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Release of ICTP and CTX telopeptides from demineralized dentin matrices: Effect of time, mass and surface area

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ABSTRACT

Objective. The present study evaluated the influence of time, mass and surface area of demineralized dentin collagen matrices on telopeptides release. The hypotheses tested were that the rates of ICTP and CTX release by matrix bound endogenous proteases are 1) not time-dependent, 2) unrelated to specimen mass, 3) unrelated to specimen surface area.
Methods. Non-carious human molars (N = 24) were collected and randomly assigned to three groups. Dentin slabs with three different thicknesses: 0.37 mm, 0.75 mm, and 1.50 mm were completely demineralized and stored in artificial saliva for one week. Collagen degradation was evaluated by sampling storage media for ICTP and CTX telopeptidases. Activity of MMPs in the aging medium was evaluated using fluorometric activity assay kit.
Results. A statistically significant ($p < 0.05$) decrease in the release of both ICTP and CTX fragments over time was observed irrespective of the specimen thickness. When data were normalized by the specimen mass, no significant differences were observed. Releases of ICTP and CTX were significantly related to the aging time as a function of surface area for the first 12 h. Total MMP activity, mainly related to MMP-2 and -9, decreased with time ($p < 0.05$).
Significance. Because the release of collagen fragments was influenced by specimen storage time and surface area, it is likely that cleaved collagen fragments closer to the specimen surface diffuse into the incubation medium; those further away from the exposed surface are still entrapped within the demineralized dentin matrix. Bound MMPs can only degrade the substrate within the limited zone of their molecular mobility.

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1. Introduction

Dentin consists of inorganic apatite crystallites embedded in a collagen-rich organic matrix. The dentin organic matrix is a complex connective tissue comprising type I collagen (≈90%), and non-collagenous proteins, such as proteoglycans, phospholipids, and enzymes (≈10%) [1,2].

Martin De Las Heras et al. reported the presence of matrix metalloproteases (MMPs) in peripheral dentin as early as 2000 [3]. To date, the functional significance of these endogenous proteases in the dentin matrix remains unclear. Different MMPs, including MMP-2 [4], MMP-3 [5], MMP-8 [6], MMP-9 [4] and MMP-20 [7,8], have been identified from peripheral dentin. In 2004, Pashley et al. [9] reported that demineralized dentin degraded *in vitro* over time; this degradation could be prevented using protease inhibitors, such as chlorhexidine. At the same time, Armstrong et al. [10] published the first transmission electron micrographs of degrading hybrid layers showing loss of insoluble collagen fibrils and their conversion to gelatin fibrils. Since then, many investigators have associated the hydrolytic activity of dentin MMPs with decrease in bond strength of resin–dentin bonds [9,10]. Following the discoveries that pro-forms of dentin MMPs could be exposed and activated by acid-etchants used in adhesive bonding [4,9,11], many investigators came to the conclusion that hybrid layer degradation *in vivo* is due to the combined activities of MMP-2, -3, -8 and -9 [2,12–15]. Osorio et al. [16] demonstrated that MMP-2 is a telopeptidase, in addition to its role as a gelatinase. The authors measured the release of the collagen telopeptide fragment "cross-linked carboxyterminal telopeptide of type I collagen" (ICTP) from demineralized dentin. They found that there was a significant increase in ICTP release when exogenous MMP-2 was added to the incubation medium. Furthermore, Bigg et al. [17] reported that MMP-9 hydrolyzes type I collagen. These studies confirmed the observations of Garnero et al. [18], who reported that MMPs were the only source of ICTP telopeptide fragments in demineralized bone.

The recent discovery that peripheral dentin also contains cysteine cathepsins [19–22] expanded the list of potential dentin proteases that are capable of degrading dentin matrices. The activity of these cysteine proteinases, in particular cathepsin K, generates a linear eight amino acid sequence on the C-telopeptide end, which goes under the acronym "C-terminal crosslinked telopeptide of type I collagen" (CTX) [18,23,24]. Tezvergil-Mutluay et al. [24] and Turco et al. [25] reported that demineralized dentin releases CTX telopeptides, albeit at lower concentrations compared with the release of ICTP by endogenous MMPs.

To investigate the release of collagen fragments during the degradation of dentin collagen matrices, it is important to understand the basic structure of collagen, and the mechanisms in which endogenous proteases interact with collagen. A single collagen molecule consists of two α1 and one α2 chain intertwined into a triple helix [26–28]. The α2 chain is the most susceptive to proteolysis due to its position and a greater dissociation from the helix at the cleavage site [29]. Owing to the diagonal position of the collagen molecules in collagen fibrils, the C-terminal end of the collagen molecule is located close to the surface, in a manner which sterically blocks the access of true collagenases to α2 collagen chain [18]. Telopeptidases, such as MMP-2 and -9 are very important for the process of collagenolysis. In fact, they are necessary to enable the access of true collagenases to the catalytic site on the collagen triple helix. According to Nagase and Fushimi [30] the fibronectin and hemopexin binding segment of the MMP-2 are very close to its active site. Therefore, the enzyme can attack the surrounding collagen it is bound to, allowing access only to the surrounding molecules. Indeed, when telopeptidases remove the C-terminal end of the collagen molecule, the steric restrictions are removed allowing access to true collagenases. Further, true collagenases unwind the triple helix on specific sites and cleave the collagen molecules into 1/4 C-terminal and 3/4 N-terminal fragments [29]. However, since dentin collagen does not turn over, and is the most cross-linked collagen tissue in the body, those fragments can remain covalently cross-linked to adjacent collagen molecules [25]. Unlike MMPs, cathepsin K can cleave collagen at multiple sites, generating multiple fragments [31]. As much as 98% of cathepsin protease activity can be attributed to cathepsin K, which can cleave helical collagen [31], while other cathepsins can only cleave the non-helical telopeptide part [32].

If the release of ICTP and CTX are to be used to follow the rate of degradation of demineralized dentin matrices over time, much more information is needed regarding the rates of release of these telopeptides over time. Furthermore, to fully determine the adequate protocol for *in vitro* assessment of dentin collagen degradation, it is necessary to investigate other parameters that could influence the release of ICTP and CTX telopeptides, such as the geometrical features of the specimens, *i.e.* thickness, surface area and mass. Accordingly, the null hypotheses tested in the present study were that the rates of ICTP and CTX release by matrix-bound endogenous proteases are 1) not time-dependent, 2) unrelated to specimen mass, 3) unrelated to specimen surface area.

2. Materials and methods

2.1. *Specimen preparation*

Twenty-four extracted non-carious human molars were collected after obtaining patients' informed consent for using their extracted teeth for research purposes, under a protocol approved by the Institutional Review Board of the University of Trieste (Italy). Teeth were extracted for orthodontic reasons from young patients (20–30 years of age) and were examined at receipt for signs of caries or cracks. The teeth without signs of carious lesions or mechanical degradation were stored at 4 °C in 0.5% chloramine-T solution for no more than 1 month before use. Enamel, cementum, and pulpal soft tissues were completely removed from each tooth. Dentin slabs with three different thicknesses: 0.37 mm, 0.75 mm and 1.50 ± 0.10 mm were obtained from the mid-coronal portion of each tooth using a slow-speed diamond saw (Isomet 5000, Buehler Ltd., Lake Bluff, IL, USA) under continuous water-cooling.

The dentin slabs were completely demineralized in 10 wt% phosphoric acid (pH 1) at 25 °C for 24 h. Demineralized dentin slabs were thoroughly rinsed in deionized water under constant stirring at 4 °C for 72 h [33]. The demineralized slabs

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