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Dentin caries progression and the role of metalloproteinases: an update

ABSTRACT

Aim *This review aims to summarise our understanding of the destructive role of acid environment and metalloproteinases in dentin caries progression using a review process.*

Method *The acids resulting from consumption of sugars by acidogenic and aciduric bacteria can cause demineralisation of the tooth surface, but are not able to cause caries-like lesions. The appearance of such lesions requires the activation of enzymatic proteolysis in an acidic environment for degradation of the dentin organic matrix, leading to cavity formation. Bacterial collagenases have long been considered responsible for organic matrix destruction; host cell-derived matrix metalloproteinases (MMPs) have recently been considered to be involved in the dentinal matrix destruction of carious lesions.*

Discussion and conlusion *MMPs are initially synthesised as inactive zymogens to be activated in acid environment of dentinal fluid during the carious process, resulting in destruction of the collagenous matrix. The role of acid environment on enamel and dentin demineralisation and the role of salivary and dentinal MMPs in dentin progression of caries has encouraged general dentists to include the monitoring of oral environment not only by control of bacterial oral flora in caries treatment protocol, but mainly by inhibition of dentinal and salivary MMPs through the use of toothpaste and/or mouthwash containing specific active agents.*

Keywords *Caries, Dentin, Organic matrix, Metalloproteinase*

Introduction

Caries is an infectious and transmittable tooth disease that results from the action of certain bacteria present within the oral cavity; it is the major cause of pulpal inflammation and infection. Nowadays caries treatment seeks not only to restore destroyed tissues but also to treat bacterial infection, with the identification of early caries lesions resulting in a more conservative therapy, such as remineralisation; these treatments represent the basis for further advances in dental care [Perillo et al., 2011; Montasser et al., 2015]. Caries progression in pulpitis is a dynamic process that depends both on the invading bacterial antigens or metabolic by-products and on inadequate host responses with inflammation and immune reactions in the dental pulp [Hahn and Liewehr, 2007]. Microbial acid formation determines the rate of caries progression in enamel, where the process consists almost purely of dissolution of the mineral phase. When the process reaches the dentin, the micro-environment changes and mechanisms of progression are different. In fact, the concentration of organic components in dentin accounts for 20% dry weight in comparison with the 1.3% dry weight of the enamel.

Progression of bacterial lesions in dentin

The dynamic process of demineralisation from the acid environment is usually balanced by saliva (buffer, flow rate, inorganic content etc.) that allow remineralisation to occur. The initial carious lesions in enamel or cementum progress to the underlying dentin when this balance is lost with predominance of pathological factors. Demineralisation occurs when the pH in dental plaque falls below 5.5 within minutes after sugar ingestion until neutralised by salivary buffers. Unlike enamel, of which only about 0.4% to 0.6% is made up of the organic matrix, dentinal tissue contains an important organic matrix (30% vol) containing collagen (90%) and non-collagenous proteins (10%). The organic components of dentin do not allow acids alone to cause caries-like lesions in dentin; rather, the appearance of such lesions requires the activation of enzymatic proteolysis, by metalloproteinases (MMPs), in mildly acidic conditions, leading to cavity formation. The dentin demineralisation rate decreases when the amount of degradable collagen increases, whereby the demineralised matrix is attributed to the hampering of ionic diffusion in and out of the demineralising area [Chaussain-Miller et al., 2006]. The dentin matrix contains mainly type I collagen, but also a small amount of type V collagen and non-collagenous proteins, such as dentin matrix protein I, dentin phosphophoryn, and dentin sialoprotein. The non-collagenous components of the dentin organic matrix are altered during caries progression even before bacterial invasion, while the collagenous matrix remains well protected when it is embedded in mineral components [Shimada et al., 2009]. Bacterial collagenase degrades

the dentin matrix in a remineralisation solution but not in a demineralisation solution, suggesting that proteolysis occurs during the remineralisation phase. The optimum pH for bacterial enzyme is close to neutral, while the activity is directly reduced by the acidic pH [Hahn and Liewehr, 2007; Buzalaf et al., 2010]. Proteolytic enzymes of host and/or microbial origin with collagenase-like activity, as well as exopeptidase and endopeptidase activities, have been detected in carious dentin, especially in the dentinal tubules. In the deep layers of dentin carious lesions with partial demineralisation, not yet colonised by bacteria, the cross-striated pattern of organic matrix of the collagen fibrils is recoverable. Therefore they can undergo remineralisation, thus protecting the organic matrix from degradation [Macedo et al., 2009]. The extracellular matrix macromolecules (ECM) of dentin are important for creating the cellular environment required not only during development and morphogenesis, but also during the remodelling of tissues that occurs throughout life. This remodelling of ECM generally needs the activity of MMPs synthesising and secreting into the ECM from cells of connective tissue (e.g., fibroblasts, osteoblasts, and odontoblasts) [Chaussain-Miller et al., 2006]. In normal conditions, these MMPs are finely regulated and expressed only when required for tissue remodelling; a loss of activity control is usually associated with the tissue destruction observed in many pathological conditions, such as rheumatoid arthritis, cancer, periodontitis, tissue fibrosis, and ulcers. During the caries process, the dissolution of the mineral part of the dentin exposes the ECM to breakdown by bacterial enzymes and by host-derived enzymes such as the MMPs present within the dentin. MMPs derived from saliva may also be involved, for direct access of saliva to dentin in the carious cavity [Takahashi and Nyvad, 2011].

Matrix metalloproteinases

MMPs, collectively called matrixins, constitute a large family of zinc- and calcium-dependent endopeptidases, that contribute in ECM degradation, participate in tooth development, dentin-caries progression and hybrid layer degradation in resin–dentin bonded restorations. MMPs are synthesised and secreted as inactive zymogens with a propeptide domain that must be removed before the enzyme is active via stepwise mechanisms as inactive zymogens. On the basis of substrate specificity, sequence similarity, and domain organisation, vertebrate MMPs can be divided into six groups: collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), transmembrane MMPs or MT-MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and others (MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, MMP-27, and MMP-28) [Shimada et al., 2009]. These enzymes are widely distributed in the organism and are involved in physiologic as well as pathologic processes such as rheumatoid arthritis, atherosclerotic plaques, and cancer metastasis. Various vertebrate MMPs are present in whole saliva and in dental plaque. These include, at least, MMP-8 (human neutrophil collagenase, PMN-MMP-8, collagenase-2), MMP-2 (72-kDa gelatinase/type IV collagenase, gelatinase A), and MMP-9 (92-kDa gelatinase/type IV collagenase, gelatinase B). Both collagenases and gelatinases have been detected in whole saliva and may originate either from the gingival crevicular fluid (GCF), a transudate of plasma through the sulcular epithelium, or from salivary gland secretions [Tjäderhane et al.,1998; Boushell et al., 2008]. However, the GCF appears to be the major source of the MMPs found in the saliva, which also contains α2-macroglobulin, a non-specific inhibitor of MMP, which in physiological conditions keeps the MMPs in inactive form [Birkedal-Hansen, 1993]. The MMPs in their zymogen form (proMMPs) are trapped or bound throughout the dentin, even if they are mainly located along the enamel-dentin junction and in the predentin, during their formation and can be catalysed to active enzymes by lowering the pH to 4.5 or below such as it occurs in dentinal fluid during the carious process. Therefore increased MMPs presence along the dentin-enamel junction may contribute to the widening of caries as it progresses into the dentin through the destruction of the collagenous matrix along this junction [Osorio, 2011]. The N-terminus of the these MMP enzymes is folded over to block the active catalyst site so Zn or Ca ions cannot bind to activate MMPs. This N-terminus part of the molecule can be cleaved off the pro-MMP by specific extracellular matrix proteins, also released from the dentin or present in saliva, to form an active MMP that can bind Zn and Ca ions from the extra cellular environment. The N-terminus chain contains a conserved cysteine residue, that interacts with the zinc ion in the active site and prevents binding and cleavage of the substrate, keeping the enzyme in an inactive form (cysteine switch). The sequence homology with collagenase 1 (MMP-1), the cysteine switch motif PRCGXPD in the propeptide that maintains proMMPs, and the zinc-binding motif HEXGHXXGXXH in the catalytic domain are the signatures used to assign proteinases to this family. The exception is MMP-23, which lacks the cysteine switch motif, but its amino acid sequence of the catalytic domain is related to MMP-1. MMPs generally consist of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain [Visse, Nagase, 2003]. MMPs can be either secreted from the cell or anchored to the plasma membrane. The release and increased activation of MMPs can occur for acid treatment of dentin with lactic and citric acids and remain active even if the pH is neutralised, while phosphoric acid greatly decreases activation [Murphy, 1999]. This suggests that organic acidic molecules can play a remarkable role in activating proMMPs. The lactic acid released by bacteria in a carious lesion can be involved in activation of pro MMPs that break down collagen matrix during the caries process [Moon, 2010]. More than the acidic environment, it is the pH alternation of acid and the subsequent neutralisation to cause activation of MMPs. In fact this alternating periods between dentin demineralisation in pH below 5.5 and the periods of neutral pH, due to salivary buffers, provides the sequence

in which the collagen fibres of the dentin organic matrix are first exposed and then degraded by MMPs [Kawasaki and Featherstone, 1997]. This explains why MMPs, although activated, cannot degrade the organic matrix of dentin at acidic pH. Afterwards, in the caries process, the pH drop is followed by pH neutralisation due to the salivary buffer systems, and this momentary increase in pH allows the pH-activated MMPs to degrade the organic matrix. Furthermore, the phosphorylated proteins released by bacterial acids during dentin matrix demineralisation could interact with tissue inhibitor of metalloproteinases (TIMP)-inhibited host MMPs within the lesion and reactivate them, hence enhancing the degrading activity [Tjäderhane, 1998].

Various MMPs have been identified in the carious lesions, including MMP-2 (gelatinase), MMP-8 (collagenase), MMP-9 (gelatinase), and MMP-20 (enamelysin) from either odontoblast and pulp tissue or from saliva.

The dentin protein matrix is composed of 90% collagen (primarily type I) and 10% non-collagen proteins. The collagen proteins can be clipped into pieces by MMP-8 and further degraded by MMP-2 and MMP-9 after acid demineralisation of dentin within the carious lesion. These MMPs may be present in the saliva or pulp or may be sequestered in dentin, to be released in the local environment during caries destruction of dentin. The MMPs are located throughout the dentin even if intensively located along the enamel-dentin junction and in the predentin. Increased MMP presence along the dentin-enamel junction may contribute to the widening of caries along this junction as caries progresses into the dentin [Moon et al., 2010; Sulkala et al., 2002]. The expression and the biological activity of MMPs are precisely regulated at the level of gene transcription, secretion, activation of the precursor zymogens, interaction with specific ECM components, and inhibition by endogenous TIMPs. Transcription can be induced by various signals, including cytokines, growth factors, mechanical stress, and changes in the extracellular matrix leading to modification in cell-matrix interactions [Visse and Nagase, 2003; Tjaderhane et al., 2001]. All MMPs except MMP-23 are expressed and translated with an amino-terminal signal peptide, which targets the protein to the rough endoplasmic reticulum, and most of them are secreted. The ultimate level of MMP synthesis is also regulated by mRNA processing. After translation, most MMPs are readily secreted, but at least MMP-7, MMP-8, and MMP-9 can be stored intracellularly. The spatial restriction of MMP activity, often to the pericellular regions, is achieved by the expression of membrane-bound MMPs and cell surface receptors for MMPs or MMP-activating enzymes [Sternlicht and Werb, 2001]. Because MMPs are secreted as inactive zymogens, they must be activated, usually by proteolytic cleavage of their NH2-terminal prodomains. Some MMPs are activated by serine proteases (such as plasmin, tissue kallikrein, and furin), by bacterial proteinases, or by other members of the MMP family [Chaussain-Miller et al., 2006]. The distribution and expression levels of MMP inhibitors can also regulate and balance local MMP activity. Three non-collagen proteins of the dentin matrix, identified as bone sialoprotein (BSP), osteopontin (OPN), and dentin matrix protein 1 (DMP1), and belonging to the small integrin binding ligand N-linked glycoproteins (SIBLING) gene family, may bind and activate specific MMPs when they are released by the acid environment in caries or potentially during acid etching for bonding. The MMPs and the binding proteins pair specifically together in the following groups for activation: MMP-2 with BSP, MMP-3 with OPN, and MMP-9 with DMP1 [Moon et al., 2010].

MMP activation

MMPs are multidomain enzymes that contain a catalytic domain with a zinc ion bound to three histidines, the "cysteine switch", in the non-activated form, to the cysteine residue of the pro-domain, and an additional structural zinc ion and two to three calcium ions required for enzymatic activity and stability. In all secreted MMPs except MMP-7 and MMP-26 the catalytic domain is followed by a C-terminal hemopexin-like or vitronectin-like domain contributing to substrate and TIMP binding, proteolytic activity, and membrane activation; in most cases there is a connecting hinge region between these domains. In MT-MMPs the C-terminal domain attaches the molecule to the plasma membrane [Visse and Nagase, 2003]. Cells produce and secrete MMPs in a latent pro-form in which a cysteine sulphydryl group in the amino-terminal pro-domain interacts with the zinc ion and blocks the active site. Removal of the propeptide (about 10 kDa) from the active site by proteolysis, leads to activation of the enzymes [Hu at al., 2007]. MT-MMPs may have an additional transmembrane domain site, either a glycosylphosphatidylinositol anchor site or an Ig-like domain that determines the localisation on the cell surface [Bozzuto et al., 2010]. The activation of proMMPs to active form is a complex and dynamic process, for which several mechanisms have been proposed. Some MMPs are intracellularly processed, whereas a few are processed at the cell membrane, into fully active enzymes [Kotra et al., 2001]. Non-proteolytic compounds such as organomercurial chemicals (4-amino¬phenyl¬mercuric acetate, or APMA), denaturants (SDS), and conformational perturbants (detergents) or proteases may open the cysteine to zinc switch to trigger proMMP activation. Changes in pH also affect zymogen activation, because an acidic condition followed by neutralisation has been shown to activate several MMPs. The prodomain of an MMP can be removed in an autocatalytical manner or by proteases, and the cysteine is replaced by a water molecule to allow enzyme catalysis to proceed [Murphy et al., 1999]. Proteolytic activation of MMPs is stepwise in many cases. The initial proteolytic attack occurs at an exposed loop region between the first and the second helices of the propeptide. The cleavage specificity of the bait region is dictated by the sequence found in each MMP. Removal of a part of the propeptide probably destabilises the rest of the propeptide, including the cysteine switch-zinc interaction, which allows intermolecular processing by partially activated MMP intermediates or other

active MMPs. Various host and bacterial proteinases are capable of initiating proteolytic activation by cleaving the proteinase-susceptible 'bait" region in the middle of the propeptide. This cleavage is followed by further processing of the prodomain, often by another MMP, and MMPs form complexed interaction networks by these activation cascades [Visse and Nagase, 2003]. Another similar mechanism is that of MMP binding to a proMMP, which could induce the N-terminus end to be displaced so that the Zn or Ca binding site is no longer blocked. A combination of these mechanisms may act in unison where another MMP molecule binds to change the molecular configuration to partially activate the MMP or displace the N-terminus, allowing the reaction of water with the active site, which promotes the removal of the N-terminus by protease enzymes [Jain et al,. 2008]. As mentioned earlier, all MMPs save for MMP-23 are expressed and translated with an amino-terminal signal peptide, and most of them are secreted. After the signal peptide, the MMPs contain a highly conserved prodomain, which generally maintains the enzyme in the zymogen form, and a catalytic domain responsible for substrate hydrolysis and autolytic cleavages of the MMP molecule [Sternlicht and Werb, 2001]. The proMMP-2 activation procedure is well recognized; it proceeds at the cell membrane through an MT-MMP–mediated cascade. In addition, PMN-derived elastase, cathepsin G, and proteinase-3 can also activate proMMP-2 through an MT1-MMP–dependent route. Essentially, in low concentrations of TIMP-2, the MT1-MMP–mediated proMMP-2 activation includes the formation of the MT1-MMP and TIMP-2 complex, serving as a receptor for proMMP-2 binding. The negatively charged C-terminal domain of TIMP-2 facilitates non-inhibitory binding to the proMMP-2 C-terminal domain via electrostatic interactions to allow an adjacent free MT1-MMP to activate proMMP-2 [Visse and Nagase, 2007]. At high concentrations, however, TIMP-2 inhibits the activation of proMMP-2 by bonding to both the proMMp-2/MT1-MMP complex and free MT1-MMP. There are many examples of MMPs activating each other [Shimada et al., 2009].

MMP inhibitors

MMP activity can be blocked by endogenous or exogenous inhibitors.

Endogenous inhibitors

TIMPs are major endogenous physiological inhibitors, but their regulation of MMPs is not well known. TIMPs exhibit variable and non-specific actions against different MMP members and display different tissue expression patterns and modes of regulation [Bake et al,. 2002]. Four TIMPs are recognised (TIMP-1 to TIMP-4), and all are secreted proteins that form complexes with MMPs, inhibiting the active forms of all MMPs. TIMP-1, TIMP-2, and TIMP-4 are found at the cell surface in close association with membrane-bound proteins. In contrast, TIMP-3 is sequestered within the ECM by binding to heparan-sulphate [de Souza et al., 2000]. The overall activity of MMPs is correlated to balance between the levels of activated enzymes and free TIMPs. Maintaining this balance is essential, and any disturbance results in proteolysis. Therefore, TIMPs counteract MMPs, inhibiting MMP activity and thereby restricting ECM remodelling and breakdown [Chaussain-Miller et al., 2006].

The wide-scale expression of MMPs and TIMPs by mature human odontoblasts and pulp tissue suggests that they participate in dentinal matrix organisation prior to mineralisation, and that growth factors may further control dentinal matrix modelling by differentially regulating individual MMPs [Dayan and Vered, 2010]. At least TIMP-1 is present in parotid and submandibular saliva and gingival crevicular fluid. Even though TIMP-1 is present only at low levels, it could have a regulatory effect on MMP activity in caries lesions *in vivo*, especially because salivary TIMP-1 has been shown to retain stability at low pH. Another mechanism of MMP activation, which takes place along with a momentary increase in pH or heat occurring at the spots of demineralised dentin to degrade the organic matrix, could be that of phosphorylated proteins, which are released by bacterial acids during dentin matrix demineralisation. These proteins interact with TIMP-inhibited host MMPs within the lesion and reactivate them, hence enhancing the degrading activity [Boushell et al., 2008; de Souza et al., 2000].

Exogenous inhibitors

MMP inhibition by natural synthetic substances in gel and/or mouthwash could provide a potential therapeutic pathway to avoid caries progression in dentin, facilitating the processes of remineralisation or prolonging longevity of dental restorations. Most exogenous and synthetic MMP inhibitors prevent MMP activity by chelating or replacing the active-site zinc ion. The effects of naturally derived substances on MMP/TIMP balance could be considered in the treatment of several diseases and in the control of caries progression. Avocado and soya bean unsaponifiables show MMP-inhibiting properties *in vitro*, inhibiting IL-1β-induced MMP-2, MMP-3, and TIMP-1 release by gingival fibroblasts. Oleic acid has also been shown to inhibit the activity of several MMPs, as well as the activation of MMP-3 by plasmin [Woessner and Nagase , 2000; Henrotin et al., 2006; Huet et al., 2004].

Green tea polyphenols, especially epigallocatechin gallate (EGCG), show potent and distinct inhibitory activity against MT1-MMP, causing the decrease of proMMP-2 activation. EGCG seems to exhibit hydrogen bonding and hydrophobic interaction with collagenases, which is responsible for the change in the secondary structure of collagenases and consequently for their inhibition. Furthermore, MMP-2 and MMP-9, as well as macrophage and neutrophil MMP-12 activities, can be directly inhibited by green tea polyphenols [Kato et al., 2010; Kato et al., 2009]. The MMP-inhibitory effect of tetracycline and its derivatives was first observed with minocycline, which inhibits, in the extracellular environment, the collagenolytic activity of gingival crevicular fluid in the absence of bacteria, while intracellularly down-regulating transcriptional MMP levels. Chemically modified tetracyclines (CMTs) without

antibiotic activities have several potential advantages over conventional tetracyclines. CMTs are the most potent MMP inhibitors and are among the few MMP inhibitors found to be safe and effective, particularly after oral hygiene. CMTs inhibit both the activity and the secretion of MMPs and are thought to act through Ca2+ chelation. Tetracyclines and their semi-synthetic forms, doxicycline and minocycline, inhibit MMP-1, MMP-2, and MMP-2, both *in vitro* and *in vivo* [Kato et al., 2010]. Bisphosphonates, pyrophosphate analogues with a high affinity for hydroxyapatite crystals, can be considered MMP inhibitors by chelating action on zinc or calcium ion [Widler et al. 2002]. Chlorhexidine digluconate reduces collagen degradation by inhibiting dentin MMPs binding electrostatically to demineralised dentin collagen and by zinc chelator effect. A high zinc concentration strongly reduces collagen degradation. Larsen and Auld [1991]. have shown that zinc inhibits carboxypeptidase A by the formation of zinc monohydroxide, which bridges the catalytic zinc ion to a side chain in the active side of the enzyme [Osorio et al., 2011].

Discussion and conclusion

The observation that carious dentin contained large amounts of organic acids supports the view that the acids are involved in the process of dentin caries. The acid environment is responsible of demineralisation of enamel and of inorganic dentin but it is not able to progress in organic dentin [Ballini et al., 2012]. The degradation of the collagenous organic matrix of dentin during caries progression is an enzymatic process. Some of the enzymes involved in this process may be of host origin, and specifically host MMPs were here hypothesised to take part in the breakdown of the dentin organic matrix. The acid environment in dentin induced by lactic acid from catabolism of acidogenic bacteria is responsible of MMPs activation. MMPs are important components in many biological and pathological processes because of their ability to degrade ECM components. In the future, the restorative protocol in conservative dentistry will have to consider the aims related to balancing the pH of saliva and the control of oral bacteria [Paolantonio et al., 2009; Agarwal et al., 2014; Paglia et al., 2016]. Future prospects are concentrated in search of synthetic or natural inhibitors of dentinal caries formation in initial phase [Fleming, 2015; Tayab et al., 2012; Femiano et al., 2014]. MMP inhibitors in gel or mouthwash could be used, in combination of a remineralising gel, in order to stop the process of caries and to repair the lost tissue avoiding small restorations.

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