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Role of Dentin MMPs in Caries Progression and Bond Stability

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Abstract

Dentin can be described as a biological composite with collagen matrix embedded with nanosized hydroxyapatite mineral crystallites. Matrix metalloproteinases (MMPs) and cysteine cathepsins are families of endopeptidases. Enzymes of both families are present in dentin and collectively capable of degrading virtually all extracellular matrix components. This review describes these enzymes and their presence in dentin, mainly focusing on their role in dentin caries pathogenesis and loss of collagen in the adhesive hybrid layer under composite restorations. MMPs and cysteine cathepsins present in saliva, mineralized dentin, and/or dentinal fluid may affect the dentin caries process at the early phases of demineralization. Changes in collagen and noncollagenous protein structure may participate in observed decreases in mechanical properties of caries-affected dentin and reduce the ability of caries-affected dentin to remineralize. These endogenous enzymes also remain entrapped within the hybrid layer during the resin infiltration process, and the acidic bonding agents themselves (irrespective of whether they are etch-and-rinse or self-etch) can activate these endogenous protease proforms. Since resin impregnation is frequently incomplete, denuded collagen matrices associated with free water (which serves as a collagen cleavage reagent for these endogenous hydrolase enzymes) can be enzymatically disrupted, finally contributing to the degradation of the hybrid layer. There are multiple in vitro and in vivo reports showing that the longevity of the adhesive interface is increased when nonspecific enzyme-inhibiting strategies are used. Different chemicals (i.e., chlorhexidine, galardin, and benzalkonium chloride) or collagen cross-linker agents have been successfully employed as therapeutic primers in the bonding procedure. In addition, the incorporation of enzyme inhibitors (i.e., quaternary ammonium methacrylates) into the resin blends has been recently promoted. This review will describe MMP functions in caries and hybrid layer degradation and explore the potential therapeutic role of MMP inhibitors for the development of improved intervention strategies for MMP-related oral diseases.

Keywords: tooth, enzymes, collagen, cathepsins, dentin bonding agents, degradation

Evidence of MMPs in Dentin

Dentin is a collagen-based mineralized tissue consisting of inorganic apatite crystallites embedded in an extracellular matrix (ECM). Type I collagen is the main component of the ECM compartment of dentin, representing up to 90% of the organic material (Linde 1984). In addition, several proteins, collectively referred to as noncollagenous proteins, constitute approximately 10% of the matrix. The noncollagenous dentin proteins include proteoglycans, phospholipids, and enzymes. Among the dentin enzymes, matrix metalloproteinases (MMPs) have recently gained much attention because of their possible roles in several physiological and pathological processes in dentin.

MMPs are endogenous Zn^{2+}- and Ca^{2+}-dependent enzymes, capable of degrading almost all ECM components. In humans, the MMP family has 23 members, classified into 6 groups based on substrate specificity and homology (Visse and Nagase 2003). MMPs consist of a prodomain, a catalytic domain, as well as other domains governing factors such as substrate specificity, recognition,

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and interaction (Visse and Nagase 2003). They are usually expressed as inactive zymogens, and the prodomain must be dissociated from the catalytic one for its activation (Hannas et al. 2007). In nonactivated MMPs, the unpaired cysteine in the prodomain forms a bridge with the catalytic zinc (referred to as the "cysteine switch" mechanism), preventing enzymatic activity and acting as a ligand for the catalytic zinc atom in the active site, excluding water molecules and rendering the enzyme inactive (Tjäderhane et al. 2013a). Regulation of MMP activity by cleavage of the propeptide may occur at multiple levels, including autolysis, serine protease plasmin, or other MMPs (Visse and Nagase 2003). Furthermore, tissue inhibitors of MMPs (TIMPs) are involved in the local control of MMP activities in tissues, representing the main inhibitors of MMPs. The TIMP family consists of 4 members (TIMP1-4) that collectively inhibit MMP activities and restrict ECM breakdown (Ishiguro et al. 1994; Palosaari et al. 2003).

The first evidence of collagenolytic activity in dentin was reported in the early 1980s both in carious and intact dentin (Dayan et al. 1983). More recently, MMPs were identified as being responsible for that activity (Tjäderhane et al. 1998), and to date, the presence of gelatinases MMP-2 and -9 (Fig. 1), collagenase MMP-8, stromelysin MMP-3, and MMP-20 have been reported (Martin-De Las Heras et al. 2000; Sulkala et al. 2002; Mazzoni et al. 2007; Sulkala et al. 2007; Boukpessi et al. 2008; Mazzoni et al. 2009; Santos et al. 2009; Boushell et al. 2011; Mazzoni, Papa, et al. 2011).

Dentin undergoes modifications by physiological aging and disease processes to produce different dentin forms, and this process affects its biomechanics and biochemistry (Marshall et al. 1997). Although the physiological roles of MMPs in dentin are not well understood, they have been suggested to participate in peritubular and tertiary dentin formation and in the release of dentinal growth factors (Tjäderhane et al. 2001; Hannas et al. 2007; Charadram et al. 2012). Unlike bone, mineralized dentin does not undergo significant restructuring; nevertheless, predentin and nonmineralized dentin inside the dentinal tubules seem to be modified in response to the functional demands. The mechanical properties of dentin (i.e., flexural strength and resistance to fatigue) decrease significantly with age (Arola 2008), and changes in the intertubular collagen matrix have been suggested to contribute to these structural responses. Dentinal MMPs may act in concert to slowly modify the structure and mechanical properties of the mineralized dentin caused by physiological and pathological processes (Fig. 2A–C).

More recently, another important family of proteases, the cysteine cathepsins, was reported to be present in dentin (Tersariol et al. 2010; Nascimento et al. 2011). In terms of protein expression, the variety of these proteases in the dentin-pulp complex has been claimed to be comparable with that described for MMPs (Palosaari et al. 2003; Tersariol et al. 2010; Tjäderhane et al. 2013a). Similar to MMPs, cathepsins also participate in ECM degradation in physiological and pathological processes (Fig. 2). Within the oral cavity, cathepsins have also been associated with caries progression and failure of adhesive restorations over time (Breschi et al. 2008; Liu et al. 2011; Nascimento et al. 2011; Tjäderhane et al. 2013a; Vidal et al. 2014).

MMPs present in dentin will be reviewed, focusing on their involvement in dentin caries pathogenesis and progression and collagen degradation in the adhesive hybrid layer (HL) under composite restorations.

Dental Caries and Role of MMPs in Caries Progression

Dental caries is an irreversible disease of calcified tissue of teeth, characterized by demineralization and subsequent destruction of the organic substance of the tooth, finally leading to cavitation. The progression of caries into dentin requires bacterial invasion along the dentin-enamel junction. The lengthening of the initial lesion with the destruction of the mantle dentin has also been described (Fig. 3, upper; Chaussain-Miller et al. 2006).

During the demineralization phase of dental caries, hydroxyapatite is solubilized by organic acids produced by oral bacteria. Bacterial organic acids can diffuse into calcified dental tissues when the local pH falls to below 5.5, leading to dissolution of the mineral crystals (Chaussain-Miller et al. 2006). The dynamic process of demineralization that occurs numerous times daily is usually balanced by the buffering potential of the saliva that allows remineralization to occur. However, if this balance is lost, pathological factors predominate, and caries progression takes place (Chaussain-Miller et al. 2006). Caries progression induces several modifications to dentin (reduction of mineral content, increase in micro- and nano-porosities due to changes in dentin collagen structure and distribution and noncollagenous protein), synergistically contributing to reductions in physical and mechanical dentin properties (Fig. 3A–D; Tjäderhane et al. 2013a).

In dentin, demineralization is followed by destruction of the collagenous organic matrix of dentin, long thought to be caused by bacterial proteases. However, cariogenic bacteria do not degrade dentin matrix after they have demineralized it (van Strijp et al. 1997). Furthermore, the bacteria collected from dentinal lesions created in situ are not capable of degrading collagen in vitro, and even purified bacterial collagenases have low activity in acidic environments (van Strijp et al. 1997). Since the evidence of bacterial input to the degradation of the organic matrix of carious dentin is lacking, it has more recently been thought to be mediated mainly by host-derived MMPs (Tjäderhane et al. 1998; Hannas et al. 2007).

The MMPs present in dentin are produced by odontoblasts (Palosaari et al. 2003) during secretion of dentin matrix and are suggested to be involved in dentin formation. After mineralization of the collagen matrix, the inactive proforms of MMPs remain trapped within the calcified matrix (Hannas et al. 2007), where they can be re-exposed and potentially activated during the dentin caries process. The acidic environment created by bacterial acids can facilitate the activation of endogenous MMPs. Low pH leads to the cleavage of prodomain and thus facilitates the functional activity of MMPs (Tjäderhane et al. 1998; Sulkala et al. 2001). However, although activated MMPs are stable in acidic pH, they function best at neutral pH. Neutralization of the acids can be obtained by the dentinal buffering mechanisms or through the salivary buffer systems, thus allowing the pH-activated MMPs to cleave matrix components (Tjäderhane et al. 1998; Chaussain et al. 2013).

The MMPs that have the potential to be proteolytically active during the carious process include collagenases (MMP-1, MMP-8), gelatinases (MMP-2, MMP-9, which also have telopeptidase activity; Fig. 2), stromelysin (MMP-3), and enamelysin (MMP-20; Tjäderhane et al. 1998; Sulkala et al. 2002; van Strijp et al. 2003; Chaussain-Miller et al. 2006; Sulkala et al. 2007; Boukpessi et al. 2008; Shimada et al. 2009; Toledano et al. 2010; Mazzoni, Papa, et al. 2011; Vidal et al. 2014). Carious dentin was reported to contain latent and active forms of MMP-2, -9, -8, and -3 (Tjäderhane et al. 1998; Boukpessi et al. 2008). As a true collagenase, MMP-8 is most effective in hydrolyzing type I collagen fibrils, while MMP-9 was the predominant gelatinolytic enzyme detected in carious lesions (Tjäderhane et al. 1998). Also, cysteine cathepsins may participate in dentinal caries development. Cysteine cathepsin activity is different in the various compartments of the caries, depending on the localization and lesion activity (Nascimento et al. 2011; Vidal et al. 2014). The significant increase of cysteine cathepsin activity in carious dentin with increasing depth toward the pulp (Nascimento et al. 2011) indicates that odontoblast or pulp-derived cysteine cathepsins may be important in expediting active caries lesions.

Dentinal fluid may be an important source of MMPs in carious tissues. Even in healthy teeth, dentinal tubules have high gelatinolytic activity (Mazzoni et al. 2012), and MMP-2 has been demonstrated in dentinal fluid (Zehnder et al. 2011). Caries stimulates MMP-2 expression in human odontoblasts in vivo (Charadram et al. 2012). MMP-2 seems to be actively secreted by odontoblasts in response to carious insult (Boushell et al. 2011), resulting in the differential expression of this protease in sound, caries-affected, and caries-infected dentin (Toledano et al. 2010; Charadram et al. 2012). Increased levels of MMP-9 (Zehnder et al. 2011) and MMP-20 (Sulkala et al. 2002) in dentinal tubules under caries lesions have also been demonstrated. Once active carious lesions begin to demineralize and remineralize, their pHs cycle from pH 5.0, the optimum pH for cathepsin K but not for MMPs, to pH 7.0, the optimum pH for MMPs but not for cathepsin K. Thus, there is significant proteolytic activity occurring for long periods of time.

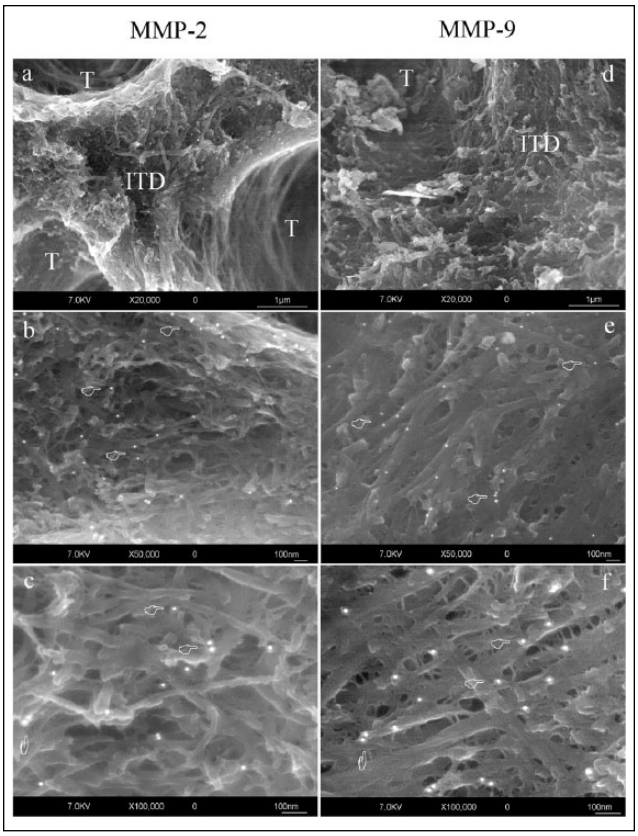

Figure 1. Field emission in-lens scanning electron micrographs (FEI-SEMs) of unfixed, partially decalcified dentin, after a preembedding immunolabeling procedure with monoclonal antibodies for matrix metalloproteinase–2 (MMP-2) or MMP-9. The images were obtained by a combination of secondary electron and backscattered electron signals to simultaneously reveal immunogold labeling and related substrate morphology. Labeling can be identified as electron-dense white spots under the electron beam (pointers). (**A, D**) Low magnification view (20,000×) of the partially decalcified dentin surface showing open tubular orifices (T) surrounded by a thick collar of fibrillar organic matrix and intertubular porous dentin (ITD). MMP-2 and -9 labeling can be identified as mainly localized in peritubular dentin. (**B, E**) A higher magnification view (50,000×) of the partially decalcified surface: positive immunohistochemical staining identifying MMP-2 (B) and -9 (E) antibodies located along collagen fibrils. (**C, F**) High-magnification FEI-SEM micrographs (100,000×), revealing the relationship between MMP-2 and -9 and the collagen meshwork. The specimen shows a moderate labeling for MMP-9 (F) uniformly weaker than MMP-2 staining (C). Reprinted with permission from Mazzoni et al. (2009).

An additional contribution to organic matrix degradation could be derived from proteases contained in saliva (Tjäderhane et al. 1998; van Strijp et al. 2003; Chaussain

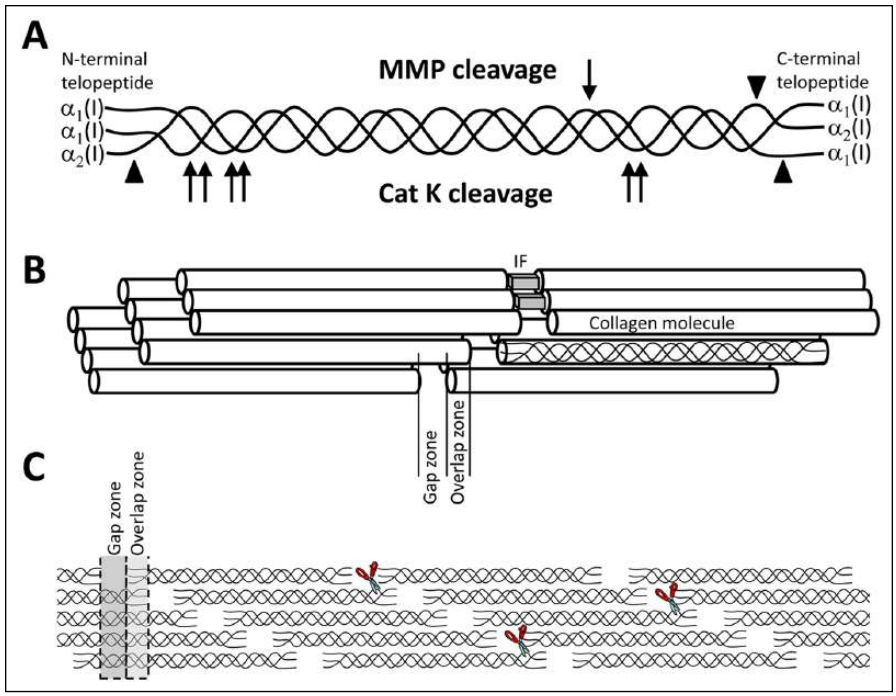

Figure 2. Cleavage mechanisms of matrix metalloproteinases (MMPs) and cathepsins. (**A**) The type I collagen molecule with approximate locations of the MMP cleavage sites (arrows above) and cysteine cathepsin K cleavage sites (arrows below). Arrowheads indicate telopeptidase cleavage activities, resulting in the loss of terminal telopeptides and release of the longer ICTP (by MMPs) and shorter CTX (by cathepsins) fragments. Collagenolytic MMPs always cleave the triple-helical part of the molecule into ¾ N-terminal and ¼ C-terminal fragments. Cysteine cathepsin K has multiple triple-helical cleavage sites, which makes it the most potent mammalian collagenolytic enzyme. (**B**) Orientation of type I collagen molecules in hard tissues. N- and C-terminal ends of successive molecules are separated by the gap zone, which is the site of intrafibrillar minerals (IF; Bertassoni et al. 2009). (**C**) The characteristic periodicity of collagen is caused by the overlapping of 4 (gap zone) or 5 (overlap zone) individual molecules. N- and C-terminal telopeptides reside in the overlap zone; therefore, their cleavage by enzymes with telopeptidase activity results in gradual loss of collagen periodicity.

et al. 2013). Saliva has been reported to contain several MMPs derived from both gingival crevicular fluid and from salivary glands (van Strijp et al. 2003). Salivary MMPs can efficiently degrade exposed dentinal collagen matrix (Tjäderhane et al. 1998). MMP-8 and MMP-9 are the most abundant salivary MMPs and predominate in dentin caries lesions (Tjäderhane et al. 1998), especially in the outer caries compared with the inner caries (caries-affected) layer (Fig. 3; Shimada et al. 2009). Significantly higher MMP activities are seen in actively progressing caries compared with chronic caries lesions (Nascimento et al. 2011). Together, these findings may indicate saliva as the source of these enzymes. Saliva also contains cysteine cathepsins (Nascimento et al. 2011), at least cathepsin B (van Strijp et al. 2003).

The increase of MMPs (and possibly cysteine cathepsins) in dentinal fluid under caries lesions and MMPs originating from saliva may be behind the markedly higher enzyme activity levels in carious than in normal dentin (Toledano et al. 2010; Nascimento et al. 2011; Vidal et al. 2014). While salivary enzymes may readily access outer, caries-infected dentin, it is unlikely that they would contribute to destruction of caries-affected dentin (Toledano et al. 2010; Vidal et al. 2014), in which dentinal fluid rather than saliva (due to the hydrostatic pressure working in favor of an outward flow of dentinal fluid) may be the source of increased activities. Alternatively, dentin matrix-bound enzymes may not necessarily be readily activated after simple in vitro demineralization. For example, while phosphoric acid–etched dentin may demonstrate very low MMP activity, treating the acid-etched dentin with adhesives causes significant reactivation (Mazzoni et al. 2006), and repeated freezing-thawing without any other treatment can cause about 30- and 60-fold increases in the gelatinolytic and collagenolytic of mineralized dentin activities (Nishitani et al. 2006). Thus, it is possible that the actual amounts of MMPs in intact dentin have been underestimated. In caries lesions, de- and remineralization processes alternate, and matrix exposure may be relatively slow. Therefore, the liberation and activation of enzymes and their effect on the matrix components may be slow and progressive.

Dentin collagen matrix in caries-affected dentin is believed to remain mostly intact, until heavily demineralized, and it is believed to retain its ability to remineralize even after up to half of the mineral is lost. However, true remineralization requires that nanometer-sized apatite crystals regrow in the gap zones of collagen fibrils, which has proven to be a difficult task (Bertassoni et al. 2009). Recent evidence indicates that the dentin collagen matrix may not necessarily remain as intact as believed during caries demineralization (Suppa et al. 2006; Tjäderhane et al. 2013a; Vidal et al. 2014). A significant reduction in immunohistological detection of intact type I collagen and proteoglycans (Suppa et al. 2006) and almost complete loss of autofluorescent signal emitted by well-structured collagen (Vidal et al. 2014)

in caries-affected dentin (Fig. 4A–F), compared with normal dentin (Fig. 4G, H), indicate molecular changes in collagen still believed to be remineralizable.

Dentin collagen may show structural changes after relatively mild demineralization in caries lesions, such as gradual loss of characteristic collagen periodicity (Deyhle et al. 2011). Since the collagen periodicity relates to the presence of terminal telopeptides next to the gap junction (the site of

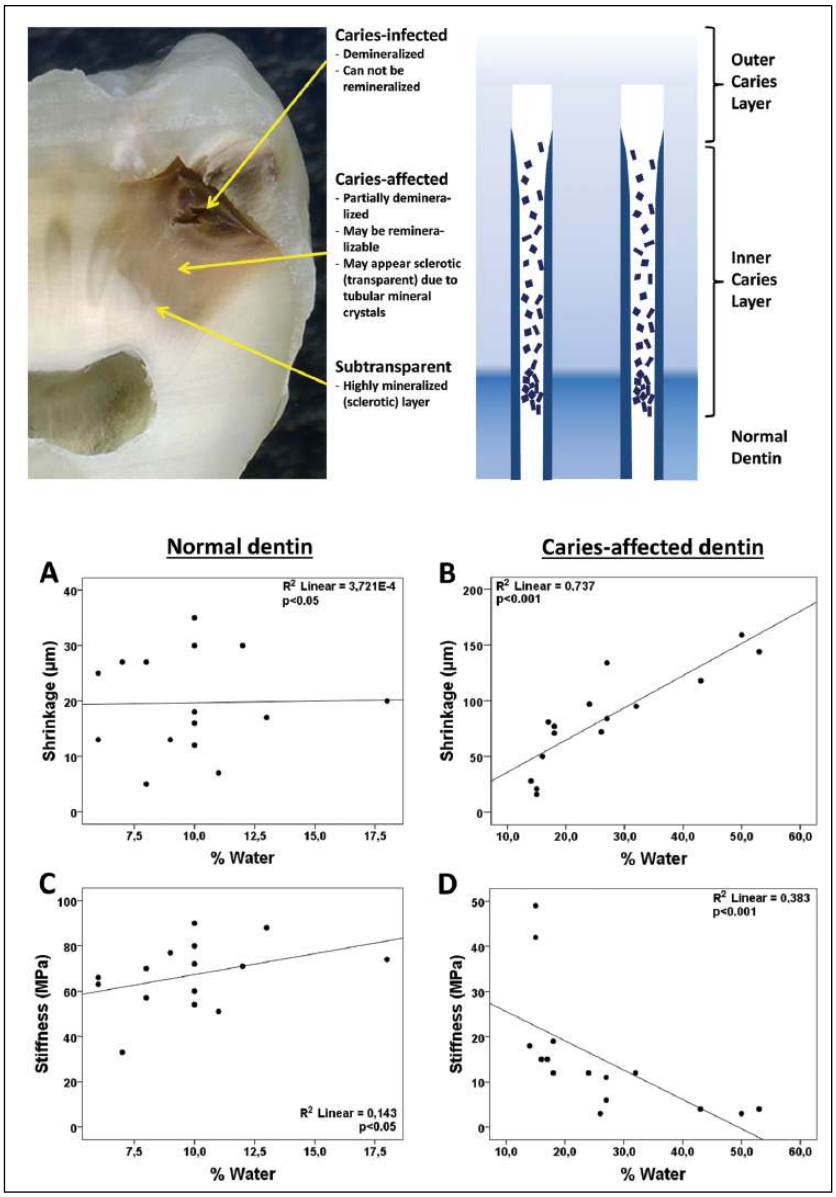

Figure 3. Clinical and schematic view of carious dentin zones as they have been described in the literature (upper), and the effect of caries on dentin mechanical properties and wetness (lower). (Upper) Outer caries layer, usually called caries-infected dentin, has lost most of its mineral component, and the collagen matrix structure is decomposed. The tubular structure of the outermost part has disappeared, and the deeper part of this layer has lost its peritubular dentin. The inner caries layer, also called the caries-affected dentin, may have several zones depending on the distance from the lesion surface (upper left). Closer to the surface, the peritubular and intertubular dentin are partially demineralized. The width of the peritubular dentin, the mineral content of the intertubular dentin, and the amount of intratubular mineral crystals increase toward the pulp, and the dentin matrix is believed to be able to remineralize (upper right). While this part of the caries-affected dentin may appear transparent and is sometimes called sclerotic, the overall mineral content is markedly lower than in normal dentin, despite the presence of intratubular mineral crystals. The deepest part of the caries-affected dentin is a narrow subtransparent layer. The subtransparent layer may have little or no demineralization of the intertubular dentin, normal peritubular dentin, and a relatively high level of intratubular mineral crystals. The mineral content and the hardness of the subtransparent layer has been found to be higher than in normal dentin, and it has therefore been described as the "real sclerotic layer" (upper right). It should be noted that the absolute and relative width of the layers may vary significantly between the lesions and even within a lesion, and all the layers are not found in all dentinal caries lesions. (Lower) Shrinkage of normal dentin (**A**) is independent of water content, while caries-affected dentin (**B**) demonstrates a highly significant correlation between shrinkage and water content. Note differences in scales for both shrinkage and water content between normal and caries-affected dentin. The increase in water content and shrinkage of caries-affected dentin are dependent on the rate of demineralization, which can vary even within caries-affected dentin. The highly significant correlation between stiffness (**D**) and water content can also be seen in caries-affected but not in normal dentin (**C**). Again, note the differences in scales. The graphs demonstrate the problems potentially faced when bonding to caries-infected or -affected dentin: increased wetness, shrinkage following drying, and low mechanical strength. Caries-affected dentin has been shown to contain more detectable matrix metalloproteinases and cysteine cathepsins (Nascimento et al. 2011; Vidal et al. 2014), and poor infiltration of carious dentin (due to tubule occlusion of tubules) during adhesive procedures has also been demonstrated. This result in much unprotected collagen (more than in normal, intact dentin), whose proteases are fully activated collagen-degrading enzymes in and under the hybrid and adhesive layers. Figure 3A and B data adapted from Ito et al. (2005). Spearman bivariate correlation was used for the statistical analysis.

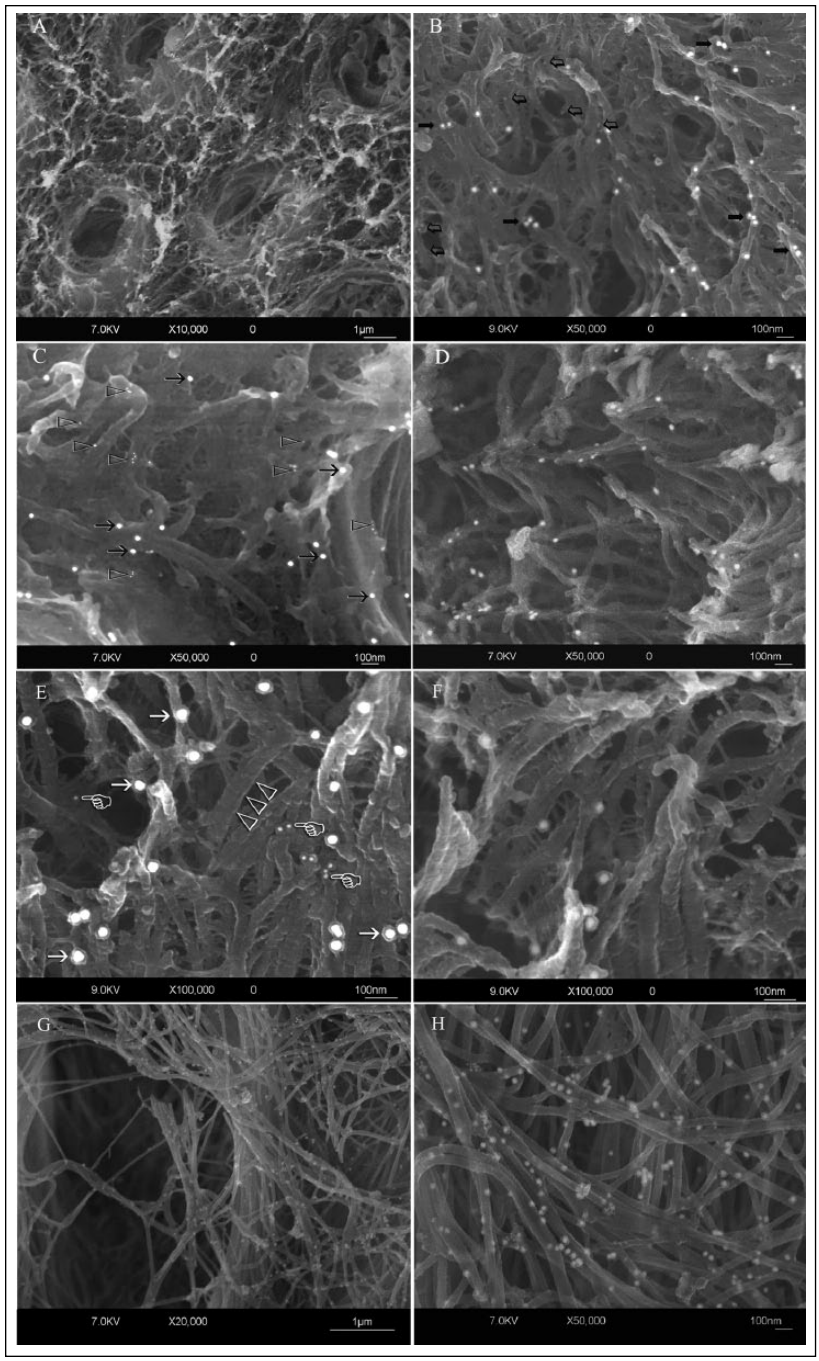

Figure 4. Field emission in-lens scanning electron micrographs (FEI-SEM) of sclerotic dentin after double immunolabeling with monoclonal antibodies for type I collagen and proteoglycans. The images were obtained by a combination of secondary electron and back-scattered electron signals. (**A**) Low magnification view of the surface of sclerotic dentin revealed partially patent tubular orifices that were surrounded by thick collars of peritubular fibrillar structures. Intertubular dentin was highly porous and was inhomogenously covered with the large (30-nm) gold particles used for labeling of antigenically intact collagen fibrils. Gold nanoparticles (15 nm) used for labeling chondroitin sulfate could not be discerned at this magnification. (**B**) A higher magnification view of the sclerotic intertubular dentin, showing the labeling patterns for type I collagen (solid arrows) and chondroitin sulphate (open arrows). Only a few nanoparticles specific for chondroitin sulfate could be visualized at this level of magnification. (**C**) Higher magnification view taken from the peritubular regions of sclerotic dentin after immunolabeling. Labeling for collagen fibrils (black arrows) was sparse in this region. A few clusters of 15-nm nanoparticles (open arrowheads) that were bound to the anti-chondroitin sulphate monoclonal antibodies could also be identified. (**D**) Another specimen showing sparse labeling for antigenically intact type I collagen. Labeling for proteoglycans could not be observed. (**E**) Very high magnification view of representative sclerotic intertubular dentin specimens after immunolabeling showing a specimen that exhibited moderately intense labeling for type I collagen and proteoglycans. Labeling of type I collagen was represented by the identification of larger (30-nm), discrete gold nanoparticles along the surface of the collagen fibrils (arrows). Labeling of proteoglycans was represented by smaller (15-nm) gold nanoparticles that appeared either as discrete particles or in clusters of 2 to 3 particles (pointers). Collagen banding was infrequently observed on the collagen fibrils in sclerotic dentin and, when present, appeared as very vague surface elevations (open arrowheads). The collagen fibrils appeared collapsed and swollen and exhibited extensive branching when compared with those observed in normal hard dentin (see G, H). (**F**) A collapsed and swollen collagen network from another specimen of sclerotic dentin that exhibited less intense immunolabeling of both type I collagen and proteoglycans. A banded collagen fibril could be seen in the foreground (open arrowheads; no open arrowheads in F). (**G, H**) FEI-SEM micrographs of the collagen fibrillar network in normal mineralized dentin after immunolabeling. Collagen fibrils appeared unmodified, with surface cross-banding features (arrow) and gold nanoparticles (pointers) along the fibrils. Gold nanoparticles specific for proteoglycans appeared as clusters of smaller electron-lucent particles around the collagen fibrils (open arrowheads). These clusters could be seen only at high magnification. Reprinted with permission from Suppa et al. (2006).

intrafibrillar mineral; Fig. 2B), the loss of telopeptides (seen as the loss of periodicity) may mean that intrafibrillar remineralization cannot occur, even though the total mineral loss is still relatively low (Tjäderhane et al. 2013a).

As at least MMP-2 and -9 and cysteine cathepsin K have telopeptidase activity (ability to cleave off the C-terminal end of the collagen molecule), dentinal

MMPs and cysteine cathepsins are considered responsible for the release of telopeptide fragments called ICTP (carboxyterminal telopeptides of type I collagen released by MMPs; Fig. 2A) and CTX (carboxyterminal telopeptides released by cathepsins; Fig. 2A). ICTP and CTX release has been used to demonstrate degradation of demineralized dentin in several in vitro studies (Tezvergil-Mutluay et al. 2013). It is therefore possible that dentinal proteolytic enzymes can actually cause small but important modifications in carious dentin matrix early in demineralization, which may affect the repair of the tissue even if demineralization can be stopped (Tjäderhane et al. 2013b).

Role of MMPs in HL Degradation

During restorative procedures, acid-etching prior to or concomitant with the application of a primer/adhesive system is performed to allow resin infiltration into the dentin. Acid solutions or acidic monomers remove or interact with the mineral content depending on the selected bonding strategy (i.e., etch-and-rinse or self-etch, respectively), and the exposed dentin matrix is impregnated with adhesive monomers forming the so-called hybrid layer (HL). Therefore, the stability of the synthetic resin polymers and collagen biopolymers is crucial for the longevity of the adhesive-dentin interface, because degradation of either can weaken adhesion and lead to gaps between teeth and restoratives (Breschi et al. 2008).

In vivo and in vitro studies revealed that the HLs created by dentin bonding systems are unstable in aqueous environments because of the hydrolytic degradation phenomena of both resins (Wang et al. 2003; Breschi et al. 2008; Tjäderhane et al. 2013b) and collagen fibrils that disappear over time (Carrilho et al. 2007; Breschi, Martin, et al. 2010; Breschi, Mazzoni, et al. 2010). It has been reported that the endogenous MMPs bound to the dentin organic matrix can potentially degrade the exposed collagen fibrils within the HL if unprotected by adhesive monomers. Since MMPs are claimed to be involved during progression of caries lesions in the breakdown of the dentin matrix, it is rational to consider that these enzymes are directly involved in the disintegration of the collagen fibrils within the HL created by contemporary etch-and-rinse and self-etch adhesives (Carrilho et al. 2007; Mazzoni, Scaffa, et al. 2013; Tjäderhane et al. 2013b).

Evidence of collagenolytic and gelatinolytic activities in partially demineralized dentin treated with either etch-and-rinse or self-etch adhesives initially supported the potential involvement of these proteases in the disruption of incompletely resin-infiltrated collagen fibrils within HLs (Mazzoni et al. 2006; Nishitani et al. 2006). These results were more recently confirmed with specific assays of MMP-2 and MMP-9 in dentin matrices treated with both etch-and-rinse and self-etch adhesives (Mazzoni, Carrilho, et al. 2011; Mazzoni, Scaffa, et al. 2013). In addition, a self-etch adhesive has been shown to increase MMP-2 synthesis in human odontoblasts (Lehmann et al. 2009), possibly increasing MMP-2 penetration into the HL via dentinal fluid. MMPs can be activated in low-pH environments (Tjäderhane et al. 1998; Sulkala et al. 2001) by inducing the cysteine switch (Chaussain-Miller et al. 2006). The acidic resin monomers contained either in etch-and-rinse or self-etch adhesives may, in fact, activate latent forms of MMPs (pro-MMPs) via the cysteine-switch mechanism that exposes the catalytic domains of these enzymes that were blocked by propeptides (Mazzoni, Scaffa, et al. 2013).

To further clarify this issue, our group recently developed an in situ zymography technique to provide direct evidence of the activity of endogenous dentin MMPs within resin-bonded HLs (Fig. 5A, B; Mazzoni et al. 2012; Mazzoni et al. 2014). Although previous studies demonstrated the presence and activity of endogenous proteolytic enzymes in human dentin, evidence of the activity of endogenous dentin MMPs within resin-bonded HLs was lacking. This analysis showed active host-derived gelatinases localized within the HL after bonding treatments (Mazzoni et al. 2012). The enzyme activity was located at the bottom of the HL, and this correlates well with the layer of demineralized, uninfiltrated collagen previously detected with a highly sensitive immunogold labeling technique (Breschi et al. 2004) and by confocal microscopy in adhesive interfaces created by simplified etch-and-rinse adhesives. Interestingly, the areas of exposed collagen also seem to correlate well with the morphological characteristic of nanoporosities of the HL defined as interfacial silver nanoleakage expression. These are initial areas of degradation of the HL over time.

In addition to acidic activation induced by etch-and-rinse and self-etch adhesives, an adjunctive contribution to the increase in MMP-2 and -9 protein levels and activity in adhesive-treated dentin could be related to the loss of inhibiting activity of TIMPs. TIMPs are endogenous inhibitors that bind MMPs, and the balance between them is crucial for maintaining the inhibitory activity of healthy tissues. Mildly acidic resin monomers can, in fact, activate MMPs by inhibiting tissue inhibitor of metalloproteinases-1 (TIMP-1; Ishiguro et al. 1994) in TIMP-MMP complexes, thereby producing active MMPs (Tjäderhane et al. 1998; Sulkala et al. 2001).

The low-pH environment induced by adhesives also provides excellent conditions for the activation of dentin cysteine cathepsins (Tersariol et al. 2010). Previous studies also supported a positive correlation between the proteolytic activities of MMPs and cysteine cathepsins (Cox et al. 2006). For these reasons, during the application of acidic monomers, not only MMPs but also cysteine cathepsins are activated and might be involved in the degradation of HL over time (Tersariol et al. 2010; Tjäderhane et al. 2013b).

According to these observations, preservation of collagen matrix integrity by inhibition of endogenous dentin

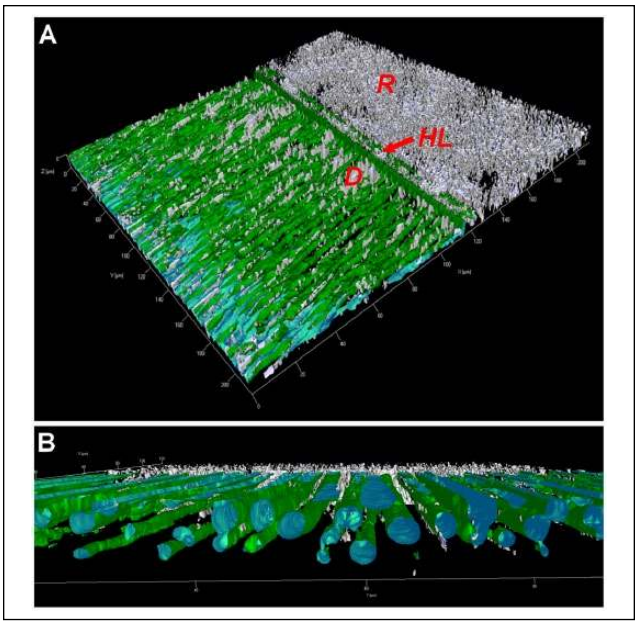

Figure 5. In situ zymogramic views of dentin, hybrid, and adhesive layer showing the endogenous enzymatic activity. (**A**) Tridimensional model of the acquired image in the green channel of the multiphoton confocal microscope superposed on images obtained with differential interference contrast showing intense fluorescence, produced by gelatin hydrolysis and expression of MMP activity, throughout the entire extension of the hybrid layer created with Scotchbond 1 XT (3M ESPE). (**B**) Higher magnification image model showing the gelatinolytic activity inside dentinal tubules visible as cylindrical tubes in deep dentin. The high tubule density is related to a very deep dentin portion. R = resin composite; HL = hybrid layer; D = dentin. Reprinted with permission from Mazzoni et al. (2012).

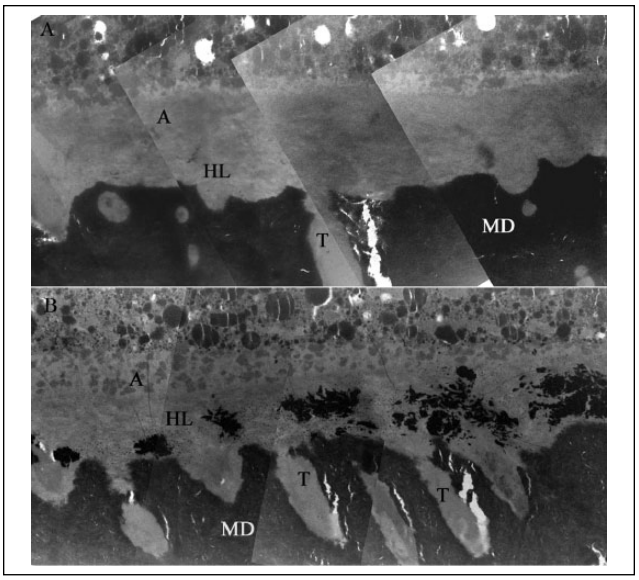

Figure 6. Interfacial nanoleakage expression of a hybrid layer created with or without the use of CHX as additional therapeutic primer. (**A**) Transmission electron microscopy (TEM) image obtained by combining numerous micrographs of a representative specimen treated with 0.2% chlorhexidine for 30 s, then bonded with Scotchbond 1 XT (3M ESPE) and stored for 2 y in artificial saliva at 37 °C. The adhesive (A) interface revealed only very few scattered particles of silver nanoleakage within the hybrid layers (HLs). MD = mineralized dentin; T = dentinal tubules; A = filled adhesive. Bar = 2 µm. (**B**) TEM image obtained combining numerous micrographs of a representative control specimen bonded with Scotchbond 1 XT and stored for 2 y in artificial saliva at 37 °C. This control adhesive interface reveals extensive interfacial silver nanoleakage due to individual silver grains and large clusters of silver deposits within the collagen fibrils of the HL. The presence of silver deposits reveals water-rich regions where collagen fibrils were hydrolyzed and replaced by water. Reprinted with permission from Breschi, Mazzoni, et al. (2010).

proteases is crucial to improve dentin-bonding durability and create stable and long-lasting adhesive restoration. The most common cause of replacement of the resin composite restorations is, in fact, secondary caries. Hence, novel treatment strategies to reinforce dentin structure have been recently suggested, including MMP inhibitors.

Therapeutic MMP Inhibitors in Caries Progression and Bond Stability

Lately, a growing interest in dental research has been focused on screening MMP inhibitors from different sources and how they might promote dental caries prevention and remineralization (Chaussain-Miller et al. 2006; Liu et al. 2011; Chaussain et al. 2013). In addition, several strategies to retain the integrity of the HL and improve the long-term dentin bond strength have been proposed (Liu et al. 2011; Tjäderhane et al. 2013b; Bedran-Russo et al. 2014).

MMP activity in dentin matrices can be reduced by endogenous and exogenous inhibitors. Endogenous inhibitors originate from different human cells, while exogenous inhibitors are synthesized as therapeutic agents. Most of these inhibitors chelate calcium or replace the zinc ions at the active site and/or interact with the MMP propeptide fragment, while others may prevent MMP access and inhibit activity by coating the substrate (Mazzoni et al. 2012; Tjäderhane et al. 2013b).

It has been previously shown in animal experiments that MMP inhibition can reduce dentinal caries progression (Tjäderhane et al. 1998; Sulkala et al. 2001). Chemically modified tetracycline-3 (CMT-3) and zoledronate (a bisphosphonate with MMP-inhibiting activity) reduced rat molar dentinal caries by 60% to 87% (Tjäderhane et al. 1999; Sulkala et al. 2001). CMT-5, a tetracycline analogue with a very low MMP-inhibitory effect compared with CMT-3, had practically no effect of rat molar caries (Tjäderhane et al. 1999; Sulkala et al. 2001). CMT-3 also eliminates the human salivary gelatinase activity (Sulkala et al. 2001), and systemic doxycycline medication has a

similar effect on salivary collagenase activity (Lauhio et al. 1995). These data further support the importance of MMPs in the development and progression of dentin caries.

Similarly, indirect proof of the role of MMPs in the stability of the HL over time has been reported by several studies, showing increased bond strength and reduced interfacial degradation over time if MMP inhibitors were used during the bonding procedure (Tjäderhane et al. 2013b). Chlorhexidine (CHX; Carrilho et al. 2007; Breschi, Mazzoni, et al. 2010), tetracycline, galardin (Breschi, Martin, et al. 2010), benzalkonium chloride (Tezvergil-Mutluay, Mutluay, et al. 2011), and quaternary ammonium methacrylates (Tezvergil-Mutluay, Agee, et al. 2011) are just some of the tested MMP inhibitors showing positive effects on bond strength stability. Indeed, the most tested inhibitor is CHX, which effectively reduces the activity of MMP-2, -9, and -8 (Breschi, Mazzoni, et al. 2010) and cysteine cathepsins (Scaffa et al. 2012). Even at concentrations as low as 0.2%, CHX showed bond strength preservation and reduced interfacial degradation (i.e., reduced nanoleakage expression; Fig. 6; Breschi, Mazzoni, et al. 2010). CHX inhibition of proteases may be related to its cation chelating property, and calcium ions released by adhesive primers may be responsible for the loss of inhibition by CHX over time. Because of this limitation (leaching and recharge of ions), collagen cross-linker agents have recently been proposed as a more permanent way of inactivating protease enzymes (Bedran-Russo et al. 2014).

Cross-linking reagents, or cross-linkers, are molecules that contain 1 or more reactive terminations capable of chemically attaching to specific functional groups (primary amines, sulfhydryls, etc.) on proteins or other molecules. The potential of cross-linkers is related to the possibility of improving the mechanical strength of the collagen network (Bedran-Russo et al. 2014), improving the resistance to enzymatic degradation, and inactivating exposed MMPs bound to matrix collagen (Liu et al. 2011; Tjäderhane et al. 2013b). Together, this leads to a stable dentin matrix network, which ultimately could determine the formation of a stable HL (Cova et al. 2011; Mazzoni, Angeloni, et al. 2013; Tjäderhane et al. 2013b; Bedran-Russo et al. 2014; Mazzoni et al. 2014).

In conclusion, because of the involvement of MMPs in caries progression and bond stability, enzyme inhibitors may play a crucial role in innovative therapeutic and preventive protocols. However, further studies are needed on new chemicals capable of inhibiting MMP activity and effectively contributing to caries prevention and improved stability of the adhesive interface.

Author Contributions

A. Mazzoni, L. Tjäderhane, L. Breschi, contributed to conception and design, drafted the manuscript; V. Checchi, contributed to conception, drafted the manuscript; R. Di Lenarda, T. Salo, contributed to data acquisition and analysis, critically revised the manuscript; F.R. Tay, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; D.H. Pashley, contributed to data acquisition, analysis, and interpretation, drafted the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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