., 2008), clinical observation of depth during excavation, and evidence (if any) of radiographic dentin tissue repair. Four intact third molars were used as a dentin control group. After extraction, all teeth were immediately placed in 10% formalin for 7 days, sectioned in half, and demineralized in 5% formic acid for 7 additional days. The hemi-teeth were sectioned into slabs 4 μm thick, which were placed on slides in a Microm Cryostat II with the Cryo-Jane System. Tooth slabs were washed in PBS and bathed in 0.3% H_2O_2 in absolute methanol to inhibit endogenous peroxidase activity. Then, they were boiled in 10 mM sodium citrate buffer (2 x 12 min) to retrieve the antigen, incubated with primary polyclonal antibody for cathepsin B (1:80; anti-cathepsin B, Sigma-Aldrich, St. Louis, MO, USA) for 24 hrs at 4ºC, then with secondary biotinylating antibody and streptavidin-biotin-peroxidase (LSAB; Dakocytomation, Glostrup, Denmark) for 30 min each. The negative control tooth slabs were incubated with non-immune serum instead of primary antibody. The reaction product was revealed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), counterstained with Harris' hematoxylin, and coverslipped with Entellan (Sigma-Aldrich). Image acquisition and the *p*-values indicating the reliability of densitometric analysis in six different areas of predentin and dentin for each specimen were calculated with Qcapture Pro™ software (QImaging, Surrey, BC, Canada).

Cysteine Cathepsin and MMP Activities in Carious Dentin and Saliva

The first set of carious dentin specimens consisted of caries lesions in the teeth of patients (n = 42, ages ranging from 10 to 55 yrs, 1 lesion *per* patient) with either chronic or active caries lesions. Lesion activity was diagnosed as previously described. The lesions were assigned to one of the following groups: chronic lesions, no pulp exposure (n = 11); chronic lesions, pulp exposure (n = 9); active lesions, no pulp exposure (n = 10); and active lesions, pulp exposure (n = 12). The second set of carious dentin specimens was collected from patients with active lesions in molars (n = 17; mean age, 22 yrs; range, 20 to 30 yrs old). Lesions were classified in one of the following groups: superficial lesion in dentin (depth up to 2-3 mm) (n = 6); deep lesion in dentin (depth up to 3-5 mm) (n = 6); and lesion with pulp exposure (n = 5). Carious dentin was removed from all cavities with sterile spoon excavators by one operator and was immediately immersed in 1.0 mL of 50 mM Tris-HCl buffer, pH 7.4, NaCl 100 mM, and stored at -20°C. For analysis, caries lesions were homogenized as previously described (Tersariol *et al*., 2010). Sublingual pooled whole saliva (0.5 mL) was also collected from patients with a Luer syringe, diluted in 0.5 mL of Tris-HCl, 50 mM, pH 7.4, NaCl 100 mM buffer, and stored at -20°C.

The total cysteine cathepsin activities in saliva and carious dentin were monitored spectrofluorometrically with the cysteine cathepsin-specific fluorogenic substrate Z-FR-MCA, as previously described in detail (Tersariol *et al*., 2010). To verify the specificity of the cysteine proteinase activity, we carried out the assays in the presence or absence of classic proteinase inhibitors: 5 μM E-64 (cysteine cathepsin inhibitor); 100 μM PMSF (serine proteinase inhibitor); 2 μM pepstatin A (aspartyl proteinase inhibitor); 1 mM 1,10 phenanthroline, 5 mM EDTA, and 10 μM phosphoramidon (metalloproteinase inhibitors).

The total collagenolytic/gelatinolytic MMP activity in saliva and carious dentin was monitored spectrofluorometrically with the synthetic internally quenched fluorescent peptide substrate Abz-GPLGLWARG-EDDnp, as previously described (Tersariol *et al*., 2010).

We analyzed data on cysteine cathepsin and MMP specific activities (mean ± SE) of independent experiments, run in triplicate, by ANOVA with Tukey's *post hoc* test to evaluate the statistical significances of dentin cysteine cathepsin and MMP activities between the samples grouped by age, and type and depth of lesion. We calculated Pearson coefficients to evaluate the correlations between the enzymatic activities of cathepsin or MMP and the age of patients for active lesions, as well as the correlation between the cathepsin and MMP activities in dentin.

RESULTS

Cathepsin B Immunostaining

Intense and consistent cathepsin B immunostaining was observed in odontoblasts and dentinal tubules of caries lesions (Figs. 1A, 1B), with less intensity in healthy teeth (Fig. 1C), and no staining in negative controls (Fig. 1D). Staining quantification by densitometric analysis showed a statistically significant difference between intact and carious dentin ($p < 0.05$), whether the lesion was coronal or cervical (Fig. 1E).

Enzyme Activities in Carious Dentin

A statistically significant increase in cysteine proteinase activity was observed with the increasing depth of the dentinal caries lesions, especially in lesions with pulp exposure (Fig. 2A). In contrast, with MMP activities, only a slight and non-significant decrease was observed (Fig. 2A).

When the dentinal caries lesions were classified according to lesion activity and patient age, cysteine cathepsin activities demonstrated a significant decrease with age in active, but an increase in chronic, lesions (Fig. 2B). With MMP activities, a significant decrease with age in both active and chronic lesions

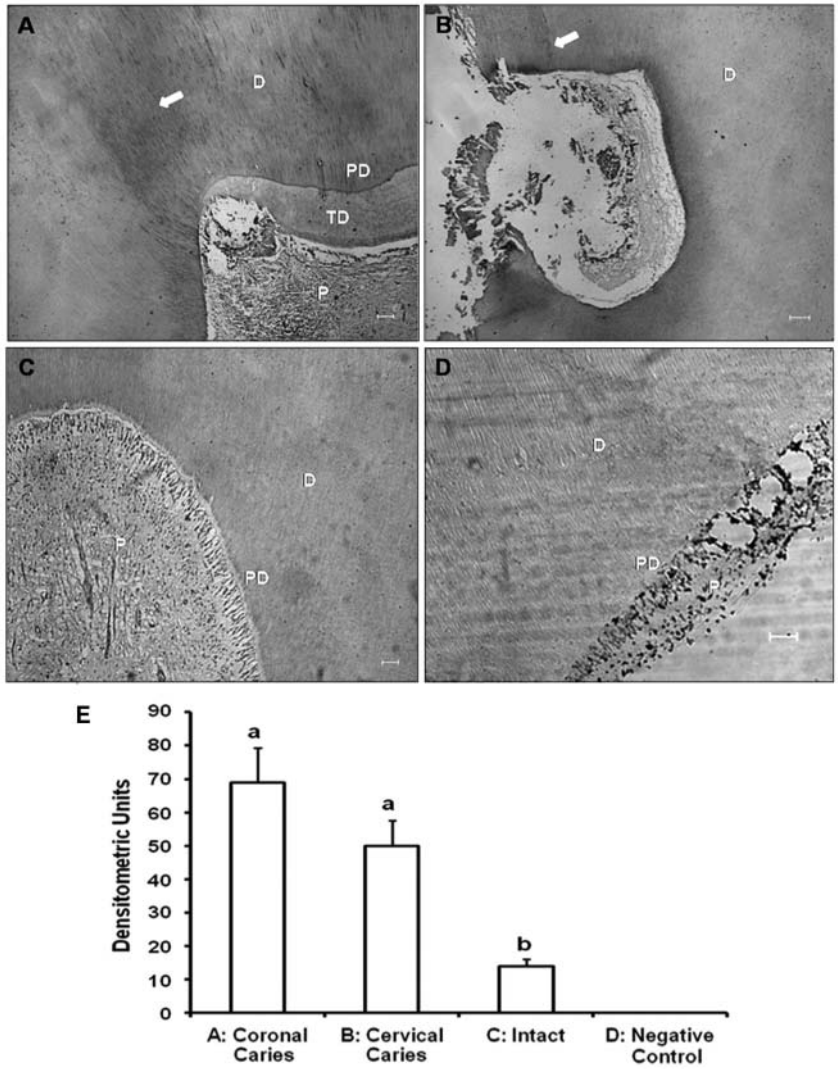

Figure 1. Cathepsin B immunostaining in the carious dentin-pulp complex. Cathepsin B was immunolabeled in coronal **(A)** and cervical carious teeth **(B)**. Panels **C** and **D** show, respectively, an intact tooth with scarcely any staining and the negative control, which was incubated with non-immune serum replacing the primary antibody. The presence of cathepsin B is represented by the dark label in dentin (solid white arrows), odontoblasts, and the pulp tissue. The counterstaining was performed with hematoxylin (D = dentin; PD = predentin; TD = tertiary dentin; P = pulp tissue). Panel **E** represents the staining quantification relative to **1A-1D**, calculated by densitometry. Different lower-case letters indicate statistically significant differences between densitometric values ($p < 0.05$). The scale bars correspond to 100 μm.

was observed (Fig. 2B). The negative correlations between the age and enzyme activities in active caries lesions were strong for both the cysteine cathepsins and MMPs (Pearson correlation coefficient -0.870 and -0.948 for cysteine cathepsin and MMP activities, respectively, with $p < 0.001$ in both cases) (Figs. 2C, 2D). However, the linearity of correlation was not as evident with cysteine cathepsin as with MMP activity, the sharpest decrease being observed in the youngest (< 25 yrs) patients (Fig. 2C). A strong positive correlation was observed with cysteine cathepsin and MMP activities (Pearson correlation coefficient 0.903, $p < 0.001$) (Fig. 2E). The similar linear relationship between intact and carious dentin activities for both cysteine cathepsins and MMPs in relation to the age of patients is presented in Appendix 1.

Enzyme Activities in Saliva

Salivary cysteine proteinase activities did not show any differences between the age groups (Fig. 3A) or when the lesions were classified according to lesion activity or depth (Figs. 3B, 3C). Also, with MMP activities, no differences were observed between the age groups (Fig. 3A). However, salivary MMP activities were significantly higher in patients with active than with chronic lesions (Fig. 3B), and a slight increase in salivary MMP activities was also observed with increasing lesion depth, the difference being statistically significant between the smallest lesion and pulp exposure (Fig. 3C).

Cysteine Cathepsin Specificity Assay

The activity analysis of saliva and different carious dentin samples against cysteine cathepsin-specific substrate Z-FR-MCA demonstrated strong inhibition with cysteine cathepsin inhibitor E-64 ($p < 0.05$ for saliva and both active and chronic lesions). Other proteinase inhibitors did not differ from the untreated control samples (Fig. 4).

DISCUSSION

The consistent detection of cysteine cathepsin activity in carious dentinal tissue allows the hypothesis to be accepted. Markedly higher activity in carious than in intact dentin and a highly significant correlation with MMP activities indicate that, along with MMPs, dentinal cysteine cathepsins have an important role in dentinal caries pathogenesis. The significant increase of cysteine cathepsin activity in carious dentin with increasing depth (approaching the pulp) further indicates that odontoblast- or pulp-derived cysteine cathepsins may be important in active caries lesions, especially with young patients. Dentin tubules of younger patients are wider and more numerous (Pashley, 1996), allowing for more dentinal fluid flow to the caries lesions. This is supported by the higher levels of cysteine cathepsin activities in caries lesions of young patients. At least MMP-20 (Sulkala *et al*. 2002), MMP-2 (Boushell *et al*. 2008), and possibly also cathepsin B (Tersariol *et al*. 2010) are present

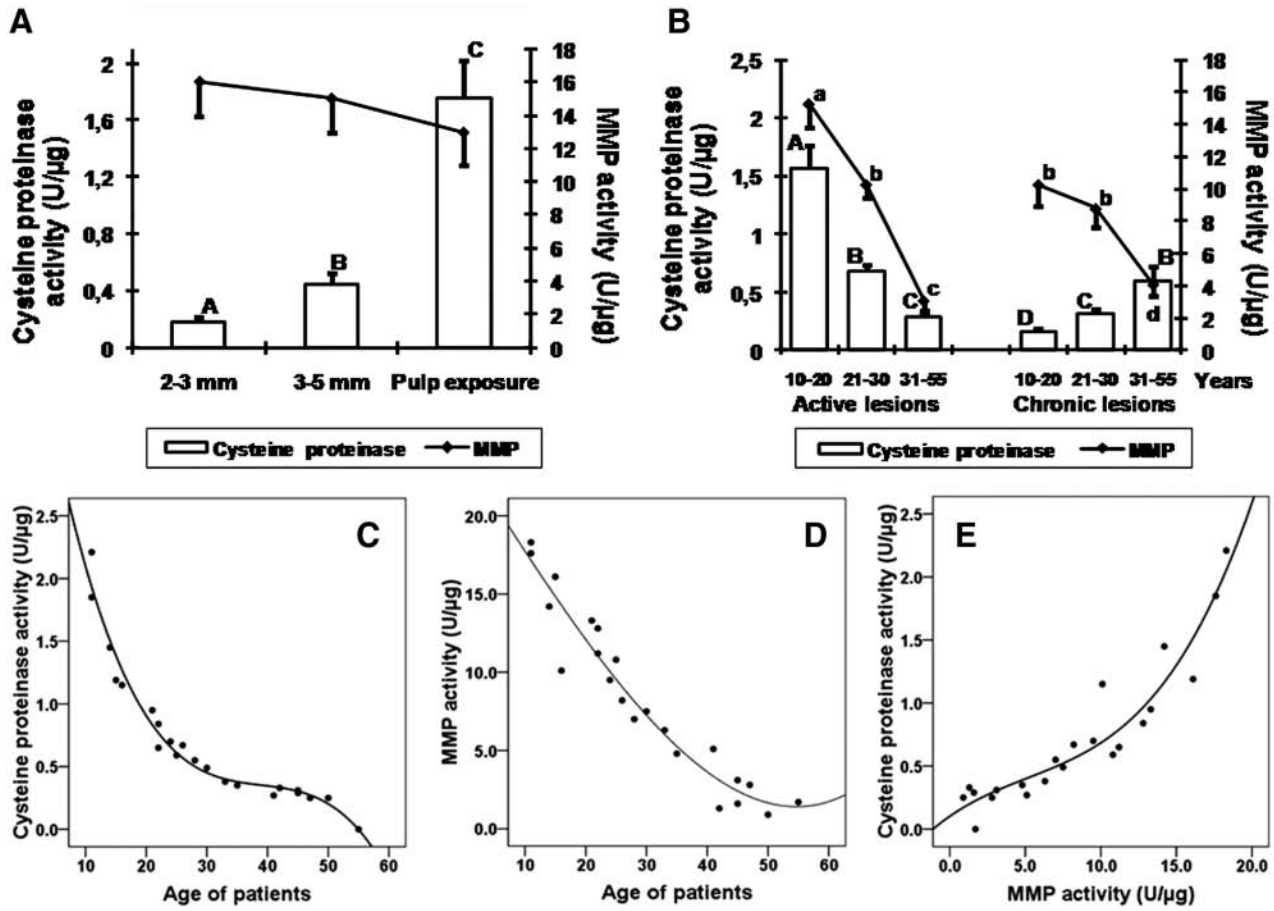

Figure 2. Proteinases in caries lesions. The bars represent cysteine cathepsin activity and line MMP activity (degradation unit/µg of sample; mean with SE in both cases). **(A)** Proteinase activities according to the depth of the lesion. Bars with different upper-case letters are significantly different from each other with respect to cysteine cathepsin activities (A is significantly lower than B, which in turn is significantly lower than C) ($p < 0.05$). There were no statistically significant differences in MMP activities between lesions with different depths. **(B)** Proteinase activities according to status of the lesion, active or chronic, and age. Bars with different upper-case letters are significantly different from each other with respect to cysteine cathepsin activities ($p < 0.05$), as described above; respective statistically significant differences between the MMP activities are indicated with different lower-case letters ($a > b > c > d$; $p < 0.05$). Panels **C** to **E** show correlations between proteinase activities in relation to the patient's age in active lesions. **(C)** Correlation between cysteine cathepsin activity and age in active lesions ($r = -0.870$; $p < 0.001$). **(D)** Correlation between MMP activity and age in active lesions ($r = -0.948$; $p < 0.001$). **(E)** Correlation between cysteine cathepsin and MMP activities ($r = 0.903$; $p < 0.001$).

in dentinal fluid, and they may contribute to the lesion activity in areas with wide dentinal tubules. There are actually experimental data indicating that devitalization of teeth, diminishing dentinal fluid flow, may reduce dentinal caries progression (Brown and Lefkowitz, 1966; Steinman *et al*., 1980; Steinman, 1985). The increased cathepsin B immunostaining in carious dentin compared with intact dentin may indicate that there is at least one cysteine cathepsin responsible for the activity in carious dentin. However, since odontoblasts express a wide variety of cysteine cathepsin genes (Tersariol *et al*. 2010), it is possible that other cysteine cathepsins also participate in the observed activity.

The reason for the significantly higher cysteine cathepsin activity in active than in chronic lesions in two younger age groups is presently unknown, but may relate to lesion pH or demineralization rate. Since the cysteine cathepsins function better in an acidic milieu (Turk *et al*., 2000), the markedly lower pH in active than in the chronic dentinal caries lesion (Hojo *et al*., 1994) may contribute to the higher activity observed in this study. Since the lesion turns from active to chronic when the excessive acidity is eliminated, *e.g*., by improved hygiene measures, the loss of activity may be related to remaining cathepsins being bound to partially demineralized and possibly partially remineralized collagen in an inactive form. Conversely, the relatively high levels of cysteine cathepsin activity in chronic lesions in the oldest patient group may be related to the presence of dentin tissue repair. Cysteine cathepsin proteinases can, *e.g*., modulate tertiary dentinogenesis by release and activation of latent TGF-β or other growth factors from the dentin matrix (Smith, 2003). It must be noted, however, that the age-related changes in cysteine cathepsin activities in chronic lesions are much less pronounced than those in active lesions. The lack of correlation between

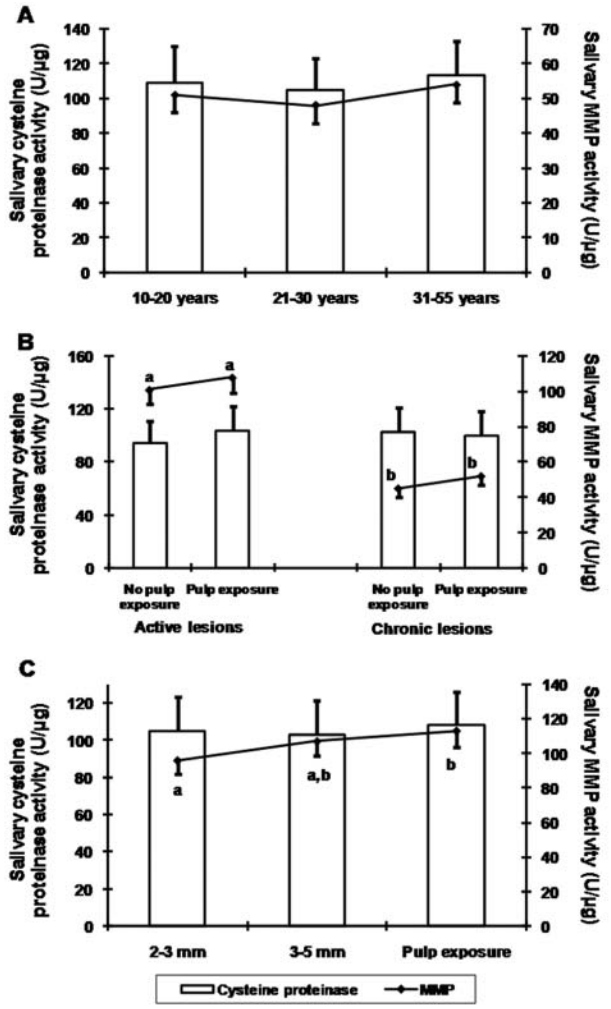

Figure 3. Salivary cysteine and MMP proteinase activities. **(A)** Salivary proteinase activities with respect to age. Neither the cysteine cathepsin nor the MMP activities differed significantly between the age groups. **(B)** Salivary proteinase activities with respect to the caries lesion activities. There were no statistically significant differences between the cysteine cathepsin activities. With MMP, the activities in active caries lesions were significantly higher than in chronic lesions ('a' is significantly higher than 'b'; $p < 0.05$). **(C)** Salivary proteinase activities with respect to lesion depth. Similarly to 2B, lower-case letters indicate statistically significant differences between the MMP activities in lesions with different depths ('a' is significantly different from 'b'; $p < 0.05$).

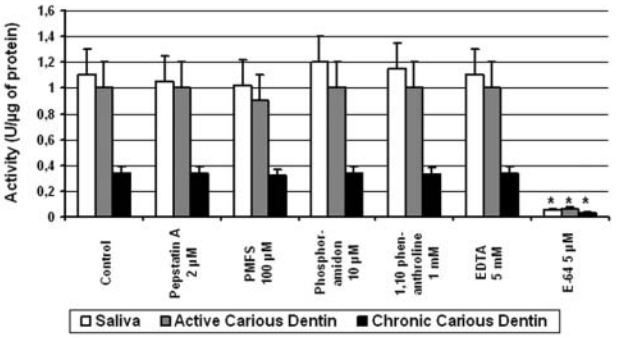

Figure 4. The effects of proteinase inhibitors on cysteine proteinase-specific Z-FR-MCA hydrolysis activity. Saliva sample values are 1/100 of the originals to fit the bars to the scale. *Statistically significant difference from the controls and all the other inhibitors ($p < 0.05$).

salivary cysteine cathepsin activities and patient age, lesion depth, and activity suggests that salivary cysteine proteinases do not contribute to dentin caries lesions, supporting previous findings (van Strijp *et al*., 2003).

The slight, non-significant decrease in MMP activities with increasing lesion depth, together with the linearity of correlation between MMP and age both in intact dentin (Tersariol *et al*., 2010) and in active caries lesions, supports the conclusion that dentin-bound, rather than pulp-derived, MMPs are the major source for MMP activity in caries. It is possible, however, that salivary MMPs contribute to total caries enzyme activity, since the enamel-dentin complex allows for molecular penetration from the oral cavity all the way to the pulp in young intact teeth and teeth with external mechanical damage (Byers and Lin, 2003). Since salivary MMP activity was significantly higher in relation to active than chronic lesions, and since salivary MMP activity in patients with deep active lesions was significantly higher, it is tempting to speculate that salivary MMPs, unlike salivary cysteine cathepsins, may participate in dentin caries pathogenesis. This assumption is supported by the results of previous studies demonstrating *in vitro* cavity formation only with externally added collagenase (Katz *et al*., 1987), the degradation of exposed dentin collagen by acid-activated salivary MMPs (Tjäderhane *et al*., 1998), and, most importantly, the decrease in dentin caries progression by locally administered MMP inhibitors (Sulkala *et al*., 2001; Tjäderhane *et al*., 2006).

The lack of correlation between salivary cysteine cathepsins and lesion depth and activity may be related to their acidic pH-related function. Cysteine proteinases are auto-activated and functional in low pH, and most (including cathepsin B) are considered unstable and inactive in neutral pH (Turk *et al*., 2000). MMPs are neutral proteinases; the best functional activity observed around neutral pH, but latent, salivary proMMPs can be activated with acidic pH followed by neutralization (Tjäderhane *et al*., 1998; Sulkala *et al*., 2001). This activation may be caused by pH-related autoactivation, but may also be due to salivary cysteine cathepsins, since cathepsins activate MMPs (Nagase, 1997). The potential pH-related interactions and synergistic effects of cysteine cathepsins and MMPs in dentinal caries pathogenesis are presented in Appendix 2.

In conclusion, analysis of the data indicates that, along with the previously demonstrated role of MMPs in caries pathogenesis (Tjäderhane *et al*., 1998; Sulkala *et al*., 2001), cysteine cathepsin proteinases are also important. This may be especially true in deep and active caries, due to the pulpal origin of cysteine cathepsins in caries lesions. MMP inhibition has been implicated in decreased caries lesion progression in dentin (Tjäderhane *et al*., 1999, 2006; Sulkala *et al*., 2001). Since MMPs and cysteine cathepsins may well have synergistic and adjunctive effects on caries pathogenesis, further research is needed to see if cysteine cathepsin inhibitors could also be used as therapeutic modes to retard or inhibit caries progression.

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