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REVIEW ARTICLE

Matrix metalloproteinases (MMPs) in oral diseases

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Matrix metalloproteinases (MMPs) are a group of enzymes that in concert are responsible for the degradation of most extracellular matrix proteins during organogenesis, growth and normal tissue turnover. The expression and activity of MMPs in adult tissues is normally quite low, but increases significantly in various pathological conditions that may lead into unwanted tissue destruction, such as inflammatory diseases, tumour growth and metastasis. MMPs have a marked role also in tissue destructive oral diseases. The role of collagenases, especially MMP-8, in periodontitis and peri-implantitis is the best-known example of the unwanted tissue destruction related to increased presence and activity of MMPs at the site of disease, but evidence has been brought forward to indicate that MMPs may be involved also in other oral diseases, such as dental caries and oral cancer. This brief review describes some of the history, the current status and the future aspects of the work mainly of our research groups looking at the presence and activity of various MMPs in different oral diseases, as well as some of the MMP-related aspects that may facilitate the development of new means of diagnosis and treatment of oral diseases.
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Introduction

Since the microbial nature of many oral diseases has been recognized long ago, for decades research has aimed to fight the microbes behind the diseases. More recently it has been realized that the host-related factors may be the keys to the fundamental understanding of the disease processes in many oral diseases. One of these host factors is a family of enzymes called matrix metalloproteinases (MMPs). Our research groups have for years been working to evaluate the presence, activity, function and regulation of MMPs in healthy and diseased oral tissues. In collaboration with other groups around the world, the work has resulted into the development of pharmacological agents for MMP inhibition in the treatment of oral diseases, as well as utilizing MMP measurements as diagnostic tools.

MMPs are a family of structurally related but genetically distinct enzymes that degrade extracellular matrix (ECM) and basement membrane (BM) components. This group of 23 human enzymes is classified into collagenases, gelatinases, stromelysins, membrane-type MMPs and other MMPs, mainly based on the substrate specificity and molecular structure. MMPs are involved in physiological processes such as tissue development, remodelling and wound healing (Uitto *et al*, 2003), and play important roles in the regulation of cellular communication, molecular shedding and immune functions by processing bioactive molecules including cell surface receptors, cytokines, hormones, defensins, adhesion molecules and growth factors. MMP activity is controlled by changes in the delicate balance between the expression and synthesis of MMPs and their major endogenous inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). The catalytic competence of MMPs is controlled through the activation of proenzymes, and the inhibition of the activation or activity by TIMPs (Uitto *et al*, 2003).

As the roles of MMPs in tissue degenerative diseases have became evident, attempts to control their activities by pharmacological means have gained much attention. Although the exact roles of individual MMPs in various diseases are not fully understood, it is clear that MMPs are often up-regulated in groups forming activation cascades both in the inflammatory and malignant diseases (Uitto *et al*, 2003).

MMP activation and inhibition

MMPs are mostly produced in latent, non-active form, and activation through a so-called cysteine switch is required for the enzyme function. In most cases, activation involves removal of the prodomain, resulting into lower molecular weight active forms (reviewed by

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Nagase, 1997), although the most recent studies indicate that *in vivo*, the proforms of at least certain MMPs may also be active while in full-size or in complex with certain proteins (Bannikov *et al*, 2002; Fedarko *et al*, 2004). Non-proteolytic activation of MMP proforms can be accomplished *in vitro*, e.g. by SH-reactive agents, such as mercurial compounds, detergents, gold(I)-combounds, or oxidation. Proteolytic activation can be attained by several proteolytic enzymes, including serine proteinases together with other MMPs. Most of the activation data has been gained from *in vitro* experiments, and the activation processes *in vivo* are not well characterized. Most likely MMP activation *in vivo* involves tissue and plasma proteinases and bacterial proteinases together with oxidative stress (Nagase, 1997; see also review by Van den Steen *et al*, 2002). Secreted MMPs are usually activated extracellularly or at the cell surface, the best-known example of cell surface activation being the activation of MMP-2 in a MMP-2/TIMP-2/MT1-MMP complex. Several MMPs may also be activated intracellularly by furin or related proprotein convertases (Pei and Weiss, 1995; Nagase, 1997).

MMP activation and activity can be controlled by inhibition in several ways: proteolytic degradation and inactivation, non-specific endogenous inhibitors such as α2-macroglobulin, and especially by specific tissue inhibitors of MMPs, TIMPs (reviewed by Brew *et al*, 2000). Currently, four TIMPs (TIMP 1–4) are known to be expressed in vertebrates. TIMPs inhibit MMPs by forming 1:1 stoichiometric enzyme-inhibitor complexes. TIMP-1, -2 and -4 are secreted, while TIMP-3 is sequestred to the ECM. The substrate specificity of TIMPs varies (Brew *et al*, 2000).

Synthetic inhibition of MMPs offer an interesting possibility to control MMP-related diseases in which extensive tissue destruction is involved (reviewed by Overall and Lopez-Otin, 2002). One approach in MMP inhibition is aimed at chelation of the enzyme's active site, Zn^{2+} ion (reviewed by Hidalgo and Eckhardt, 2001; Coussens and Fingleton, 2002). The first MMP inhibitors to enter clinical trials in tumour treatment, batimastat and marimastat, base their MMP inhibitory effect on chelation (Coussens and Fingleton, 2002). Tetracyclines and their non-antimicrobial analoques, chemically modified tetracyclines (CMTs), inhibit MMPs through several mechanisms. In addition to Zn^{2+} chelation, they can down-regulate MMP mRNA expression, interfere with the protein processing during activation, and render the MMPs more susceptible for degradation (reviewed by Golub *et al*, 1998).

The clinical studies, conducted so far mostly in cancer patients, and the results, have not been very convincing. However, if the key MMPs in different diseases can be reliably determined, development of effective, target-specific MMP inhibitors may facilitate effective treatment with minimal side effects (Hidalgo and Eckhardt, 2001). One promising product is so-called CTT-peptide, a synthetic decapeptide, that specifically and selectively inhibits gelatinases. It has given promising results in animal models, targeting the vascular tumor tissue and efficiently reducing the growth of several carcinoma types in nude mice (Koivunen *et al*, 1999; Medina *et al*, 2001).

MMPs in the pathogenesis of periodontitis and peri-implantitis

It was only 4 years after Gross and Lapiere (1962) discovered the tadpole collagenase (MMP-1) when the human gingival collagenase was identified by Fullmer *et al*., (1966). Now, more than 35 years later, significant evidence exists to show that collagenases, along with other MMPs, play an important role in the periodontal destruction. Tissue extracts and cultured tissue explants of inflamed human gingiva were shown to contain more collagenase activity than extracts and explants from healthy human gingiva. Collagenase activity in gingival crevicular fluid (GCF) also increased and correlated with the severity of periodontal disease. Experimental gingivitis and periodontitis also increased collagenase activity in diseased gingiva and GCF (reviewed by Uitto *et al*, 2003).

Since putative periodontopathogenic bacteria are always present in periodontitis, it was logical to assume that collagenases in periodontal disease would originate from microbial sources (Sorsa *et al*, 1987). However, gingival tissue extracts, GCF and other samples produce the type I collagen cleavage pattern characteristic of mammalian, not bacterial collagenases (Sorsa *et al*, 1988). Briefly, mammalian collagenases (MMP-1, -8 and -13) and other collagenolytic MMPs cleave native collagen at a single locus, resulting in formation of two distinct fragments (Uitto *et al*, 2003). Instead, the bacterial collagenolytic proteases attack native collagen at multiple sites, producing many short peptide fragments (Sorsa *et al*, 1987). Sorsa *et al* (1988) demonstrated that the major collagenase in periodontitis was human collagenase-2, MMP-8, accompanied by MMP-9 (Sorsa *et al*, 1995). These findings have been confirmed and extended by the studies utilizing a wide range of RNA- and protein analysing techniques specific for MMPs and TIMPs (Tervahartiala *et al*, 2000; Kiili *et al*, 2002). Previously it was thought that the expression and release of MMP-8 was limited to neutrophils (Uitto *et al*, 2003), but at present it is clear that many non-PMN-lineage cell types present in the normal and diseased human periodontium (gingival sulcular epithelial cells, fibroblasts and endothelial cells, monocyte/macrophages and plasma cells) can be induced to express distinct MMPs including MMP-8 (Hanemaaijer *et al*, 1997; Tervahartiala *et al*, 2000; Wahlgren *et al*, 2001; Kiili *et al*, 2002; Prikk *et al*, 2002).

It also early became evident that TIMPs are not sufficient to down-regulate the pathologically elevated MMPs (Ingman *et al*, 1996). Therefore, the possibility of selective MMP inhibition by synthetic inhibitors as a method to avoid or limit the periodontal tissue destruction was advanced. In series of collaborative studies with Professor Lorne M. Golub's group we have demonstrated that MMP-8, -9, -13 and -14, all considered as (at least potentially) important in periodontitis, are far more sensitive to inhibition by doxycycline and

chemically modified non-antimicrobial tetracycline (CMT)-derivatives than MMP-1 and -2 (Golub *et al*, 1998). Further work demonstrated that the inhibition can be obtained by therapeutically attainable serum concentrations of these drugs (Golub *et al*, 1998). While MMP inhibition is a promising approach in periodontal disease treatment, further work including other approaches need to be evaluated (reviewed by Reddy *et al*, 2003).

The classical periodontal diagnosis determines previous periodontal tissue destruction. Evaluation of disease activity before significant destruction, and measurement of successful treatment or disease arrest, would allow the treatment to be directed to the right patient, possibly even the site of high disease activity, at the right moment (reviewed by Eley and Cox, 1998). Thus, there is a need for a chair-side test for diagnosis and monitoring of periodontal diseases. MMP-8 is a potential candidate for such a test (Sorsa *et al*, 1988, 1999; Kiili *et al*, 2002). With that goal in mind, we have developed monoclonal antibodies for MMP-8 to be utilized in a chair-side dip-stick test for MMP-8 that allowed the development of a novel sensitive, specific, rapid and practical immunological chair-side dip-stick test for MMP-8 in GCF and peri-implant sulcular fluid (PISF) (Kivelä-Rajamäki *et al*, 2003a,b; Mäntylä *et al*, 2003). The test, bearing resemblance to pregnancy home test kits (Figure 1), can be performed by a dentist without specific equipment, and measures the GCF MMP-8 level in 5 min (Mäntylä *et al*, 2003). It differentiates healthy and gingivitis sites from periodontitis sites (Mäntylä *et al*, 2003), and reduction of GCF MMP-8 levels can be observed after successful periodontal treatment (Mäntylä *et al*, 2003). GCF MMP-8 level testing is a very useful tool to monitor the beneficial effects of adjunctive sub-antimicrobial doxycycline-medication for periodontitis patients (Emingil *et al*, 2004a,b). This rapid point-of-care test developed for periodontitis is obviously a useful tool also for monitoring of peri-implantitis (Sorsa *et al*, 1999; Kivelä-Rajamäki *et al*, 2003a,b).

MMPs in caries, pulp and periapical pathogenesis

Demineralization in caries lesions is caused by microbial acids, and lesion progression in dentin is accompanied with degradation of dentin organic matrix to the point beyond remineralization. Traditionally, microbial enzymes have been held responsible for this matrix degradation, although research has questioned this concept (Tjäderhane *et al*, 1998b, 2001). The idea of the possible role of MMPs in dentin matrix degradation lead us to search for the evidence in the mid-1990s. Indeed, we were able to demonstrate the presence of both pro- and active forms of MMP-8, -2 and -9 in human dentinal caries lesions (Tjäderhane *et al*, 1998b). Since the active forms are short-lived, the presence of active MMPs indicates activation in site, suggesting their active role in the dentin matrix degradation. This was supported by the finding that the pH changes taking place in caries lesion are extremely powerful activators for MMPs. These findings formed a base to the theory of a sequencial demineralization-MMP activation and dentin matrix degradation taking place in the dentin lesion (Tjäderhane *et al*, 1998b). The importance of MMPs in lesion progression was further supported by *in vivo* study in which the MMP inhibition significantly down-regulated the dentinal caries lesion progression (Sulkala *et al*, 2001) (Figure 2).

There are two possible sources for the MMPs in caries lesions. Salivary and GCF MMPs are reserved in plaque (Sorsa *et al*, 1995), which is also the potential site for acid activation. MMPs are also produced by odontoblasts (Tjäderhane *et al*, 1998a; Palosaari *et al*, 2000,

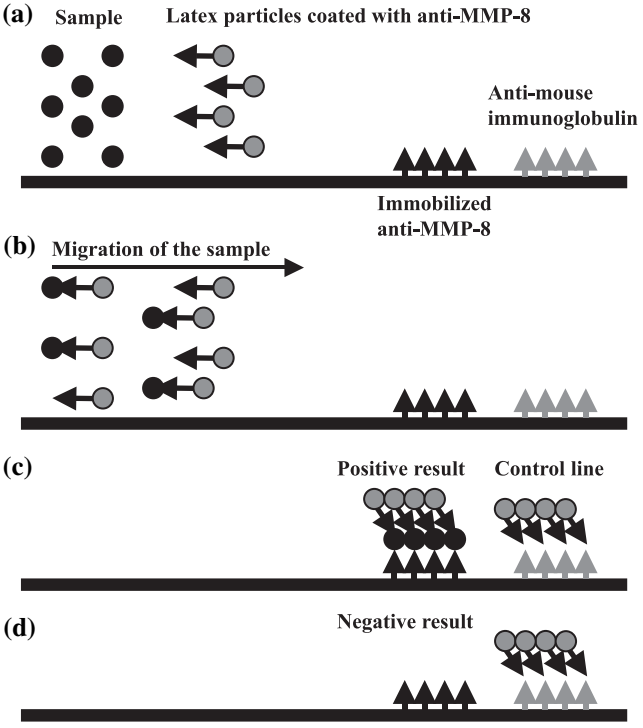

Figure 1 Principle of the immunochromathographic MMP-8 dip-stick chair-side test in gingival crevicular fluid (GCF), peri-implant sulcular fluid (PISF) and other oral fluids (i.e. mouth-rinse samples, saliva, etc.). The MMP-8 test stick is based on the immunochromatography principle that uses two monoclonal antibodies specific for different epitopes of MMP-8. One is immobilized onto a nitrocellulose membrane to form a catching zone and the other onto blue latex particles. When the sample collected from the gingival crevice with a strip is diluted into the test kit buffer, the tip of an MMP-8 test stick is then immersed in the buffer for about 10 s. Fluid is absorbed into the stick, and MMP-8 in buffer migrates along the test stick and binds to the blue latex-labelled MMP-8 antibody on the stick (phase a). The complexes then migrate with the sample fluid across the nitrocellulose membrane over the catching zone containing the other MMP-8 antibody (phase b). A sufficient MMP-8 concentration in the sample results in a blue line within 5 min in this zone when MMP-8 carrying latex is bound to it (phase c). The stick also contains latex-labelled mouse immunoglobulin, that after migration attaches to the anti-mouse immunoglobulin zone, demonstrationg two blue lines to indicate successful performance of the test (phase c). When the MMP-8 concentration in the sample is below the detection level, only one (control) blue line appears, indicating a successfully performed test with negative result (phase d). The immunoassay can be adapted for any MMPs, TIMPs, PMN elastase, PMN lipocalin etc. alone and/or in any combinations with tissue degradation products.

2002, 2003; Sulkala *et al*, 2002) and they are present in mineralized human dentin (Martin-De Las Heras *et al*, 2000; Sulkala *et al*, 2002; M. Sulkala *et al*, unpublished data). Interestingly, dentin matrix protein-1, osteopontin and bone sialoprotein, all members of SIBLING protein family and present in dentin, have been suggested to have a role in eliciting the functional activity in MMP proforms (Fedarko *et al*, 2004). Indeed, the studies identifying MMPs in mineralized dentin have shown enzyme activity immediately after extraction (Martin-De Las Heras *et al*, 2000; Sulkala *et al*, 2002; Hashimoto *et al*, 2004; M. Sulkala *et al*, unpublished data). Whether this is due to the complex formation with proteins inducing functional activity of proMMPs (Fedarko *et al*, 2004) or other reasons remains to be studied.

Although the physiological role of odontoblast-derived MMPs mostly remains to be studied (Tjäderhane *et al*, 2001), they may be involved in dentinal caries pathogenesis (Tjäderhane *et al*, 1998b; Martin-De Las Heras *et al*, 2000; Sulkala *et al*, 2001, 2002). Recently, an increasing number of *in vitro* and *in vivo* studies have demonstrated a hydrolytic loss of collagen fibers in and under the adhesive layers of composite restorations (e.g. Sano *et al*, 1999; Hashimoto *et al*, 2002, 2003a,b), and MMPs present in dentin have been indicated to be responsible for the degradation (Carrilho *et al*, 2004; Hashimoto *et al*, 2004; Pashley *et al*, 2004). The future work will show if MMP inhibition can be used to prevent the loss of adhesion, as suggested (Hashimoto *et al*, 2004; Pashley *et al*, 2004).

Like in other inflamed tissues, MMPs are present in inflamed dental pulp tissue (Wahlgren *et al*, 2002) and periapical lesions (Wahlgren *et al*, 2001, 2002). The level of MMP-8 in periapical exudates decreases during successful root canal treatment, while in cases with persistent inflammation the levels remain high, indicating that MMP-8 dip-stick analysis from periapical exudate could be used to monitor inflammatory activity and the success of treatment in teeth with periapical lesions (Wahlgren *et al*, 2002).

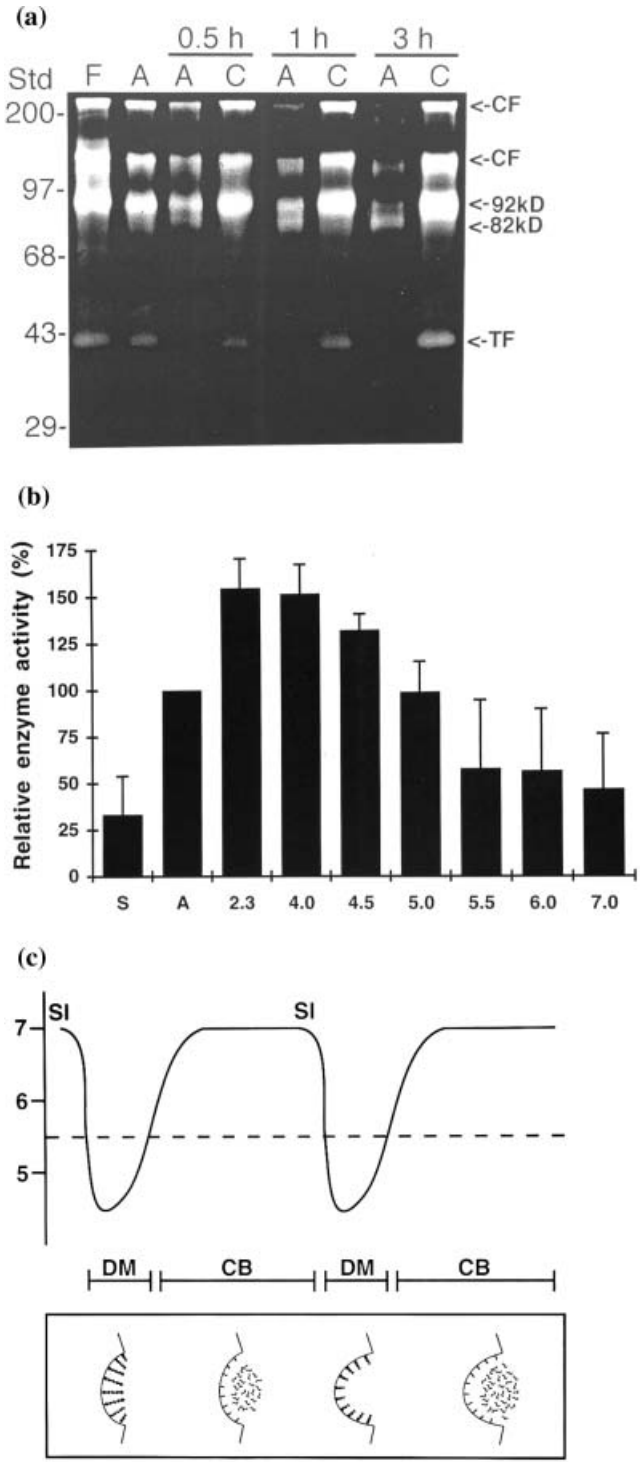

Figure 2 The activation and role of MMPs in dentinal caries lesions (reproduced from Tjäderhane *et al* (1998b), with permission). (**a**) Enzymography of saliva before, during and after acid activation, describing the activation of salivary gelatinases, mainly MMP-9. There are no apparent differences between filtered saliva without any handling (F) and immediately after incubation in pH 4.5 for 37°C followed by neutralization (**a**: acid activation resembling the pH changes in caries lesion). With increasing incubation time at 37°C after neutralization, a shift of 92 kDa latent proMMP-9 into 82 kDa active form can be observed with no changes in the control saliva (**c**). At the same time, the decrease in 135 and >200 kDa complexed forms of gelatinolytic MMPs (CF) and 42 kDa truncated form (TF) can be seen. (**b**) Relative gelatinolytic activity of saliva after APMA (**a**)- or acid activation saliva at various pHs (from 2.3 to 7.0) followed by neutralization, as observed in the functional activity assay with ^{125}I-labeled gelatine as a substrate. The gelatinolytic activity after APMA (often considered as a super-activator of MMPs) is used as a reference (100%). The bars (average + s.d.; $n = 5$) demonstrate the extreme MMP activation efficiency of pH changes observed in caries lesions (decrease of pH into 4.0–5.0, followed by neutralization). S: non-activated saliva. (**c**) The schematic presentation of the alternating sequences of demineralization and degradation of the organic matrix in dentin caries lesion, demonstrating the pH changes (modified Stephan curve) and corresponding changes in the caries lesion (box). Immediately after sugar ingestion (SI), the pH decreases below the level in which demineralization occurs (pH 5.5, dashed line). During this demineralization period (DM), dentin matrix is exposed (first figure in the box), and latent salivary and/or dentin-bound MMPs are converted into active forms. Due to the buffering capacity of saliva, the pH slowly returns into neutral. With increasing pH, the MMP activity increases (since MMPs are neutral proteases, with the ultimate activity in pH close to neutral), degrading the dentin organic matrix exposed during demineralization (CB: collagen breakdown). The sequence is repeated after each ingestion of carbohydrates

MMPs in oral cancer

It took 18 years before the second member of the MMP family was discovered. This metalloproteinase, specifically degrading BM type IV collagen but also other ECM components, was later named as gelatinase A or MMP-2. MMP-2 was purified and partially characterized from the melanoma tissue in early 1980s in Dr Karl Tryggvason's laboratory (Salo *et al*, 1982, 1983). Since MMP-2 was secreted by numerous cultured malignant cell lines, we originally speculated that it is the key enzyme in cancer growth and metastasis (Salo *et al*, 1982). However, already in mid-1980s MMP-2 was identified also in cultured non-neoplastic fibroblasts, but still the activity was induced by the tumor promoter phorbol ester, PMA (Salo *et al*, 1985). Using immunohistochemistry and *in situ* hybridization MMP-2 was, however, shown to be secreted not by the carcinoma cells but by the surrounding stromal fibroblasts (Sutinen *et al*, 1998). The expression is similar to that found in healing mucosal wounds (Salo *et al*, 1994). The protein is located in carcinoma cell membranes linked to MT1-MMP and TIMP-2 complex. This complex is required for the full local, pericellular MMP-2 activity in cancer tissue (Thomas *et al*, 1999). Unlike MMP-2, MMP-9 (gelatinase-B) is mainly synthesized by carcinoma and inflammatory cells of the carcinoma tissue (Thomas *et al*, 1999). Numerous studies have now shown the induced expression of other MMPs in head and neck carcinomas produced both by tumor and surrounding mesenchymal cells (Sutinen *et al*, 1998; Väänänen *et al*, 2001; Moilanen *et al*, 2002) (Figure 3). Although several MMPs are up-regulated in oral tumors (reviewed by Thomas *et al*, 1999), oral carcinoma patients with elevated MMP-2 and -9 activity have shorter disease-free survival after treatment than patients with low gelatinase tumor activities (Yorioka *et al*, 2002). Future work will be needed to determine the key MMPs in different oral cancers, as is the situation also with other tissues (reviewed by Curran and Murray, 1999).

A tumor associated trypsin-2 (TAT-2) and its specific inhibitor (TATI) were originally discovered in professor Ulf-Håkan Stenman's laboratory (Stenman *et al*, 1982; Koivunen *et al*, 1991). They are co-expressed in many tumors, including oral carcinomas (T. Sorsa, L. Tjäderhane, T. Salo, unpublished observation) and increased TATI serum concentration exists in several cancers (Stenman, 2002). TAT-2 is a highly potent activator of proteases associated with cancer invasion, like gelatinases, collagenases and stromelysin-1 (MMP-3) (Sorsa *et al*, 1997; Moilanen *et al*, 2003). Thus, TAT-2 can be regarded as the initial activator serine proteinase of proMMP-activation cascade (Sorsa *et al*, 1997; Moilanen *et al*, 2003). Interestingly, TAT-2 itself is able to fragment native type I collagen (Moilanen *et al*, 2003). We have demonstrated that TAT-2 transfected tongue carcinoma cells secrete activated MMP-9, indicating *in vivo* capacity of MMP-9 activation by TAT-2. This TAT-2-dependent MMP-9 activation also correlates with the induced invasive potential of tongue carcinoma cells (Nyberg *et al*, 2002). This demonstrates clearly an important concert action not only of various MMPs, but also other (serine) proteinases and their cascades in the carcinoma growth processes.

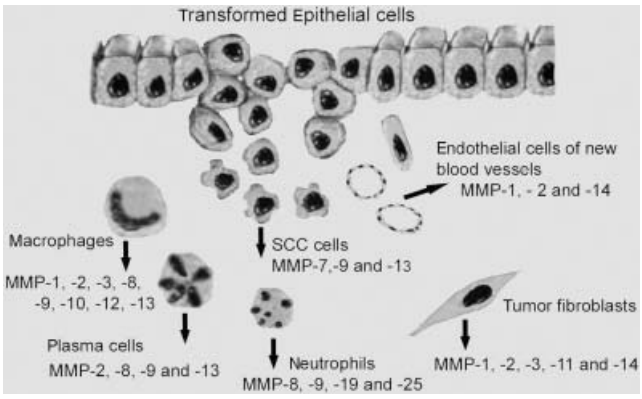

Figure 3 Schematic drawing of MMPs that can be produced in oral squamous cell carcinoma (SCC) tissues. MMPs are secreted either by SCC cells themselves, surrounding mesenchymal tumor fibroblasts, different inflammatory cells, or endothelial cells

Endostatin is an antiangiogenic 20 kDa fragment derived proteolytically from hemidesmosomal type XVIII collagen (O'Reilly *et al*, 1997). We recently found that endostatin also inhibits *in vivo* invasive capacity of tongue carcinoma cells, based at least partially on its capacity to inhibit the activation of MMP-2, -9 and -13 (Nyberg *et al*, 2003). This reveals that endostatin is acting at least in two levels in oral cancer growth, by inhibiting endothelial cell growth and by reducing cancer cell capacity to modulate surrounding matrix by active MMPs. However, endostatin cannot inhibit MMP-8 (Nyberg *et al*, 2003). Interestingly, MMP-8 seems to have an anti-tumor activity in breast carcinomas (Agarwal *et al*., 2003) and skin tumors (Balbin *et al*, 2003). The role of MMP-8, present in trace amounts also in oral SCC tissues and carcinoma cell lines (Moilanen *et al*, 2002), is unknown.

It is evident that gelatinase activity correlates with the invasive capacity of the oral cancer, and the antitumor effect of gelatinase-specific MMP inhibitor CTT-peptide on tongue carcinoma was recently demonstrated using CAM-assay (Nyberg *et al*, 2002). However, the spectrum of MMPs and other enzymes orchestrating tongue carcinoma growth is most likely larger and more complex than anticipated and therefore the usefulness of MMPIs, like CTT-peptide in combination with other cytostatic or cytotoxic cancer drugs, should further be studied in preclinical trials. The possible usefulness of specifically selected MMPIs, including tetracycline derivatives (Cianfrocca *et al*, 2002) or bisphosphonates (Heikkilä *et al*, 2002) would be worthy of investigation also as chemopreventive agents in patients at high risk of developing oral cancer.

The future of MMPs and oral diseases

The story of MMP family members in oral diseases is far from complete – it seems that it has only just begun. For example, several molecular forms of MMP-8 isoenzymes

and their multiple active forms in periodontits plaque, GCF and PISF have been identified (Sorsa *et al*, 1995; Kiili *et al*, 2002; Kivelä-Rajamäki *et al*, 2003a,b). Future studies should examine the synthesis, role and inhibition of how these different MMP-8 isoenzymes function *in vivo*. The possibility to use the local MMP inhibition to prevent dentinal caries progression or loss of adhesive restorations remains to be studied. The role of MMPs in oral cancer still requires basic research to find the right combination of MMP inhibitors or possible activators. Further development of diagnostic technology may allow the use of one or more MMPs, TIMPs and tissue degradation product(s) in a combination chair-side tests or mouthrinse screening-tests for periodontitis and peri-implantitis (Kivelä-Rajamäki *et al*, 2003a,b; Emingil *et al*, 2004a,b), and may also be adapted for monitoring of other diseases (Prikk *et al*, 2002; Apajalahti *et al*, 2003; Holopainen *et al*, 2003).

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