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D.H. Pashley[1]*, F.R. Tay[2], C. Yiu[2], M. Hashimoto[3], L. Breschi[4], R.M. Carvalho[5], and S. Ito[6]

[1]Department of Oral Biology and Maxillofacial Pathology, Medical College of Georgia, Augusta, GA 30912, USA; [2]Paediatric Dentistry and Orthodontics, University of Hong Kong, Hong Kong, SAR, China; [3]Division of Pediatric Dentistry, Hokkaido University, Graduate School of Dental Medicine, Kita 13 Nishi 7, Kita-ku, Sapporo 060-8586, Japan; [4]Department of Surgical Sciences, University of Trieste, Italy; [5]Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo, Brazil; and [6]Department of Operative Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa, Tobetsu, 061-0293, Japan; *corresponding author, dpashley@mail.mcg.edu

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Collagen Degradation by Host-derived Enzymes during Aging

ABSTRACT

Incompletely infiltrated collagen fibrils in acid-etched dentin are susceptible to degradation. We hypothesize that degradation can occur in the absence of bacteria. Partially demineralized collagen matrices (DCMs) prepared from human dentin were stored in artificial saliva. Control specimens were stored in artificial saliva containing proteolytic enzyme inhibitors, or pure mineral oil. We retrieved them at 24 hrs, 90 and 250 days to examine the extent of degradation of DCM. In the 24-hour experimental and 90- and 250-day control specimens, we observed 5- to 6-μm-thick layers of DCM containing banded collagen fibrils. DCMs were almost completely destroyed in the 250-day experimental specimens, but not when incubated with enzyme inhibitors or mineral oil. Functional enzyme analysis of dentin powder revealed low levels of collagenolytic activity that was inhibited by protease inhibitors or 0.2% chlorhexidine. We hypothesize that collagen degradation occurred over time, *via* host-derived matrix metalloproteinases that are released slowly over time.

KEY WORDS: dentin matrix, matrix metalloproteinases (MMPs), gelatinase.

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INTRODUCTION

There is a general consensus that resin-dentin bonds created by contemporary hydrophilic dentin adhesives deteriorate over time (Gwinnett and Yu, 1995; Burrow *et al.*, 1996; Armstrong *et al.*, 2001; De Munck *et al.*, 2003). For total-etch adhesives, a decreasing gradient of resin monomer diffusion within the acid-etched dentin (Wang and Spencer, 2002) results in incompletely infiltrated zones along the bottom of hybrid layers that contain denuded collagen fibrils (Armstrong *et al.*, 2001; Hashimoto *et al.*, 2002). These zones corresponded with the sites of different modes of silver nanoleakage within the hybrid layers (Tay *et al.*, 2002). For self-etch adhesives, incomplete resin infiltration was also observed as nanoleakage within hybrid layers (Sano *et al.*, 1995), despite the ability of these adhesives to etch and prime simultaneously. This has been attributed to the incomplete removal of water that is associated with the hydrophilic resin monomers *via* hydrogen bonding.

Several *in vivo* studies have provided morphologic evidence of resin elution and/or hydrolytic degradation of collagen matrices in aged resin-dentin bonds (Sano *et al.*, 1999; Hashimoto *et al.*, 2000, 2003; Takahashi *et al.*, 2002). Resin elution from hydrolytically unstable polymeric hydrogels within hybrid layers (Wang and Spencer, 2003) may continue to occur through the nanoleakage channels during aging, rendering the previously resin-infiltrated collagen matrices susceptible to attack by proteolytic enzymes. This probably accounted for the almost complete disappearance of portions of hybrid layers from resin-dentin bonds that were aged for 4 yrs in water (De Munck *et al.* 2003). Exposed collagen matrices from acid-etched dentin were also found to be dissolved down to the demineralization front after the specimens were aged in water for 500 days (Hashimoto *et al.*, 2003).

Recent studies revealed the contributions of host-derived proteinases to the breakdown of the collagen matrices in the pathogenesis of dentin caries (Tjäderhane *et al.*, 1998; Sulkala *et al.*, 2002; van Strijp *et al.*, 2003) and periodontal disease (Lee at al., 1995). They have potential implications in dentin bonding. Since nanoleakage can occur in the absence of frank gaps along resin-dentin interfaces created *in vivo* (Ferrari and Tay, 2003), the results of these studies suggest that degradation of incompletely infiltrated zones within the hybridized dentin by host-derived matrix metalloproteinases within the dentin matrix may proceed in the absence of bacterial enzymes. *In situ* collagen degradation within incompletely infiltrated hybrid layers may also adversely affect the remineralization potential of the denuded collagen fibrils *in vivo* (Mukai and ten Cate, 2002). Thus, the objective of this study was to determine if acid-etched dentin matrices can be degraded by dentin-derived proteolytic enzymes, in the absence of bacterial colonization over time. The null hypothesis tested was that there is no difference among acid-etched dentin matrices that were aged in artificial saliva, in artificial saliva containing proteolytic enzyme inhibitors, and in non-aqueous mineral oil in which hydrolytic degradation

cannot proceed in the absence of water.

MATERIALS & METHODS

Twenty-seven non-carious human third molars were collected after the patients' informed consent had been obtained under a protocol reviewed and approved by the institutional review board of the Medical College of Georgia, USA. Within 1 mo of extraction, the occlusal enamel and roots of these teeth were removed by means of a slow-speed saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water-cooling. The exposed dentin surfaces were polished with wet 180-grit silicon carbide papers.

Experimental Design

Since it was difficult to produce zones of incomplete resin infiltration with consistent dimensions in hybrid layers, we created 5- to 6-μm-thick layers of demineralized collagen matrix (Ferrari and Tay, 2003) by etching each tooth surface with a silica-free 32% phosphoric acid gel (Bisco Inc., Schaumburg, IL, USA) for 15 sec, but without the application of a dentin adhesive. This served as a model for observation of the degradation of denuded acid-etched collagen fibrils over time.

The specimens were randomly divided into 3 groups of 9 teeth each, according to the type of storage medium used. In the experimental group, each tooth was stored in a 5-mL aliquot of artificial saliva containing sodium azide to prevent bacterial growth. The rationale for using artificial saliva was that the presence of calcium and phosphate ions would prevent additional demineralization that could alter the depth of the acid-etched dentin during aging. The artificial saliva contained (mmoles/L): $CaCl_2$ (0.7), $MgCl_2 \cdot 6H_2O$ (0.2), KH_2PO_4 (4.0), KCl (30), NaN_3 (0.3), and HEPES buffer (20). The protease inhibitors (mmoles/L) were: benzamidine HCl (2.5), ε-amino-*n*-caproic acid (50), N-ethylmaleimide (0.5), and phenylmethylsufonyl fluoride (0.3).

In the first control group, each tooth was stored in a 5-mL aliquot of pure mineral oil. The rationale for this control was that hydrolytic degradation of collagen fibrils, regardless of the origin of proteolytic enzymes, could not proceed in the absence of water. In the second control group, each tooth was stored in a 5-mL aliquot of artificial saliva containing proteolytic enzyme inhibitors (Martin de Las Heras *et al.*, 2000). Both matrix metalloproteinase (MMP) inhibitor (benzamidine HCl) and cysteine proteinase inhibitors (N-ethylmaleimide, ε-amino-*n*-caproic acid) and serine protease inhibitors (phenylmethylsufonyl fluoride) were included to prevent digestion of the collagen fibrils and remnant non-collagenous proteins (Everts *et al.*, 1998) that were present in the demineralized dentin matrix. The storage medium was replaced weekly to maintain the activity of the inhibitors.

Three teeth were retrieved from each group at 24 hrs, 90 and 250 days for transmission electron microscopic (TEM) examination of the thickness of the remaining demineralized collagen matrix (DCM) and the status of collagen fibrils. Both undemineralized and

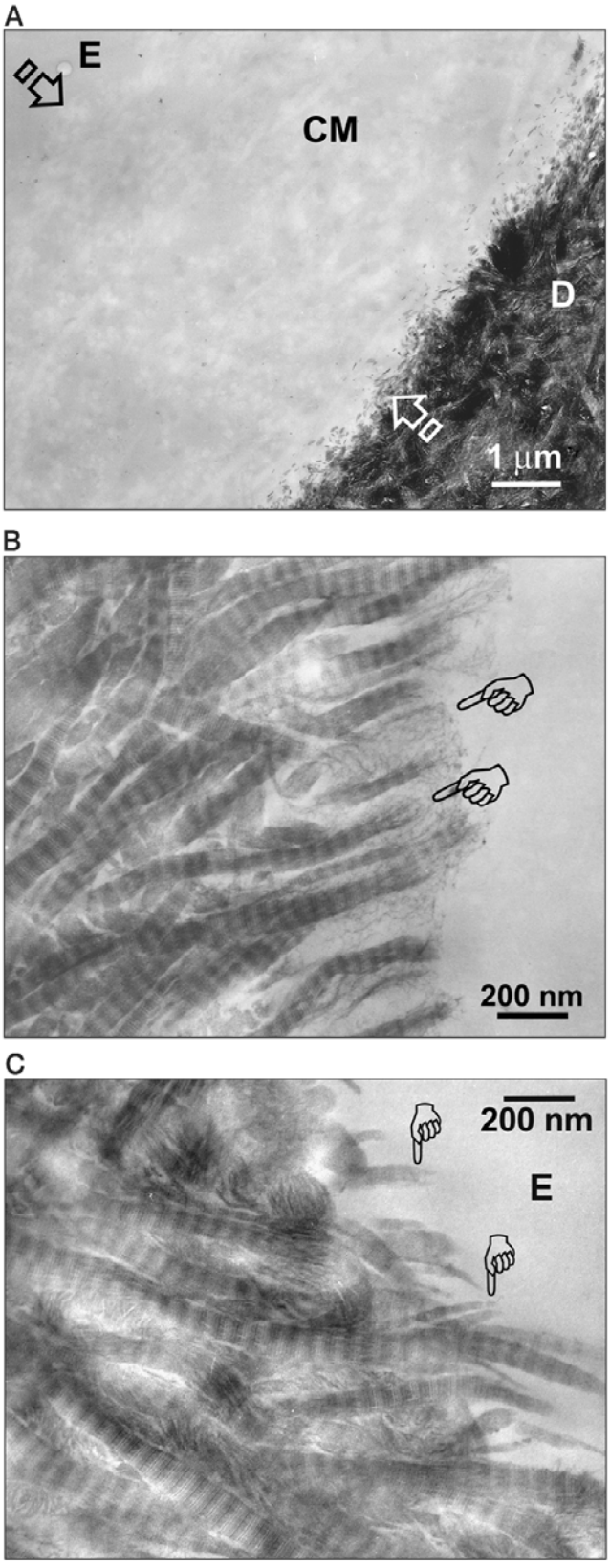
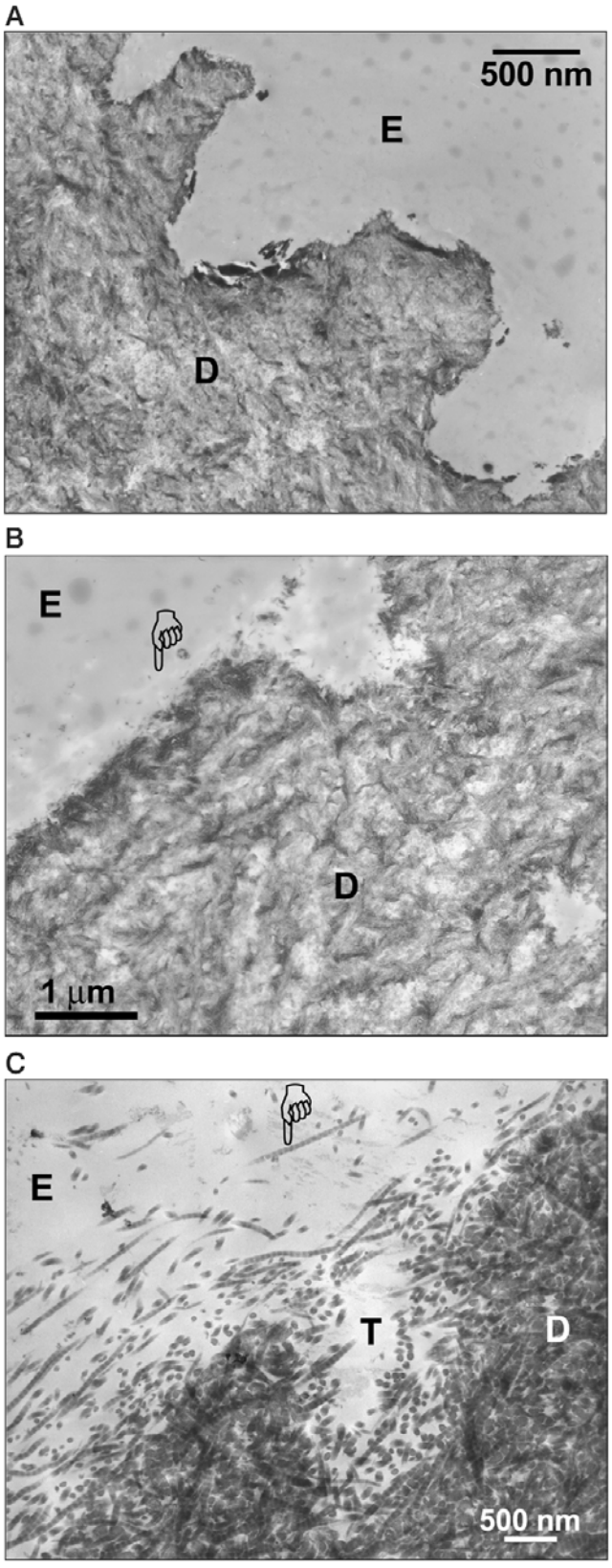


Figure 1. Transmission electron micrographs of control acid-etched dentin. **(A)** Unstained, undemineralized TEM micrograph of phosphoric-acid-etched dentin after being aged in artificial saliva for 24 hrs. A 5- to 6-μm-thick layer (between open arrows) of demineralized collagen matrix (CM) can be observed. D, undemineralized dentin; E, epoxy resin. **(B)** Higher magnification of the completely demineralized surface region of Fig. 1A, but which was stained for collagen. Unraveling of the cut collagen fibrils (pointers) resulted in the appearance of microfibrillar strands (*i.e.,* partially denatured collagen) along the surface of the cut dentin. Cross-banding can be identified from the underlying intact collagen fibrils. **(C)** Stained demineralized TEM micrograph of the surface of phosphoric-acid-etched dentin after being aged in artificial saliva for 90 days. The thickness of the collagen matrix was first identified from unstained, undemineralized sections (not shown) and was found to be similar to that observed in the 24-hour specimens. The unraveled microfibrils disappeared from the surface of the cut dentin, and the exposed ends of the collagen fibrils appeared blunted (pointers).

demineralized, epoxy-resin-embedded thin sections were prepared according to the TEM protocol of Tay *et al.* (1999). Two 2x2 mm blocks were examined for each tooth. The depth of remaining DCM was first determined with the use of 90- to 100-nm-thick, unstained, undemineralized sections. Thereafter, the status of the intertubular collagen fibrils was examined with 60- to 80-nm-thick demineralized sections that were double-stained with 1% phosphotungstic acid (PA) and 2% uranyl acetate for 20 min each. They were examined with the use of a TEM (Philips EM208S, Philips, Eindhoven, The Netherlands) operating at 80 kV.



Assay for Collagenolytic Activity in Dentin

The functional collagenolytic activity of dentin was measured by means of the EnzChek collagenase assay kit (Cat. E-12055, supplemented with type I bovine soluble skin collagen-fluorescein conjugate; Cat. D-12060, Molecular Probes, Eugene, OR, USA). We reduced the human coronal dentin discs to fine powder (*ca.* 10- to 20-μm-diameter particles) by freezing the dentin in liquid nitrogen and triturating it in a stainless steel mixer mill at -120°C (Retsch, Model MM301, Newtown, PA, USA) for 6 min at 30 Hz. The powder was then sieved through a 20-μm screen and kept dry and frozen until used. The assay uses fluorescein-labeled soluble collagen that is internally quenched. When the collagen is solubilized, the cleavage products become fluorescent and can be read in a 96-well fluorescent plate reader operated at an absorption maxima at 495 nm and a fluorescence emission maxima at 515 nm. Because the collagenolytic activity was very low, the reactions were run for 24 hrs at 25°C prior to fluorescence measurements. The assays were run twice with quadruplicate samples. Activation of the enzyme by 4-amino-phenylmercuric acetate was not done, since preliminary studies found no difference in enzyme activity with or without activation. We used a one-way ANOVA to seek significant differences among untreated dentin powder, powder pre-incubated with 4 protease inhibitors listed above, or pre-incubated with 0.2% chlorohexidine, or acid-etched with 37% phosphoric acid for 15 sec. Multple comparisons were made by Tukey's test at $\alpha = 0.05$.

RESULTS

TEM Studies

Specimens retrieved from artificial saliva after 24 hrs and 90 days revealed DCMs that were 5 to 6 μm thick (Fig. 1A). In the 24-hour specimens, collagen fibrils along the dentin surface were partially denatured at their severed ends, with unraveling of their microfibrillar architecture (Ottani *et al.*, 2002) (Fig. 1B). These microfibrillar strands disappeared at 90 days, with the exposed ends of the collagen fibrils appearing blunted (Fig. 1C).

All specimens that were retrieved from artificial saliva after 250 days exhibited complete (Fig. 2A) or partial loss (Fig. 2B) of the DCM, revealing the rough demineralization fronts that were created by phosphoric acid-etching. Remnant collagen fibrils were sparsely distributed, but retained their original

Figure 2. Transmission electron micrographs of acid-etched dentin incubated in artificial saliva for 250 days. **(A)** Unstained, undemineralized micrograph of phosphoric-acid-etched dentin that was aged in artificial saliva for 250 days. The entire thickness of collagen matrix completely disappeared, exposing the highly irregular demineralization front of the original acid-etched dentin. D, undemineralized dentin; E, epoxy resin. **(B)** Another unstained, undemineralized micrograph from the 250-day artificial saliva group showing the presence of a thin remnant layer of unstained collagen fibrils (pointer) above the demineralized matrix. D, undemineralized dentin; E, epoxy resin. **(C)** Stained, demineralized micrograph taken from the same group, showing the disappearance of the bulk of the demineralized collagen matrix. A 1-μm-thick remnant zone of sparsely arranged, banded collagen fibrils (pointer) can be observed over the previously unetched, laboratory-demineralized dentin (D). E, epoxy resin; T, dentinal tubule.

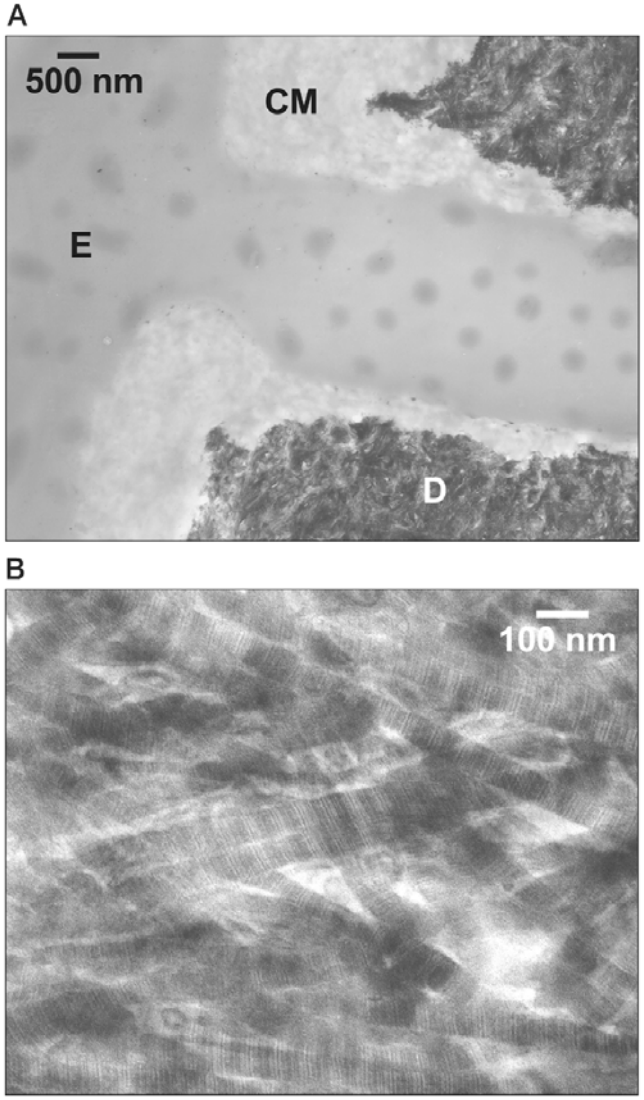


Figure 3. Transmission electron micrographs of acid-etched dentin incubated in oil for 250 days. **(A)** Unstained, undemineralized micrograph of phosphoric-acid-etched dentin that was aged in mineral oil for 250 days. The demineralized collagen matrix (CM) was retained, but was collapsed and appeared thinner due to dehydration in the mineral oil. D, undemineralized dentin. Slightly electron-dense patches that appeared on the epoxy resin (E) were water marks that represented minute water droplets that evaporated from the surface of the hydrophobic epoxy resin following retrieval of the carbon- and formvar-coated copper grid from the water reservoir of the diamond knife. **(B)** Higher magnification of the matrix in CM in Fig. 3A, after double-staining with phosphotungstic acid (PTA) and uranyl acetate to reveal structural detail of collagen fibrils. Intact intertubular collagen fibrils observed 0.5 μm beneath the cut dentin surface revealed a three-dimensional arrangement with intact cross-banding.

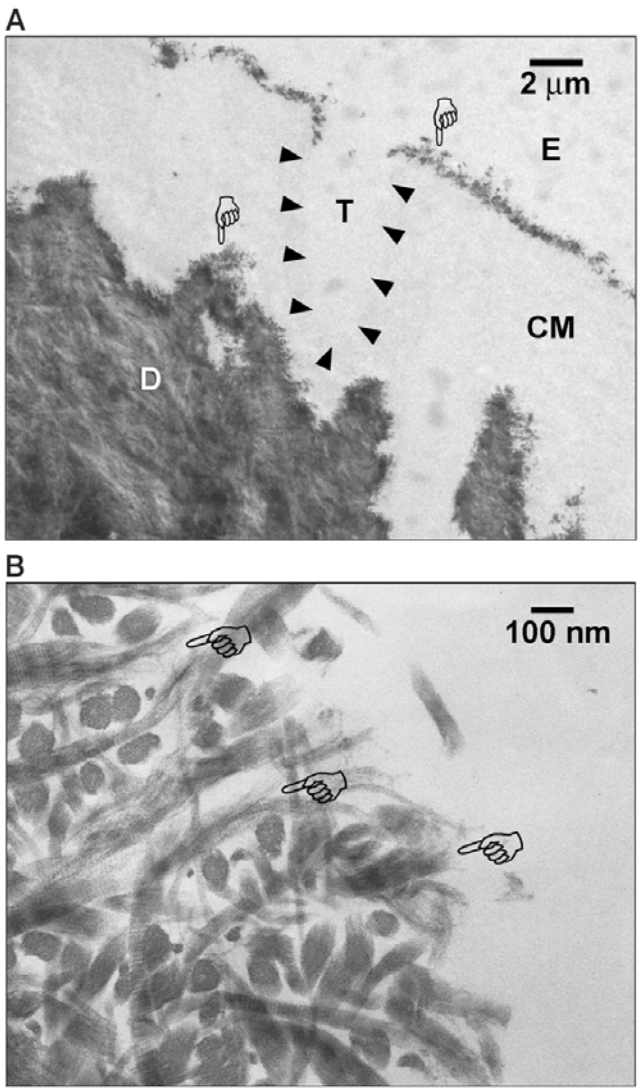


Figure 4. Transmission electron micrographs of acid-etched dentin incubated in protease inhibitors for 250 days. **(A)** Unstained, undemineralized micrograph of phosphoric-acid-etched dentin that was aged in artificial saliva containing protease inhibitors for 250 days. Unlike specimens that were aged for the same period in artificial saliva without protease inhibitors, in which the bulk of the demineralized collagen matrix was dissolved, the demineralized collagen matrix (CM) in the proteolytic enzyme inhibitor group was preserved, and there were crystallites (pointers) deposited along the cut surface of the dentin as well as along the demineralization front (pointers). The internal surfaces (demarcated by arrowheads) of the dentinal tubules (T) were devoid of these crystallites. D, undemineralized dentin; E, epoxy resin. **(B)** Higher magnification of completely demineralized sections stained for collagen, taken from the surface of 250-day protease-inhibited, aged, acid-etched dentin. Similar to specimens that were aged for 24 hrs (control) in artificial saliva, microfibrillar strands (pointers) that were derived from the unraveling of the collagen fibrils were preserved. The acidity of the PTA stain dissolved the surface crystals.

cross-banding staining patterns (Fig. 2C).

In contrast, specimens that were aged in pure mineral oil (Fig. 3A) retained their DCMs, although they appeared collapsed (*ca.* 2-4 μm thick) due to specimen dehydration. Unraveled microfibrillar strands from the severed surface collagen fibrils appeared crust-like (not shown). Subsurface intertubular collagen fibrils revealed intact cross-banding patterns but with minimal interfibrillar spaces (Fig. 3B), caused by the collapse of the three-dimensional matrix following dehydration in mineral oil. Cross-banding staining characteristics were also retained within the intratubular collagen bundles that were found in some dentinal tubules (not shown).

Control specimens that were retrieved from the artificial saliva containing proteolytic enzyme inhibitors after 250 days revealed normal full thicknesses (*i.e.*, 5-6 μm) of the DCMs

Table. Collagenolytic Activity of Dentin Powder

Assay Condition	RFU[d]/80 mg-24 hrs
Coronal dentin powder	196 ± 31 (8)[a]
Powder incubated with 4 protease inhibitors	53 ± 21 (8)[b]
Powder incubated with 0.2% chlorhexidine	3 ± 4 (8)[c]
Powder acid-etched with 37% PA, 15 sec	69 ± 12 (8)[b]

[a-c] Groups identified by different superscript letters are significantly different ($p < 0.05$) by Tukey's test.
[d] RFU, relative fluorescence units.

(Fig. 4A). Higher magnification of stained sections revealed normal collagen fibril dimensions and organization (Fig. 4B).

Functional Collagenolytic Assay

The results of the functional collagenolytic assay indicated that mineralized dentin powder contains a low but measurable level of intrinsic activity (Table). When dentin powder was pre-incubated in the same concentrations of the 4 protease inhibitors used in the morphologic study, the collagenolytic activity was inhibited by 73% ($p < 0.05$). Pre-incubation of mineralized dentin powder with 0.2% chlorhexidine gluconate for 60 sec inhibited the collagenolytic activity to near-zero levels ($p < 0.05$, Table). Acid-etching the mineralized dentin powder with 37% phosphoric acid (PA, Table) for 15 sec reduced the collagenolytic activity by 65% ($p < 0.05$).

DISCUSSION

Since the thicknesses of the remnant DCMs and the status of the collagen fibrils were different when acid-etched dentin was aged in the experimental and the two control storage media, we have to reject the null hypothesis and assert that hydrolytic degradation of denuded collagen fibrils occurs in the absence of bacterial colonization.

Although collagenolytic activity identified from bacteria such as *Streptococcus mutans* (Jackson *et al.*, 1997) may contribute to the hydrolytic degradation of the dentinal matrices in the caries process, results from recent studies suggest that host-derived proteinases, in the form of different types of MMPs present in the saliva and released from the dentin matrix, play an equally important role in dentin caries pathogenesis (Dung *et al.*, 1995; Tjäderhane *et al.*, 1998; van Strijp *et al.*, 2003). MMPs are a family of zinc-dependent proteolytic enzymes that are capable of degrading the dentin organic matrix after demineralization (Tjäderhane *et al.*, 1998). Enzymes with gelatinolytic (MMP-2 and MMP-20) activities are present within intact dentinal matrix (Dayan *et al.*, 1983; Martin de Las Heras *et al.*, 2000) and in carious dentin (Tjäderhane *et al.*, 1998). They may be inhibited *in situ* by tissue inhibitors of metalloproteinases such as TIMP-1 (Ishiguro *et al.*, 1994), or they may be released from mineralized dentin matrix from which they can be activated by low pH (Tjäderhane *et al.*, 1998; Vuotila *et al.*, 2002) and may cause degradation of the demineralized dentin matrix under different physiological and pathological conditions (Tjäderhane *et al.*, 1998).

Since 37% phosphoric acid decreased the functional collagenolytic actvity of dentin (Table), we speculate that the degradation of the demineralized dentin matrix that was observed by TEM was due to enzymes that were from the underlying mineralized matrix slowly released during the 250-day incubation. The partial to complete disappearance of the DCM in specimens that were retrieved from the artificial saliva after 250 days provided morphologic evidence of the effectiveness (Fig. 2C) of the collagenolytic activity (Table) assayed in powdered dentin. Since no collagenase (MMP-1, -8, -13, or -18) has been identified in dentin, but the measured collagenolytic activity could be inhibited by chlorhexidine (Table), the collagenolytic activity may come from MMP-2 (Gendron *et al.*, 1999), which is known to degrade collagen types I, II, and III, albeit more slowly than collagenases (Tjäderhane *et al.*, 2002). No bacteria were observed in any of the specimens or in the artificial saliva used in the experimental group. Since destruction of the DCM occurred in the absence of proteolytic enzyme inhibitors but did not occur in their presence, we propose that the degradation of the DCMs in our experimental group was due to the slow release of active MMP-2 (or other proteolytic enzymes) from the mineralized dentin matrices during storage.

It has been recently shown that denuded collagen fibrils that were found within hybrid layers became less susceptible to staining after being aged (De Munck *et al.*, 2003). This suggested that some forms of hydrolytic degradation do occur within hybrid layers over time. In the present study, we used a model of DCM to provide a more consistent way of examining the degradation of denuded, acid-etched collagen fibrils during aging. Although we have shown that proteolytic enzyme inhibitors are capable of preventing the degradation of exposed collagen fibrils, this experiment should be repeated in the future on dentin specimens that are bonded with either total-etch or self-etch adhesives that may restrict such enzyme activity.

The results of this study seemed to contradict those of our previous study, that showed no change in either the mechanical properties or in the TEM appearance of dentin beams that were completely demineralized in EDTA and stored in water for 48 mos (Carvalho *et al.*, 2000). These apparent contradictory results can be reconciled by the knowledge that the EDTA treatment used in that experiment both extracts and inactivates dentin-bound MMPs (Martin de Las Heras *et al.*, 2000).

From a clinical perspective, it would be advantageous to be able to prevent the degradation of incompletely resin-infiltrated collagen fibrils by host-derived MMPs in dentin hybrid layers. However, even if proteolytic enzyme inhibitors such as those used in this study can be subsequently shown to prevent the degradation of hybrid layers, these poisonous organic salts cannot be applied to acid-etched dentin in routine clinical bonding procedures. Conversely, it has been recently shown that chlorhexidine possesses desirable MMP-inhibitory properties, even at low concentrations (Gendron *et al.*, 1999). Complete inhibition of MMP-2 and MMP-9 gelatinase activities occurred at chlorhexidine concentrations as low as 0.03% (Gendron *et al.*, 1999). Thus, the currently accepted technique of applying a chlorhexidine disinfecting solution to acid-etched dentin prior to the use of total-etch adhesives may have additional potential merits in preventing the degradation of collagen fibrils in dentin hybrid layers, apart from its widely known antimicrobial property. Further *in vitro* and *in vivo* studies should be performed to validate the concept that MMP

inhibition may prevent collagen degradation in resin-dentin bonds, thereby improving their longevity.

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