Matrix Metalloproteinases and Other Matrix Proteinases in Relation to Cariology: The Era of ‘Dentin Degradomics’

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Key Words

Caries · Cathepsin · Collagen · Dentin · Enzyme · Erosion · Hybrid layer · Matrix metalloproteinase

Abstract

Dentin organic matrix, with type I collagen as the main component, is exposed after demineralization in dental caries, erosion or acidic conditioning during adhesive composite restorative treatment. This exposed matrix is prone to slow hydrolytic degradation by host collagenolytic enzymes, matrix metalloproteinases (MMPs) and cysteine cathepsins. Here we review the recent findings demonstrating that inhibition of salivary or dentin endogenous collagenolytic enzymes may provide preventive means against progression of caries or erosion, just as they have been shown to retain the integrity and improve the longevity of resin composite filling bonding to dentin. This paper also presents the case that the organic matrix in caries-affected dentin may not be preserved as intact as previously considered. In partially demineralized dentin, MMPs and cysteine cathepsins with the ability to cleave off the terminal non-helical ends of collagen molecules (telopeptides) may lead to the gradual loss of intramolecular gap areas. This would seriously compromise the matrix ability for intrafibrillar remineralization, which is

considered essential in restoring the dentin's mechanical properties. More detailed data of the enzymes responsible and their detailed function in dentin-destructive conditions may not only help to find new and better preventive means, but better preservation of demineralized dentin collagenous matrix may also facilitate true biological remineralization for the better restoration of tooth structural and mechanical integrity and mechanical properties.

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Dental caries, erosion and creation of the hybrid layer for the bonding of composite restorations all have two things in common. In all of them dentin is demineralized, revealing the collagenous organic matrix, and the degradation of this matrix is believed to be detrimental to the tissue if not properly protected. The majority of research in all these conditions has focused on demineralization. In caries and erosion, demineralization has been almost held as a synonym for disease progression and severity. In adhesive dentistry, partial or complete demineralization is a prerequisite for successful bonding with contemporary adhesives. Infiltration of dentin collagen with adhesive monomers creates the collagen-adhesive hybrid layer responsible for the composite-dentin bond. The role

of enzymatic loss of collagen component of the hybrid layer by dentinal enzymes, at least partially responsible for the time-related loss of bond strength, has been recognized and is under active research [Tjäderhane et al., 2013a, b]. Recently the role of the dentinal matrix also in caries and erosion has gained more attention both in the pathological processes and as a means to improve preventive measures.

Degradomics is an application of genomic and proteomic approaches to identify the protease and protease substrate repertoires [López-Otín and Overall, 2002] both in physiological and pathological conditions. The determination of protease activity in clinical specimens can offer therapeutic options in different diseases [Findelsen and Neumaier, 2012]. 'Dentin degradomics' refers to the field of research aiming to identify the enzymes and their specific roles, substrates and end products in dentin-pulp complex physiology and pathologies. The aim of this paper, based on presentations given at the European Organisation for Caries Research (ORCA) Saturday Afternoon Symposium in Liverpool, UK in July 2013, is (1) to provide an overview of the current knowledge of the dentin matrix role and fate in dentin pathologies, and (2) to discuss how the dentin degradomic approach could help to understand and manage these conditions.

Dentin and Dentinal Proteolytic Enzymes

Dentin is composed of approximately 70 weight percent/50 volume percent minerals (biological hydroxyapatite) and the organic matrix (about 20 weight percent/30 volume percent), the remaining fraction being water. Structurally, dentin can be described as a nanocrystalline reinforced composite, while enamel would be a dense ceramic with impurities [Tjäderhane et al., 2012]. About 90% of the dentin organic matrix is collagen, consisting almost exclusively of type I collagen. The remaining 10% of dentin organic matrix consists of non-collagenous proteins (NCPs) and lipids (<2%). NCPs are a heterogeneous group of proteoglycans, phosphoproteins, glycoproteins, serum proteins, enzymes and growth factors. While several members of the NCPs may have significant impact on dentin-pulp complex physiology and pathologies (for example, the role of growth factors liberated from dentin affecting the reactionary or reparative dentinogenesis), with the exception of the enzymes and some of their substrates in mineralized dentin, their functions are mainly beyond the focus of this article. For detailed overviews, the readers are referred to the recent review articles [Maz-

zoni et al., 2012a; Orsini et al., 2012; Tjäderhane and Haapasalo, 2012; Chaussain et al., 2013].

Since dentin is the most abundant mineralized tissue in tooth, its mechanical performance is considered to have major significance to the overall function of teeth [Bertassoni et al., 2009]. Dentin collagen is mineralized in mineralization front after the organization of collagenous matrix in predentin [Tjäderhane and Haapasalo, 2012]. Proteoglycans are believed to concentrate in the collagen gap zone, with the protein core surrounding the collagen fibril, and glycosaminoglycan chains penetrating inside the fibrils. Inside, calcium ions related to the glycosaminoglycan chains would start the crystal nucleation [Dechihi et al., 2007]. Extrafibrillar mineralization in the spaces separating the collagen fibrils follows the intrafibrillar mineralization. Intrafibrillar mineral has the major role in dentin mechanical properties [Kinney et al., 2003; Balooch et al., 2008; Bertassoni et al., 2009, 2011]. Dentinogenesis imperfecta type II (DI-II) dentin lacks intrafibrillar mineral, with approximately 33% less mineral than normal dentin [Kinney et al., 2001, 2003]. However, the relative reductions in elastic modulus (74%) and hardness (76%) of DI-II dentin compared to normal dentin indicate the importance of intrafibrillar mineral, which stiffens the collagen fibrils, to the dentin mechanical (elastic) behavior under load [Kinney et al., 2003].

Dentinal Enzymes Involved in Dentin Matrix Degradation

Matrix metalloproteinases (MMPs) are a family of Zn^{2+} - and Ca^{2+} -dependent enzymes, which are important in many biological and pathological processes because collectively they are able to degrade practically all extracellular matrix components. In humans, the 23 members of the MMP family are frequently divided into six groups – collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs – based on substrate specificity and homology. Even though this classification is practical, it underestimates the complexity of MMP functions and biological activities, as most MMPs can – at least in vitro – degrade several substrates with variable specificities. For example, collagenase-1 and collagenase-3 (MMP-1 and MMP-13) also degrade gelatin at a slow rate, and gelatinases MMP-2 and MMP-9 degrade several types of collagen with telopeptidase activity against type I collagen. Therefore, more recently MMPs have often been classified according to their molecular structure [Mazzoni et al., 2012a; Tjäderhane et al., 2013a]. MMPs are synthesized and mostly secreted as inactive proenzymes (zymogens), in which the prodomain pre-

Fig. 1. General structure of MMPs. The cysteine residue (C) of the MMP prodomain co-ordinates with the catalytic domain Zn^{2+} ion, forming a so-called ‘cysteine switch’ and hiding the functional site of the enzyme. When the prodomain is removed, the catalytic site is exposed and the enzyme is activated. The hemopexin domain ‘propeller’ is present in most MMPs and attached to the catalytic domain by a flexible hinge domain. The hemopexin domain contributes to substrate recognition, enzyme activation and protease localization. The ovals within the catalytic domain represent fibronectin type II repeats present in gelatinases MMP-2 and MMP-9. They are responsible for the gelatin substrate recognition and binding, providing high affinity of gelatinases against gelatin.

vents the functional activity of the catalytic domain. Activation occurs when the prodomain bridge with the catalytic zinc (the so-called ‘cysteine switch’) is disrupted by prodomain cleavage by other MMPs, cysteine cathepsins or other proteinases, or chemically, e.g. by pH changes (fig. 1).

There are 11 human cysteine cathepsins, the cathepsins B, C, F, H, K, L, O, S, V, X and W. The majority of cathepsins are ubiquitously expressed in human tissues, but cathepsins K, W and S show more restricted cell- or tissue-specific distribution. Cysteine cathepsins participate intracellularly in diverse processes, such as normal protein turnover, pro-protein processing and apoptosis. Extracellularly, they can contribute directly to the degradation of the extracellular matrix, and participate in proteolytic cascades that amplify the degradative capacity. Unlike MMPs, which have the optimum functional activity in a neutral environment, cysteine cathepsins have optimal activity in a slightly acidic pH, generally between 5 and 6. However, pH optimum may depend on the substrate: for example, cathepsin K degrades collagen most efficiently at a pH between 5 and 6, but gelatin still at a neutral pH. Cathepsin B endopeptidase activity has a pH optimum around 7.4. Cysteine cathepsins are usually considered to be unstable or denatured in neutral and alkaline pH, but this may also be condition-dependent: for example, cathepsin K stability in neutral pH is greatly in-

creased by chondroitin-4-sulphate [Dickinson, 2002; Mazzoni et al., 2012a; Turk et al., 2012; Tjäderhane et al., 2013a].

Cathepsin K is the major protease responsible for bone resorption and remodeling, and cathepsin K can degrade collagen at various sites inside the molecule’s helical region. This property is similar to bacterial collagenases and unique among mammalian proteinases, as all collagenolytic MMPs cleave the collagen molecule helical part at one distinct site. Several cathepsins can degrade gelatin, and at least cathepsins K, B and L cleave collagen in the non-helical telopeptide extensions. In tissues, glycosaminoglycans form complexes with cathepsin and may affect their function in a highly complex manner [Mazzoni et al., 2012a; Turk et al., 2012; Tjäderhane et al., 2013a].

To date, intact human dentin matrix has been shown to contain MMP-8 (collagenase 2) [Sulkala et al., 2007], MMP-2 and MMP-9 (gelatinases) [Martin-De Las Heras et al., 2000; Mazzoni et al., 2007; Boushell et al., 2008; Mazzoni et al., 2009; Santos et al., 2009; Vidal et al., 2014], MMP-3 (stromelysin-1) [Boukpepsi et al., 2008; Mazzoni et al., 2011], MMP-20 [Shimada et al., 2009], cysteine cathepsin B [Tersariol et al., 2010; Vidal et al., 2014] and cysteine cathepsin K [Vidal et al., 2014]. In addition to abundant gelatinolytic enzymes in dentinal tubules [Mazzoni et al., 2012b; Pessoa et al., 2013], at least MMP-20 is present in dentinal fluid [Sulkala et al., 2002]. Tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 and MMP inhibitors α 2-macroglobulin and fetuin-A are also present in marked quantities in dentin [Mazzoni et al., 2012a; Tjäderhane et al., 2013a].

The physiological roles of collagenolytic enzymes in dentin are not well known. They may participate e.g. in peritubular and tertiary dentin formation, and in the release of dentinal growth factors which in turn would regulate defensive reactions in the pulp [Tjäderhane et al., 2002; Hannas et al., 2007; Charadram et al., 2012; Mazzoni et al., 2012a; Muromachi et al., 2012; Chaussain et al., 2013; Tjäderhane et al., 2013a]. They are, however, capable and sufficient to degrade demineralized dentin in vitro [Carrilho et al., 2009; Tezvergil-Mutluay et al., 2010, 2013; Toledano et al., 2013].

Host Proteases in Caries

Already in 1983, Dayan et al. [1983] demonstrated that intact, but especially carious human dentin had collagenolytic activity against synthetic peptide. This finding was mostly ignored, possibly because carious dentin was be-

Fig. 2. Correlation between the age of patients and MMP (a) and cathepsin (b) activities in carious and intact dentin. The original values of intact dentin were multiplied by 10 to fit the curves into same graphs with carious dentin. For both MMP and cathepsin activities, the correlations between age and activities are highly sig-

nificant ($p < 0.001$ in all cases; Pearson correlation test), and the activities in carious dentin are significantly higher ($p < 0.01$ for cathepsins and $p < 0.001$ for MMPs, t test). Data adapted from Tersariol et al. [2010] and Nascimento et al. [2011].

lieved to contain bacterial collagenases, and because the knowledge of MMPs was still very limited. Only 15 years later, mature human odontoblasts were shown to express MMP-2 and MMP-9 [Tjäderhane et al., 1998b], MMP-2, MMP-9 and MMP-8 were identified in carious dentin, and the role of MMPs in the degradation of dentin collagen was demonstrated [Tjäderhane et al., 1998a]. At the same time, the activation of latent salivary pro-MMPs by exposure to low pH followed by neutralization (so-called acid activation) was also shown [Tjäderhane et al., 1998a; Sulkala et al., 2001]. The same phenomenon was later demonstrated with dentinal MMPs [Mazzoni et al., 2006; Nishitani et al., 2006]. The acid activation occurs in pH changes relevant to caries, indicating the role of bacterial acids in the process.

Other studies have confirmed the presence of these MMPs in caries-affected dentin [van Strijp et al., 2003; Boushell et al., 2008; Shimada et al., 2009; Toledano et al., 2010; Charadram et al., 2012]. The cysteine cathepsins were also identified in carious dentin [Nascimento et al., 2011; Vidal et al., 2014]. Recent studies have also indicated MMPs among susceptibility genes for dental caries [Tannure et al., 2012a, b; Wang et al., 2013].

Based on current knowledge, it is not possible to determine with certainty the exact role and importance of salivary, dentinal or pulp-derived (via dentinal fluid) en-

zymes in dentinal caries lesions. Saliva contains several MMPs, which have been experimentally shown to efficiently degrade exposed dentinal collagen matrix [Tjäderhane et al., 1998a]. MMP-9 is perhaps the most abundant salivary MMP and predominant in dentin caries lesions [Tjäderhane et al., 1998a]. High relative presence of MMP-8 and MMP-9 in outer caries layer compared to inner (caries-affected) layer [Shimada et al., 2009] indicates saliva as a source of these enzymes, supported by the strong correlation of salivary MMP-8 with caries [Hedenbjörk-Lager et al., 2015]. Saliva also contains cysteine cathepsins [Nascimento et al., 2011], at least cathepsin B [van Strijp et al., 2003]. Interestingly, significantly higher levels of the cathepsin inhibitors cystatin and lipocalin-1 were found in saliva and pellicle of caries-free subjects than those with high DMFT [Vitorino et al., 2006].

Decreasing intensity in MMP-2 staining from the pulp towards the enamel [Niu et al., 2011] and decreasing detection and activities of collagenolytic enzymes with age [Martin-de Las Heras et al., 2000; Tersariol et al., 2010] have raised the question of the true importance of these enzymes in caries. It has to be emphasized that even though this negative correlation can be seen in both intact and carious dentin, the activities are markedly (3- to 11-fold) higher in carious tissue (fig. 2), which may indicate the role of dentinal fluid as a source of the enzymes. This

is supported by a recent finding with intensively increased detection of cathepsin K and cathepsin B as well as MMP-2 and MMP-9 in caries-affected dentin close to the dentin-pulp border [Vidal et al., 2014]. Extremely intense gelatinolytic activity in mantle dentin [Pessoa et al., 2013] presumably due to MMP-2 [Boushell et al., 2008; Tole-dano et al., 2010; Boushell et al., 2011; Niu et al., 2011] may indicate that MMP-2 is involved at least in the early lateral spreading of caries under the dentin-enamel junction [Hietala et al., 1993; Tjäderhane et al., 1995]. Inten-sive gelatinolytic activity has been observed in dentinal tubules [Mazzoni et al., 2012b; Pessoa et al., 2013]. In ad-dition, TGF- β (indicated to regulate dentin-pulp com-plex response to caries [Cooper et al., 2011; Charadram et al., 2012] and identified as a caries susceptibility gene [Wang et al., 2013]) increases MMP-9 synthesis in ma-ture human odontoblasts in vitro [Tjäderhane et al., 1998b], and MMP-2 [Boushell et al., 2011], MMP-9 [Zehnder et al., 2011] and MMP-20 [Sulkala et al., 2002] increase in dentinal tubules of carious teeth. MMP-2 and TIMP-2 expressions are also significantly up-regulated in odontoblasts under the carious lesion [Charadram et al., 2012]. The granular appearance of gelatinases close to the dentin-pulp border but not in the vicinity of the dentin-enamel junction [Pessoa et al., 2013] may indicate that MMPs are packed into granules or matrix vesicles for transportation in tubules. Matrix vesicles, known to be important in biomineralization, contain MMP-2, MMP-3 and MMP-13 [Golub, 2009]. And finally, a significant increase in the cathepsin activity with increasing lesion depth [Nascimento et al., 2011] may be caused by the in-flux of odontoblast- or pulp-derived enzymes via dentin-al tubules. Dentinal fluid as a continuous source of col-lagenolytic enzymes indicates that age- or dentin depth-related differences in the presence of enzyme in intertubular matrix [Tersariol et al., 2010; Niu et al., 2011] may not be that significant in respect to the caries process.

There are numerous natural and synthetic MMP in-hibitors that have been examined or proposed to be useful to eliminate or reduce dentin matrix degradation [Tjä-derhane et al., 2013b]. Tetracycline and its derivatives have long been known to inhibit MMPs, and they have been extensively studied especially in the treatment of peri-odontitis [Sorsa et al., 2006; Hannas et al., 2007]. Animal experiments have indicated that MMP inhibition by in-traoral administration of chemical MMP inhibitors could reduce caries progression in dentin [Tjäderhane et al., 1999; Sulkala et al., 2001]. Chemically modified tetracy-cline-3 (CMT-3) (tetracycline analogue without antimi-crobial effect [Sorsa et al., 2006]) can reduce the progres-

sion of rat molar dentinal caries by 60–87% [Tjäderhane et al., 1999; Sulkala et al., 2001]. Zoledronate (a bisphos-phonate with MMP-inhibiting activity) was equally effec-tive [Sulkala et al., 2001], while with CMT-5 (another tet-racycline analogue lacking most of the MMP-inhibitory effect of CMT-3) the effect was markedly lower or absent [Tjäderhane et al., 1999; Sulkala et al., 2001]. CMT-3 was also shown to effectively eliminate human salivary gela-tinase activity [Sulkala et al., 2001], and systemic doxycy-cline medication has a similar effect on salivary collage-nase activity [Lauhio et al., 1995]. Several synthetic MMP inhibitors are based on their zinc/calcium-chelating groups, since MMPs require metal ions for their catalytic activity [Gendron et al., 1999]. For example, ethylenedi-aminetetraacetic acid (EDTA) inhibits dentin endoge-nous MMPs [Thompson et al., 2012]. Potent MMP-in-hibiting activity of chlorhexidine (CHX) involves a calci-um-chelating mechanism [Gendron et al., 1999], even if inhibition of cysteine cathepsins may depend on molecu-lar docking to the enzyme active site [Scaffa et al., 2012].

Experimental data demonstrating the reduction of dentin collagen matrix degradation with different MMP inhibitors are overwhelming [for reviews, see Tjäderhane et al., 2013a, b]. However, practically all of these experi-ments have used simple dentin demineralization proto-cols with sound dentin. As discussed below in detail, nat-ural caries lesions develop over extended time with nu-merous pH changes, de- and remineralization cycles and slowly progressing loss of minerals. The changes caused by enzymatic activities in the dentin organic matrix may be slow and gradual. There is an urgent need for experi-mental in vitro models that would mimic natural progres-sion of dentinal lesions, including the time factor, to study the effect of collagenolytic enzymes and their inhibition in caries.

Fate of Dentin Collagen during Caries

Demineralization – Role of Host Enzymes

During initial demineralization the extrafibrillar min-eral is more rapidly dissolved [Balooch et al., 2008], and remaining intrafibrillar mineral crystallites should act as nucleation sites for remineralization [Bertassoni et al., 2011]. However, experimental studies with rapid de-rem-ineralization cycles do not allow potential time-related changes in the dentin organic matrix that might occur in natural lesions. As pointed out by Ito et al. [2005], den-tinal carious lesions develop over months and even years, after thousands of episodes of demineralization, neutral-ization and remineralization. Quantitative characteriza-tions have shown that carious lesions are far from identi-

Fig. 3. Analysis of dentin organic matrix components in sound and carious dentin. **a** Comparison of type I collagen and glycosaminoglycan side chains of proteoglycans in sound and caries-affected dentin using monoclonal antibodies and immunogold labeling. * Significantly different from sound dentin ($p < 0.05$). Data from Suppa et al. [2006]. **b** Correlation between collagen periodicity and mineral content in sound, caries-affected and caries-infected dentin. The correlation is highly significant ($p < 0.001$, Pearson correlation test). Data adapted from Deyhle et al. [2011]. **c, d** Collagen periodicity values (**c**) and mineral content (**d**) in sound, caries-affected and caries-infected dentin. ** $p < 0.01$; *** $p < 0.001$. Data adapted from Deyhle et al. [2011]. SAXS = Small-angle X-ray scattering.

cal [Marshall et al., 2001; Ito et al., 2005]. Attempts to create 'natural' caries lesions with caries-infected and -affected dentin by demineralizing normal dentin in vitro have not been very successful.

There is a convincing amount of evidence that the recovery of dentin mechanical properties depends not only on total mineral reformed, but also on the mineral location and interaction with the matrix. In DI-II dentin the absence of intrafibrillar mineral (approximately 33% of total mineral) reduces elastic modulus and hardness by about 75% [Kinney et al., 2003]. Attempts to remineralize demineralized dentin should thus provide for crystallization in the gap zones [Balooch et al., 2008; Bertassoni et al., 2011]. Even though direct comparison cannot be done, it is interesting to note that caries-affected (sclerotic) dentin has similar relative reduction in stiffness (79%) and increase in shrinkage after drying (76%) compared to normal dentin [Ito et al., 2005].

Dentin collagen matrix in caries-affected dentin has usually been considered to remain mostly intact, retaining its ability to remineralize even when up to half of the mineral content has been lost. However, this assumption

has been largely based on limited data with descriptive findings, such as transmission electron microscopy demonstration of occasional banded collagen fibrils in caries-affected dentin [Ohgushi and Fusayama, 1975] or combined scanning electron microscopy and microradiographic analysis [Arends et al., 1989]. The analyses of collagen-associated amino acids and cross-links between caries-infected, caries-affected and normal dentin have provided conflicting results [Kuboki et al., 1977; Nakornchai et al., 2004].

Contrary to earlier findings, Suppa et al. [2006] demonstrated significantly reduced detection of intact type I collagen and proteoglycans in caries-affected compared to normal dentin (fig. 3a). The authors raise concern whether the intrafibrillar remineralization of caries-affected dentin would be possible. More recently, Deyhle et al. [2011] used spatially resolved small-angle X-ray scattering, detecting both collagen fibril periodicity and mineralization level in caries-infected, caries-affected and sound dentin. Even though the authors concluded that the collagen network is conserved in abundance and orientation even after relatively high (up to 30%) loss of min-

Fig. 4. Basic structure of type I collagen. **a** Intact type I collagen molecule (tropocollagen) with two $\alpha_1(I)$ chains and one $\alpha_2(I)$ chain. The non-helical terminal ends, telopeptides, are required for correct molecular registration and major cross-link formation to develop fibrillar structures. Cleavage of telopeptides with telopeptidase activity leaves behind the helical part of the molecule (atelocollagen). Loss of telopeptides or impaired cross-linking can significantly affect connective tissue structural integrity [Orgel et

al., 2000], as atelocollagen is less thermally stable and more prone to local unfolding and degradation by non-specific proteases. Loss of C-terminal telopeptide has also been shown to expose the MMP binding site and subsequently lead to degradation [Perumal et al., 2008]. **b** The periodicity of collagen is based on overlapping arrangement of individual collagen molecules. Collagen molecular packing allows for the formation of microfibrils, a gap region with four and an overlap region with five collagen molecular segments.

erals, the outcome may be more complicated. Re-evaluation of the data presented in that article demonstrates a highly significant correlation between the loss of mineral component and the loss of collagen periodicity (fig. 3b). The statistically significant loss of minerals already in caries-affected dentin is accompanied by a respective loss of collagen periodicity signal [Deyhle et al., 2011] (fig. 3c, d). Vidal et al. [2014] show that the autofluorescent signal emitted by the molecularly well-structured collagen in sound dentin is almost completely lost in caries-affected dentin. Thus, these data actually indicate that dentin collagen may be prone to structural changes already after mild demineralization in natural caries lesions.

In principle, the loss of collagen periodicity in caries-affected and caries-infected dentin may be attributable to the telopeptidase activity of endogenous or salivary MMPs and/or cysteine cathepsins. In collagen fibril, the five collagen molecules that make up the microfibril each have N- and C-terminal non-helical telopeptides (fig. 4a). In the overlap region, all five molecules overlap with each other, one of them with the C-terminal telopeptide: the gap region (where intrafibrillar mineral resides) is located between the N- and C-terminal telopeptides of adjacent molecules (fig. 4b). Cleavage of the C- and N-terminal telopeptide (containing the carboxy- and aminoterminal cross-links, respectively [Orgel et al., 2000]) results in the loss of terminal ends of the collagen molecule. The en-

zymes capable of cleaving telopeptides are collectively called telopeptidases, and in relation to dentin pathologies include at least MMP-2 and MMP-9 [Okada et al., 1995; Garnero et al., 1998, 2003; Osorio et al., 2011], cathepsin K [Garnero et al., 1998, 2003], pepsin [Walton et al., 2010] and trypsin [Mirigian et al., 2013]. Type I collagen telopeptide contains recognition sites for the gelatinase fibronectin-like domain [Steffensen et al., 1995], indicating that MMP-2 and MMP-9 telopeptidase activity may be totally separate from the gelatinolytic activity of these enzymes. It is interesting to note that neither pepsin nor trypsin have any marked cleavage activity against type I collagen helical parts [Walton et al., 2010; Mirigian et al., 2013]. Therefore, the role of gastric enzymes in the matrix degradation in erosion [Schlueter et al., 2010, 2012b] may relate to telopeptidase activity and activation of other collagenolytic enzymes [Mirigian et al., 2013], e.g. MMPs or cysteine cathepsins.

In mineralized dentin, the gap region contains intrafibrillar mineral crystals, and collagen periodicity is retained (fig. 5a, b). When dentin is experimentally demineralized e.g. with acids, the extrafibrillar mineral is removed faster [Kinney et al., 1995; Balooch et al., 2008] and the fibril periodicity is still retained [Balooch et al., 2008]. In natural caries, however, with slowly advancing demineralization and repeated demineralization-remineralization phases, removal of extrafibrillar mineral may

Fig. 5. Proposed effect of telopeptidase activity on dentin collagen structure during demineralization. **a, b** Fully mineralized dentin collagen has normal type I collagen periodicity (**a**) with intrafibrillar mineral crystals (approximately 33% of total dentin mineral) located in the gap regions between adjacent N- and C-terminal telopeptides, and larger extrafibrillar mineral crystals protecting the gap regions (**b**). The C-terminal telopeptide (red squares) contains the intermolecular cross-link (red lines) to the adjacent molecule's helical part (black squares). **c** Early demineralization occurs mainly in the interfibrillar space, possibly exposing some gap regions. Localized loss of intrafibrillar mineral enables local telopeptidase activity to cleave C-terminal telopeptide, resulting in minor loss of collagen periodicity and partial loss of intermolecular cross-links. **d** Advancing demineralization increases the rate of extra- and intrafibrillar demineralization, subsequently leading to loss of telopeptides with cross-links, and increases the loss of periodicity. Loss of intrafibrillar mineral and C-terminal telopeptide (increasing the width of the gap region) may decrease the ability for intrafibrillar remineralization.

expose intrafibrillar mineral, allowing its removal at least partially. At the same time, the pH changes can activate endogenous MMPs and cysteine cathepsins with telopeptidase activity (at least MMP-2, MMP-9 and cathepsin K), which may cleave the C-terminal telopeptide (fig. 5c). This would lead to the loss of the fifth molecular component of the overlap region, resulting in the local loss of collagen periodicity. When demineralization advances, more intrafibrillar mineral and C-terminal telopeptides are lost with the advancing loss of periodicity (fig. 5d).

It is also possible that the activation and telopeptidase activity occurs after the removal of extrafibrillar but before the loss of intrafibrillar mineral. This is at least indirectly supported by the increase in detection of proteases with telopeptidase activity (cathepsin K, MMP-2 and MMP-9) together with the loss of collagen detection in caries-affected dentin [Vidal et al., 2014]. The loss of C-terminal telopeptide would then cause the loss of intrafibrillar mineral from the widened gap region. This could explain the gradual loss of collagen periodicity and its correlation with mineral loss in caries-affected dentin [Deyhle et al., 2011]. Since cathepsins, but not MMPs, are able to cleave N-terminal telopeptide [Garnero et al., 1998; Kafienah et al., 1998] and MMP telopeptidase activity seems to be markedly (about 10-fold) higher than cathepsin-related activity in dentin [Tezvergil-Mutluer et al., 2013], the retention of N-terminal cross-links may prevent the complete disassembly of collagen fibrils at this stage. Only later, after gradual degradation of the helical part of the collagen molecule by endogenous or – in case of caries – salivary or bacterial collagenases, will the destruction of dentin collagen matrix be completed.

Suppa et al. [2006] demonstrated reduced proteoglycan antigenicity together with loss of collagen antigenicity in caries-affected dentin. MMP-3, present in dentin [Boukpepsi et al., 2008; Mazzoni et al., 2011] and known to degrade proteoglycans, has been shown to extract the proteoglycans decorin and biglycan and selectively also some phosphorylated proteins, whose presence might be important for remineralization [Boukpepsi et al., 2008; Khaddam et al., 2014]. Inhibition of external MMP-3 by grape seed extracts totally and by fluorides partially abolished the release of decorin, biglycan and dentin sialoprotein from human dentin [Khaddam et al., 2014]. As papain gel, also known to extract proteoglycans, decreases the mechanical properties of demineralized dentin collagen and might even affect the collagen fibril structure [Bertassoni and Marshall, 2009], MMP-3 might contribute to the loss of carious dentin matrix mechanical properties and reduce the ability for natural remineralization.

Host Proteases and Erosion

A sequence similar to that in caries [Tjäderhane et al., 1998a] occurs in erosive challenges. The pH fall and neutralization by salivary buffers leads to demineralization, exposing the organic matrix, and is at the same time expected to lead to the activation and functional activity of proteases. These active proteases can degrade the demineralized organic matrix (DOM), thus increasing the rate of erosion progression [Kleter et al., 1994; Ganss et al., 2001]. Since erosive demineralization does not involve bacteria, host-derived proteases (from either saliva or dentin) might be more involved in the degradation of the DOM in erosive lesions. Considering that cysteine cathepsins can degrade collagen at acidic pH while MMPs are functional at neutral environment and also that cysteine cathepsins can activate MMPs [Nagase, 1997], the interplay between these collagenolytic enzymes might govern the fate of the DOM during erosive demineralization. However, the relative contribution of each of these classes of collagenolytic enzymes for the progression of erosion is not known so far. The use of specific inhibitors for these distinct classes of enzymes might be a good tool to better understand their role in the progression of erosive lesions.

The role of collagenolytic enzymes for the progression of erosive demineralization is supported by clinical data showing higher activity of collagenase in saliva of bulimic volunteers with erosion when compared with controls [Schlueter et al., 2012a]. Thus, a logical approach that was recently proposed to reduce dentin erosive demineralization is the use of protease inhibitors to preserve the DOM (fig. 6), which has been shown to reduce further mineral loss [Buzalaf et al., 2012; Kato et al., 2012].

Among the different types of inhibitors of collagenolytic enzymes, CHX and epigallocatechin gallate (EGCG) have been evaluated as part of preventive strategies to reduce erosive dentin demineralization, using in situ protocols. CHX is able to inhibit both MMPs [Gendron et al., 1999] and cysteine cathepsins [Scaffa et al., 2012], while EGCG inhibits MMPs [Demeule et al., 2000] but not cysteine cathepsins [Katunuma et al., 2006]. In these studies, the inhibitors were included in rinse solutions [Kato et al., 2009; Magalhães et al., 2009] or gels for topical application [Kato et al., 2010]. When the inhibitors were included in rinse solutions, significant (around 30–40%) reduction of dentin loss was achieved when compared with control for the rinses performed for 1 min after each erosive challenge [Kato et al., 2009; Magalhães et al., 2009]. However, the need of rinsing immediately after each ero-

Fig. 6. Mechanism of action of protease inhibitors against dentin erosive demineralization. Spheres indicate inactive proteases and asterisks indicate active proteases. **a** Sound dentin. After erosive challenge, the dentin surface is demineralized, exposing the collagen fibrils (**b**). At the same time, salivary and dentin proteases are

activated (**c**) and degrade the DOM (**d**), allowing erosion to progress. When protease inhibitors are used (**b'**), the DOM is not degraded (**c'**) and prevents further demineralization during the subsequent erosive challenges (**d'**).

sive challenge is not practical for clinical application. To increase the contact of the protease inhibitors with dentin, they were included in gel formulations that were applied on the specimens for 1 min only once, before the first erosive challenge. This single 1-minute treatment was able to completely prevent dentin loss evaluated by profilometry [Kato et al., 2010]. Thus, it seems that the increased time of contact of the inhibitors with dentin by the use of gel formulations plays an important role for the preventive effect of the product. Further studies should evaluate for how long the protective effect of the gels lasts, in order to propose protocols for clinical use.

The above-mentioned in situ studies indicate that compounds classically known for their ability to inhibit collagenolytic enzymes are effective to prevent dentin erosive demineralization. However, in these studies, the response variable employed was profilometry that evaluates dentin loss (mineralized plus organic tissue), but not the degradation of the DOM itself. A recent in vitro proof-of-concept study showed that release of hydroxyproline in the incubation solution (artificial saliva containing bacterial collagenase from *Clostridium histolyticum*) was significantly reduced when the specimens previously demineralized with citric acid (0.87 M, pH 2.3, 36 h) were

treated with gels containing EGCG (400 μ M) or CHX (0.012%) for 1 min [Kato et al., 2012]. This study provides evidence that the protease inhibitors evaluated are able to reduce the DOM degradation.

An interesting finding of the study by Kato et al. [2012] was that treatment with a gel containing 1.23% NaF, included as a control, significantly reduced the DOM degradation. Due to this, it was hypothesized that besides its well-known action on the de-remineralization processes [Buzalaf et al., 2011], fluoride could also inhibit MMPs. This hypothesis was confirmed using gelatin zymography that revealed decreased activities of proactive and active forms of salivary and purified human MMPs by incubation with NaF in a dose-response manner [Kato et al., 2014]. These findings might help to explain why the effect of fluoride against dentin erosion depends on the maintenance of the DOM [Ganss et al., 2004] and provide new insights into the mechanism of action of fluoride for the prevention of both caries and erosion in dentin.

In summary, the use of protease inhibitors seems to emerge as a potential preventive tool against dentin erosive demineralization. However, the knowledge available so far comes from in vitro and in situ studies. Clinical trials are needed to confirm the relevance of this preventive

measure and to establish protocols for its clinical use. Moreover, studies unraveling the relative importance of distinct collagenolytic enzymes for the degradation of the DOM under conditions of erosive demineralization are necessary.

Host Proteases and Composite Resin Bonding

Restoration of caries lesions is increasingly accomplished by using methylmethacrylate-based resins. Resin-dentin bonding mechanisms, whether etch-and-rinse (ER) or self-etch (SE), are based on the formation of a hybrid layer that couples methylmethacrylate dental adhesives/resin composites to the underlying dentin. Except for the resin tags extending some micrometers into dentinal tubules, which usually comprise just a few percent of bonded surface areas, the only physical continuity between the hybrid layer and the underlying dentin (either sound or caries-affected) are the collagen fibrils.

Chemical amendments of adhesive formulations by the inclusion of more hydrophilic monomers and acidic resin monomers, combined with solvents, rendered the dental adhesives more compatible to interact with the naturally moist dentin surface, yielding significant improvements in the immediate bonding effectiveness of most current adhesive systems [Tay and Pashley, 2003]. However, the incorporation of hydrophilic and acidic resin monomers in contemporary ER and SE adhesives are claimed to bring potential problems to the long-term performance of final restorations. Several laboratory and *ex vivo/in vivo* studies have reported remarkable drops of resin-dentin bond strength after short- and long-term investigations [Sano et al., 1999; Hashimoto et al., 2000; De Munck et al., 2003; Armstrong et al., 2004; Hebling et al., 2005; Carrilho et al., 2007a, b; Abdalla, 2010; Garcia-Godoy et al., 2010; Hashimoto et al., 2010; Heintze et al., 2010]. Dramatic loss of effectiveness of some adhesives in terms of mechanical cohesion and/or dentinal sealing capacity has been also shown when non-caries cervical lesions were evaluated [Peumans et al., 2005; Heintze et al., 2010]. Failure of resin-adhesive restorations is often followed by secondary caries [Tyas, 2005; Sunnegårdh-Grönberg et al., 2009], further loss of tooth structure, increased rate of pulp destruction, and eventually need for endodontic, restorative and prosthetic treatments [Abbott, 2004; Hickel et al., 2005]. Depending on factors such as the size of the restoration, tooth location and the patient's general health/features, the life span of resin composite restorations is roughly half of that reported for

those with similar features but made of amalgam [Mjör et al., 2000; Van Nieuwenhuysen et al., 2003].

The durability of hybrid layers ultimately depends on the intrinsic resistance of their individual components with regard to degradation mechanisms. Although the exact mechanism responsible for hybrid layer degradation is not completely understood, it has been thought that it involves extraction of poorly converted resins from the bonded dentin matrix via water-filled nanometer-sized voids within hybrid layers, followed by an enzymatic attack of exposed collagen fibrils [Tjäderhane et al., 2013b].

A series of recent studies have identified potential and relevant implications of dentin endogenous enzymes in the lack of integrity of resin-dentin bonded interfaces. By assaying the collagenolytic activity of mineralized dentin powder using fluorescein-labeled type I collagen from bovine skin, Pashley et al. [2004] demonstrated an intrinsic proteolytic activity for human mineralized dentin. These authors speculated that such proteolytic activity could be exerted by dentinal MMPs, which on that occasion had already been shown to be potentially expressed in the dentin-pulp complex. When some years later Tersariol et al. [2010] and Nascimento et al. [2011] demonstrated that cysteine cathepsins could also be expressed by mature human odontoblasts and pulp tissues extracted from sound and carious teeth, the participation of this class of proteinases in the enzymatic degradation of denuded collagen within hybrid layers started also being hypothesized. In addition, the study by Tersariol et al. [2010] showed a positive correlation between the enzymatic activity of dentinal cysteine cathepsins and MMPs, which suggests that these classes of enzymes may act synergistically in degradation of dentin matrix in different physiological and pathological processes.

Over the last decade, after the groundbreaking report by Pashley et al. [2004] that mineralized dentin does have an intrinsic proteolytic activity, several papers have described different techniques to confirm that host-derived enzymes play a role in the degradation of the hybrid layer collagenous matrix. The finding that the well-known antimicrobial CHX exhibits also a potent anti-MMP-2, anti-MMP-8 and anti-MMP-9 activity [Gendron et al., 1999] encouraged the determination of whether CHX could stabilize the organic matrix of resin-dentin bonds. This led to numerous *ex vivo* [Carrilho et al., 2007a; Breschi et al., 2009; Stanislawczuk et al., 2009; Breschi et al., 2010; Manfro et al., 2012] and *in vivo* studies [Hebling et al., 2005; Brackett et al., 2007; Carrilho et al., 2007b; Brackett et al., 2009] demonstrating that CHX has beneficial ef-

fects on the preservation of resin-dentin bonds, thereby offering a valuable alternative to clinicians who seek to delay the degradation process of adhesive restorations. The success of CHX in preserving the integrity of resin-dentin bonds was firstly credited to its ability to inhibit the proteolytic activity of MMPs, but it was also shown to be effective in controlling the activity of dentinal cysteine cathepsins [Scaffa et al., 2012].

Zymography assays performed on protein dentin extracts after treating dentin powder with different procedures that mimic bonding procedures (i.e. acid-etching, primer or adhesive treatment) have shown that the gelatinolytic activity of dentin can be remarkably increased with regard to that of mineralized dentin [Breschi et al., 2010]. On the contrary, when dentin is treated with proteinase inhibitors before being resin-hybridized (i.e. CHX, galardin, EDTA, tetracyclines and derivatives, quaternary ammonium compounds), it exhibits consistently lower or no proteolytic activity if compared to that of mineralized dentin [Carrilho, 2012; Tjäderhane et al., 2013b].

Since MMPs and cysteine cathepsins are secreted to the extracellular matrix compartment as proenzymes, thus being inactive, it is not expected that in physiological conditions they can exert a substantial function. Nonetheless, when dentin is prepared to be hybridized with dental resins, it is subjected to a series of acidic chemicals that may be implicated as possible activators of dentinal proteinases [Tjäderhane et al., 2013b]. Although MMPs are prone to cleave collagen and other extracellular matrix components at neutral pH, they were shown to be activated at low pH around 2.5–4.5 [Tjäderhane et al., 1998a]. Conversely, cysteine cathepsins are auto-activated and functional in low pH, and most are considered unstable and inactive in neutral pH [Turk et al., 2000]. This low pH dependence may explain why mild acidic treatment of dentin with primer solutions of ER or SE dental adhesives can indeed activate dentin proteinases, making them likely apt to degrade the collagen, which is eventually exposed and/or denatured with the hybrid layers [Mazzoni et al., 2006; Nishitani et al., 2006; Mazzoni et al., 2013].

Evidence for the involvement of MMPs (much more numerous) and cysteine cathepsins in the degradation of collagen within hybrid layers has been mostly attained from indirect demonstration, such as by treating dentin with proteinase inhibitors prior to or during the bonding procedures. However, using a correlative approach with an ELISA assay and *in situ* zymography, Mazzoni et al. [2012b] were able to quantify and localize MMP activity within the hybrid layer created by an ER adhesive. This was performed

using quenched fluorescent-labeled gelatin showing intense and precise fluorescent localization, indicating enzyme activity within the hybrid layer [Mazzoni et al., 2012b]. Even though these techniques have not been yet performed with SE dental adhesives or to detect the activity of cysteine cathepsins, we truly believe that they could show similar results for both experimental conditions.

Understanding the function of the enzymes responsible for the hydrolysis of collagen within hybrid layers has prompted several approaches to retain hybrid layer integrity and strong dentin bonding. Undoubtedly CHX has been so far the most successful substance used for this purpose. The excellent results of CHX application are consistent mainly because they have been independently obtained from laboratories all over the world, which provides a reliable body of evidence on its effect on hybrid layer preservation. For a comprehensive overview of current knowledge on the effect of exogenous proteinase inhibitors and different other approaches to preserve the integrity of hybrid layers, the reader is referred to recent reviews [Carrilho, 2012; Reis et al., 2013; Tjäderhane et al., 2013b]. In a nutshell, these reviews show that mainly with regard to sound dentin, the prevention of collagen matrix degradation may be achievable with clinically applicable techniques and commercially available materials.

Most of the studies, not to say all, which have been performed to understand the role of proteinases in the lack on durability of hybrid layers have chosen sound dentin as the major object of investigation. Nevertheless it should be kept in mind that with the recent and growing acceptance of minimally invasive dentistry, many bonding protocols are actually performed in caries-affected dentin. Therefore, we should start concentrating on how to enhance bonding performance of commercial adhesives to this substrate, and on alternatives to make this resin-adhesive interface resilient to degradation over time. We truly believe that by improving our knowledge of the role and interplay of proteases in this substrate may help us to determine clinical strategies to produce durable adhesive restorations, regardless whether the bonding procedures are restricted to sound dentin or are extended to caries-affected one.

Conclusions and Future Perspectives

The discovery of the presence, identification and functional activity assessment of endogenous collagenolytic dentinal enzymes has marked the beginning of the era of dentin degradomics. The evidence of the significant role

of these enzymes in the conditions involving the complete or partial exposure of dentin collagenous matrix is steadily increasing. A more detailed picture of the events will further improve our understanding of the processes and the players involved. That, in turn, will help us to benefit from the inhibition of unwanted and the enhancement of beneficial processes.

To achieve this goal, the enzyme function has to be studied in or as close as possible to their natural environment, where the natural substrates are available. For example, what is – if any – the role of dentin-bound dentin phosphoprotein (DPP) in the remineralization of caries-affected or erosion-demineralized dentin? If it is important, how do we best preserve it in partially or totally demineralized dentin? The binding of DPP on collagen is highly selective: in turkey tendon fibril it binds to the collagen gap region, where it has been suggested to regulate mineral deposition within the gap regions [Traub et al., 1992]. Separation of DPP from dentin collagen is relatively difficult and requires acid phosphatase [Saito et al., 1997]. Even though MMP-2 and MMP-20 have been shown to process dentin sialophosphoprotein into DPP, these enzymes fail to further degrade DPP [Yamakoshi et al., 2006], indicating that MMP inhibition would have no direct relevance to DPP retention in dentin. However, the loss of dentinal telopeptides may well result in the loss of DPP, thus seriously reducing the dentin remineralization capacity. The role of telopeptides in remineralization is supported by the inability of cross-linked DPP to mineralize collagen in which telopeptides have been removed with pepsin, in contrast to native collagen [Saito et al., 2000]. Therefore, inhibition of enzymes with telopeptidase activity might help to preserve the collagen telopeptides and retain the natural ability for physiological remineralization.

Different enzymes may have a different role or relative importance depending on the situation. Demineralization of dentin in caries lesions may take a very long time, while in erosion or dentin acid etching in restorative processes it occurs rapidly, even within seconds. The sequence of degradation may be totally different, with different enzymes playing differential roles at different phases of the process. In the dentin degradomic approach, the selection of substrate may be equally important as the enzyme or inhibitor to be studied. The activity of cathepsin K, for example, is highly regulated by the presence of different glycosaminoglycans [Mazzoni et al., 2012a]. If this is also the case in dentin, stromelysins with proteoglycanase activity (e.g. MMP-3) might regulate cathepsin K activity by selectively degrading dentin NCPs. Since not only the enzymes but also other NCP components may vary between species [Tjäderhane et al., 2012], use of dentin – especially human dentin – whenever possible is essential to allow correct interpretation of the results.

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