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ABSTRACT

The recent paradigm that endogenous collagenolytic and gelatinolytic activities derived from acid-etched dentin result in degradation of hybrid layers requires *in vivo* validation. This study tested the null hypothesis that there is no difference between the degradation of dentin bonded with an etch-and-rinse adhesive and that in conjunction with chlorhexidine, an MMP inhibitor, applied after phosphoric-acid-etching. Contralateral pairs of bonded Class I restorations in primary molars of clinical subjects were retrieved after a six-month period of intra-oral functioning and processed for transmission electron microscopy. Hybrid layers from the chlorhexidine-treated teeth exhibited normal structural integrity of the collagen network. Conversely, abnormal hybrid layers were seen in the control teeth, with progressive disintegration of the fibrillar network, to the extent that it was beyond detection by collagen staining. Self-destruction of collagen matrices occurs rapidly in resin-infiltrated dentin *in vivo* and may be arrested with the use of chlorhexidine as an MMP inhibitor.

KEY WORDS: primary dentin, hybrid layer, collagenolysis, gelatinolysis, MMPs.

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INTRODUCTION

Considerable evidence has accumulated over the past decade, based on both *in vitro* and *in vivo* work, that bonds created in dentin by resin-based adhesives may not be as durable as was previously conjectured (De Munck *et al.*, 2005; Frankenberger *et al.*, 2005). Degradation of these bonds occurs *via* the interaction of the components above and below the adhesive interface. Although the current strategies of incorporating ionic and hydrophilic resinous components into total-etch and self-etch adhesives arise from the need to bond to an intrinsically wet substrate, they create potentially unstable resin matrices that slowly degrade *via* water sorption (Yiu *et al.*, 2004). This is particularly so when resin-dentin bonds are not protected by enamel (De Munck *et al.*, 2003), and when the durability of these bonds is challenged by the reduction of adhesive interfaces into smaller portions, to expedite aging effects and increase their interactions with water (Shono *et al.*, 1999; Hashimoto *et al.*, 2002; DeMunck *et al.*, 2003).

A potential bond degradation mechanism that is derived from beneath the bonded interface is the instability of the demineralized dentin collagen matrix. This was manifested as the thinning or disappearance of collagen fibrils from aged, unbonded, or bonded dentin (Hashimoto *et al.*, 2003a,b; Ferrari *et al.*, 2004; Yoshida *et al.*, 2004), or the failure of aged hybrid layers to take up heavy metal stains (De Munck *et al.*, 2003; Armstrong *et al.*, 2004). The issue of collagen instability has potential consequence in the pathogenesis of dentinal caries, with the breakdown of acid-demineralized collagen matrices by host-derived matrix metalloproteinases (MMPs), a class of zinc- and calcium-dependent endopeptidases (Tjäderhane *et al.*, 1998; Sulkala *et al.*, 2001; van Strijp *et al.*, 2003).

In the context of dentin bonding, residual collagenolytic activity was observed in mineralized dentin powder produced from extracted teeth that accounted for the disintegration of collagen fibrils from unbonded, aged acid-etched dentin, in the absence of the contribution from bacterial or salivary MMPs. This low but persistent endogenous collagenolytic activity was completely inhibited by the use of protease inhibitors, the incorporation of which preserved the structural integrity of the collagen fibrils (Pashley *et al.*, 2004). In that study, phosphoric-acid-etching reduced, but did not completely inhibit, the inherent collagenolytic activity of mineralized dentin, while the use of chlorhexidine, in even very low concentrations, strongly inhibited such activity. Thus, apart from being a commonly known disinfectant, chlorhexidine also functions as a potent MMP inhibitor (Gendron *et al.*, 1999).

These reported results, however, are of clinical significance only if proteolysis of the resin-infiltrated collagen network can be demonstrated *in vivo*. To ascertain that endogenous enzymatic activities are involved, we must identify such activities from aged, adhesive-bonded dentin that is completely surrounded by enamel (De Munck *et al.*, 2003). Such an *in vivo* study should also demonstrate that such activities can be arrested *via* the use

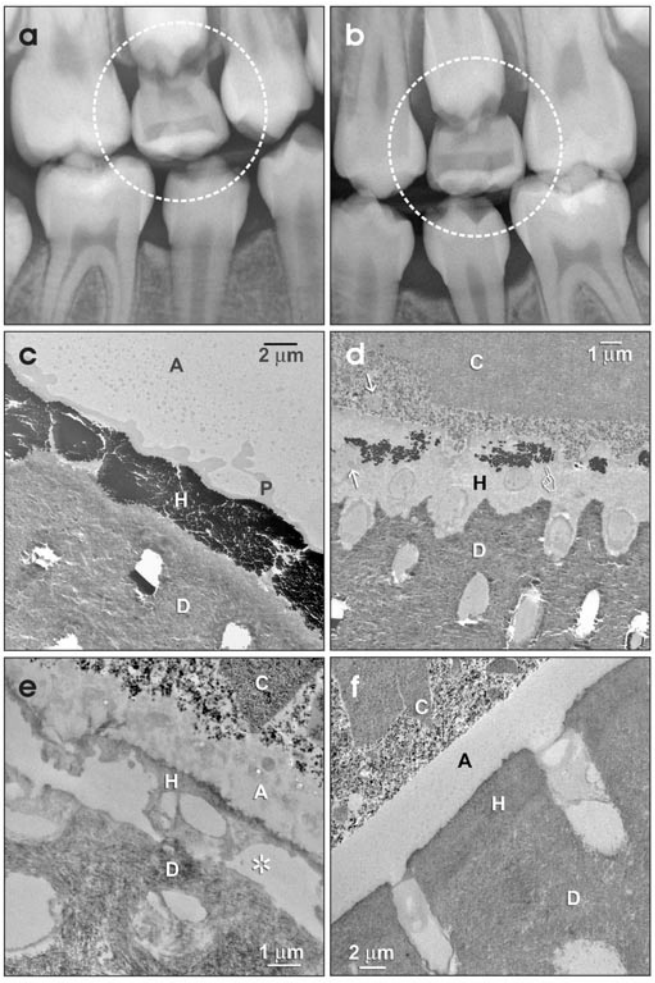

Figure 1. Radiographs and TEM micrographs of the primary molars that were retrieved from the first clinical subject. C, resin composite; A, adhesive; H, hybrid layer; D, dentin. Class I restorations with circumferential enamel cavosurface margins were placed with Single Bond as the dentin adhesive. In the control group **(a,c,e)**, the adhesive was applied to phosphoric-acid-etched dentin. In the experimental group **(b,d,f),** 2% chlorhexidine was applied to the phosphoric-acid-etched dentin before the use of Single Bond. **(a)** Bitewing radiograph showing the Class I restoration in tooth 55 (control, circled). **(b)** Bitewing radiograph showing the Class I restoration in tooth 65 (experimental, circled). **(c)** Undemineralized, unstained, silver-impregnated section from the control tooth showing extensive, black silver deposits that almost completely obscured the hybrid layer. P, polyalkenoic acid copolymer. **(d)**. The same type of section from the experimental tooth, showing sparsely distributed silver deposits (pointer) within the hybrid layer. Arrows: polyalkenoic acid copolymer within the dentinal tubules and resin composite. **(e)** Demineralized section from the control tooth that was stained with phosphotungstic acid and uranyl acetate. The hybrid layer was partially degenerated and missing, with empty regions occupied by epoxy resin (asterisk). These empty regions corresponded to the regions of extensive silver deposits depicted in Fig. 1c. **(f)** The same type of section from the experimental tooth, showing an intact normal hybrid layer.

of potential non-toxic MMP inhibitors, such as chlorhexidine or doxycycline (Grenier *et al.*, 2002), that can be safely applied intra-orally, unlike the highly toxic protease inhibitors that were utilized in the previous *in vitro* study (Pashley *et al.*, 2004). Thus, this study tested the null hypothesis that there is no difference between the ultrastructure of vital human dentin in primary molars bonded with an etch-and-rinse adhesive only and that of dentin bonded in conjunction with the use of chlorhexidine as an MMP inhibitor applied after phosphoric-acid-etching but before adhesive application.

MATERIALS & METHODS

Clinical Procedures

The clinical subjects included in this work represented those who satisfied all the criteria established during the initial screening of 137 primary-school children between the ages of 8 and 12 yrs. Twenty-eight subjects—each with a contralateral pair of carious, non-exposed primary molars that could be conservatively restored with Class I cavity preparations and complete enamel cavosurface margins—were selected as potential candidates. Only 11 subjects were recruited for the study, with the expectation that the restored teeth would be extracted after 6 mos of intra-oral functioning. Informed consent was received from the parents of these subjects, under a protocol approved by the Ethical Committee for Human Studies, Araraquara Dental School, São Paulo, Brazil. Of these 11 subjects, eight had at least 1 of the restored teeth exfoliated naturally and were excluded from the study. Only three subjects remained with both restored molars intact on re-examination after 6 mos.

For each recruited subject, the contralateral pair of primary molars was rendered caries-free under local anesthesia, rubber dam isolation, and the use of a caries detector dye. The control tooth was acid-etched with phosphoric acid gel for 15 sec, bonded with Single Bond (3M ESPE, St. Paul, MN, USA) with a moist-bonding technique, and restored incrementally with a microfilled resin composite (EPIC-TMPT, Parkell Inc., Farmingdale, NY, USA). A layer of hybrid resin composite (Z250, 3M ESPE) was used as the last increment. The experimental tooth was subjected to a similar treatment, with the exception that 2% chlorhexidine digluconate solution (Cavity Cleanser, Bisco Inc., Schaumburg, IL, USA) was applied generously to the cavity after acid-etching. The solution was gently blot-dried after a dwell time of 30 sec prior to the placement of the same adhesive and resin composite. Post-operative bitewing radiographs of the restored teeth were taken as records.

Following the six-month period of intra-oral functioning, the primary molars of the three subjects who completely satisfied all criteria were evaluated for their marginal integrity, according to USPHS criteria. All the restorations were intact, with no associated clinical symptoms, no chipping of the composites, and no recurrent caries, and their marginal integrities were graded as 'alpha'. These teeth were extracted under local anesthesia and immediately fixed in 2.5% glutaraldehyde. Each pair of contralateral specimens was stored in 0.2% sodium azide solution in coded glass vials, so that subsequent specimen-processing was performed blindly with respect to the group designations for the control and experimental teeth of each respective subject.

Laboratory Procedures

When the specimens were received, each tooth was sectioned buccolingualy into 4 1-mm-thick serial sections, each containing a part of the restoration. Two sections from each tooth were randomly selected and immersed in a 50 wt% ammoniacal silver nitrate solution for 24 hrs, according to the tracer protocol for nanoleakage examination reported by Tay *et al.* (2002). These specimens were processed for TEM examination without further laboratory demineralization. The other 2 sections were completely

demineralized in ethylene diamine tetra-acetic acid. Both undemineralized and demineralized, epoxy-resin-embedded, 90-nm-thick sections were prepared according to the TEM protocol of Tay *et al.* (2002). Only the resin-dentin interfaces from the cavity floors of the control and experimental teeth were examined, since they represented the parts of the restorations that were the most secluded from the oral environment.

Undemineralized sections were examined without being stained further. Demineralized sections were stained with 2% uranyl acetate and Reynolds' lead citrate for examination of the characteristics of the resin-dentin interfaces, and with 1% phosphotungstic acid and 2% uranyl acetate for examination of the status of the collagen fibrils. The sections were examined in a TEM (Philips EM208S, Philips, Eindhoven, The Netherlands) operating at 80 kV.

RESULTS

Radiographs and TEM micrographs of the contralateral pairs of control and experimental primary molars retrieved from the three clinical subjects are shown in Figs. 1-3. All restorations were placed on Class I cavities with enamel cavosurface margins (Figs. 1a, 1b, 2a, 2b, 3a, 3b). In general, more extensive silver deposits could be identified from unstained, undemineralized, silver-impregnated sections of the control teeth (Figs. 1c, 2c, 3c). The heavy silver deposits in the control teeth had a tendency to obscure the additional electronlucent, silver-free zones that were concomitantly present within these hybrid layers (Figs. 3d, 4f). These electronlucent silver-free zones were absent from hybrid layers of the experimental teeth (Figs. 1d, 2d, 3d).

Demineralized sections stained with either phosphotungstic acid/uranyl acetate or uranyl acetate/lead citrate prepared from the experimental teeth revealed intact, normal hybrid layers when chlorhexidine was used as an MMP inhibitor after phosphoric-acid-etching (Figs. 1f, 2f, 3f). In contrast, all specimens from the control teeth exhibited evidence of degradation within the hybrid layers. Different patterns could be seen, ranging from nearly complete disintegration of the entire collagen matrix (Fig. 1e), to partial degradation along the surface part of the hybrid layer (Fig. 2e), to partial, vertical degradation of parts of the hybrid layer, with these zones appearing abruptly among intact, non-degraded regions (Fig. 3e).

Figs. 4a-4e depict the variation in the extent of degradation from different regions of the control teeth. They ranged from discontinuous (Fig. 4a) to coalesced voids (Fig. 4b), to larger patches with microfibrillar strands (Fig. 4c), to regions with complete absence of collagen fibrils (Fig. 4d), and, ultimately, to the loss of the bulk of the stainable portion of the hybrid layer (Fig. 4e). No TEM evidence of bacteria was seen in the bonded dentin or within the restorations.

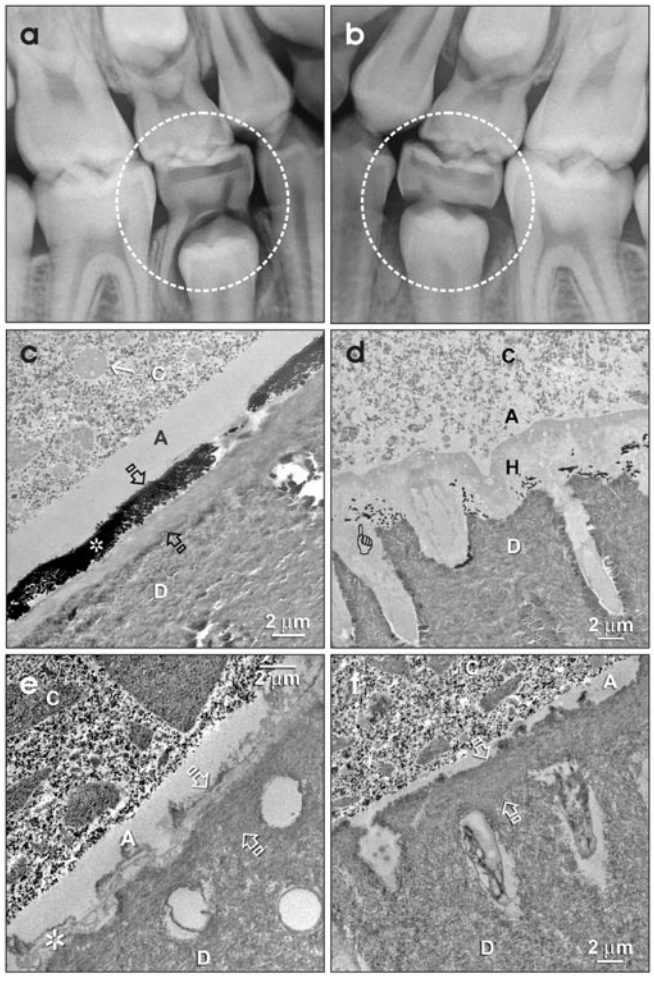

Figure 2. A horizontal pattern of degradation within the hybrid layer, as illustrated by the radiographs and TEMs of primary molars that were retrieved from the second clinical subject (Control group, **a, c,** and **e**; Experimental chlorhexidine group, **b, d,** and **f**). C, resin composite; A, adhesive; H, hybrid layer; D, dentin. **(a)** Bitewing radiograph showing the Class I restoration in tooth 85 (control, circled). **(b)** Bitewing radiograph showing the Class I restoration in tooth 75 (experimental, circled). **(c)** Undemineralized, unstained, silver-impregnated section from the control tooth, showing that the top 1.5-2 µm (asterisk) of the hybrid layer (between open arrows) was almost completely impregnated with silver deposits. **(d)** The same type of section from the experimental tooth, showing sparsely distributed silver deposits (pointer) that occurred predominantly at the base of the hybrid layer (between open arrows). **(e)** Demineralized section from the control tooth that was stained with phosphotungstic acid and uranyl acetate. Partial degradation of the collagen fibrils along the top 1-1.5 µm (asterisk) into non-banded, microfibrillar strands (*i.e.*, gelatin; high magnification not shown) of the hybrid layer (between open arrowheads) corresponded to the region of heavy silver deposits depicted in Fig. 2c. **(f)** The same type of section from the experimental tooth, showing the absence of degradation from the hybrid layer (between open arrowheads) when chlorhexidine was used as an MMP inhibitor.

DISCUSSION

The actual number of *in vivo* specimens was scanty, due to the stringent criteria required for this controlled single-blind clinical study. However, the conditions of the resin-dentin interfaces from different parts of the occlusal floor of each specimen were examined with 4-7 different TEM blocks *per* tooth, depending on the size of the restoration. Since pronounced differences in the extent of nanoleakage, interfacial staining characteristics, and the conditions of the collagen fibrils within the hybrid layers could be universally identified between the control bonded acid-etched dentin and the experimental bonded, chlorhexidine-treated, acid-etched dentin from all the clinical subjects, the null hypothesis has to be rejected.

It is disturbing to observe that subclinical deterioration of

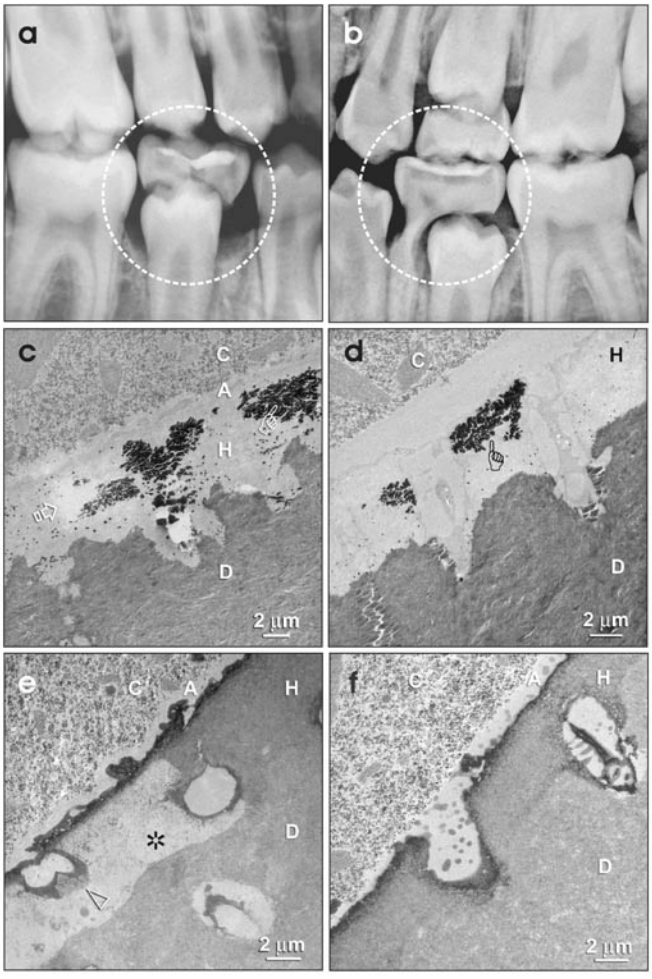

Figure 3. An abrupt, vertical pattern of degradation within the hybrid layer, as illustrated by the radiographs and TEMs of primary molars that were retrieved from the third clinical subject (Control group, **a, c**, and **e**; Experimental chlorhexidine group, **b, d,** and **f**). C, resin composite; A, adhesive; H, hybrid layer; D, dentin. **(a)** Bitewing radiograph showing the Class I restoration in tooth 85 (control, circled). **(b)** Bitewing radiograph showing the Class I restoration in tooth 75 (experimental, circled). **(c)** Undemineralized, unstained, silver-impregnated section from the control tooth. The hybrid layer exhibited a pale electron density (gray), with regions containing silver deposits (pointer). In addition, there were electronlucent (white) zones within the hybrid layer that did not contain silver deposits (open arrow). Since silver impregnation was performed prior to epoxy resin infiltration, these white zones probably contained materials that were more highly penetrable by the electron beam than the original resin-infiltrated collagen fibrils. **(d)** The same type of section from the experimental tooth, showing similar silver deposits (pointer) within the hybrid layer, but with the lack of those electronlucent regions depicted in Fig. 3c. **(e)** Demineralized section from the control tooth that was stained with uranyl acetate and lead citrate. Vertical regions (asterisk) were found abruptly within the hybrid layer in which the bulk of the collagen fibrils have degraded, leaving behind strands of loosely arranged microfibrils (*i.e.*, partially degraded gelatin; see Fig. 4e). Resin tags that were surrounded by islands of intact collagen fibrils (open arrowhead) remained within these abrupt regions of degradation. **(f)** The same type of section from the experimental tooth, showing the absence of degradation from the hybrid layer (between open arrowheads) with chlorhexidine as an MMP inhibitor.

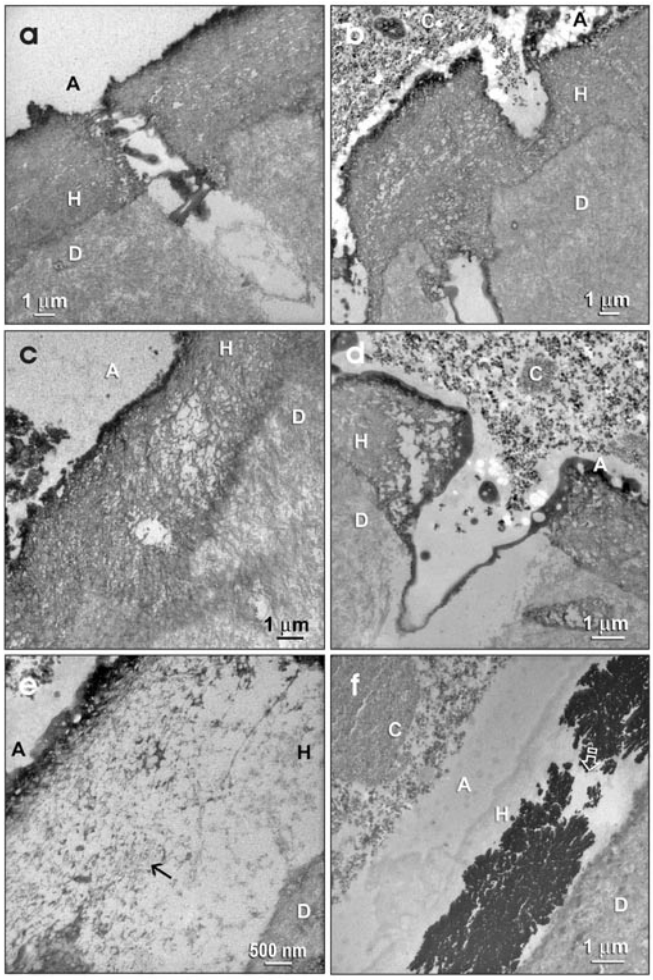

Figure 4. Increasing severity of degradation within hybrid layers. **(a-e)** A series of TEM micrographs, taken from demineralized, stained sections of the other parts of the control teeth of the three clinical subjects, showing an increasing severity in the degradation that occurred within the hybrid layers after 6 mos of intra-oral functioning. In **(e)**, most of the collagen fibrils within the hybrid layer have degraded, and only loose strands of microfibrils remain (arrow). **(f)** An undemineralized, unstained, silver-impregnated section depicting, from a different part of the control tooth, the electronlucent (white), silver-free zone within the hybrid layer (open arrow). These white zones probably corresponded to the regions in Fig. 4e, in which the collagen fibrils have degraded into gelatin strands. Further degradation of the denatured gelatin into polypeptides and amino acid residues could have resulted in the leaching of these smaller molecules out of the hybrid layer, with the resultant empty spaces being infiltrated by the silver deposits.

the hybrid layers occurred so quickly *in vivo*, in the absence of adjunctive clinical signs and symptoms. Unlike previous *in vitro* studies (De Munck *et al.*, 2003; Armstrong *et al.*, 2004), the resin-dentin interfaces examined were protected by bonded enamel cavosurface margins and were not sectioned into beams for an accelerated aging effect. Under the conditions used in this study, it appeared that clinical degradation of hybrid layers by endogenous, host-derived MMPs, at least in exfoliating primary molars, occurred at a rate much faster than the 3-5 years of accelerated aging required for such conditions to be identified *in vitro*. Initially, we speculated that degradation of hybrid layers by endogenous MMPs would occur more rapidly under *in vitro* conditions, due to the possible disturbance of the balance between MMPs and their natural inhibitors, TIMPs (tissue inhibitors of metalloproteinases), when extracted teeth

are used for aging experiments. It is known that both TIMP-1 and TIMP-2 can complex with active MMP-2 and inhibit proteolytic activity (Ward *et al.*, 1991; Palosaari *et al.*, 2003), and both of them (especially TIMP-1) are expressed by human odontoblasts (Palosaari *et al.*, 2003). Since these natural MMP inhibitors have shorter half-lives (Bode *et al.*, 1999) than MMPs, prolonged interruption of MMP-TIMP interaction, such as the cessation of dentinal fluid flow (*i.e.*, *in vitro* conditions), may prevent the replenishment of pulpal TIMPs out into peripheral dentin. Apparently, these initial assumptions were not fully supported by our *in vivo* results.

The reason for the rapid degradation compared with *in vitro* studies may be that bonding was performed on caries-affected dentin. Caries-affected dentin demonstrates decreased collagen cross-linking (Kuboki *et al.*, 1977). Since carious dentin exhibits increased collagenolytic activity compared with intact dentin (Dayan *et al.*, 1983), the speed of degradation may partially be caused by the increased MMP activity of caries-affected dentin. Moreover, the observations in the control group *vs.* the experimental group may, in part, be the result of chlorhexidine, being an effective antimicrobial agent, in controlling remnant bacteria that were present within the caries-affected dentin. Unlike previous *in vitro* studies that demonstrated the almost complete absence of staining from hybrid layers, due to the longer period of accelerated aging, a series of progressively advancing patterns of degradation could be identified from the present *in vivo* results (Fig. 4), probably due to the shorter period between intra-oral bonding and specimen retrieval. The differences in the degradation patterns may also reflect the local differences in the extent of the caries process, thus affecting the local MMP activity.

We demonstrated, in control specimens, the existence of discontinuous patches of grossly disintegrated microfibrillar fragments that remained silver-free despite the loss of the electron density which is characteristic of the normal hybrid layer. These fragments probably represented remnant 3/4- and 1/4-length fragments that resulted from collagenolysis (Gross and Nagai, 1965), but were retained by the adhesive resins within the hybrid layer. The additional moderate to severe nanoleakage (*i.e.*, silver uptake) in the hybrid layers of the control specimens probably represented regions wherein leaching of the degraded fragments occurred with concurrent hydrolysis of the hydrophilic resin components within the hybrid layers. Conversely, the silver-free electronlucent areas in the undemineralized control sections that corresponded to regions with a complete lack of stainable fibrillar components in demineralized sections suggested that, in these regions, the degenerated microfibrillar fragments have further been degraded beyond detection. Such a process may occur *via* gelatinolytic MMPs released from the underlying mineralized dentin, with the gelatin breaking down into peptides of lower molecular weight (kDa). This phenomenon is analogous to the appearance of clear bands in Coomassie-blue-stained gels, when the cleavage products of gelatin were subjected to Western blotting after treatment with MMP-2 (Gelatinase A) or MMP-9 (Gelatinase B) (Gendron *et al.*, 1999; Smith *et al.*, 2004). Subsequent to the report of collagenolytic activity in mineralized dentin powder (Pashley *et al.*, 2004), we have also identified separate gelatinolytic activity from the same substrate, *via* the use of fluorescein-labeled gelatin derived from porcine skin (Pashley *et al.*, unpublished results).

The ultrastructural nature of the present study precluded the identification of the exact classes of MMPs involved in *in vivo* degradation of dentin hybrid layers. Collagenase MMP-8 has been shown to exist in carious human dentin (Tjäderhane *et al.*, 1998), and it seems to be also present in intact dentin (Sulkala *et al.*, unpublished results). The gelatinase MMP-2 has been shown to be present in human dentin (Martin-De Las Heras *et al.*, 2000). Apart from its gelatinolytic activity, MMP-2 is also capable of collagenolysis (Aimes and Quigley, 1995), albeit at a slower rate (Patterson *et al.*, 2001). Thus, even in the absence of an endogenous collagenase source, endogenous MMP-2 from the dentin matrix can apparently result in the slow but complete cleavage of the entire resin-infiltrated collagen network.

Since conservative dentistry does not involve only the use of etch-and-rinse adhesives on primary dentin, further studies are urgently needed to validate these results using both etch-and-rinse and self-etch adhesives on vital normal and non-vital coronal, extraradicular, and intraradicular adult dentin. Although the results of the present study are clinically provocative, it is reassuring that the use of chlorhexidine as an MMP inhibitor resulted in the arrest of the *in vivo* degradation of the hybrid layers. Such a protocol may also be of clinical value to prevent collagen degradation in root canals (Ferrari *et al.*, 2004), in light of the recent upsurge of interest in the application of bondable root-filling materials to intraradicular dentin (Shipper *et al.*, 2004; Zmener, 2004).

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