MATRIX METALLOPROTEINASES (MMPS) IN ORAL CARCINOMAS

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University of Oulu

OULU 2005
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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Auditorium 1 of the Institute of Dentistry, on May 28th, 2005, at 12 noon

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2005
Oulu, Finland

Abstract
Matrix metalloproteinases, MMPs, are a family of enzymes capable of modulating connective tissue
components. The expression of several MMPs is increased in oral squamous cell carcinomas
(OSCCs). They are assumed to have an important role in the development and progression of OSCCs.
However, the exact role and mechanism of the regulation of MMPs in malignant transformation are
still largely unknown.

In this study, tumour-associated trypsin-2 (TAT-2) was detected in OSCC tissue sections, and its
role in MMP-2 and -9 regulation in carcinoma cells was evaluated. The TAT-2 gene was transfected
into two different OSCC cell lines and one immortalized oral epithelial cell line. In TAT-2-
transfected cells, MMP-9 activation increased OSCC cell invasion in chicken chorionallantoic
membrane assay. Increased invasation was prevented by tumour-associated trypsin inhibitor or
specific gelatinase-inhibiting CTT-peptide. TAT-2 also converted MMP-1, -8, -13 and -3 into smaller
molecular weight forms in vitro. However, TAT-2-transfected OSCC cells showed no conversion.
TAT-2 was demonstrated to degrade powerfully type I collagen into small fragments in vitro. The cell
surface receptor \(\alpha_v\beta_6\) integrin is strongly up-regulated in OSCCs. By using \(\beta_6\)-transfected OSCC
cells, it was demonstrated that \(\alpha_v\beta_6\) integrin down-regulates MMP-13 expression. However, this
integrin did not regulate other collagenses or TIMP-1. \(\beta_6\)-transfected cells invaded more efficiently
through the basement membrane matrix, but their migration through type I collagen remained
unchanged. MMP-8 expression was detected for the first time in head and neck squamous cell
carcinoma (HNSCC) cell lines and corresponding cultured dermal and tumour fibroblasts. The
localization of MMP-8 in HNSCC was determined by immunohistochemical stainings and in situ
hybridization. MMP-8 production levels in carcinoma cells were faint and sporadic in HNSCCs
sections. Ninety-two primary mobile tongue SCCs were subjected to MMP-8 immunohistochemical
staining, and the staining results were compared to survival rates. MMP-8 was associated with
improved disease-free survival in females but not in males.

Keywords: carcinoma; squamous cell, collagen type I, extracellular matrix, head and neck
neoplasms, integrin \(\alpha_v\beta_6\), matrix metalloproteinases, mouth neoplasms, neoplasm
invasiveness, survival, trypsinogen
To Pekka and Lauri
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Oulu, March 2005

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<table>
<thead>
<tr>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MEM</td>
<td>alpha-medium</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ADAM</td>
<td>a disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>a disintegrin and metalloproteinase with thrombospondin-1-like motif</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>API</td>
<td>α1-protease inhibitor or α1-antitrypsin</td>
</tr>
<tr>
<td>APMA</td>
<td>p-aminophenylmercuric acetate</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C-</td>
<td>carboxy-</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CAM</td>
<td>chorioallantoic membrane</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned medium</td>
</tr>
<tr>
<td>CME</td>
<td>cell membrane extract</td>
</tr>
<tr>
<td>CMT</td>
<td>chemically modified tetracycline</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>CTT</td>
<td>CTTHWGFTLC peptide</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DF</td>
<td>dermal fibroblast</td>
</tr>
<tr>
<td>D-MEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DDT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMMPRIN</td>
<td>extracellular matrix metalloproteinase inducer</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGM</td>
<td>fibroblast growth medium</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GTG</td>
<td>gold thioglucose</td>
</tr>
<tr>
<td>HFF</td>
<td>human foreskin fibroblast</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor, scatter factor</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>IFMA</td>
<td>immunofluorometric method</td>
</tr>
<tr>
<td>IL-</td>
<td>interleukin-</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KGM</td>
<td>keratinocyte growth medium</td>
</tr>
<tr>
<td>LAP</td>
<td>latency associated peptide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MMPI</td>
<td>matrix metalloproteinase inhibitor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane-type matrix metalloproteinase</td>
</tr>
<tr>
<td>OB</td>
<td>odontoblast</td>
</tr>
<tr>
<td>OSCC</td>
<td>oral squamous cell carcinoma</td>
</tr>
<tr>
<td>PC</td>
<td>proprotein convertase</td>
</tr>
<tr>
<td>PAR-</td>
<td>proteinase-activated receptor-</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SF</td>
<td>scatter factor, hepatocyte growth factor</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSC</td>
<td>standardized saline citrate</td>
</tr>
<tr>
<td>TAT-</td>
<td>tumour-associated trypsinogen-</td>
</tr>
<tr>
<td>TATI</td>
<td>tumour-associated trypsin inhibitor</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TGF-</td>
<td>transforming growth factor-</td>
</tr>
<tr>
<td>TF</td>
<td>tumour fibroblast</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>TP</td>
<td>Trizol® purified total protein</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl phorphol 13-acetate</td>
</tr>
<tr>
<td>TPCK</td>
<td>N-Tosyl-L-phenylalanyl chloromethyl ketone</td>
</tr>
<tr>
<td>TRITC-</td>
<td>tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.


In addition, some unpublished results are presented.

Article I is reprinted from Journal of Dental Research, 81: 831–835 (2002) with permission from the International Association of Dental Research / American Association of Dental Research.

Article II is reproduced with permission from Biochemistry 42: 5414–5420. Copyright 2003 American Chemical Society.

Article IV is reproduced from Journal of Pathology, 197: 72–81 (2002) with permission. Copyright John Wiley and Sons Ltd.
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References
1 Introduction

Oral squamous cell carcinoma (OSCC) is an aggressive disease, which invades and metastasizes very efficiently, and the prognosis of OSCC patients is thus often poor. In OSCC tissue, the delicate balance between the production, activation and inhibition of proteolytic enzymes is often irreversibly disturbed by conditions enabling uncontrolled growth of tumour tissue.

In this thesis, OSCC covers all epithelial cancers whose primary tumour is inside the mouth, including the tongue and other sites of oral mucosa. Head and neck squamous cell carcinomas (HNSCCs) were also used as study material in this thesis. HNSCCs include carcinomas of the larynx and pharynx in addition to OSCCs.

Matrix metalloproteinases (MMPs) are a genetically distinct but structurally related family of zinc-dependent proteolytic enzymes, which very efficiently degrade almost all extracellular matrix (ECM) components and play a pivotal role in tumorigenesis. The role of MMPs in tumour biology is wider than has previously been assumed. They do not only facilitate the breakdown of ECM but also participate in apoptosis, angiogenesis, release of growth-promoting signals and modulation of immune responses. (Nabeshima et al. 2002, Egebald & Werb 2002.) Moreover, some MMPs modulate basement membrane by cleaving the γ2-chain of laminin-5, which, in turn, contributes to cancer cell migration (Katayama & Sekiguchi 2004). In several cancers the production and activity levels of various MMPs are increased compared to normal tissues. However, the exact role and regulation mechanisms of each MMP in OSCC are not known.

Tumour-associated trypsinogen-2 (TAT-2, trypsinogen-2) is a tumour-derived trypsinogen, which shares many properties with its pancreatic counterpart (Koivunen et al. 1989). TAT-2 exists in many cancers, including ovarian carcinomas, but also in normal tissues, such as the male genital tract (Koivunen et al. 1989, Paju et al. 2000). The role of TAT-2 in cancers is not known, but one role could be an ability to act as an activator of other enzymes. TAT-2 has been found to be an efficient activator of MMP-9 (Sorsa et al. 1997).

Integrin αvβ6 is a cell surface receptor, which is highly up-regulated during mucocutaneous wound healing and malignant epithelial transformation of the kind seen in OSCC. Integrin αvβ6 is not expressed in normal oral mucosa. It participates, together with other integrins, in binding cancer cells to ECM components, and it has been
assumed that $\alpha_v\beta_6$ integrin has a role in OSCC invasion. $\alpha_v\beta_6$ integrin can promote the expression of proteolytic enzymes, e.g. MMP-3 and -9, in OSCC cells. (Breuss et al. 1995, Thomas et al. 2001b, Ramos et al. 2002.)

In this work, the role of TAT-2 in MMP-9 activation was studied in an in vivo invasion model by using tongue carcinoma cells. Moreover, the role of TAT-2 in collagenase (MMP-1, -8, -13) and MMP-3 activations was analyzed. The role of $\alpha_v\beta_6$ integrin in collagenase and TIMP-1 regulation was determined by using $\beta_6$-transfected and control OSCC cells and clinical OSCC samples. The expression and regulation of MMP-8 in OSCC were evaluated by analyzing the MMP-8 mRNA expression of established HNSCC cells and subjecting one cell line to TGF-β1 and PMA, using immunohistochemical stainings and in situ hybridization of OSCC tissue sections. Finally, the role of MMP-8 in tongue cancer was studied by comparing immunohistochemical stainings with clinicopathological variables.
2 Review of the literature

2.1 Concise classification of proteolytic enzymes

Proteolytic enzymes, peptidases or proteases are currently classified based on three major criteria: 1) the reaction catalysed by the protease, 2) the chemical nature of the catalytic site, and 3) the evolutionary relationship of the protease primary structure. When the classification is based on the catalysed reaction, proteases can be divided into two groups, exopeptidases and endopeptidases. Exopeptidases act only near the ends of polypeptide chains, but endopeptidases act rather in the inner regions of peptide chains. Endopeptidases are further separated into five main groups: serine, threonine, cysteine, aspartic and metalloproteinases. The metzincin superfamily is one of the five subfamilies of metalloproteinases, and metzincins are further subdivided into four enzyme families: serralysins, actacins, adamalysins and matrix metalloproteinases (MMPs) (Table 1). (Barret 1994, Barret et al. 1998, Strenlicht & Werb 2001.)

Table 1. Classification of endopeptidases and some examples of the known enzyme members of the main groups (Barret et al. 1998, Sternlicht & Werb 2001).

<table>
<thead>
<tr>
<th>Main groups of endopeptidases</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine peptidases</td>
<td>TAT-2, chymotrypsin, uPA, tPA, plasmin</td>
</tr>
<tr>
<td>Threonine peptidases</td>
<td>archaean proteasome</td>
</tr>
<tr>
<td>Cysteine peptidases</td>
<td>papain, cathepsins B, L, S, K, H and T, caspases</td>
</tr>
<tr>
<td>Aspartic peptidases</td>
<td>pepsin, cathepsins D and E, renin</td>
</tr>
<tr>
<td>Metalloproteinases</td>
<td></td>
</tr>
<tr>
<td>Metzincin superfamily</td>
<td>bacterial enzymes</td>
</tr>
<tr>
<td>Serralysins</td>
<td>bone morphogenetic protein-1 (BMP-1)</td>
</tr>
<tr>
<td>Astacins</td>
<td>ADAMs and ADAMTSs</td>
</tr>
<tr>
<td>Adamalysins</td>
<td>MMPs and MT-MMPs</td>
</tr>
</tbody>
</table>
2.1.1 Matrix metalloproteinases (MMPs)

Nowadays, MMPs are classified into subclasses in two ways based on either substrate specificity or structure. The classification based on substrate specificity is called “classical”, and it is used in this dissertation. The classical classification divides the 23 human MMPs into collagenases, gelatinases, stromelysins, matrilysins, membrane-type matrix metalloproteinases (MT-MMPs) and other MMPs (Table 2).

MMPs contain several different functional domains (Figure 1). 1) The N-terminal signal peptide or predomain directs MMP synthesis inside the cell, and it is removed before MMPs are secreted. The N-terminal end of MMP-23 has a unique transmembrane domain (signal anchor) targeting it to the cell membrane (Pei et al. 2000). 2) The prodomain (about 80 amino acids) maintains the enzyme in an inactive state by a conserved PRCXXPD sequence whose cysteine ligates to the catalytic zinc of the catalytic domain. According to Marchenco and coworkers (Marchenco et al. 2002), MMP-26 has a unique PHCGXXD cysteine switch, which interacts with the zinc ion of the catalytic domain. Some MMPs (MMP-11, -21, -23, -28 and MT-MMPs) contain a furin cleavage site RX(R/K)R in a prodomain, indicating that these proenzymes can be activated intracellularly by furin and other proprotein convertases (PCs) (Pei et al. 2000, Lohi et al. 2001, Ahokas et al. 2002). 3) Catalytic domain (about 175 amino acids other than gelatinases) contains a conserved zinc-binding region HEBXHXBGXH (typically consisting of three histidines, which bind the catalytic zinc ion) and a conserved methionine (“Met-turn”). In addition, the catalytic domain typically consists of an additional structural zinc ion and 2-3 calcium ions. The catalytic domain determines the substrate specificity of MMPs through its active site cleft, through specific sub-site pockets that bind amino acid residues immediately adjacent to the scissile peptide bond, and through secondary substrate-binding exosites located outside the active site itself. MMP-2 and MMP-9 have three fibronectin-type-II domain inserts in the catalytic domain. 4) A hinge or linker domain (up to 70 amino acids) connects the catalytic domain to the hemopexin domain. It also influences substrate specificity. MMP-21 and MMP-23 have no linker domain. 5) The hemopexin domain (about 195 amino acids) binds TIMPs and certain substrates and participates in membrane activation and some proteolytic activities. MMP-7 and -26 have no hemopexin domain. Cysteine-rich, proline-rich and immunoglobulin (Ig)-like domains replace the hemopexin domain in MMP-23. 6) The transmembrane domain is a specific domain for MT-MMPs. In addition, MT-MMPs have a short cytoplasmic C-terminal tail (MT1-, MT2-, MT3-, MT5-MMP) or a C-terminal cytoplasmic region, which acts as a glycosphatidylinositol (GPI) membrane-anchoring signal (MT4- and MT6-MMP). MMPs may be glycosylated to different extents and different sites, which influences their molecular weight. (Hanemaaijer et al. 1997, Nagase & Woessner 1999, Sternlicht & Werb 2001, Bode & Maskos 2003, Stamenkovic 2003.)
2.1.2 Regulation of MMPs

Most MMPs are produced at low levels or not at all in resting-state adult tissues. However, during physiological and pathological events, such as embryogenesis,
ovulation, tooth eruption, arthritis and malignancy, their production is induced. MMP expression is a strictly regulated, complex event, and it is controlled at several different levels, including transcription, activation and inhibition of the active enzyme, see figure 2.

Fig. 2. Regulatory levels of MMPs. Different regulatory signals interact with specific receptors at the cell surface and initiate a cascade of cellular events leading to the generation of functional MMPs, which are localized to the cell surface (MT-MMPs) or secreted into the extracellular space. Secreted MMPs can be further activated, inhibited or bound to cell surface receptors. (Modified from Overall & López-Otín 2002)

2.1.2.1 Transcriptional regulation

Transcriptional MMP regulation is a consequence of the binding of various cytokines, growth factors, hormones and other soluble or solid cell surface mediators to receptors. These mediators usually act by paracrine or direct cell-cell interactions with target receptors. The signal from target receptors to the promoter region of MMP genes can use several specific protein kinase cascades, such as mitogen-activated protein kinases (MAPKs) or serine/threonine kinases, which activate specific transcription factors in the nucleus. Finally, these transcription factors bind to specific regulatory elements in the promoter region of the MMP gene and achieve transcriptional changes. Though there are many similarities between the MMP promoters, there also are some differences between the different MMPs. The most striking differences are seen in the number of individual transcription factor binding sites and their spatial arrangement and mutual proximity in the gene promoter region. Activator protein-1 (AP-1) and polyoma enhancer A-binding protein-3 (PEA-3) are examples of these regulatory elements. AP-1 constitutes the
phorbol ester-responsive element (TRE) and binds dimers of the Fos and Jun transcription factor families, and responses to, for instance, phorbol esters, including PMA. AP-1, are shown by several MMP promoters, except MMP-2, -11, -21, -26, -28 and MT1-MMP. The PEA-3 element, in turn, binds members of ETS transcription factors, and it is found in almost all inducible MMP promoters except the MMP-12 promoter. There are also several other “common” regulatory elements of MMPs, such as the classical TATA-binding site, and some MMP-specific regulatory elements, such as the osteoblast-specific element-2 (OSE-2) in the MMP-13 gene promoter. (Benbow & Brinckerhoff 1997, Lohi et al. 2000, 2001, Sternlicht & Werb 2001, Vincenti 2001, Chakraborti et al. 2003, Marchenko et al. 2003.) Single nucleotide polymorphisms (SNPs) and other genetic variations in the promoter region of MMP genes can influence the transcriptional regulation of MMPs (Ye 2000).

MMP regulation is also modulated at the post-transcriptional level. For example, MMP-1 and MMP-3 mRNA transcripts are stabilized if signaling is mediated through p38α (Reunanen et al. 2002). There are also mechanisms that inhibit translation. For example, the TIAR-a protein (T-cell-restricted intracellular antigen-related protein) affects the 3'-untranslated region in the MMP-13 transcript and inhibits translation (Yu et al. 2003). MMP expression can also be regulated by alternative splicing, as in MMP-8, or by alternative polyadenylation. (Hu et al. 1999, Sternlicht & Werb 2001, Vincenti 2001.)

2.1.2.2 Activation of MMPs

MMPs can be activated in either extracellular or intracellular space, depending on the structure of MMP. Extracellular activation can also occur in association with the cell membrane. Most MMPs are secreted from the cell in a proenzyme form, in which an unpaired cysteine (Cys73) sulfhydryl group acts as a fourth ligand in addition to the three histidines for the active site zinc ion of the catalytic domain. Activation requires that this cysteine-to-zinc switch is opened and/or removed proteolytically or non-proteolytically. Once this switch is opened, the Cys73-thiol group is replaced by a H2O molecule, which allows interactions between MMP and its activators/substrates. (Nagase 1997, Sternlicht & Werb 2001.)

MMP activation usually occurs in a stepwise manner. In a non-proteolytical activation cascade (activators in vitro include SH reactive agents, denaturants, heat treatment and in vivo reactive oxygen species), several intermediates can be generated. This usually means initial conformational changes in MMPs or cleavage or removal of a propeptide. Finally, a fully active MMP is achieved by applying another proteolytic enzyme, often another MMP. Activation of proMMPs by proteases also generates intermediates. An activator protease attacks the proteinase-susceptible “bait” region located in the middle of the propeptide. This cleavage induces conformational changes and renders the final activation, in which the second protease (often another MMP) cleaves the propeptide away. In both non-proteolytical and proteolytical activation, MMP can be autoactivated after an initial intermediate form. The fully active MMP usually has phenylalanine (Phe) or tyrosine (Tyr) at the N-termini of the first amino acid. (Van Wart & Birkedal-Hansen 1990, Nagase 1997, Sternlicht & Werb 2001, Chakraborti et al. 2003.) There is also
evidence that only the opening of the cysteine-to-zinc switch or the binding of MMP to its substrate achieves MMP activation without a loss of molecular weight (Sang et al. 1995, Bannikov et al. 2002, Fedarko et al. 2004).

Tumour-associated trypsin-2, also called trypsin-2 or TAT-2, activates very efficiently proMMP-9 and, to a lesser extent, proMMP-2 in vitro (Sorsa et al. 1997). Moreover, this trypsin also activates proMMP-20 (Väinämönen et al. 2001). TAT-1 and -2 activate and can be activated by the uPA/plasmin system in vitro (Koivunen et al. 1989, Uchima et al. 2003). The uPA/plasmin system converts several proMMPs into their active forms (Table 2).

There is accumulating evidence to suggest that the cell surface association may be critical for optimal MMP function. Activation of MMP-2 is the classical example of cell membrane-associated MMP activation. MMP-2 is associated with the MT1-MMP/TIMP-2 complex, following initial activation achieved by an adjacent MT1-MMP. Partially activated MMP-2 is fully activated by another MMP-2 molecule. (Strongin et al. 1993, Sternlicht & Werb 2001.) Furthermore, MT1-MMP can activate MMP-8 and -13 (Knäuper et al. 1996, Holopainen et al. 2003). Other cell surface binding sites have been found for various MMPs, i.e. EMMPRIN for MMP-1, CD44, αvβ3 integrin and the α2(IV) chain of collagen for MMP-9, αvβ3 integrin for MMP-2 and CD44 for MMP-7 (Brooks et al. 1996, Olson et al. 1998, Yu & Stamenkovic 1999, Guo et al. 2000, Rolli et al. 2003). Recent findings indicate that MMP-8 can also be membrane-bound on PMN cells. Membrane-bound MMP-8 presents in both pro and active forms, but no MMP-8-binding ligand is known (Owen et al. 2004).

At least MMP-11, -21, -23, -28 and MT-MMPs can be activated intracellularly by furin or other proprotein convertases (PCs). These MMPs include a furin cleavage site in their prodomain. (Van Wart & Birkedal-Hansen 1990, Nagase 1997, Pei et al. 2000, Sternlicht & Werb 2001, Lohi et al. 2001, Ahokas et al. 2002.)

There is evidence that microbiological proteinases can activate human proMMPs and thus induce tissue destruction in bacterial infections. For example, certain proteases of periodontopathogens can activate proMMP-1, -3, -8 and -9. (Sorsa et al. 1992, DeCarlo et al. 1997).
Table 2. Human MMPs, their subgroups (according to substrate specificity), molecular sizes, activators and activating capacity (in vitro).

<table>
<thead>
<tr>
<th>Name (specific name)</th>
<th>Size latent/active (kDa)</th>
<th>Activated by</th>
<th>Activators of</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collagenases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1 (collagenase-1, tissue collagenase, interstitial collagenase, FIB-CL)</td>
<td>53/43</td>
<td>MMP-3, -10, -7 plasmin, kallikrein chymase, trypsin</td>
<td>MMP-2, -9</td>
</tr>
<tr>
<td>MMP-8 (collagenase-2, neutrophil collagenase, PMN-CL)</td>
<td>85/64, 75/58, 55/46, 42</td>
<td>MMP-3, -10, MMP-14, cathepsin G, trypsin, chymotrypsin, kallikrein</td>
<td>MMP-2, -9</td>
</tr>
<tr>
<td>MMP-13 (collagenase-3, Col-3)</td>
<td>65/55, 48</td>
<td>MMP-2, -3, -10, -14, -15, plasmin</td>
<td>MMP-2, -9</td>
</tr>
<tr>
<td><strong>Gelatinases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2 (gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase)</td>
<td>72/67, 62</td>
<td>MMP-1, -7, -13, -14, -15, -16, -17, -24, -25, bone sialoprotein, thrombin</td>
<td>MMP-9, -13</td>
</tr>
<tr>
<td>MMP-9 (gelatinase B, 92-kDa gelatinase)</td>
<td>92/67, 65</td>
<td>MMP-1, -2, -3, -7, -10, -13, -26, cathepsin G, dentin matrix protein-1, TAT-2, trypsin, α-chymotrypsin, plasmin</td>
<td></td>
</tr>
<tr>
<td><strong>Stromelysins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-3 (stromelysin-1, proteoglycanase, SL-1, transin-1, collagenase activator protein)</td>
<td>60, 57/45, 28</td>
<td>plasmin, kallikrein, chymase, tryptase, trypsin, osteopontin</td>
<td>MMP-1, -7, -8, -9, -13</td>
</tr>
<tr>
<td>MMP-10 (stromelysin-2, SL-2, transin-2)</td>
<td>56/47, 24</td>
<td>elastase, cathepsin G, plasmin</td>
<td>MMP-1, -7, -8, -9, -13</td>
</tr>
<tr>
<td>MMP-11 (stromelysin-3, SL-3)</td>
<td>58/28</td>
<td>furin, PACE4</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 Continued.

<table>
<thead>
<tr>
<th>Name (specific name)</th>
<th>Size latent/active (kDa)</th>
<th>Activated by</th>
<th>Activators of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrilysins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-7 (matrilysin, PUMP-1, Mat, uterine metalloproteinase)</td>
<td>28/19</td>
<td>MMP-3, -10, leukocyte elastase, trypsin, plasmin</td>
<td>MMP-2-, 8, ADAM28, MMP-9, MMP-1, MMP-9/TIMP-1 complex</td>
</tr>
<tr>
<td>MMP-26 (matrilysin-2, endometase)</td>
<td>29/19</td>
<td>trypsin, plasmin</td>
<td>MMP-9</td>
</tr>
<tr>
<td>MT-MMPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1-MMP (MMP-14)</td>
<td>66/60</td>
<td>plasmin, furin</td>
<td>MMP-2-, 8, 13</td>
</tr>
<tr>
<td>MT2-MMP (MMP-15)</td>
<td>68/62</td>
<td>furin</td>
<td>MMP-2</td>
</tr>
<tr>
<td>MT3-MMP (MMP-16)</td>
<td>63/59</td>
<td>furin</td>
<td>MMP-2</td>
</tr>
<tr>
<td>MT4-MMP (MMP-17)</td>
<td>70,67</td>
<td>furin</td>
<td>MMP-2, ADAMTS-4</td>
</tr>
<tr>
<td>MT5-MMP (MMP-24)</td>
<td>73/64</td>
<td>furin</td>
<td>MMP-2</td>
</tr>
<tr>
<td>MT6-MMP (MMP-25, leukolysin)</td>
<td>56/45,38</td>
<td>furin</td>
<td>MMP-2</td>
</tr>
<tr>
<td>Other MMPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-12 (metalloelastase, macrophage elastase, MME)</td>
<td>54/45, 29, 22</td>
<td>trypsin, plasmin, thrombin</td>
<td></td>
</tr>
<tr>
<td>MMP-19 (RASIC, stromelysin-4)</td>
<td>57</td>
<td>trypsin</td>
<td></td>
</tr>
<tr>
<td>MMP-20 (enameleycin)</td>
<td>54/43</td>
<td>TAT-2, M14</td>
<td>kallikrein-4</td>
</tr>
<tr>
<td>MMP-21</td>
<td>62/49</td>
<td>furin</td>
<td></td>
</tr>
<tr>
<td>MMP-23 (A and B, CA-MMP)</td>
<td>56, 50, 46</td>
<td>furin</td>
<td></td>
</tr>
<tr>
<td>MMP-27</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-28 (Epilysin)</td>
<td>58, 55, 52/48</td>
<td>furin</td>
<td></td>
</tr>
</tbody>
</table>

2.1.2.3 Inhibition of MMPs

The activity of MMPs can be inhibited by endogenous and exogenous inhibitors. Endogenous inhibitors originate from different human cells, and exogenous inhibitors are synthetized as therapeutic agents. In this chapter, endogenous inhibitors are presented briefly. Later, in chapter 2.1.5.2, different exogenous synthetic inhibitors will be presented.

MMPs have several endogenous inhibitors, among which tissue inhibitors of metalloproteinases (TIMPs) make up the main group. There are four mammalian TIMPs, TIMP-1 to -4. The TIMPs have molecular weights of 21–28 kDa and are variably glycosylated. They have a two-domain structure, which is formed of 12 separated cysteine residues with six disulphide bonds. The inhibitory activity of the TIMPs resides almost exclusively in the N-terminal domain. However, both domains take part in enzyme-inhibitor binding. The C-terminal domain usually binds to the hemopexin domain of MMPs and the N-terminal domain to the catalytic domain of MMPs. Like MMPs, TIMPs can also associate with cell membrane proteins or ECM proteins, with the exception of TIMP-1, which is only found in an unbound form. TIMP-3 is always associated with ECM proteins, for example, heparan-sulphate-containing proteoglycans. TIMPs inhibit MMPs reversibly in a 1:1 stoichiometric fashion. Their ability to inhibit different MMPs varies. TIMP-1 and -3 can also inhibit some ADAM and ADAMTS enzymes. TIMPs also have other functions apart from MMP inhibition. They can affect cell proliferation, apoptosis and angiogenesis and stimulate tumour metastasis formation. (Sternlicht & Werb 2001, Baker et al. 2002.)

α2-macroglobulin is an abundant plasma protein and the major inhibitor of MMPs in tissue fluids. It is mainly synthesized in the liver by hepatocytes. α2-macroglobulin forms complexes with MMPs, and these complexes are removed by scavenger receptor-mediated endocytosis. One scavenger receptor is LDLR-related protein (LRP), which acts as a receptor for MMP-2, MMP-9 and MMP-13 (Hertz & Strickland 2001, Yang et al. 2001, Hahn-Dantona et al. 2001). MT1-MMP can degrade LRP and may thus regulate extracellular protease activity (Rozanov et al. 2004). Type I collagen C-proteinase enhancer protein (PCPE), tissue factor pathway inhibitor 2 (TFPI-2), noncollagenous NC1 domain of type IV collagen and thrombospondin-1 and -2 are also endogenous MMP inhibitors. RECK (reversion inducing cysteine-rich protein with Kazal motifs) inhibits MMP-2 and MMP-9 activation. (Sternlicht & Werb 2001, Baker et al. 2002.)

2.1.3 MMPs in oral squamous cell carcinoma (OSCC)

MMPs have several roles in malignant transformation and tumour progression. They can modulate different proteins and thereby regulate cancer biology at several levels (Figure 3). For example, MMPs release cell-membrane bound precursors of growth factors, modulate apoptosis mediators, produce angiogenesis inhibitors by cleavage or allow endothelial cell invasion into tumour tissue. They also degrade ECM and BM
components, allowing cancer cells to migrate and to invade, and modulate the immune response by suppressing lymphocyte proliferation. (Egeblad & Werb 2002.) Moreover, genetic alterations in the promoter region of MMPs can modulate MMP transcription and change expression significantly (Ye 2000).

MMPs contribute to epithelial and carcinoma cell migration by cleaving laminin-5, which is a major component of hemidesmosomes in BM and promotes static adhesion between the epithelium and BM. Laminin-5 consists of α3-, β3- and γ2-chains. At least MMP-2, -3, -13, -20 and MT1-MMP can cleave the γ2-chain. The cleaved γ2-chain may stimulate migration of carcinoma cells by binding to EGFR (Shenk et al. 2003, Koshikawa et al. 2005). Immunohistological and in situ hybridization studies have shown that the laminin-5 γ2-chain is expressed in the cytoplasm of carcinoma cells at the advancing edge of the tumour. This expression correlates with invasiveness of the tumour. Thus, it has been assumed that MMP-mediated processing of laminin-5 may be important for carcinoma progression. (Koshikawa et al. 2000, Pirilä et al. 2003, Katayama & Sekiguchi 2004.)

In the following chapter, the expression and role of each MMP group will be reviewed individually based on the publications describing their involvement in head and neck squamous cell carcinomas (HNSCCs), including oral squamous cell carcinomas (OSCC). Based on the literature, nothing definitive is known about the exact roles of MMPs in OSCC. In fact, the research findings are partly contradictory, and the number of analyzed cases is often small and the methods variable (Werner et al. 2002).

![Fig. 3. MMPs modulate cancer biology at different levels. (Modified from Overall & López-Otín 2002 and Katayama & Sekiguchi 2004)](image-url)
2.1.3.1 Collagenases

No MMP-1 mRNA or protein production has been seen in normal oral mucosa. In situ carcinomas and dysplasias show low levels of MMP-1 production in stromal fibroblasts and epithelial cells. In OSCC, MMP-1 mRNA and protein levels are higher than in in situ carcinomas and dysplasias, and MMP-1 localizes mainly in fibroblastic cells of tumoral stroma and only occasionally in cancer cells. 57-100% of OSCCs are positive for MMP-1 mRNA or protein. (Polette et al. 1991, Muller et al. 1991, Gray et al. 1992, Charous et al. 1997, Sutinen et al. 1998, Ziober et al. 2000, Shimada et al. 2000, Franchi et al. 2002.) Nonetheless, some authors have reported that the MMP-1 protein localizes mainly in tumour cells (Kurahara et al. 1999). MMP-1 expression has also been detected in stromal and cancer cells in lymph node metastases of OSCC (Sutinen et al. 1998). When 96 biopsy samples were analyzed by immunohistological staining, increased MMP-1 expression was detected in the highly invasive cases compared to less invasive ones. The number of stained cells correlated positively with metastatic lymph node involvement. (Kurahara et al. 1999.) When analyzed by cDNA microarray analysis, the amount of MMP-1 mRNA has been shown to be greater in OSCCs with lymph node involvement than in non-metastatic cancers. Intense expression of MMP-1 mRNA has been proposed to predict the risk of cervical lymph node metastasis in OSCCs. (Nagata et al. 2003.) O-charoenrat et al. (2001) found a statistically significant relationship between MMP-1 mRNA expression and higher T stage tumour classification (T3-T4) but not between MMP-1 expression and lymph node involvement. The earlier studies have revealed no correlation between the amount of MMP-1 mRNA and the T stage or lymph node involvement (Muller et al. 1991, Charous et al. 1997). Recently, an association between MMP-1 2G polymorphism and the presence of OSCC or HNSCC lesion was discovered (Hashimoto et al. 2004, Lin et al. 2004a). Especially non-buccal OSCC patients have a 2G genotype in the MMP-1 promoter region (Lin et al. 2004a). However, differing from these findings, Zinzindohoué et al. (2004) found the MMP-1 2G genotype to be associated with a lower risk of HNSCC, especially in cases which also involve MMP-3 6A polymorphism at position –1171 bp. Two alleles, 2G and 1G, were formed by the insertion/deletion of a guanine at position –1607 bp. The 2G polymorphism increases significantly MMP-1 transcriptional activity.

MMP-13 is not produced in normal oral mucosa. MMP-13 mRNA has been found in HNSCCs, including OSCCs, by Northern blot and in situ hybridizations. MMP-13
mRNA is mainly seen in cancer cells at the invasive front and, to a lesser extent, in stromal fibroblasts. (Johansson et al. 1997.) Recent findings indicate that the MMP-13 protein also localizes to malignant keratinocytes (Impola et al. 2004). Johansson et al. (1997) demonstrated that MMP-13 may be associated with a high invasive capacity of SCCs, especially in early primary tumour development. Moreover, Impola et al. (2004) suggested that the number of MMP-13-positive cells increases as the tumour becomes more invasive. Nonetheless, O-charoenrat et al. (2001) failed to find an association between the expression of MMP-13 mRNA and any of the clinicopathological variables they studied.

2.1.3.2 Gelatinases

MMP-2 mRNA is detected mainly in fibroblasts near the invasive front of OSCC cells and, to a lesser extent, in endothelial cells by using in situ hybridization. MMP-2 transcript is also seen in lymph nodes around tumour cells. MMP-2 transcript is conspicuously absent within tumour cells, and normal oral mucosa is negative for MMP-2 mRNA. (Charous et al. 1997, Sutinen et al. 1998.) Imanishi et al. (2000) also found MMP-2 mRNA in tumour cells. Using a semiquantitative RT-PCR method, O-charoenrat et al. (2001) found the amount of MMP-2 mRNA to correlate weakly with lymph node involvement and advanced stage of HNSCC tumours. Charous et al. (1997) and Imanishi et al. (2000) did not find a statistically significant relationships between MMP-2 mRNA levels and tumour size or lymph node status. However, Imanishi et al. (2000) found that MMP-2 mRNA levels are higher in carcinomas of histologic grade G3 and G2 than in G1 tumours. Recently, it has been demonstrated that subjects carrying CC polymorphism at -1306 in the MMP-2 promoter region have an approximately two-fold risk for developing OSCC and an even higher risk for non-buccal OSCC. C (cytocine) transition to T (tymidine) in the promoter region of MMP-2 eliminates the promoter activity. (Lin et al. 2004b.)

MMP-2 protein localizes mainly in tumour cells and, to a lesser extent, in fibroblasts surrounding the cancer nests and endothelial cells, as detected by immunohistological staining. In metastasized lymph nodes some cancer cells are immunopositive for MMP-2. Normal oral epithelium is either negative or faintly immunopositive for MMP-2. 39.2–100% of OSCCs are MMP-2-positive. When analyzed by gelatin zymography, the total amount of MMP-2 protein is higher in OSCC than in normal oral mucosa tissue. Using in situ zymography, gelatinolytic activity localizes in the carcinoma cell nests, and weaker activity is also detected in the stromal components adjacent to the carcinoma. (Kusukawa et al. 1993, Sutinen et al. 1998, Kurahara et al. 1999, Pickett et al. 1999, Shimada et al. 2000, Hong et al. 2000, Imanishi et al. 2000, Yoshizaki et al. 2001, Franchi et al. 2002, Ondruschka et al. 2002, Impola et al. 2004, Katayama et al. 2004.) Immunostaining for type IV collagen is decreased in the basement membrane zone in the OSCCs in which immunostaining is increased for MMP-2 and MMP-9 (Kurahara et al. 1999).

Increased MMP-2 immunostaining has been found to be associated with lymph node involvement and diffuse invasion of HNSCCs, OSCCs and tongue SCCs (Kusukawa et al. 1993, Kurahara et al. 1999, Yoshizaki et al. 2001). MMP-2 immunoreactivity is
significantly related to MT1-MMP expression (Kurahara et al. 1999, Yoshizaki et al. 2001). In tongue SCC, MMP-2 immunoreactivity also correlates with advanced clinical stage, and tongue cancer patients with low expression levels of MMP-2, MT1-MMP and TIMP-2 have significantly longer disease-free survival (Yoshizaki et al. 2001). However, Ondruschka et al. (2002), Imanishi et al. (2000) and Hong et al. (2000) failed to detect a statistically significant correlation between MMP-2 immunostaining and lymph node involvement. Instead, they found the expression of MMP-2 at the invasive front to be a marker of poor survival, which appears to be associated with early recurrence in initially lymph node-negative patients and also correlates with the histologic grade of OSCC.

Several studies have aimed to resolve whether the degree of MMP-2 activation associates with the metastatic potential or other clinicopathological factors of OSCC. Shimada et al. (2000), Tokumaru et al. (2000) and Kawamata et al. (1998) found that the MMP-2 activation ratio (active/active+proMMP-2) is significantly higher in metastatic than in non-metastatic cancers. However, according to Ikebe et al. (1999), Yorioka et al. (2002) and Hong et al. (2000), there is no such association between the degree of MMP-2 activation and the metastatic potential. Yorioka et al. (2002) demonstrated that patients whose tumours produce elevated MMP-2 activity have significantly shorter disease-free survival than patients with low activity. Furthermore, they found that MMP-2 and MMP-9 activities do not associate with gender, tumour stage, tumour localization, tumour differentiation, death or proliferative potential of the tumour. Yoshizaki et al. (2001) did not find active MMP-2 in normal or non-metastatic tissues, but only in tumours and metastatic lymph nodes in tongue SCC, and the degree of MMP-2 activation assessed by gelatin zymography showed a significant correlation with the immunohistochemical staining of MMP-2, MT1-MMP and TIMP-2.

In normal oral epithelia, trace amounts of MMP-9 protein and mRNA are seen in gelatin zymography, RT-PCR and immunohistological staining. (Charous et al. 1997, Sutinen et al. 1998, Pickett et al. 1999, Hong et al. 2000, O-charoenrat et al. 2001, Franchi et al. 2002, Tsai et al. 2003, Impola et al. 2004.) MMP-9 mRNA and protein levels are significantly higher in OSCC tissues than in normal oral mucosa (Sutinen et al. 1998, Shimada et al. 2000, O-charoenrat et al. 2001). MMP-9 mRNA localizes in both tumour and stromal cells (Charous et al. 1997). According to O-charoenrat et al. (2001), the mRNA levels of both MMP-2 and MMP-9 are higher in advanced disease compared with early SCC lesions. Furthermore, in primary lesions, higher MMP-9 mRNA expression correlates with higher T classification, an infiltrating pattern of growth and the presence of lymph node involvement. (O-charoenrat et al. 2001.) In a study of ten OSCC patients assessed by RT-PCR from laser-captured microdissection of cancer tissue and immunomagnetic separation of circulating cancer cells, MMP-9 was detected in 50% of cancer tissues and was the only analyzed MMP (out of MMP-1, -2, -7 and -9) in circulating cancer cells (10%) (Ito et al. 2003). Semi-quantitative RT-PCR revealed that the mRNA levels of epidermal growth factor receptor (EGFR) and c-erbB-2, which belong to the c-erbB receptor family, correlate strongly with MMP-9 expression and also with MMP-2, -7, -10 and MMP-2, -10, -11, -13 expressions in HNSCCs, respectively (O-charoenrat et al. 2002a).

cancer cells in invading cancer nests and, to a lesser extent, in stromal cells, including inflammatory and endothelial cells. In one study, however, immunostaining occurred mainly in stromal cells (Kurahara et al. 1999). Hong et al. (2000), and Kurahara et al. (1999) reported that cancers with lymph node metastasis show stronger immunopositivity for MMP-9 than cancers without metastasis. Moreover, Kurahara et al. (1999) demonstrated that lymph node involvement probably increases when the expression levels of several MMPs (MMP-1, -2, -3, -9 and MT1-MMP) increase simultaneously. Ruokolainen et al. (2004) did not find an association between MMP-9 immunopositivity and the stage or grade of disease, tumor size or cervical lymph node involvement. Interestingly, they found during a long follow-up period (5 years) that MMP-9 immunoreactivity associates with shorter relapse-free and cause-specific survival in HNSCC. Riedel et al. (2000a) also found that MMP-9 immunoreactivity correlates with poorer survival of HNSCC patients and, moreover, with microvessel density and vascular endothelial growth factor (VEGF) immunoreactivities, suggesting that MMP-9 participates in the regulation of tumor angiogenesis. Franchi et al. (2002) documented the presence of a correlation between MMP-9 expression, microvessel density, activity of the nitric oxide pathway and p53 mutation. Riedel et al. (2000a) compared the localization, T stage, N stage and histological grading of 52 HNSCC tumours to MMP-9 immunostaining and found no association.

Elevated MMP-9 activity detected by gelatin zymography associates with short disease-free survival, and cancers with an aggressive invasion mode possess elevated proMMP-9 levels (Ikebe et al. 1999, Yorioka et al. 2002). Hong et al. (2000) pointed out that OSCCs with metastasis produce an activated form of MMP-9 at significantly higher levels than normal mucosa or OSCCs without metastasis. Riedel et al. (2000b) found MMP-9 serum concentrations to be significantly higher in 86 HNSCC patients than controls. Moreover, MMP-9 levels are higher in patients with an advanced stage of tumour than in patients with an early stage of cancer. However, there are no differences in serum levels between positive and negative lymph node tumours. MMP-2 serum concentrations do not differ between HNSCC patients and controls. Ranuncolo et al. (2002) also found elevated MMP-9 levels from the plasma of HNSCC patients compared to controls.

Robinson et al. (2003) investigated whether the amounts of MMP-2 and -9 in OSCC cells cultured on plastic correlate with their cellular invasion and motility in vitro and tumour behaviour in vivo. The expression of MMP-2 and -9 correlated weakly with tumour cell invasion and motility in vitro, but did not correlate with tumorigenicity and metastatic potential in an in vivo mouse model.

### 2.1.3.3 Stromelysins

MMP-3 mRNA and protein are produced by OSCCs, but normal oral epithelium is almost negative (Kusukawa et al. 1995, Impola et al. 2004). MMP-3 mRNA localizes in epithelial cancer cells inside the tumour and at the invasive front. The stromal cells around the tumour also express MMP-3 mRNA. (Impola et al. 2004.) According to O-charoenrat et al. (2001), there is no association between MMP-3 mRNA levels and any of
the clinicopathological factors studied, although MMP-3 mRNA levels are significantly higher in primary tumours than in normal oral mucosa and metastasized lymph nodes. Using cDNA microarray analysis, Nagata et al. (2003) showed that MMP-3 mRNA levels are 15 times higher in OSCC tissues compared with normal oral mucosa. In addition, MMP-3 is one of the genes whose levels increase in OSCC tissues with lymph node metastasis compared with OSCCs without metastasis. (Nagata et al. 2003.) In immunohistochemical studies, 42.6–88.5% of OSCCs are MMP-3-positive (Kusukawa et al. 1995, 1996, Kurahara et al. 1999). Kusukawa et al. (1995, 1996) observed that MMP-3 protein localizes to small cancer nests at the advancing front of tumour tissue, but Kurahara et al. (1999) found MMP-3 immunoreactivity mainly in stromal cells. Immunoreactivity is faint or negative in normal oral epithelia. Many associations have been demonstrated in OSCCs between MMP-3 protein expression and other factors, including the clinical stage of the tumour, tumour thickness, diffuse invasion type, lymph node metastasis, quantity of EGF receptors and TIMP-1 protein expression (Kusukawa et al. 1995, 1996, Kurahara et al. 1999).

MMP-10 has been demonstrated in OSCC tissue by Northern blot hybridization, in situ hybridization and RT-PCR methods (Muller et al. 1991, Polette et al. 1991, Birkedal-Hansen et al. 2000, O-charoenrat et al. 2001). In Northern blot and in situ hybridization, the probes recognized both MMP-3 and MMP-10 mRNAs, and the exact localization of MMP-10 is therefore not known based on these studies. In spite of this unspecificity, Muller et al. (1991) and Polette et al. (1991) concluded that MMP-10 mRNA is not present in normal oral mucosa. In carcinoma tissues, MMP-10 localizes in stromal fibroblasts and tumour cells. Birkedal-Hansen et al. (2000) detected MMP-10 mRNA in 2 out of 7 adjacent normal tissues, when all tumor samples expressed MMP-10 mRNA. Muller et al. (1991), using Northern blot hybridization, demonstrated that high levels of MMP-10 mRNA correlate with a high degree of differentiation and high local invasiveness of tumours. However, using RT-PCR, O-charoenrat et al. (2001) failed to find any associations between MMP-10 expression and clinicopathological variables.

MMP-11 mRNA and protein localize mainly in fibroblasts adjacent to carcinoma cells and, to some extent, in spindle-shaped epithelial tumour cells. MMP-11 transcript has not been detected in stromal cells surrounding in situ carcinomas. (Muller et al. 1993, Polette et al. 1993, Soni et al. 2003.) Soni et al. (2003) did not find detectable levels of MMP-11 protein in normal oral mucosa, whereas Birkedal-Hansen et al. (2000) detected MMP-11 mRNA in 6 out of 7 normal mucosa samples. The expression pattern of MMP-11 protein is assumed to depend on the differentiation stage of cancer; in well-differentiated OSCCs MMP-11 protein localizes in stromal cells in close contact with keratin-positive tumour cells, while in poorly differentiated OSCCs MMP-11 protein is widespread throughout the tumour. 57% of precancerous oral lesions and 70% of OSCCs are MMP-11-immunopositive according to Soni et al. 2003. Muller et al. (1993) showed that there is a highly positive correlation between the MMP-11 mRNA levels and the local invasiveness of the tumour. A positive correlation between MMP-11 protein and mRNA expression and lymph node metastasis has also been found, whereas no significant association between MMP-11 immunopositivity and survival has been observed. (O-charoenrat et al. 2001, Soni et al. 2003.) Moreover, Soni et al. (2003) assumed that MMP-11 expression correlates with increased microvessel density in precancerous and carcinomatous lesions. Spindle-shaped cells, which express MMP-11, also express
fibroblastic and epithelial cell markers, suggesting that these cells might have undergone epithelial-to-mesenchymal conversion.

2.1.3.4 Matrilysins

MMP-7 mRNA and protein have been detected in carcinoma cells, metastatic lymph nodes and normal oral mucosa in OSCCs. The amounts of MMP-7 mRNA and protein are higher in carcinoma tissues than in normal oral mucosa. (Muller et al. 1991, Birkedal-Hansen et al. 2000, O-charoenrat et al. 2001, Impola et al. 2004.) 50% of OSCC samples express MMP-7 (Muller et al. 1991). Impola et al. (2004) found that MMP-7 protein locates in epithelial cells, particularly in well-differentiated areas at the invasive front of cancer cell nests and also in stromal fibroblastic-like cells. MMP-7 mRNA correlates weakly with lymph node metastasis (O-charoenrat et al. 2001). Using Northern blot hybridization, Pacheco et al. (2002) showed that c-jun mRNA expression correlates directly with MMP-7 and -9 mRNA levels, indicating that c-jun may be involved in the regulation of these MMPs.

MMP-26 protein locates at the edge of invasive cancer nests of well-differentiated tumours, while in verrucous carcinomas it is expressed in basal keratinocytes (Impola et al. 2004).

2.1.3.5 Membrane-type MMPs

The first in situ hybridization study with MT1-MMP probe in HNSCCs revealed that MT1-MMP transcript locates only in stromal fibroblast-like cells (Okada et al. 1995), but MT1-MMP mRNA has since been found mainly in carcinoma cells and, to a lesser extent, in stromal fibroblasts and endothelial cells surrounding cancer nests. Normal oral mucosa does not show any hybridization signals. (Imanishi et al. 2000, Shimada et al. 2000, Myoung et al. 2002.) Using RT-PCR or Northern blot analysis, MT1-MMP has been revealed in 59.5–100% of tumour tissues and 16.7–71% of normal mucosa (Birkedal-Hansen et al. 2000, Imanishi et al. 2000, Shimada et al. 2000). O-Charoenrat et al. (2001) did not find differences between MT1-MMP mRNA levels in tumours and control tissues. Moreover, they did not find associations between the expression of MT1-MMP and any of the clinicopathological variables studied. Differing from these findings, Imanishi et al. (2000) and Shimada et al. (2000) found that OSCCs with lymph node metastasis produce higher levels of MT1-MMP mRNA than cancers without metastasis. In addition, the mRNA levels of MT1-MMP correlated directly with the degree of proMMP-2 activation. (Imanishi et al. 2000, Shimada et al. 2000.) The immunostaining pattern of MT1-MMP is similar to that in in situ hybridization analysis; the stain locates in tumour cells and in some fibroblasts and endothelial cells. 45.7–95.8% of OSCCs and 35.3% of tongue SCCs are immunopositive for MT1-MMP. The increased intensity of MT1-MMP immunostaining correlates positively with lymph node metastasis, advanced clinical stage and invasion grade, a high degree of differentiation and the expression of
2.1.3.6 Other MMPs

MMP-12 mRNA has been found in macrophages and, to a lesser extent, cancer cells analyzed by in situ hybridization (Impola et al. 2004). MMP-19 has been demonstrated in cultured OSCC cells by the RT-PCR method (Grant et al. 1999) and in OSCC tissue samples by immunohistochemical stainings (Impola et al. 2004). MMP-19 localizes in epithelial keratinocytes in hyperproliferative areas, but it is down-regulated in invasive cancer cell nests. MMP-19 is also expressed by endothelial cells and fibroblasts. (Impola et al. 2004.) MMP-20 mRNA and protein have been found in cultured tongue SCC cells by RT-PCR and Western blotting (Väänänen et al. 2001).

2.1.4 Regulation of MMP level and activity in OSCC

Not much is known about actual in vivo regulation and function of MMPs. Most published studies and those discussed below are based on in vitro experiments.

2.1.4.1 Transcriptional regulation in OSCCs

Stromal fibroblasts and tumour cells communicate with each other through different factors, such as cytokines, receptors and other molecules. Westermarck et al. (2000) showed that OSCC cells secrete soluble factor(s), which initiate the MMP-1 production by fibroblasts. EMMPRIN, a highly glycosylated transmembrane molecule, belongs to the immunoglobulin superfamily and is produced by carcinomatous and epithelial cells. This causes at least MMP-2 synthesis in fibroblasts. (Bordador et al. 2000.) Fibroblasts can also initiate MMP production by OSCC cells. Lengyel et al. (1995) were the first to report that fibroblasts secrete certain protein(s) which cause MMP-9 production in OSCC cells. Mucosal fibroblasts produce hepatocyte growth factor (HGF), also known as scatter factor, which stimulates the invasion of OSCC cells and leads to the expression of E1AF transcription factor in OSCC cells. Increased E1AF mRNA production correlates with increased production of MMP-1, -3 and -9 mRNAs. (Matsumoto et al. 1994, Shindoh et al. 1996, Hanzawa et al. 2000.) Bennett et al. (2000) found that HGF stimulates MMP-2 and -9 production in normal and malignant oral keratinocytes, and that these cells also synthesize c-met mRNA. c-met is a cell surface receptor for HGF. Hayashido et al. (2003a) demonstrated that fibroblasts induce MT1-MMP production on the OSCC cell
surface by secreting soluble factor(s). Thus, fibroblasts enhance the abilities of OSCC cells to bind and activate MMP-2. Hayashido et al. (2003b) observed that thrombospondin-1, which is a mainly fibroblast-derived glycoprotein, stimulates MMP-9 synthesis in OSCC cells. On the other hand, treatment of fibroblasts with a conditioned medium of OSCC cells leads to marked induction of thrombospondin-1 synthesis. (Hayashido et al. 2003b.)

Different cell-surface receptors regulate MMP synthesis in OSCC. Cadherins are a family of cell surface adhesion molecules, which participate in Ca²⁺-dependent cell-cell adhesion (Goodwin & Yap 2004). Munshi et al. (2002a) pointed out that, when E-cadherin-mediated adhesive contacts between cells begin to form, the expression of MMP-9 and uPA is suppressed in premalignant oral keratinocytes. E-cadherin forced expression is also associated with decreased MT1-MMP mRNA production and MMP-2 activation in tongue SCC cells after E-cadherin transfection, and this alteration might be mediated through suppression of the MAPK cascade and/or cytoskeletal organization. (Ara et al. 2000.) Snail is one of the recently identified E-cadherin repressors and a zinc finger transcription factor that triggers the epithelial-mesenchymal transition (Nieto 2002). Yokoyama et al. (2003) transfected the Snail vector to E-cadherin-positive OSCC cells and found increased expressions of MMP-2 and vimentin (mesenchymal marker), decreased expression of E-cadherin, alteration of cell morphology towards a spindle shape and increased invasion capacity. Bair et al. (2001) used co-cultures of human tongue SCC cells and foreskin fibroblasts to demonstrate that cell-cell and cell-matrix interactions are important for MMP-7 expression, and that cell-cell interactions need calcium for MMP-7 production. Moreover, they found that N- and E-cadherins and β₁ integrins participate in the expression of MMP-7. (Bair et al. 2001.) Type I receptor tyrosine kinases (EGFR, c-erbB-1, c-erbB-2, c-erbB-3 and c-erbB-4) are frequently involved in the genesis and progression of human tumours. It has been found that HNSCC cells produce ligands for these receptors, and ligand binding leads to up-regulation of MMP-1, 3, 7, 9, 10, 11, 13 mRNAs, but they have minimal or no effects on the synthesis of MMP-2, MT1-MMP, TIMP-1 and TIMP-2 mRNAs. MMP-9 mRNA appears to rise to a higher level than the other MMPs. Moreover, parts of these ligands increase invasion through Matrigel in proportion to their MMP up-regulation. In addition, a strong correlation has been found between the level of EGFR expression and the in vitro invasive capacity of HNSCC cells. (O-charoenrat et al. 1999, 2000a, 2000b.) Others have also reported that EGF causes MMP-1 protein production in tongue SCC cells (Ziober et al. 2000). Tsuzuki et al. (1998), Tomita et al. (2000) and Sugimoto et al. (2001) demonstrated that OSCC cells can produce granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating (GM-CSF) receptors, and that recombinant G-CSF or GM-CSF initiates the synthesis of MMP-2 or MMP-2 and MT1-MMP, respectively, in OSCC cells. The presence of G-CSF receptor correlates with lower disease-free and survival rates in OSCCs (Tsuzuki et al. 1998). It has been found that OSCC cells produce interleukin-8 (IL-8) and IL-8 receptors. IL-8 leads to the production of MMP-7 but not MMP-2 and -9 at mRNA and protein levels (Watanabe et al. 2002).

Thomas et al. (2001a, 2001b) have demonstrated that αvβ₆ integrin expression leads to increased MMP-9 production and somewhat also MMP-2 secretion in OSCC cells and in normal oral keratinocytes. Ramos et al. (2002) found that β₆ integrin transfection into tongue SCC cells is associated with increased MMP-3 production significantly and does
not affect MMP-2 or -9 expression. Li et al. (2003) demonstrated that the ligation of β6 integrin with fibronectin activates the transcription of MMP-3.

Gum et al. (1997) and Simon et al. (1998) showed that regulation of MMP-9 expression requires a protein kinase family, MAPK, in OSCC cells. The MAPK family is composed of the ERKs, JNKs and the p38 MAPK subfamilies, all of which have partially their own signal pathways and substrate specificities and participate in the regulation of MMP-9. At least the ERK and JNK pathways regulate MMP-9 via AP-1 transcription factor. p38 MAPK has been shown to regulate PMA-induced MMP-9 secretion and MMP-9-related invasion of OSCC cells. (Gum et al. 1997, Simon et al. 1998.) It has been proposed that overexpression of JunB alters the dynamics of the AP-1 complex and achieves, among the other effects, increased expression of MMP-9 (Robinson et al. 2001). The p38 MAPK and JNK-2 pathways also mediate MMP-1 production in fibroblasts (Westernmarck et al. 2000). It has been assumed that proteasomes mediate TNF-α-induced MMP-9 production, and that the transcription-activating factor NF-κB mediates this cascade (Ikebe et al. 1998). Wilson et al. (2003) demonstrated that endostatin activates the transcription factors NF-κB and AP-1 in a cell line-dependent fashion in OSCC cells, and this decreases the expression of α2, β1 integrins and MMP-10 cell line-dependently and diminishes cell migration and invasion in vitro. Acetaldehyde, the first metabolite of ethanol, markedly increases MMP-11 mRNA expression and activates Jun/AP-1 expression and its DNA binding activity in HPV-immortalized oral keratinocytes (Timmons et al. 2002).

2.1.4.2 Regulation of MMP activity in OSCC

Cancer cells activate fibroblast-derived proMMP-2 probably by MT1-MMP (Tokumaru et al. 2000). Higher calcium concentrations also lead to MMP-2 activation in a dose-dependent manner without altering zymogen production, and this activation is MT1-MMP-dependent (Munshi et al. 2002b). Type I collagen has been found to activate proMMP-2 in two- and three-dimensional OSCC cultures (Kinsenn et al. 2003). Integrin α, can mediate the binding of active MMP-2 to the surface of OSCC cells (Hayashido et al. 2003a).

Bassi et al. (2001a) observed that furin levels correlate positively with the increased invasive potential of HNSCC cells and tumour samples, and furin expression also correlates with the conversion of MT1-MMP to an active form. Later, Bassi et al. (2001b, 2003) transfected a selective furin inhibitor, alpha 1-PDX, or furin to HNSCC cells and found that alpha 1-PDX diminishes the activation of MMP-2 and MT1-MMP, and overexpression of furin increases the activation of MT1-MMP, MMP-2 and, surprisingly, also MMP-9. By controlling the degree of activation of MT1-MMP by modulating the furin amount, it is possible either to diminish or to increase the invasion of HNSCC cells in vitro and in vivo. Aznavoorian et al. (2001) and Rosenthal et al. (1999) presented that an active MT1-MMP/MMP-2 system is essential for the degradation of interstitial type I collagen and participates in SCC invasion.

Dang et al. (2004) transfected β6 integrin into tongue SCC cells and found that the expression of β6 is associated with increased activation of the uPA→MMP-3→MMP-9
pathway. On the contrary, Dalvi et al. (2004) found that \( \beta_6 \) transfection into OSCC cells reduces uPAR expression and down-regulates cell surface plasmin generation.

Nyberg et al. (2003) demonstrated that endostatin, which is a 20 kDa C-terminal fragment of type XVIII collagen, binds to recombinant proMMP-9 and inhibits the activation of proMMP-9 and the formation of smaller (~40 kDa) activation products of MMP-2 in tongue SCC cells. Endostatin also inhibits the activation of recombinant MMP-13, but has no effect on recombinant MMP-8. Endostatin inhibits the migration of tongue SCC cells in vitro and intravasation in the chicken chorioallantoic membrane (CAM) model.

### 2.1.5 Inhibition of MMPs in OSCC

#### 2.1.5.1 TIMPs in OSCC

The first published results on the localization and expression of TIMP-1 mRNA in OSCC or HNSCC differed significantly. Gray et al. (1992) found low TIMP-1 mRNA expression in fibrous connective tissue, and Polette et al. (1993) found TIMP-1 mRNA in well-differentiated invasive cancer clusters and in some endothelial cells. In later studies, TIMP-1 mRNA and protein localized mainly in stromal and endothelial cells (Charous et al. 1997, Sutinen et al. 1998, Kurahara et al. 1999). TIMP-1 immunostaining has been detected in both tumour epithelia and peritumoural stroma (Ondruschka et al. 2002). TIMP-1 transcript has not been found in normal oral mucosa, lichen planus or dysplastic lesions by in situ hybridization (Sutinen et al. 1998). However, by using RT-PCR and sandwich enzyme immunoassay, TIMP-1 mRNA and protein have been found in both tumour and normal mucosa samples, although tumour tissue produces TIMP-1 significantly more than normal oral mucosa (Shimada et al. 2000, Birkedal-Hansen et al. 2000, O-charoenrat et al. 2001). In lymph node metastasis, TIMP-1 mRNA locates in stromal cells surrounding cancer cells (Sutinen et al. 1998). Ikebe et al. (1999) demonstrated that OSCCs with high protein levels of gelatinases and low levels of TIMP-1 may have a tendency to metastasize. However, Schmalbach et al. (2004) analyzed metastatic and non-metastatic SCCs of oral cavity and oral pharynx by an oligonucleotide microarray method and found that TIMP-1 mRNA is expressed over three times more intensely in metastatic carcinomas than in non-metastatic carcinomas. Moreover, elevated levels of TIMP-1 and TIMP-2 in immunostainings correlate positively with the total malignancy score, which grades the degree of keratinization and nuclear polymorphism, tumour-stroma interaction, pattern of invasion and lymphocytic infiltration (Ondruschka et al. 2002). O-charoenrat et al. (2001) found that increased TIMP-1 mRNA expression in primary HNSCC shows a statistically significant relationship with a higher tumour T classification (T3-T4), and Kurahara et al. (1999) found a relationship between the degree of TIMP-1 staining, the presence of lymph node metastasis and the expression of MMP-1, -3 and -9. Based on cell culture studies, O-charoenrat et al. (2000a) demonstrated that the EGFR signalling pathway might play an important role in the invasive behaviour of HNSCC cells via, for instance, specific down-regulation of TIMP-
1. Nii et al. (2000) also demonstrated with cell experiments that TIMP-1 overexpression decreases the metastatic and invasive abilities of OSCC cells.

TIMP-2 mRNA localizes in stromal fibroblasts, some inflammatory cells and endothelial cells in HNSCCs. Normal tissue samples are negative in in situ hybridization. (Polette et al. 1993.) Sutinen et al. (1998) did not find TIMP-2 mRNA or protein in OSCC samples. According to Kurahara et al. (1999) and Shimada et al. (2000), TIMP-2 protein localizes mainly in tumour cells. Localization in both stroma and tumour epithelia has also been described (Ondruschka et al. 2002). In tongue SCC, 43% of samples are TIMP-2-immunopositive. The stain locates mainly in tumour cells, but also in endothelial cells and stromal fibroblasts. (Yoshizaki et al. 2001.) The TIMP-2 mRNA and protein levels in tumour tissue and control epithelia have been found to be similar (Birkedal-Hansen et al. 2000, Shimada et al. 2000, O-charoenrat et al. 2001). On the other hand, there has been a statistically significant correlation between TIMP-2 expression and local tumour invasion (Ondruschka et al. 2002). In HNSCCs, there is no significant relationship between the degree of TIMP-2 staining and nodal involvement or between MMP-2 and MT1-MMP expression and TIMP-2 expression (Kurahara et al. 1999). Moreover, O-charoenrat et al. (2001) failed to find an association between TIMP-2 expression and any of the clinicopathological variables studied. In tongue SCC, however, TIMP-2 expression correlates with nodal status, clinical stage and MMP-2 and MT1-MMP expressions together. Tongue SCCs with high TIMP-2 expression in immunohistological stainings produce abundant active MMP-2 in gelatin zymography. Tongue SCC patients with low expression of MMP-2, MT1-MMP and TIMP-2 have longer disease-free survival compared to patients with high expression levels. (Yoshizaki et al. 2001.) Munshi et al. (2002b) demonstrated that calcium decreases soluble TIMP-2 levels in a dose-dependent manner in OSCC cells, affecting TIMP-2 regulation at the post-translational level. MT1-MMP participates in TIMP-2 degradation in the presence of a high calcium concentration. (Munshi et al. 2002b.)

TIMP-3 protein and mRNA localize in stromal cells surrounding cancer nests (Sutinen et al. 1998). Birkedal-Hansen et al. (2000) found TIMP-3 mRNA expression in both tumour tissue and adjacent normal mucosa by RT-PCR. Almost all cancer samples express high levels of TIMP-3 mRNA, but less than half of normal mucosa samples contain it. The same samples do not contain TIMP-4 mRNA.

The use of protein-based TIMP therapy as cancer treatment is limited because TIMPs suffer from poor pharmacokinetics and their administration is difficult. Moreover, TIMPs are multifunctional proteins, which means that they may have paradoxical effects during treatment (Overall & López-Otín 2002).

2.1.5.2 Synthetic MMP inhibitors

There are many theoretical approaches to inhibit MMPs either at the gene level or by blocking MMP activation. MMP gene transcription can be prevented by influencing the extracellular factors that involved in MMP synthesis or by activating the suppressors of MMP expression. Moreover, the signalling pathways of cytokines and growth factors, which up-regulate MMPs, can be hindered. It is also possible to affect MMP transcription
by inhibiting the signal transduction pathways that mediate MMP induction. One strategy is to influence either general or MMP-specific nuclear factors that regulate MMP genes. Gene therapy methods, such as ribozymes or antisense constructs, can also be used to regulate MMP production. ProMMP activation can be inhibited by blocking MMP-activating proteases, e.g. plasmin, proprotein convertases, such as furin, or trypsins. Another strategy to block MMP activation is to use factors that inhibit directly proMMP activation, such as endostatin, or factors that promote MMP catabolism or clearance. (Overall & López-Otín 2002.)

The first synthesized MMPIs mimic the structure of collagen at the site where MMPs bind and cleave collagen. These peptidomimetic MMPIs have a hydroxamic acid zinc-binding group, which potently, competitively and reversibly binds to the active site of MMPs. Peptidomimetic MMPIs include at least batimastat (BB-94), marimastat (BB-2516), GM6001, CT1746 and KB-R7785. These peptidomimetics inhibit a broad spectrum of MMPs. Non-peptidomimetic inhibitors were designed by using a structurally based approach that allows the discovery of more selective compounds. This group contains at least prinomastat (AG3340), BMS275291, BAY12-9566, Ro 32-3555, and CGS 27023A. Unfortunately, most of these MMPIs have been ineffective or have had serious side effects during clinical trials, and their development has therefore been discontinued. Some of these MMPIs have been used only in pre-clinical trials. (Pavlaki & Zucker 2003, Peterson 2004.)

There are also other MMPIs, such as neovastat, bisphosphonates, tetracyclines, chemically modified tetracyclines (CMTs) and a CTTHWGFTLC peptide. Neovastat has been isolated from shark cartilage, and it inhibits collagenases and gelatinases and further inhibits the VEGF signalling pathway. Bisphosphonates and tetracyclines, which were originally designed for other clinical purposes, also have inhibitory efficacy against MMPs. Bisphosphonates affect the zinc-coordinating properties of MMPs and inhibit a broad spectrum of MMPs. Tetracyclines inhibit both the synthesis and the activity of MMPs, and they inhibit at least collagenases as well as MMP-2, -9 and -12, but not other proteinase classes, such as serine proteinases. A tetracycline analog, Periostat®, which lacks anti-bacterial activity, but inhibits MMP activities, is the only synthetic MMPI that has been licensed. The indication for this drug is periodontitis. CMTs have been developed from tetracyclines by removing the side-chain required for antimicrobial activity. Currently, at least 10 different CMTs have been generated with different properties. (Golub et al. 1998, Overall & López-Otín 2002, Pavlaki & Zucker 2003, Peterson 2004.)

A synthetic decapeptide, CTTHWGFTLC (CTT peptide), inhibits selectively MMP-2 and MMP-9 and has no effect on MMP-8, -13 and MT1-MMP or on serine proteinases trypsin-2, elastase and cathepsin-G. CTT peptide has been isolated from phage display peptide libraries. It has a cyclic form, and it contains the HWGF sequence, which is a selective inhibitor of MMP-2 and -9. It inhibits the migration of HT1080 fibrosarcoma, C8161 melanoma, SKOV-3 ovarian carcinoma and KS1767 Kaposi’s sarcoma as well as endothelial cell lines. Moreover, it inhibits in vitro invasion of HT1080 and C8161 cells. In an in vivo mouse model, CTT peptide delays tumour formation and growth induced by breast carcinoma, Kaposi’s sarcoma or ovarian carcinoma cells and increases the survival of tumour-bearing animals. This peptide also has an ability to home and target to tumours. In cell and animal experiments, CTT peptide has not been found to have toxic
effects. (Koivunen et al. 1999.) Because CTT peptide is hydrophobic, it incorporates in phospholipids and liposomes. It has been shown that gelatinase-expressing carcinoma cells effectively take up CTT-bearing liposomes. When adriamycin, a widely used anticancer drug, is encapsulated into CTT liposomes, it helps to target this therapeutic agent to tumour cells. (Medina et al. 2001.) In addition, CTT peptide blocks β₂ integrin-dependent leukocyte migration in vitro (Stefanidakis et al. 2003).

2.1.5.3 Synthetic MMPIs in OSCC

No clinical trials on synthetic MMPIs in the treatment of OSCC have been published. The known MMPIs and various other compounds have been tested in OSCC cell cultures and animal experiments and evaluated for efficacy in view of MMP levels, cancer cell behaviour or tumour growth. Many different MMPI compounds have been used in these studies, but there are few repeated studies with the same compounds and methods, and it is therefore difficult to summarize the results. In OSCC cell experiments, marimastat, batimastat, protein kinase C inhibitors staurosporine and H7, immunosuppressive factors like dexamethasone and interleukin-4, inostamycin (inhibitor of phosphatidylinositol turnover) and genestein (a tyrosine kinase inhibitor) have been tested (Rosenthal et al. 1998, Baba et al. 2000, Beppu et al. 2002, O-charoenrat et al. 2002b, Kinsenn et al. 2003, Tsai et al. 2003, Myoung et al. 2003). Marimastat inhibits MMP-2 activation in both two- and three-dimensional type I collagen cultures, inhibits the colony formation of OSCC cells and inhibits the proliferation of HNSCC cells by preventing the release of EGFR ligands (O-charoenrat et al. 2002b, Kinsenn et al. 2003). Batimastat suppresses the invasion of EGF- and SF-stimulated OSCC cells without affecting MMP-1, -3, -9 and MT1-MMP expression or cell motility across collagen-coated surfaces (Rosenthal et al. 1998).

Mouse experiments have been made to test, for instance, genestein, ONO-4817, ERK1 inhibitor (PD 098059) and marimastat (Simon et al. 1999, Maekawa et al. 2002, Myoung et al. 2003, Yamashita et al. 2003). ONO-4817 suppresses the formation of cervical lymph node metastasis and MMP-9 activation (Yamashita et al. 2003). ERK1 inhibitor (PD 098059) reduces the invasion of OSCC cells, and this reduction is associated with diminished amounts of MMP-9 in tumour tissue (Simon et al. 1999). Marimastat does not affect tumour size. However, marimastat-treated mice have less lymph node metastasis and longer survival than control mice without marimastat treatment. Moreover, tumours in marimastat-treated mice show a lower degree of proliferation and MMP-2 activation than tumours in control mice. (Maekawa et al. 2002.)

Gene therapy methods regulate MMP levels in HNSCC cells. When wild-type p53 gene is delivered to HNSCC cells with mutated p53 by adenoviral gene delivery, MMP-1 and MMP-13 productions decrease remarkably and invasion through Matrigel is prevented by 35% (Ala-aho et al. 2002). The targeted inhibition of MMP-13 by antisense ribozyme in cutaneous HNSCC cells inhibits invasion through Matrigel and promotes apoptosis. Targeted MMP-13 inhibition also suppresses the growth of carcinoma and the proliferation of cancer cells in vivo. Moreover, the gelatinolytic activity of carcinomas
diminishes in vivo. The idea of the antisense ribozyme method is that it specifically cleaves the human MMP-13 transcript. (Ala-aho et al. 2004.)

2.1.6 Tumour-associated trypsinogens (TATs, trypsinogens) and their inhibitor TATI in cancer growth

2.1.6.1 Trypsinogens

The trypsinogens-1, -2 and -3 are the major trypsinogen genes in the human pancreas (Sheele et al. 1981). The fourth trypsinogen, trypsinogen-4, was originally identified in human brain and may be a splice variant of trypsinogen-3. It is also expressed in pancreas. (Wiegand et al. 1993.) Trypsinogens belong to the serine protease superfamily, and they are activated to trypsins by membrane-bound peptide enterokinase (also called enteropeptidase) in the small intestine (Lu et al. 1999). Trypsins catalyse the hydrolysis of the peptide bond on the carboxyl side of lysine (K) or arginine (R) residues (Barret et al. 1998). Stenman et al. (1988) detected and Koivunen et al. (1989) subsequently purified and sequenced from ovarian tumour cyst fluid two trypsinogens that are probably encoded by the same genes as the corresponding pancreatic trypsinogen-1 and -2 (Sorsa et al. 1997). Moreover, the production of extrapancreatic trypsinogen-4 has been described in tumour-derived epithelial cell lines (Cottrell et al. 2004). Tumour-associated trypsinogens-1 and -2 (TAT-1 and -2 or trypsinogen-1 and -2) correspond to pancreatic trypsinogens in amino acid sequences, molecular size and immunoreactivity. However, substrate specificity, susceptibility to inhibition by protease inhibitors and isoelectric points differ between tumour-associated and pancreatic trypsinogens. The molecular sizes of trypsinogen-1 and -2 are 25 and 28 kDa, respectively. (Koivunen et al. 1989.) See Table 3. In extracellular fluids, the activity of trypsin is controlled by several inhibitors, including α1-protease inhibitor (API), also called α1-antitrypsin, and α2-macroglobulin (Ohlsson 1988). Tumour-associated trypsin inhibitor (TATI) is an efficient inhibitor of tumour-derived trypsinogens (Stenman 2002).

Extrapancreatic trypsinogen-1 and/or -2 expression has been detected in vascular endothelial cells and epithelial cells of the skin, esophagus, stomach, small intestine, lung, kidney, liver and extrahepatic bile duct as well as splenic and neuronal cells (Koshikawa et al. 1997, 1998). Paju et al. (2000) demonstrated that trypsinogen-1 and -2 are expressed in the human male genital tract. Trypsinogen-1 and/or -2 have also been found from many cancer cell lines and clinical cancer samples. Colon adenocarcinoma, erythroleukemia, fibrosarcoma, gastric, and breast cancer cell lines produce trypsinogens in a cell line-dependent manner (Koivunen et al. 1991a, Koshikawa et al. 1994, Miyata et al. 1999). At least ovarian, gastric, colorectal and biliary tract cancers as well as cholangiocarcinomas and lung neoplasms produce trypsinogen-1 and/or -2 (Koivunen et al. 1990, Hedström et al. 1996, 1999, Kawano et al. 1997, Miyata et al. 1999, Ichikawa et al. 2000, Oyama et al. 2000).
The overexpression of trypsinogens stimulates cellular growth and adhesion to fibronectin and vitronectin and correlates with the malignant phenotype of gastric cancer cells (Kato et al. 1998, Miyata et al. 1998). Trypsinogen-2 levels are higher than trypsinogen-1 levels in ovarian cyst fluids of malignant and benign tumours. Moreover, trypsinogen-1, trypsinogen-2, trypsin-1-API (trypsin-1-α1-antitrypsin-complex, which reflects the correlation of active trypsin) and trypsin-2-API levels are higher in malignant ovarian cyst fluid than in benign cyst fluids. High concentrations of active MMP-9 correlate with high concentrations of trypsinogen-2, trypsin-1-API and trypsin-2-API, whereas active MMP-2 concentrations associate inversely with trypsinogen concentrations. (Koivunen et al. 1990, Paju et al. 2001.) Moreover, the concentration of MMP-8 but not MMP-1 or -13 correlates with ovarian tumour malignancy and elevated trypsinogen-2 levels in ovarian cyst fluids (Stenman et al. 2003). Immunohistological staining has also revealed that the amount of trypsinogen-1 is higher in ovarian carcinomas than in low malignant-potential tumours, whereas benign tumours and normal ovaries are negative (Hirahara et al. 1998). Trypsinogen-1, trypsinogen-2, trypsin-1-API or trypsin-2-API concentrations are higher in the serum of patients who have linitis plastica (one aggressive subtype of gastric cancer), biliary tract cancer or cholangiocarcinoma compared to healthy patients or patients with benign disease or other types of gastric cancer (Hedström et al. 1996, 1999, Ichikawa et al. 2000). In colorectal cancer, there is no relationship between trypsinogen immunoreactivity and the clinicopathological findings (Oyama et al. 2000). Expression of trypsin-2 is also seen in diseases other than cancers, including bronchial epithelial cells, monocytes and macrophages in chronic inflammatory airway disease, labial salivary glands in Sjögren’s syndrome and bronchopulmonary dysplasia in infants (Konttinen et al. 1998, Prikk et al. 2001, Cederqvist et al. 2003).

Koivunen et al. (1991b) demonstrated that trypsin-2 can participate in the degradation of extracellular matrix in vitro. Both trypsin-1 and -2 are capable of activating pro-uPA (Koivunen et al. 1989). Moreover, uPA activates trypsin-1 in pancreatic cancer cells (Uchima et al. 2003). Trypsin-2 activates efficiently MMP-9 and, to a lesser extent, MMP-2 and degrades TIMP-1 and -2 (Sorsa et al. 1997). Trypsin-2 also activates MMP-20 (Väänänen et al. 2001). Interestingly, both tumour-derived and pancreas-derived trypsin-2 degrades and activates proteinase-activated receptor-2 (PAR-2) (Alm et al. 2000). Later, it has been found that trypsin stimulates strongly the proliferation of human colon and gastric cancer cells and stimulates integrin α5β1-dependent adhesion to fibronectin in gastric cancer cells. These events are mediated through PAR-2. (Miyata et al. 2000, Darmoul et al. 2001.) Recent findings indicate that trypsin-mediated PAR-2 activation releases TGF-α MMP-dependently. The released TGF-α activates EGFR, which further activates the downstream MAPK/ERK/1/2 cascade, leading to cell proliferation. (Darmoul et al. 2004.) Trypsinogen-4 also activates PAR-2 and, in addition, PAR-4 receptors (Cottrell et al. 2004).

There are few studies concerning the regulation of trypsinogen production and activation. It is well known that trypsinogens are activated by enterokinase. Enterokinase production has been found in tumour-derived epithelial cells lines of prostate, colon, airway and pancreas (Cottrell et al. 2004). The anti-inflammatory glucocorticoid dexamethasone down-regulates trypsinogen-2 production in HT1080 fibrosarcoma cells, but has no effect on COLO-205 colon carcinoma cells (Koivunen et al. 1991a).
Koshikawa et al. (1997) demonstrated that TPA stimulates trypsinogen-2 expression and secretion in vascular endothelial cells, whereas TNF-α, TGF-α and IL-1β do not have regulatory effects. Lukkonen et al. (2000) found that CMT-1, -3, -5, -8 and doxycycline inhibit trypsinogen-2 mRNA and protein expression and enteropeptidase-induced migration in colon carcinoma cells.

**Table 3. Tumour-associated and pancreas-derived trypsin(ogen)s.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Identified first from</th>
<th>Relative molecular mass (kDa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT-1</td>
<td>Ovarian tumours</td>
<td>25</td>
<td>Koivunen et al. 1989</td>
</tr>
<tr>
<td>TAT-2</td>
<td>Ovarian tumours</td>
<td>28</td>
<td>Koivunen et al. 1989</td>
</tr>
<tr>
<td>Trypsinogen-3</td>
<td>Pancreas</td>
<td>26.7</td>
<td>Sheele et al. 1981</td>
</tr>
<tr>
<td>Trypsinogen-4</td>
<td>Brain</td>
<td>30 (active)</td>
<td>Cottrell et al. 2004</td>
</tr>
<tr>
<td>a-form</td>
<td></td>
<td>24.218*</td>
<td>Wiegand et al. 1993</td>
</tr>
<tr>
<td>b-form</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Theoretical molecular weight of active enzyme calculated on the basis of amino acid content

### 2.1.6.2 Tumour-associated trypsin inhibitor (TATI)

Tumour-associated trypsin inhibitor (TATI) was initially isolated from the urine of a patient with ovarian cancer by Stenman et al. (1982). TATI is identical to the earlier described pancreatic secretory trypsin inhibitor (PSTI), which is also called Kazal inhibitor. The molecular weight of TATI is 6 Da. TATI is a potent inhibitor of trypsin. The interaction between TATI and trypsin is reversible, and TATI can be degraded gradually by trypsin. TATI is expressed at low concentrations in several healthy tissues, especially in the gastrointestinal and urogenital tracts, but its production increases clearly in cancers and benign diseases affecting TATI-producing organs. TATI is also considered an acute-phase reactant, and elevated TATI levels have thus been observed in response to surgical stress, serious injuries, inflammatory disease, various infections and sepsis (Ogawa 1988). TATI levels can be measured from serum and urine. Elevated TATI levels have been observed in gynecologic, gastrointestinal, urologic, lung and breast cancers. Moreover, Goumas et al. (1995) found increased TATI concentrations in the serum 66.6% of their HNSCC patients. TATI levels are significantly higher in HNSCC patients than in healthy controls or patients with inflammatory or benign pathology in the head and neck region. TATI levels are higher in patients with progressive disease compared to patients with no evidence of disease during the clinical 12-month follow-up period. (Goumas et al. 1995.) TATI is especially useful for the diagnosis of mucinous ovarian cancer, and the combined use of TATI with other specific cancer markers may improve the differentiation between malignant and benign diseases in certain cancers. TATI is co-expressed with trypsin in cancer cells, and it has been suggested that TATI protects cells from destruction induced by inadvertent activation of trypsinogen (Solakidi et al. 2003). The role of TATI...
in cancer may be more complex than mere action as a trypsin inhibitor; TATI increases the proliferation of a variety of cell lines and stimulates migration. It has been suggested that TATI has a role in maintaining epithelial integrity. (Marchbank et al. 1998, Stenman 2002.)

2.1.7 Integrins in OSCC

Integrins are transmembrane receptors composed of α and β subunits (Hynes 2002). At least 8 β and 18 α subunits are known, and they form 24 distinct integrins. Most integrins link ECM ligands to the cytoskeleton inside cells, most often to an actin-based microfilament system. The other important function of integrins is to mediate signals between the extracellular and intracellular spaces into both directions. Thus, integrins participate in many essential physiologic functions, including cell-cell adhesion, apoptosis, development, immune responses, leukocyte traffic and hemostasis. They are also implicated in many human diseases, such as cancers. Many ECM and plasma proteins are ligands for integrins. Integrins bind to their ligands through short integrin-binding motifs, including the RGD (arginine-glycine-aspartic acid) motif. The RGD sequence exists in, for example, fibronectin, vitronectin, fibrinogen, von Willebrand factor and latency-associated peptide (LAP). In addition to the subunit composition of an integrin and the presence of integrin recognition sequences in ligands, integrin-ligand interactions are determined by the amount of divalent cations and the activation state of the integrin. Many integrins are not constitutively active; they can be, and often are, expressed on cell surfaces in an inactive state. (Ruoslathi 1996, Munger et al. 1999, Arnaout et al. 2002, Hynes 2002.)

Compared to normal oral mucosa, OSCC loses the expression of some integrins but retains or up-regulates the level of the others. Usually, integrins which are down-regulated are thought to maintain stable adhesion and tissue organization, whereas retained or up-regulated integrins are considered to participate in cell migration. Moreover, integrin expression varies both between and within different areas of the same OSCC. (Thomas & Speight 2001.) However, there are contradictory results concerning the up- or down-regulation of different integrins in OSCC (Table 4).
Table 4. Integrins in OSCC. Their expression compared to normal oral mucosa and suggested role in OSCC and ligands.

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Expression in OSCC compared to normal oral mucosa</th>
<th>Suggested role in OSCC</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_2 \beta_1 )</td>
<td>↓</td>
<td>Tumour progression</td>
<td>Coll I, III, IV, V, VI, denatured Coll I, ( \alpha_2 \beta_1 )</td>
</tr>
<tr>
<td>( \alpha_3 \beta_1 )</td>
<td>↑</td>
<td>Metastasis and invasion</td>
<td></td>
</tr>
<tr>
<td>( \alpha_3 \beta_1 )</td>
<td>↓</td>
<td>Poor histological differentiation</td>
<td>Laminin-5 and -10, collagen, ( \alpha_2 \beta_1 )</td>
</tr>
<tr>
<td>( \alpha_4 )</td>
<td>↑</td>
<td>Lymph node metastasis</td>
<td>Fibronectin, VCAM</td>
</tr>
<tr>
<td>( \alpha_4 )</td>
<td>a</td>
<td>Down-regulates migration by suppressing ( \alpha_5 ) and ( \alpha_6 ) function</td>
<td>Fibronectin, fibrinogen</td>
</tr>
<tr>
<td>( \alpha_5 \beta_1 )</td>
<td>↑</td>
<td>Metastasis, migration</td>
<td>Fibronectin, fibrinogen</td>
</tr>
<tr>
<td>( \alpha_6 \beta_1 )</td>
<td>↑</td>
<td>Invasion and metastasis</td>
<td>Laminin-5, component of hemidesmosomes</td>
</tr>
<tr>
<td>( \alpha_9 )</td>
<td>↓</td>
<td>Early marker of malignancy, poor histological differentiation</td>
<td></td>
</tr>
<tr>
<td>( \alpha_9 \beta_1 )</td>
<td>a</td>
<td>Migration, cell spreading</td>
<td>Fibronectin, vitronectin, LAP-TFG-( \beta )</td>
</tr>
<tr>
<td>( \alpha_9 \beta_1 )</td>
<td>↓↑</td>
<td>Reduced terminal differentiation in OSCC cells</td>
<td>Vitronectin, osteopontin</td>
</tr>
<tr>
<td>( \alpha_9 \beta_1 )</td>
<td>↑↑</td>
<td>See below</td>
<td>Fibronectin, vitronectin, tenascin, LAP-1 and -3</td>
</tr>
<tr>
<td>( \alpha_9 \beta_1 )</td>
<td>DE</td>
<td>Cell proliferation, differentiation</td>
<td>Tenascin, osteopontin, VCAM-1</td>
</tr>
</tbody>
</table>

Reviewed from Steffensen et al. 2001, Thomas & Speight 2001, Ziober et al. 2001 and Jin & Varner 2004 with additional data from Kikkawa et al. 1998 for \( \alpha_3 \beta_1 \), Nagata et al. 2003 for \( \alpha_5 \), Zhang et al. 2004 for \( \alpha_4 \), Zhang et al. 2002 for \( \alpha_5 \beta_1 \), Koivisto et al. 2000 for \( \alpha_5 \beta_1 \), Jones et al. 1996, 1997 and Koivisto et al. 2000 for \( \alpha_5 \beta_1 \), Jones et al. 1997 and Koivisto et al. 2000 for \( \alpha_5 \beta_1 \), and Håkkinen et al. 1999 for \( \alpha_5 \beta_1 \). b Cell experiment studies with OSCC cells. b Not expressed in normal oral epithelia. ↑ integrin expression is higher in OSCC than in normal oral epithelium. ↓ integrin expression is lower in OSCC than in normal oral epithelium. DE diffuse expression in basal and suprabasal cells, normally expressed in the cell membranes of basal epithelial cells.

2.1.7.1 \( \alpha_9 \beta_6 \) integrin in OSCC

Integrin \( \alpha_9 \beta_6 \) is not expressed in normal resting oral epithelia, but is strongly up-regulated during mucocutaneous wound healing and in malignant epithelial transformation (Breuss et al. 1995). In addition to OSCC, integrin \( \alpha_9 \beta_6 \) is expressed in gastric, breast, ovarian and colon carcinomas (Agrez et al. 1994, Niu et al. 1998, Arihiro et al. 2000, Ahmed et al. 2002). Fibronectin, tenascin, vitronectin and LAP-1 and -3 are known ligands for \( \alpha_9 \beta_6 \) integrin (Busk et al. 1992, Prieto et al. 1993, Huang et al. 1998, Munger et al. 1999, Annes et al. 2002). In immunohistochemical stainings, integrin \( \alpha_9 \beta_6 \) expression has been found throughout tumour islands and especially at the invading edge of tumours. In some
cases, there are areas with weak or negative staining. There is no association between staining intensity and the stage of tumour differentiation. (Breuss et al. 1995, Jones et al. 1997, Regezi et al. 2002.) Integrin αvβ6 mRNA also localizes in tumour cells throughout OSCC tissue (Impola et al. 2004). In addition to OSCCs, integrin αvβ6 is expressed to a lesser extent in verrucous hyperplasias, verrucous carcinomas, in situ carcinomas, lichen planus and clinically diagnosed leukoplakia lesions (Hamidi et al. 2000, Regezi et al. 2002, Impola et al. 2004). Epithelial cells of inflammatory, drug-induced or idiopathic hyperplasia or chronic inflammatory lesions do not express αvβ6 integrin. It has been shown with a limited number of tissue samples that αvβ6 integrin-positive leukoplakia specimens have a greater tendency to disease progression during follow-up than leukoplakias negative for αvβ6 integrin. (Hamidi et al. 2000.) Tenascin and αvβ6 integrin are overexpressed in in situ carcinomas and invasive carcinomas of the floor of the mouth, and the expressions co-localize, which is consistent with their biological interaction (Regezi et al. 2002).

Based on cell culture studies, integrin αvβ6 has a role in binding OSCC cells to fibronectin and in the migration on fibronectin together with α5β1 and αvβ1 integrins (Koivisto et al. 2000, Xue et al. 2001, Thomas et al. 2001b, Ramos et al. 2002). However, Koivisto et al. (2000) demonstrated that αvβ6 integrin is not the primary fibronectin receptor in OSCC cells. The role of αvβ6 integrin in keratinocyte migration has also been studied in β6 integrin knockout and transgenic mouse experiments (Huang et al. 1996, Häkkinen et al. 2004). In both models, acute skin wounds heal at a normal rate and without significant scar formation, and alterations in β6 integrin expressions hence do not seem to influence keratinocyte migration. However, OSCC cells have an adaptive ability to remain capable of migration, although some integrins are inhibited (Koivisto et al. 2000). Based on cell culture studies, there are controversial results about whether αvβ6 integrin influences cell proliferation and growth (Xue et al. 2001, Thomas et al. 2001b, Ramos et al. 2002). It is assumed that αvβ6 integrin has an important role in OSCC cell invasion. Increased expression of αvβ6 in OSCC cells increases invasion through Matrigel and enhances tumour growth and invasion in an in vivo mouse model (Thomas et al. 2001b, Ramos et al. 2002). Moreover, Xue et al. (2001) demonstrated that the αvβ6 integrin antibody inhibits tumour growth in a nude-mouse model.

Integrin αvβ6 overexpression induces production of MMP-3 and -9 and, to a lesser extent, MMP-2 in both ligand-dependent and -independent manners (Thomas et al. 2001a, Ramos et al. 2002, Thomas et al. 2002). This αvβ6 integrin-mediated overexpression of MMP-9 associates with increased invasion of OSCC cells. Recent findings indicate that the amino acid sequence EKQKVDLSTDC, which is the C-terminal sequence of the integrin β6 subunit, is essential for this MMP-9-dependent invasion in OSCC cells (Morgan et al. 2004). Ligation of β6 integrin with fibronectin achieves activation of the Fyn/FAK complex, which in turn couples β6 signaling to the Raf-ERK/MAPK pathway. This pathway activates MMP-3 transcription and promotes OSCC cell proliferation and experimental metastasis in vivo. (Li et al. 2003.) There are controversial results about whether αvβ6 integrin regulates uPA. Dang et al. (2004) found that, in addition to MMP-3 and -9, integrin αvβ6 activates uPA expression at the protein level in β6-transfected OSCC cells. Moreover, inhibition of uPA suppresses the activation of MMP-3 and MMP-9 and suppression of MMP-3 inhibits MMP-9 activation. Thus, they suggest that the expression of αvβ6 integrin by OSCC cells increases the activation...
of the uPA→MMP-3→MMP-9 pathway. Dalvi et al. (2004) demonstrated that transfection of full-length β6 integrin into OSCC cells reduces uPAR expression in mRNA and at the protein level and cell surface plasmin generation. They found that C-terminal amino acids EKQKVDLSTDC of β6 subunits are required for the down-regulation of uPAR. Moreover, modulation of uPAR expression by αvβ6 integrin seems to be specific for epithelial cells. (Dalvi et al. 2004.) However, regardless of whether or not β6 integrin regulates uPAR/plasmin expression, it has been shown that plasmin promotes αvβ6 integrin-dependent migration of OSCC cells (Thomas et al. 2001a, Dalvi et al. 2004).

It is assumed that αvβ6 integrin has a role in TGF-β activation in OSCC. In OSCC cells, integrin αvβ6 mediates adhesion to a latency-associated peptide (LAP) (Xue et al. 2001, Thomas et al. 2002). The binding of αvβ6 to solid LAP induces MMP-9 up-regulation, which can be inhibited with αvβ6 antibody in OSCC cells (Thomas et al. 2002). The activation of TGF-β requires dissociation of LAP from TGF-β. Also, the latent TGF-β-binding protein-1 (LTBP) is required for αvβ6 integrin-mediated TGF-β activation (Annes et al. 2004).
3 Aims of the present study

It is known that the expression of several MMPs is altered in OSCC compared to normal oral tissue. The expression of specific MMPs is often increased in cancers, but the roles of individual MMPs are largely unknown. MMPs are primarily known by their capability to degrade almost all ECM components. Recently, it has been shown that MMPs can regulate the important cell physiological properties related to cancer by modulating cell growth, apoptosis, angiogenesis, invasion, genetic instability and immune response. However, the regulation and activation mechanisms of individual MMPs during carcinogenesis are poorly understood.

The specific aims of the study were:

1. To determine the role of TAT-2 in gelatinase (MMP-2 and -9) activation and intravasation by transfecting TAT-2 cDNA into two human tongue SCC cell lines with different metastatic capacities and one virus-immortalized human gingival keratinocyte cell line.
2. To investigate the effects of TAT-2 on in vitro conversion of collagenases (MMP-1, -8 and -13) and stromelysin-1 (MMP-3).
3. To analyze the role of αvβ6 integrin in the regulation of collagenases (MMP-1, -8, and -13) and TIMP-1 in OSCC cells.
4. To find out MMP-8 expression in HNSCC in vitro and in vivo.
5. To study the expression of MMP-8 in tongue SCC in relation to clinicopathological variables and the causes of specific survival in male and female patients in vivo.
4 Materials and Methods

More detailed descriptions of the materials and methods are presented in the original publications referred to by their Roman numerals.

4.1 Cell cultures

4.1.1 SCC-25 and HSC-3 (I, II, IV)

The tongue squamous cell carcinoma cell lines, SCC-25 (ATCC CRL, 1628, Rockville, MD, USA) and the more invasive HSC-3 (JCRB Cell Bank 0623, Osaka, Japan), were cultured in 1:1 Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL) and Ham’s Nutrient Mixture F-12 (Gibco BRL) supplemented with 10% fetal calf serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, 50 IU/mL nystatin, 0.25 µg/ml amphotericin-B, 1 mM sodium pyruvate, 2 mM L-glutamine and 4×10⁻⁵ µg/ml hydrocortisone and 1×non-essential amino acids.

4.1.2 HaCaT (III) and IHGK cells (I, IV)

HaCaT cells are immortalized human skin keratinocytes with p53 mutation (Boukamp et al. 1988), and IHGK cells are human HPV16-immortalized oral epithelial cells (Oda et al. 1996). Two different HaCaT cell lines were used. One was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL), 10% newborn calf serum (NCS), 100 IU/mL penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 50 IU/mL nystatin and 1×non-essential amino acids. The other HaCaT cell line and IHGK cells were cultured in Keratinocyte-SFM medium (Gibco BRL) with 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 IU/ml nystatin and Supplements for Keratinocytes (Gibco BRL).
4.1.2.1 Generation of TAT-2-transfected SCC-25, HSC-3 and IHGK cells (I,II)

Total RNA isolated from COLO 205 cells with Trizol reagent® (Life Technologies) was transcribed to cDNA by Superscript II RNase H- reverse transcriptase (Gibco BRL) and amplified with PCR. The PCR reaction was done according to the manufacturer’s instructions using 3 μg of total RNA and 2.8 pmol of trypsinogen-2 gene-specific primers. Dynazyme™EXT DNA polymerase (Finnzyme) was used in the PCR reaction. The antisense primer was 5’-ATG GGA TCC TTA GCT GTT GGC AGC TAT GGT-3’, and the sense primer was 5’- CTG GCT AGC ACC ATG AAT CTA CTT CTG ATC-3’. The annealing temperature for trypsinogen-2 was 54°C. The 760 bp trypsinogen-2 PCR product was purified from 1% agarose gel with the QIAEX II kit (Qiagen). The PCR product was cloned into a pCR®3.1 vector using a bi-directional TA-cloning kit (Invitrogen). The trypsinogen-2-pCR®3.1 vector was purified with the Quantum Prep kit (BioRad). To verify the correct orientation of the trypsinogen-2 gene in the vector and the correctness of the gene itself, sequencing was done with an ABI PRISM™ 377 DNA sequencer (Perkin Elmer) using the dRhod Terminator Cycle Sequencing Ready reaction kit (Perkin Elmer) and T7 and pCR®3.1 reverse primers (Invitrogen).

The trypsinogen-2 construct was stably transfected into HSC-3, SCC-25 and IHGK cells using Lipofectin Reagent (Life Technologies). 1 μg of control or trypsinogen-2 plasmid and 2.5 μl of Lipofectin reagent were incubated with 30% confluent cells in 24-well dishes for 5 h in 250 μl of serum- and antibiotic-free medium. Cells were grown in normal medium for three days and then placed under G418 selection (300 μg/ml for HSC-3 and SCC-25 cells and 600 μg/ml for IHGK cells). After the selection, the presence of trypsinogen-2 mRNA was estimated by RT-PCR before and after transfection, and the amount of trypsinogen-2 protein was measured from serum-free culture medium and from trypsinogen-2-transfected and control cells according to the immunofluorometric method (IFMA) (Itkonen et al. 1990).

HSC-2 and HSC-3/TAT-2 cells were treated with 50 ng/ml enterokinase and 10 μg/ml TATI or used in CAM assay.

4.1.3 C1 and VB6 cells (III)

VB6 cells were created from V3 OSCC cells by retroviral infection with β6 integrin cDNA, and C1 cells were control transfectant cells (Thomas et al. 2001b). Both C1 and VB6 cells express αvβ6 integrin, but VB6 cells produce it significantly more than C1 cells. The cells were grown in standard keratinocyte growth medium, including alpha medium (Gibco BRL) containing 10% FBS supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 1.8×10^{-7} adenine, 5 μg/ml insulin, 1×10^{-10} M cholera toxin, 0.5 μg/ml hydrocortisone, 10 ng/ml EGF and 7.5% sodium bicarbonate.

1.5×10^5 of C1 and VB6 cells in serum-free keratinocyte growth medium were placed on 78 cm² plastic dishes, pre-coated with 0.5 μg/ml latency-associated peptide (LAP) (Sigma) or 0.5% bovine serum albumin (BSA) (Sigma) and incubated at 37°C. RNA was
isolated after 8 and 14 h using Trizol® reagent (Invitrogen) following the manufacturer’s instructions. The isolated RNA was used in real-time PCR.

10⁵ of C1 and VB6 cells were incubated in α-MEM with 1-5 μg/ml αvβ6-antibody (10D5, Chemicon International), 1 μg/ml MMP-3 antibody (Chemicon International, AB812) or 1 μg/ml non-immune serum (Dako) for 30 min at room temperature and seeded into un- or pre-coated 24-wells with antibody medium. The wells were pre-coated with 0.25 μg/ml LAP and/or 0.1% BSA. The cells were incubated for 24 h, and the conditioned medium was collected. Later, the media were used for Western blotting.

To analyse whether MMP-8 and -13 bind to the cell membrane of C1 and VB6 cells, the subconfluent cultures were maintained for 48 h in 78 cm² dishes. The conditioned medium was sampled and the dishes with cells were incubated for 10 min on ice with 2% Triton X-100 in PBS⁺ for sampling of cell membrane extract (CME). Moreover, the total proteins (TP) of C1 and VB6 cells were extracted with Trizol® reagent (Invitrogen) after culturing for 48 h. These samples were subjected to Western blotting.

To analyse whether recombinant MMP-8 binds to the cell surface of VB6 cells, the intrinsic protein production of VB6 was blocked with cycloheximide (Sigma). Then, cells were incubated in α-MEM, which contained 1.5 μg/ml recombinant MMP-8 (Chemicon) for 1 h. The conditioned medium was collected and the cells were incubated with 2% Triton X-100 in PBS⁺ for 10 min on ice to extract CME. The amount of MMP-8 in CME samples was determined by immunofluorometric assay (IFMA) (Hanemaaijer et al. 1997).

4.1.3.1 Organotypic cultures (III)

1 ml of type I collagen gel, which contained 5×10⁵ foreskin fibroblasts (HFF), was pipetted into a 24-well plate and allowed to polymerize for 30 min at 37°C. 1 ml of fibroblast growth medium (FGM) was added to each well, and the gels were incubated for 24 h at 37°C. After that, the medium was aspirated from the wells and 5×10⁵ C1 or VB6 keratinocytes were added to each well in α-medium. 24 h later, the gels were removed from the 24-well plate using a sterile spatula and placed onto collagen-coated nylon discs resting on steel grids. This time point was defined as day 1 of organotypic culture. The steel grids were placed into 6-well plates, and a sufficient amount of KGM was added to reach the undersurface of the grid, allowing the epithelial layer to grow at an air-liquid interface. The medium was changed every 2 days. The gels were processed for histology after 14 days.

4.1.4 Established cell lines from HNSCCs (UT-SCCs, TFs and DFs) (IV)

Twenty-five squamous cell carcinoma cell lines from HNSCCs (UT-SCCs) and the corresponding seven tumour fibroblasts lines (TFs) and two dermal fibroblasts lines (DFs) were established at the Department of Medical Biochemistry and Otolaryngology-Head and Neck Surgery, University of Turku. The clinical data, tumour characteristics
and passage numbers of each cell line are shown in Table 1 (IV). Cells were cultured in DMEM, 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 IU/ml nystatin, 0.25 μg/ml amphotericin-B and 1×non-essential amino acids.

The UT-SCC-20A cell line was established from a moderately differentiated SCC of the floor of the mouth (T1N0M0) prior to any treatment. The UT-SCC-42B cell line was established from a poorly differentiated and metastasised SCC of supraglottic larynx (T4N3M0).

UT-SCC-42B cells and the corresponding TFs and DFs were cultured to subconfluency. UT-SCC-42B cells were incubated with 10 nM or 100 nM PMA or with 1 ng/ml or 10 ng/ml TGF-β1 for 24 h. DFs and TFs were incubated with 10 nM PMA for 24 h. The conditioned media were collected and subjected to MMP-8 Western blotting.

4.2 Tissue samples

4.2.1 Cancer samples (III-V)

Paraffin (n = 11) and frozen (n = 2) OSCC samples and frozen breast cancer (n = 2) samples were obtained from the Department of Pathology, University of Oulu, Finland (III). The use of routine samples for study purposes was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu, Finland.

The frozen OSCC and breast cancer samples were pulverized in liquid nitrogen (III). The pulverized tissues were diluted with Trizol® reagent (Invitrogen), and proteins were extracted. The total protein content was measured by a Bio-Rad DC Protein Assay (Bio-Rad).

Altogether nineteen squamous cell carcinoma (SCC) biopsy samples from the head and neck region were included in the study (IV). SCC biopsy samples were collected in the Department of Otorhinolaryngology, Turku University Hospital. Fifteen biopsies were from primary SCCs, two biopsies were from metastases, one was from recurrent SCC, and one was from residual SCC. Eight of the SCCs were from the larynx, one from the parotid gland, six from the tongue, two from the skin of the temporal region and two from the mandibular gingiva. Seventeen biopsy samples had a corresponding UT-SCC-cell line (IV). The Joint Commission on Ethics of the Turku University and the Turku University Central Hospital approved the use of SCC biopsy samples for research.

Paraffin-embedded primary SCCs of mobile tongue from 92 patients (mean age 62) were included in the study (V). 48 of the patients were female and 44 male. The average follow-up time was 49 months (range 2–180 months). The use of these samples for study purposes was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu, Finland.
4.3 Invasion assays

4.3.1 In vivo invasion assay (I)

The chorionallantoic membrane (CAM) assay was done according to Kim et al. 1998, except for a few modifications. \(2 \times 10^6\) cells (HSC-3, HSC-3/TAT-2, SCC-25, SCC-25/TAT-2, IHGK and IHGK/TAT-2) with or without enterokinase and TATI were inoculated into a CAM of 10-day-old chick embryos. After 50 h of inoculation, the CAMs lining the cavity of the lower eggshell were collected and frozen. The frozen CAMs were pulvérized and used for the extraction of genomic DNA. The intravasated human cells present in CAM were analysed by using radioactive PCR, in which human specific Alu primers were used: Alu sense, 5’ ACG CCT GTA ATC CCA GCA CTT 3’, and Alu antisense, 5’ TCG CCC AGG CTG GAG TGC A 3’. The primers produced a band of 224 bp. The role of MMP-9 in intravasation was studied by using a specific MMP-9 inhibitor, CTT peptide (Koivunen et al. 1999), in inoculation.

4.3.2 In vitro invasion assay (III)

Cell invasion assays were performed by using Matrigel or type I collagen-coated polycarbonate filters (8 μm pore size, Transwell®, Beckton Dickinson) as previously described (Thomas et al. 2001a, b). Matrigel or type I collagen was added to the upper membrane and allowed to gel for 1 h at 37°C. Keratinocyte growth medium (KGM) was used as a chemo-attractant in the lower chamber of the Transwell. VB6 and C1 cells were plated in the upper chamber in α-MEM and incubated at 37°C for 72 h. The cells in the lower chamber (including those attached to the undersurface of the membrane) were then trypsinised and counted on a Casy 1 counter (Sharfe System GmbH, Germany).

4.4 Methods of RNA analysis

4.4.1 Real-Time PCR (II, III)

Baseline expressions of MMP-1, -3, -13 (II) and -9 in HSC-3 and HSC-3/TAT-2 cells were analysed by real-time PCR. The analyses were performed as described by Palosaari et al. (2003).

cDNA from C1 and VB6 cells (III) was transcribed from 1 μg RNA by using the Taqman reverse transcription (RT) reagent kit. The PCR reaction was performed in a total volume of 25 μl with 1xTaqman Universal PCR Master Mix (Applied Biosystems), 200 nM of each primer, 100 nM of each probe and 5 ng of cDNA, or for 18S amplification, 1 ng of cDNA. The cycling conditions were: incubation at 50°C for 2 min followed by 10
min at 95°C and 40 cycles of 15 s at 95°C. The samples were analysed with ABI Prism 7700 Sequence Detection Systems (Applied Biosystems). The MMP results were normalised to 18S and expressed as a ratio. The primer sequences for real-time PCR were: TIMP-1 forward primer 5’-GAC GGC CTT CTG CAA TTC C-3’, reverse primer 5’-GTA TAA GGT GGT CTG GTT GAC TTC TG-3’, MMP-1 forward primer 5’-AAG ATG AAA GGT GGA CCA ACA ATT-3’, reverse primer 5’-CCA AGA GAA TGG CCG AGT TC-3’, MMP-8 forward primer 5’-CAC TCC CTC AAG ATG ACA TCG A-3’, reverse primer 5’-ACG GAG TGT GGT GAT AGC ATC A-3’, MMP-13 forward primer 5’-AAA TTA TGG AGG AGA TGC CCA TT-3’, reverse primer 5’-TCC TTG GAG TGG TCA AGA CCT AA-3’.

4.4.2 RT-PCR (IV)

For RNA analysis, total RNA was isolated from cultured UT-SCCs, HSC-3, HIGK, HaCaT and SCC-25 cells by the Trizol® method (Invitrogen). Extracted total RNA was incubated with RQ1DNAase (Promega) to eliminate DNA contaminants. 3 μg of total RNA was transcribed into cDNA using 200 units of Superscript™II reverse transcriptase enzyme (Gibco BRL, Life Technologies) and random hexamer primers. cDNA was used for PCR amplification. The specific primers for MMP-8 in RT-PCR were sense 5’AAG GCA ACC AAT ACT GGG3´ and antisense 5’ATT TTC ACG GAG GAC AGG3´, which produced a band of 522 base pairs (bp) (Tonetti et al. 1993), and those in nested PCR were sense 5’AGC AGC GTC CAA GCA ATT G 3´ and antisense 5’ AAT GCA TGC TGA ACT TCC CTT C 3’, which produced a band of 352 bp. Specific primers for the housekeeping gene, 18S ribosomal RNA, which served as a control gene, were sense 5’GGT TGA TCC TGC CAG TAG CAT ATG CTT 3’ and antisense 5’GGC AGC GAC CAA AGG AAC CAT AAC TGA T 3’, which produced a band of 125 bp (McCallum & Maden 1985). The RT- and nested PCR were performed in a 30 μl reaction containing 2 μl cDNA for RT-PCR or 0.5 μl RT-PCR product for nested PCR, 100 ng each of primers for RT-PCR and 10 pM for nested PCR and 1 μl DNA polymerase (Dynazyme 1 U/μl, Finnzymes OY). The annealing temperatures were 58°C for MMP-8 in RT-PCR, 62°C in nested-PCR and 68°C for 18S ribosomal RNA.

4.4.3 Southern blotting (IV)

The specific cDNA probe for MMP-8 was prepared using RT-PCR primers (Tonetti et al. 1993). Total RNA from established gingival fibroblasts, passage 6, was subjected to RT-PCR and separated on a 1% agarose gel. A band size of 522 bp, corresponding to MMP-8, was extracted from the gel using a QIAEX II Gel Extraction Kit (Qiagen). The extracted product was labelled with [α-32P]-dCTP and used as a cDNA probe. The PCR products from odontoblasts, UT-SCC-20A and UT-SCC-42B cells were separated on 1.5% agarose gel, transferred on to a nylon filter (Immobilon™-Ny+ Transfer Membrane, Millipore Corporation) and hybridized with the MMP-8 probe as described elsewhere (Ausubel et al. 1988).
4.4.4 In situ hybridization (IV)

In situ hybridization followed the protocol described by Arnold et al. (1992) and modified by Mäkelä et al. (1999). For in situ hybridization, all the solutions were treated with 0.1% DEPC. Eight different paraffin HNSCC sections from six patients were used.

A 95 bp Sphl fragment of human MMP-8 cDNA (Hasty et al. 1990) was transcribed to cRNA using a riboprobe transcription kit (Boehringer Mannheim GmbH) and labelled with digoxigenin-11-UTP (DIG). The tissue sections were deparaffinized, rehydrated and proteolyzed in 0.2 M HCl at room temperature and in 5 μg/ml Proteinase K-treatment (Finnzymes) at 37°C. The reaction was stopped by 100 mM glycine, and acetylation was done with 0.25% acetic anhydride in 0.1 M triethanolamine. The sections were equilibrated in 4× standardized saline citrate (SSC) for 15 min before prehybridization at 58 °C for 2 h. The hybridization buffer contained 10 mM dithiotreitol, 250 μg/ml yeast t-RNA (Boehringer Mannheim GmbH), 250 μg/ml salmon sperm DNA (Sigma), 50% deionized formamide, 4 × SSC, 10% (w/v) dextran sulphate, 0.02% (w/v) Ficoll®, 0.02% (w/v) BSA and 0.02% (w/v) polyvinylpyrrolidone. The prehybridization buffer was removed and hybridization buffer containing 600 ng/ml DIG-labeled antisense or sense probe was applied to each section and hybridized overnight at 58 °C. After that, the hybridization samples were washed and equilibrated. The digoxigenin label was detected by incubating the samples with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim GmbH) diluted 1:100 and by using Fast Red tablets as chromogen. The samples were counterstained with Mayer’s hematoxylin (Merck KGaA).

The proportion of positively labelled cells and their staining intensity were quantified by using light microscopy as described by Bachmeier et al. (2000).

4.5 Methods of protein analysis

4.5.1 Gelatin and reverse zymographies (I, III)

The presence of gelatinases was analysed by zymography using 0.75 mm 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin labelled with 2-methoxy-2,4 dephenyl-3(2H) furanone (Fluka) (Mäkelä et al. 1994). Briefly, prior to electrophoresis, proteins from cell experiments (I, III) were mixed with Laemmli’s sample buffer (Laemmli 1970), and electrophoresed. After electrophoresis, gelatinases were activated by incubating the gels in activation solution (50 mM Tris, 5 mM CaCl₂, 1 μM ZnCl₂, 0.02% NaN₃, pH 7.5) overnight at 37°C. During incubation, the gelatinases present in the samples degraded gelatin from the gel. The degradation of gelatin was visualized under long-wave UV light. The gels were also stained with 0.5% Coomassie blue R-250. Purified MMP-9 enzyme was used as a control.

The presence of different TIMPs was analysed using reverse zymography. Conditioned medium of C1 or VB6 cells and recombinant TIMP-1 as a control were mixed with Laemmli’s sample buffer. Samples were electrophoresed into a fluorescent-labeled
zymography gel according to the method described by Oliver et al. (1997). During incubation, the TIMPs present in the samples maintained gelatin bands in the gel equalling the molecular weights of the TIMPs.

### 4.5.2 Western immunoblotting (I-V)

For western blotting, three (I) to one hundred-fold (II-V) concentrated proteins from cell experiments were diluted into Laemmli’s sample buffer and incubated for 20 min at 60°C. Before that, certain samples were incubated with 1-2 mM APMA at 37°C for 30 min–one h. The incubated samples were separated according to size on a 12% SDS-polyacrylamide gel, stained with Coomassie Brilliant Blue to visualize the total protein composition in the sample and transferred onto polyvinylidene difluoride (PVDF) microporous membrane (Immobilon P Transfer membrane, Millipore). After transfer, the stain was washed away with methanol and the non-specific binding was blocked by incubation with 1×TBS supplemented with 5% non-fat dry milk for 60 min. The filters were incubated with specific antibodies (Table 5) overnight at room temperature. The filters were then incubated with biotinylated secondary antibody (1:500-1:1000, DAKO) for 1 h at room temperature. After washing, the filters were incubated with avidin-peroxidase complex (DAKO) for 45 min. The chemiluminescence reaction produced by the ECL Western Blotting detection kit (Amersham Pharmacia Biotech) was detected by autoradiography. The western blotting and gelatin zymography products were quantified with an image processing and analysis program (ScionImage PC, Scion Corporation, Frederick, MD, USA).

**Table 5. List of antibodies used in Western immunoblotting.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution/Concentration</th>
<th>Original publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Oncogene Research Products (IM67)</td>
<td>1 μg/ml</td>
<td>III</td>
</tr>
<tr>
<td>MMP-9 (active)</td>
<td>Duncan et al. 1998</td>
<td>1:10</td>
<td>I</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Oncogene Research Products (IM44L)</td>
<td>1 μg/ml</td>
<td>III</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Chemicon International Inc. (Ab 8105)</td>
<td>1:1000</td>
<td>II</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Chemicon International Inc. (Ab 811)</td>
<td>1:1000</td>
<td>II</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Bergmann et al. 1989, Michaelis et al. 1990</td>
<td>1 μg/ml</td>
<td>II-V</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Kjeldsen et al. 1993</td>
<td>1:1000</td>
<td>I</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Chemicon International Inc. (Ab 8114)</td>
<td>1:1500</td>
<td>II</td>
</tr>
</tbody>
</table>

### 4.5.3 Proteolytic activation of proMMPs and sequencing (II)

15 nM TAT-2 was mixed with native and/or recombinant proMMP-1, -3, -8 and -13 (15 μM) at various molar ratios ranging from 1:100 to 1:1000 (TAT-2/proMMP) and incubated at 37°C for 10-60 min. TAT-2 was inhibited by a fourfold amount of TATI by
incubating for 60 min at 37°C. The samples were reduced with 20 mM DTT and electrophoresed on 8% SDS-PAGE and blotted onto PVDF membrane (Bio-Rad). The blotted membrane was visualized by staining in Coomassie Brilliant Blue R-250, and appropriate protein bands were excised. Amino acid sequencing was performed in an Applied Biosystems 477A/120A pulse-liquid protein sequencer by means of Edman degradation (Baumann 1990).

4.5.4 Immunohistochemical stainings (III-V)

For immunohistochemical stainings, primary antibodies against MMP-8 (1:100, polyclonal, Bergmann et al. 1989, Michaelis et al. 1990) and -13 (1 μg/ml, monoclonal, Oncogene Research products, IM44L), α,β6 integrin (6.2G2 at 0.5μg/ml, Weinreb et al., 2004, generously provided by Biogen Corp, USA) and trypsinogen (monoclonal, Itkonen et al. 1990, 6D11, 1:500) were used.

Envision Kit (DakoCytomation) for MMP-13 (III), Vector Kit (PK-6102, Vector Labs) for α,β6 integrin (III) and Vectastain Elite ABC Kit (Vector Laboratories) for MMP-8 (IV, V) were used in immunohistochemistry. Paraffin sections (6 μm) of HNSCC tissues were deparaffinized and hydrated. Endogenous peroxidase activity was blocked. The sections were treated with 0.4% pepsin (Merck KGaA) for 5–45 min at +37 °C for α,β6 integrin and MMP-8 immunostainings. The nonspecific binding sites were blocked by incubation with normal goat serum. The sections were then incubated with primary antibody for 2 h–overnight at room temperature or +4 °C. Negative controls were incubated with non-immune serum instead of primary antibody. DAB Substrate System (Lab Vision Co) or 3-amino-9-ethylcarbazole (Sigma) were used for detection. Counterstaining was done with Mayer’s hematoxylin (Merck KGaA). Placenta and ductal breast carcinoma were used as positive controls for MMP-13.

TAT immunohistochemistry (Figure 4) was kindly done in professor Anders Bjartell’s laboratory in Malmö University Hospital, Sweden. Staining was performed as described by Paju et al. (2000). Pancreatic tissue was used as a positive control for TAT-2.

4.6 Collagen degradation assay (II, III)

The degradation of native type I collagen by MMP-1, -8, -13, conditioned media of C1 and VB6 cells or TAT-2 was determined by collagen degradation assay (Turto et al. 1977, Sorsa et al. 1994). Native human proMMP-1, -8 and -13 were pre-incubated with or without TAT-2, 0.5 mM gold thioglucose (GTG), 0.5 mM sodium hypochlorite (NaOCl) or 1 mM APMA, after which 1.5 μM type I collagen substrate was added and incubated at 20° or 4°C for 3-24 h (II). TAT-2 was pre-treated with TATI before the collagenase assay. 15 nM TAT-2 was also incubated alone or with TATI with type I collagen (II). The conditioned media of C1 and VB6 cells were incubated with human type I collagen with or without APMA at 22°C for six days (III). The cleavage products were separated on 8% SDS-PAGE and stained with Coomassie Brilliant Blue.
4.7 Immunolocalisation of MMP-8 and -13 in C1 and VB6 cells (III)

1.5×10^4 C1 or VB6 cells were plated in KGM onto 13 mm glass coverslips and incubated for 24 h at 37°C in 5% CO₂. For MMP-13 analysis, cells were incubated for 24 h in KGM containing 1 μM monensin (Sigma) to block protein secretion. Cells were rinsed, fixed and permeabilised with 0.1% Triton X-100 for 10 min, followed by incubation for 60 min in wash buffer. Anti-MMP-13 antibody (1:100, Oncogene Research products, IM44L) or anti-MMP-8 antibody (1:100, Bergmann et al. 1989, Michaelis et al. 1990) diluted in wash buffer was added for 60 min at 4°C. Bound antibody was detected with Alexa 488 - conjugated secondary antibody (1:200, Invitrogen Ltd). Actin was visualised with TRITC-conjugated phalloidin (5 ng/ml, Sigma). Coverslips were washed and mounted with MOWIOL 4-88 (0.1 g/ml of Citifluor mounting medium, Novabiochem) and viewed under a confocal laser scanning microscope (Zeiss LSM510, Welwyn Garden City).

4.8 Statistical analysis (I, III, V)

Scheffé’s test (I) and independent 1-tailed t-test (III) were used to estimate the statistical significance of differences. P values less than 0.05 were considered significant. MMP-8 immunohistological staining (V) was evaluated as described by Bachmeier et al. (2000). The grading score for the proportion of stained cells was compared to the clinicopathological variables (TNM classification and histological grade) and the survival functions. Survival curves were constructed by the Kaplan-Meier method (V).
5 Results

5.1 TAT-2 cDNA transfection into tongue carcinoma cells and virus-immortalized gingival keratinocytes and its effects on cell functions (I, II)

We first wanted to create a cell line that would overexpress the TAT-2 gene, to find out whether TAT-2 overproduction would increase gelatinase activation and have an effect on the invasion capacity of the cells, using the CAM intravasation model. Moreover, we wanted to find out whether TAT-2 activates collagenases or MMP-3 in TAT-2 overproduction cells or in vitro. Then, the type I collagen degradation capacity of TAT-2 protein in vitro was studied. Moreover, TAT-2 expression in OSCCs was studied by immunohistochemical staining.

5.1.1 TAT-2 cDNA transfection into HSC-3, SCC-25 and IHGK cells increases TAT-2 mRNA and protein production (I)

TAT-2 cDNA transfection into two human tongue SCC cell lines (HSC-3, SCC-25) and one virus-immortalized human gingival keratinocyte cell line (IHGK) led to increase TAT-2 mRNA and protein production in TAT-2-transfected cells compared to control cells. HSC-3 control cells produced TAT-2 protein 2 ng/ml (SD ± 1.2) and HSC-3/TAT-2 cells 7 ng/ml (SD ± 1.1). SCC-25 control cells did not produce TAT-2 protein or produced it at levels not detectable, whereas SCC-25/TAT-2 cells expressed 35 ng/ml (SD ± 15.9) TAT-2. The amount of TAT-2 protein was also markedly increased in IHGK/TAT-2 cells compared to IHGK control cells, 1 ng/ml and 22 ng/ml (SD ± 1.7), respectively. The amounts of produced proteins were analyzed from 48 h cultures by IFMA, and the amount of mRNA was analyzed by RT-PCR. For further studies, two clones with the maximum increase in TAT-2 production were selected.
5.1.2 TAT-2 associated proMMP-9 activation in HSC-3/TAT-2 cells (I)

In cells transfected with TAT-2 cDNA conversion of proMMP-9 to the active form was seen in HSC-3/TAT-2 cells. The serum-free, enterokinase-treated (50 ng/ml) conditioned media of HSC-3/TAT-2 and control cells were subjected to gelatin zymography and Western blotting. The ratio of 77 kDa to 92 kDa MMP-9 increased on an average 2.2-fold from 0.79 (SD + 0.26) in controls to 1.81 (SD + 0.48) in HSC-3/TAT-2 cells when analyzed from zymography bands. Addition of the TAT-2 inhibitor, TATI, to the experiments prevented the conversion of the 92 kDa form to the 77 kDa form (0.73 SD + 0.21). Western blotting with two different MMP-9 antibodies (one was specific for both the pro and active forms and the other only for the active form) confirmed the results of gelatin zymography. TAT-2 transfection had no effect on proMMP-2 activation in HSC-3/TAT-2 cells. Although the amount of secreted TAT-2 protein was increased more in SCC-25 and IHGK cells than in HSC-3 cells after TAT-2 transfection, activations of proMMP-2 and proMMP-9 were not seen in HSC-2/TAT-2 and IHGK/TAT-2 cells.

5.1.3 TAT-2 cDNA transfection into HSC-3 enhances intravasation (I)

The role of active MMP-9 in the intravasation was studied with HSC-3/TAT-2 cells. TAT-2 cDNA transfection into HSC-3 cells increased intravasation of cells in the chorioallantoic membrane (CAM) model. The ratio of intravasated cells was quantitated by human specific Alu-PCR bands. When HSC-3 control cells were inoculated with the TAT-2 activator enterokinase into eggs, intravasation of the cells increased by 24% (SD ± 11%) compared to controls without enterokinase. HSC-3/TAT-2 cells intravasated 43% (SD ± 12.4%) and HSC-3/TAT-2 with enterokinase up to 66% (SD ± 19.4%) more than control cells (p ≤ 0.001). To confirm the role of TAT-2 in intravasation, the TAT-2 inhibitor TATI was added to the experiments. When TATI was inoculated into CAMs with HSC-3/TAT-2 cells, the intravasation of cells decreased by 65% compared to HSC-3/TAT-2 without TATI (p ≤ 0.05). When TATI and enterokinase were added into CAMs with HSC-3/TAT-2 cells, the cells intravasated only 6% more than untreated control cells. The difference was highly significant compared to HSC-3/TAT-2 cells with enterokinase but without TATI (p ≤ 0.001). The TAT-2 overproduction of SCC-25/TAT-2 cells increased intravasation only slightly, and addition of enterokinase led to more efficient intravasation of SCC-25/TAT-2 cells. Virus-immortalized IHGK cells did not intravasate at all regardless of TAT-2 transfection or the presence of enterokinase. The role of MMP-9 in intravasation was analyzed by using a specific MMP-9 inhibitor, CTT peptide (Koivunen et al. 1999). CTT peptide decreased the intravasation of control HSC-3 cells in a dose-dependent manner, but the negative control peptide (Koivunen et al. 1999) had no effect.
5.1.4 Influence of TAT-2 transfection on the production of collagenases, MMP-3 and MMP-9 in HSC-3 cells (II)

Real-Time PCR analysis was performed to evaluate the influence of TAT-2 cDNA transfection on production of MMP-1, -3, -9 and -13 mRNA in HSC-3 cells. The productions of MMP-1, -3 and -13 mRNAs were decreased 3.5, 3 and 10.1 times in HSC-3/TAT-2 cells compared to control cells. MMP-9 mRNA level increased over 5 times in HSC-3/TAT-2 cells compared to control cells.

TAT-2 cDNA transfection into HSC-3 cells enhanced the protein production of MMP-1, -3, -8 and -13. MMP-1 protein increased, MMP-3 and -8 decreased, and MMP-13 remained unchanged after TAT-2 transfection. TAT-2 transfection did not cause conversion of procollagenases and proMMP-3 to their active forms.

5.1.5 TAT-2 activates procollagenases and proMMP-3 in vitro (II)

TAT-2, purified from conditioned medium of COLO 205 cells, led to conversion of native and recombinant procollagenases (MMP-1, -8 and -13) and proMMP-3 in vitro. N-terminal sequence analysis revealed that TAT-2 cleaved MMP-1 at Lys^{56}-Val^{57}, MMP-3 at His^{82}-Phe^{83}, PMN MMP-8 at Arg^{48}-Phe^{49} and Arg^{70}-Cys^{71} and MMP-13 at Glu^{84}-Tyr^{85}. The cleaving site of MMP-1 was consistent with trypsin specificity, but the conserved PRCXXPD motif, which is responsible for the latency of MMPs, was not removed. The cleavage sites of MMP-3 and MMP-13 was downstream of the PRCXXPD sequence. The cleavage of MMP-3 and -13 involved an autoproteolytic process, since TAT-2 is not able to cleave at the carboxyterminal site of histidine (H) or glutamic acid (E). Thus, the initial cleaving sites remained unidentified. PMN-derived 85 kDa MMP-8 was cleaved by TAT-2 at two tryptic sites, which located upstream and inside the PRCXXPD motif, achieving partially activated PMN MMP-8. Type I collagen degradation assay confirmed that the TAT-2-induced conversions of MMP-1, -8 and -13 were proteolytically active enzymes. Procollagenases exposed to TAT-2 were able to degrade type I collagen to the characteristic ½ (αA) and ¾ (αB) degradation products at 4°C and 20°C. When procollagenases and proMMP-3 were converted in a time-dependent manner to their active forms by TAT-2, their molecular weight diminished by approximately 10-20 kDa. The TAT-2 inhibitor TATI prevented TAT-2-induced conversions of MMP-3, -8 and -13.

5.1.6 TAT-2 degrades soluble human type I collagen (II)

Type I collagen, incubated with TAT-2 at 20°C and 4°C, was degraded efficiently to multiple-size degradation products different from typical MMP-1, -8 and -13-induced degradation products. TATI prevented TAT-2-induced type I collagen degradation at both temperatures. Human neutrophil elastase and TPCK-treated bovine pancreatic trypsin did not degrade native human type I collagen.
5.1.7 TAT-2 in OSCCs

OSCC samples were subjected to TAT-2 immunohistochemistry. TAT-2-positive cells were localized especially in the peripheral tumour cells, but also occurred throughout the cancer islands. Moreover, positive staining was found sporadically in fibroblasts of the surrounding stroma. Previously, macrophages have been reported to be trypsinogen-immunopositive (Koshikawa et al. 1998). This was confirmed by our stainings (Figure 4).

Fig. 4. Immunohistochemical analysis for the localization of TAT-2 in OSCC samples. TAT-2 localized mainly in the peripheral tumour cells (A, B), but also occurred throughout cancer cell islands and some fibroblasts of the stroma (see arrows). Macrophages were also positive (C, arrows). D is pancreas as a positive control. Original magnifications: ×100 (A), ×200 (B, D) and ×400 (C).

5.2 Influence of $\alpha_v\beta_6$ integrin on collagenases, TIMP-1 and behaviour of OSCC cells (III)

We wanted to investigate whether $\alpha_v\beta_6$ integrin, which is widely expressed in OSCCs, regulates collagenase or TIMP-1 expression levels in OSCC cells by using $\alpha_v\beta_6$ overexpressing VB6 cells and control C1 cells. We also analyzed the effects on altered MMP-13 expression in type I collagen degradation in vitro.
5.2.1 Integrin αvβ6 down-regulates MMP-13 but does not affect MMP-1 and -8 or TIMP-1 in OSCC cells (III)

The expression levels of MMP-1, -8 and -13 and TIMP-1 mRNA in C1 and VB6 cells cultured on BSA- (inert substrate) or LAP- (αvβ6 integrin ligand) coated dishes for 8 and 14 h were evaluated by real-time PCR. After 8 h incubations, MMP-13 mRNA levels were significantly higher in C1 cells than in VB6 cells on BSA (p = 0.037) and LAP (p = 0.026). After 14 h incubations, the trend was similar to that seen at 8 h (BSA p = 0.126 and LAP p = 0.127). There were no significant differences in MMP-1, MMP-8 and TIMP-1 mRNA levels between C1 and VB6 cells at any experimental point.

The MMP-1, -8, -13 and TIMP-1 protein productions were evaluated by Western blotting and reverse zymography. C1 and VB6 cells were incubated with or without different antibodies for 24 h on uncoated or LAP- or BSA-coated dishes. When C1 and VB6 cells were incubated with αvβ6 integrin antibody 10D5 (1 μg/ml), MMP-13 protein production increased remarkably in both cell lines. Without this antibody, the protein levels were under the detection limit in Western blotting. MMP-13 existed in a proform (60 kDa) and degraded forms 45 and 38 kDa. No increase in MMP-13 was seen upon incubation with MMP-3 antibody and non-immune serum. Thus, specific inhibition of αvβ6 integrin induced MMP-13 production. MMP-8 production was similar on uncoated or LAP-coated dishes with or without 10D5 (1-5 μg/ml) in both C1 and VB6 cells. MMP-8 was in a proform (50 kDa). No MMP-1 was seen on uncoated or LAP-coated dishes. 10D5 did not influence its expression. The amount of TIMP-1 expression (28 kDa) was similar in both C1 and VB6 cells, and 10D5 did not achieve changes in TIMP-1 protein production. To confirm that C1 and VB6 cells maintained their αvβ6 integrin-dependent differences in MMP-9 production (Thomas et al. 2002), gelatin zymography was done. In gelatin zymography, VB6 cells produced more MMP-9 than C1 cells and 10D5 (1 μg/ml) diminished MMP-9 production.

5.2.2 Reduced MMP-13 expression does not affect collagenolytic capacity of VB6 cells (III)

The conditioned medium of C1 and VB6 cells was incubated with native soluble type I collagen with or without APMA activation. There were no significant differences between C1 and VB6 cells in type I collagen degradation when analyzed by collagen degradation assay.

C1 and VB6 cells were subjected to invasion assay through type I collagen or Matrigel. It has been demonstrated previously that VB6 cells are significantly more invasive than C1 cells through Matrigel, and this elevated invasiveness is αvβ6 integrin-dependent (Thomas et al. 2001b). Our results confirmed this finding. However, the invasion capacities of C1 and VB6 cells through type I collagen were similar. The low level of invasion was in line with the low level of collagenase activity assessed by collagen degradation assay.
C1 and VB6 cells were grown on human foreskin fibroblast (HFF) gel for 14 days and then subjected to immunohistochemistry. The staining for MMP-13 in C1/HFF cultures confirmed higher MMP-13 levels in C1 cells. VB6/HFF cultures were almost negative. MMP-13 immunohistochemical stainings were also performed on tongue SCC samples along with \( \alpha_v \beta_6 \) integrin stainings. The tongue SCC samples that produced high levels of \( \alpha_v \beta_6 \) integrin as assessed by immunohistochemistry, stained weakly for MMP-13. Reciprocally, in cells that produced low levels of \( \alpha_v \beta_6 \) integrin stained intensively for MMP-13.

5.3 Expression and regulation of MMP-8 in HNSCCs (IV)

We wanted to study whether MMP-8, which degrades very efficiently type I collagen, is expressed in HNSCCs by using established and commercial cell lines and, in addition, HNSCC cancer samples and to find out whether PMA or TGF-\( \beta \) affects MMP-8 production.

5.3.1 MMP-8 mRNA expression (IV)

Total RNA from the cultured HNSCC cells (UT-SCCs) and the corresponding seven tumour and two dermal fibroblast lines were analyzed by nested RT-PCR for MMP-8. The 352 bp MMP-8 transcript was found from 22 of 25 UT-SCC cell lines, five of the seven corresponding tumour fibroblast lines (TF) and both of the two dermal fibroblast lines (DF). HSC-3, SCC-25, IHGK and HaCaT cells also expressed MMP-8 mRNA (IV, Table 1.) The specificity of the 552 bp RT-PCR products from UT-SCC-20A and UT-SCC-42B cells was confirmed by using Southern blotting.

A positive MMP-8 mRNA signal was seen in a few carcinoma cells in the invading tumour cells in HNSCCs analyzed by in situ hybridization. In PMN and plasma cells, hardly any mRNA signal was seen. To evaluate the in situ hybridizations quantitatively, the multiplication values were calculated for each tissue section by grading the positively labelled cells and their staining intensity as described by Bachmeier et al. 2000. The lowest and highest multiplication values were 0.1 and 1.6, respectively. The maximal value was 12, indicating that, based on this scoring, the expression of MMP-8 was faint.

5.3.2 MMP-8 protein production and regulation (IV)

HNSCC cells produced MMP-8 protein in vitro and in vivo and, furthermore, TGF-\( \beta \) and PMA regulated MMP-8 protein production. 100-fold concentrated serum-free
conditioned media from UT-SCC-42B cells and corresponding TF and DF cells were exposed to Western immunoblotting with polyclonal MMP-8 antibody. A latent 50 kDa immunoreactive band was seen in UT-SCC-42B cells and the corresponding TFs and DFs. 1 ng/ml and 10 ng/ml TGF-β1 down-regulated MMP-8 protein by approximately 30% and 60%, respectively in UT-SCC-42B cells. 10 nM and 100 nM PMA up-regulated the MMP-8 protein levels about 2- and 2.5-fold, respectively, in UT-SCC-42B cells. Furthermore, PMA activated partly proMMP-8 (50 kDa) to an active form (45 kDa) similarly to 2 mM APMA, but to a lesser extent, in UT-SCC-42B cells and the corresponding DFs and TFs. The same polyclonal MMP-8 antibody was used in immunohistochemistry and in Western blotting. MMP-8 was sporadically seen in carcinoma cell islands and stromal fibroblasts. The amount of staining was low and the staining intensity faint in cancer cells. Inflammatory cells, i.e. PMNs and plasma cells, stained more intensively than carcinoma cells and fibroblasts.

5.4 Increased expression of MMP-8 is associated with longer survival in female tongue SCC patients (V)

We wanted to investigate whether MMP-8 protein production analyzed by immunohistochemical staining associates with the clinical or pathological parameters of mobile tongue SCCs. 15% of the 92 tongue SCC samples were MMP-8-immunopositive. MMP-8 expression was independent of the stage or grade of the tumour. MMP-8 expression was associated with improved disease-specific survival in female patients (p = 0.068). Male patients showed no difference in survival between MMP-8-positive and -negative expression (p = 0.215).

5.5 MMP-8 and -13 are cell surface-associated proteins in OSCC (III, V)

We wanted to study whether MMP-8 and -13 are cell surface-associated proteins in OSCC by using immunofluorescence and Western blotting analyses of cultured OSCC cells. When the localization of MMP-8 in VB6 cells was analyzed, the immunofluorescence appeared to localize near the cell surface and in granules in the cytoplasm (V). To determine the molecular weight of MMP-8 in conditioned medium (CM), Trizol®-purified total protein (TP) and cell membrane extract (CME) samples, we found 50 kDa forms in CM, 110, 90, 70 and 50 kDa forms in TP and 110 and 90 kDa forms in CME. After cycloheximide incubation, the external recombinant MMP-8 was also seen associated with the cell surface of VB6 cells. The CMEs were collected and analyzed by IFMA. The CME without recombinant MMP-8 contained 0.009 μg/l of MMP-8, and the CME with recombinant MMP-8 contained 0.014 μg/l. This confirmed that MMP-8 is a cell surface-associated protein. VB6 cells showed less cytoplasmic immunofluorescence for MMP-13 than C1 cells (III). The concentration of MMP-13 was increased inside the cells with monensin
treatment, which prevents protein secretion from the cells. MMP-13 immunoreactivity was weak without monensin treatment. To determine the molecular weight of MMP-13 in conditioned medium (CM), Trizol®-purified total protein (TP) and cell membrane extract (CME) samples, we found 60 and 45 kDa forms in CM, 60, 76 and 110 kDa forms in TP and 76 and 110 kDa forms in CME samples. Thus, similarly to MMP-8, MMP-13 is also a cell surface-associated protein.

5.6 MMP-8 and -13 protein levels are lower in OSCC than in breast cancer (III)

We wanted to study preliminarily if there are differences in the MMP-8 and MMP-13 protein levels between OSCCs and breast cancers. Two breast cancer specimens and one tongue SCC and one gingival SCC sample were pulverized in liquid nitrogen, and the proteins were extracted. The proteins were loaded on to SDS-PAGE and subjected to Western blotting for MMP-8 and MMP-13. The immunoreactive bands were compared to the total protein content. The average relative amounts of both MMP-8 and MMP-13 were lower in the OSCC samples than in the breast cancer samples (III, Figure 5).
Fig. 5. The MMP-8 protein level is lower in OSCC tissues compared to breast cancer tissue. 13 μg of extracted protein was loaded on to 12% SDS-PAGE and subjected to Western blotting (A). 50 kDa immunoreactive products were compared against the total Coomassie blue-stained protein gel (B). The lanes 1 and 3 are breast cancer samples, lane 2 is a tongue SCC sample, lane 4 is a gingival SCC sample, and OB is odontoblast medium as a positive control. The relative amounts of MMP-8 in all samples are shown in figure C. The breast cancer sample in lane 1 has a value 1.
6 Discussion

Although there are currently over two hundred publications of MMPs in HNSCC, it is difficult to compare the results and to draw specific conclusions. Most OSCC studies have been performed on a small number of SCC sections or established cell lines from diverse regions of the head and neck. Also, the materials (e.g. antibodies) and methods have been highly variable between publications. (Werner et al. 2002.)

The aim of my thesis was to evaluate the role and regulation of some MMP family members and TAT-2 in OSCC. MMP-9 turned out to be strongly activated by TAT-2 in a TAT-2-transfected tongue HSC-3 carcinoma cell line. Transfection of TAT-2 did not lead to increased conversion of collagenases (MMP-1, -8 or -13) or stromelysin-1 (MMP-3) into active forms. However, purified TAT-2 activated collagenases and stromelysin-1 in vitro. TAT-2 also degraded efficiently fibrillar type I collagen in vitro. A TAT-2/HSC-3 cell line secreting active MMP-9 was more invasive than HSC-3 without TAT-2 in a chorionallantoic membrane intravasation assay. The induced intravasation was inhibited by a TAT-2 inhibitor TATI or by a specific MMP-9 activity-inhibiting cyclic CTT peptide. Although the transfection of integrin αvβ6 induces the production of MMP-9 (Thomas et al. 2001a) and the invasion of OSCC cells, we demonstrated that transfection of this integrin, surprisingly, down-regulated the MMP-13 level. It did not, however, affect the levels of MMP-1, -8 or TIMP-1. Our study indicated that the human OSCC expressed TAT-2 and a cell membrane-bound MMP-8. Finally, in our preliminary clinical study, we demonstrated that the expression of MMP-8 in mobile tongue correlated with longer survival of female patients. However, similar trend was not seen in male tongue SCC patients. In the following chapters, the possible significance of TAT-2, αvβ6 integrin and MMP-8 for head and neck cancer growth will be discussed in more detail.

6.1 Role of TAT-2 in OSCC

TAT-2 production has been observed in epithelial and other cell types, such as leukocytes, neurons and vascular endothelial cells (Koshikawa et al. 1997, 1998). Several cancers also produce TAT-2. Elevated TAT-2 levels have been observed in tissue sections and serum or cyst fluid samples from gastric, colon, lung and ovarian cancer patients.
We were able to demonstrate variable TAT-2 production in different OSCC cell lines (1). The highly invasive tongue HSC-3 cell line produced TAT-2, whereas the less invasive SCC-25 did not. We also detected TAT-2 immunoreactive protein in OSCC tissue in vivo. Trypsinogen localized sporadically, particularly in peripheral carcinoma cells of cancer nests, in macrophages and some fibroblasts (Figure 4). The presence of TAT-2 in OSCC is in line with the finding that the levels of the trypsin inhibitor TATI are elevated in the serum of HNSCC patients (Goumas et al. 1995). Elevated TAT-2 levels have previously also been shown to correlate with high TATI levels in mucinous ovarian carcinomas (Stenman 1990).

TAT-2 and pancreatic trypsin-2 have partially different substrate specificities (Koivunen et al. 1989). Moreover, many of the previous trypsin-related MMP activation studies have been done with bovine trypsin and not with tumour-associated trypsins (Nagase et al. 1990). Here, we demonstrated that TAT-2 converted procollagenases (MMP-1, -8, -13) and proMMP-3 into smaller forms in vitro (II). Although the PRCXXPD motif was not removed from MMP-1 after TAT-2 conversion, MMP-1 was a catalytically active enzyme, because it degraded type I collagen in vitro. MMP-3 and -13 were completely active forms after TAT-2-induced conversion, and similar cleaving sites have been described using bovine chymotrypsin and TPCK-treated bovine trypsin (Nagase et al. 1990, Knäuper et al. 1996). PMN MMP-8 was cleaved at two tryptic sites located upstream and inside the PRCXXPD motif, achieving partial activation of MMP-8. A similar activation mechanism has been described for bovine trypsin (Tschesche et al. 1992). The converted collagenases degraded type I collagen after TAT-2 incubation in vitro. However, in TAT-2-transfected HSC-3 cells, no conversions of MMP-1, -3, -8 and -13 were observed (II). Moreover, the changes in mRNA and protein levels did not correlate with each other after TAT-2 transfection. In the case of MMP-9, we found that the activation ratio of MMP-9 increased and MMP-2 remained unchanged after TAT-2 transfection in HSC-3/TAT-2 cells (I). This is in line with a previous report of TAT-2 protein activating efficiently proMMP-9, but less efficiently proMMP-2 in vitro (Sorsa et al. 1997). Thus, HSC-3/TAT-2 cells may lack some of the factors that are needed for the translation and activation of MMP-1, -3, -8 or -13 in TAT-2 induction, but not for TAT-2-induced MMP-9 regulation. Moreover, there were no changes in MMP-9 activation in TAT-2-transfected IIGK and SCC-25 cells, although there was more TAT-2 overproduction in these cells compared to HSC-3/TAT-2 cells. The HSC-3 cell line was only one of the three cell lines that naturally produced TAT-2. Thus, it is possible that this cell line has naturally existing enterokinase-like activity. Enterokinase is the trypsinogen activator known to interfere with immunofluorometric assay by digesting part of the TAT-2 molecule essential for antibody recognition (Itkonen et al. 1990). Thus, the actual concentration of TAT-2 in the cell media of HSC-3/TAT-2 cells might be higher than measured.

The ability to activate some MMPs or to regulate their expression could be one important feature of TAT-2 in vivo. In malignant ovarian cyst fluids, high concentrations of active MMP-9 correlate with high concentrations of TAT-2, TAT-1-API (tumour-associated trypsin-1-α1-antitrypsin complex, which reflects the correlation of active trypsin) and TAT-2-API. The active MMP-2 concentrations correlate inversely with TAT concentrations. (Paju et al. 2001.) Moreover, concentrations of MMP-8, but no MMP-1,
correlate with elevated TAT-2 levels and ovarian tumour malignancy stage (Stenman et al. 2003). In our experiments, TAT-2-induced MMP-9 activation in HSC-3/TAT-2 cells correlated well with the increased invasion capacity of the cells in in vivo chorioallantoic membrane assay (CAM) (Kim et al. 1998). Other TAT-2-transfected cells, where the activation ratio of MMP-9 did not change, showed poor intravasation in this assay (I). The important roles of TAT-2 and MMP-9 in OSCC cell invasion were determined by using specific TAT-2 and MMP-9 inhibitors, TATI and CTT peptide, respectively. Both inhibitors decreased the intravasation of HSC-3/TAT-2 cells efficiently in a dose-dependent manner. TAT-2 did not only activate MMP-9 but also increased mRNA expression up to fivefold in HSC-3/TAT-2 cells. These findings suggest that active MMP-9 and TAT-2 have important roles in the OSCC intavasation process.

Another role of TAT-2 in carcinoma progression could be its direct contribution to the degradation of ECM components. We demonstrated that TAT-2 degraded effectively native soluble type I collagen in vitro, generating multiple fragments differing from the classical collagenase-induced ½ (αA) and ¼ (αB) cleavage products (II). TATI inhibited this TAT-2-induced type I collagen degradation. Previously, it has been shown that TAT-2 degrades fibronectin (Koivunen et al. 1991b) and activates uPA in vitro (Koivunen et al. 1989). The uPA/plasmin system is an important activator system of several MMPs, including MMP-9 (Lijnen 2001). Via uPA/plasmin-mediated MMP activation and degradation of the MMP inhibitors TIMP-1 and -2 (Sorsa et al. 1997), ECM degradation is further enhanced by TAT-2. TAT-2 stimulates strongly the proliferation of human colon and gastric cells and also stimulates the α5β1 integrin-dependent adhesion to fibronectin by gastric cancer cells. These events are mediated through a proteinase-activated receptor (PAR-2), which can be degraded and activated by both TAT-2 and pancreas-derived trypsin-2 (Alm et al. 2000, Miyata et al. 2000, Darmoul et al. 2001). TAT-2 activates PAR-2, which in turn releases TGF-α MMP-dependently. The released TGF-α activates EGFR, which further activates the downstream part of the MAPK/ERK1/2 cascade, leading to cell proliferation. (Darmoul et al. 2004.)

Thus, based on our studies, TAT-2-activated MMP-9 may have an important role in the progression of tongue carcinomas, since in CAM assay TAT-2-induced MMP-9 activation clearly increased the intravasation of tongue OSCC cells. Also, based on the previous studies, MMP-9 production is significantly elevated at the mRNA and protein levels in OSCC compared to normal oral mucosa (Sutinen et al. 1998, Shimada et al. 2000, O-charoenrat et al. 2001). MMP-9 mRNA and protein have been found in both tumour and stromal cells and as well as in cancer cells in circulation (Charous et al. 1997, Kurahara et al. 1999, Hong et al. 2000, Franchi et al. 2002, Ondruschka et al. 2002, Ito et al. 2003). Ruokolainen et al. (2004) recently demonstrated that MMP-9 immunopositivity in HNSCCs is associated with shortened relapse-free and cause-specific survival. However, MMP-9 levels did not correlate with the stage or grade of the disease, tumour size or cervical lymph node involvement. There are also other reports demonstrating that the activation level of MMP-9 associates with shortened disease-free survival and high metastatic frequency in OSCCs (Hong et al. 2000, Yorioka et al. 2002). Therefore, the specific inhibition of MMP-9 activity might reduce the growth of SCC and improve the survival of tongue SCC patients.
6.2 αvβ6 integrin-mediated MMP regulation in OSCC

During malignant development in oral cavity, integrin αvβ6 expression is turned on (Jones et al. 1997). Sporadic up-regulation of αvβ6 integrin has been demonstrated consistently in oral epithelial dysplasias, in situ carcinomas and verrucous carcinomas. Hamidi et al. (2000) showed that αvβ6 expression in dysplastic oral epithelium correlates with the malignant progression of the lesion, suggesting that αvβ6 is important at the early stage of oral carcinogenesis. About 35-100% of OSCC tissues express high levels of αvβ6 integrin throughout the tumour islands and especially at the invading edge of the carcinoma. (Breuss et al. 1995, Jones et al. 1997, Hamidi et al. 2000, Regezi et al. 2002, Impola et al. 2004.) Although αvβ6 is associated with OSCC and other epithelial carcinomas, its role in tumourigenesis has been unknown.

Recently, Janes and Watt (2004) showed that αvβ6 integrin has a role in apoptosis. Integrin αvβ6 protects SCC cells from apoptosis (anoikis) by activating an Akt survival signal, whereas αvβ3 integrin-expressing cells undergo apoptosis. Moreover it has been suggested that αvβ6 has a role in the modulation of proteolytic enzyme levels and/or activation of TGF-β1 and -β3 as well as in the migration and invasion of OSCC cells (Munger et al. 1999, Thomas et al. 2001a, 2001b, Annes et al. 2002, Ramos et al. 2002). Expression of αvβ6 integrin is associated with the elevated expression of MMP-3 and -9 and regulation the uPA/plasmin system in OSCC cells (Thomas et al. 2001a, Ramos et al. 2002, Dang et al. 2004, Dalvi et al. 2004). However, we were surprised to find out that more invasive β6-transfected VB6 cells produced significantly lower levels of MMP-13 mRNA and protein than the control C1 cells (III), although up-regulation of MMP-13 production has been demonstrated in several studies of HNSCCs (Johansson et al. 1997, Impola et al. 2004). The changes in αvβ6 integrin levels did not affect the expression of MMP-1, -8 or TIMP-1. This is in line with the finding that the MMP-1 and TIMP-1 levels are similar in β6-transfected colon carcinoma cells and in control cells (Agrez et al. 1999). In addition to αvβ6 integrin-mediated MMP regulation, other β integrins also regulate MMPs. For example, αvβ3 integrin activates MMP-2 and uPAR (Guo & Giancotti 2004), and αvβ5 integrin has a binding site for MMP-9 ( Björklund et al. 2004).

The cytoplasmic domain of β6 integrin, including the 11 amino acids (EKQKVDLSTDC), seems to have an important role in regulation of protease production. This domain is also essential in MMP-9-dependent invasion and down-regulation of uPAR (Morgan et al. 2004, Dalvi et al. 2004). Gu et al. (2002) demonstrated that the cytoplasmic binding site for ERK-2 (extracellular signal-regulated kinase-2) is important for MMP-9 secretion in colon cancer cells. The role of the β6 integrin cytoplasmic domain in the regulation of MMP-13 production is unknown.

We used LAP as a ligand for αvβ6 integrin in our cell experiments. LAP contains a RGD sequence, which acts as a ligand for αvβ6 integrin (Munger et al. 1999). LAP and LTBP (latent TGF-β-binding protein) have been shown to be important in TGF-β activation (Annes et al. 2004). An αvβ6 integrin-expressing cell recognizes the RGD domain in LAP, whereas LTBP fixes a large latent complex to ECM. Integrin αvβ6 activates and liberates TGF-β from LAP by mechanical traction (Annes et al. 2004, Keski-Oja et al. 2004.) TGF-β has been shown to associate with αvβ6 integrin in other studies, too. Luettich & Schmidt (2003) demonstrated that the binding of mature TGF-β1
to αvβ6 integrin via the DLXXL motif (aspartate-leucine-X-X-leucine) activates c-Jun and ERK-1 via the MEKK1/p38 kinase pathway and influences cytoskeletal organization by phosphorylating proteins, which are associated with focal adhesions. This finding may indicate a link between TGF-β1, αvβ6 integrin and the metastatic behavior of cancers. Another study demonstrates that TGF-β1 binds directly to αvβ6 integrin, and activates Ras/MAP kinase pathway leading to growth inhibition in TGF-β1-sensitive cells. This alternative pathway is independent of the known SMAD pathway, which has been described to act as the main pathway in TGF-β1-mediated growth inhibition (Kracklauer et al. 2003.) Thus, interaction of αvβ6 integrin with TGF-β1 has been associated with carcinoma-promoting and growth-inhibiting properties. Another growth factor, TNF-α, is shown to promote MMP-9 and αvβ6 integrin expressions in keratinocytes during tumour formation (Scott et al. 2004).

Based on in vitro invasion and collagen degradation assays, there were no differences in collagenolytic activities between the cell lines, although C1 produced significantly more MMP-13 than β6-transfected VB6 cells (III). These findings were unexpected, since previous studies have assumed MMP-13 to be an important collagenolytic enzyme in HNSCCs and to participate in the early invasion process of the carcinomas (Johansson et al. 1997). Moreover, the intensity of MMP-13 immunostaining in cancer cells has been shown to be higher in more invasive tumours (Impola et al. 2004). In contrast, however, O-charoenrat et al. (2001) did not find any association between the level of MMP-13 mRNA expression and the clinicopathological variables in oral carcinomas. Thus, further studies are required to establish the significance of αvβ6 integrin-mediated MMP regulation and the importance of MMP-13 for OSCC progression.

There is accumulating evidence to show that soluble MMPs associate with the cell membrane, which may be critical for their activation and targeted functions. The cell – surface-associated activation of MMP-2 is well known (Strongin et al. 1993), and at least MMP-1, -7, -9, and -13 have specific cell membrane receptors (Brooks et al. 1996, Knäuper et al. 1996, Olson et al. 1998, Yu & Stamenkovic 1999, Guo et al. 2000, Rolli et al. 2003). Our results confirmed that MMP-13 is also cell membrane-associated in OSCC cells (III). Confocal microscopy demonstrated that MMP-13 localized mainly inside C1 and VB6 cells. The immunoreactivity was, however, also demonstrated in the cell membrane. Bachmeier et al. (2000) found MMP-13 staining only inside skin keratinocytes, not on cell membranes. In our study, the MMP-13 bands of the cell membrane-associated protein of OSCC cells were approximately 110 and 76 kDa and those from conditioned medium 60 and 45 kDa. However, Bachmeier et al. (2000) found only 52 and 42 kDa forms from the cell extracts of three different skin keratinocytes with different malignancy capacities (non-tumourigenic, benign, and malignant cell line) and could not identify immunoreactive MMP-13 in conditioned medium. The amounts of MMP-13 were equal in extracts of the three cell lines. The variation in the results may indicate that oral and skin keratinocytes have differences in the MMP-13 production process or their binding to the cell surface. Also, the antibodies and immunocytochemical stainings used in the different protocols differ.
6.3 Role of MMP-8 in OSCC

Bachmeier et al. (2000) were the first to demonstrate that cultured malignant and non-malignant skin keratinocytes produce MMP-8 at mRNA and protein levels. At the same time, MMP-8 was found in homogenates of OSCC cells and control mucosal epithelia at the protein level (Shimada et al. 2000). We demonstrated low levels of MMP-8 production in cultured HNSCC cells in vitro as well as in HNSCC tissue samples in vivo (IV). MMP-8 mRNA and protein was seen sporadically in carcinoma cells. In addition, tumour stromal fibroblasts, PMN and plasma cells produced MMP-8. However, in basal cell carcinoma of skin, MMP-8 presents only in stromal cells (Varani et al. 2000). MMP-8 has also been found in migratory and proliferating epithelial keratinocytes of healing skin wounds in rats (Pirilä et al. 2001).

We found that MMP-8 production in vitro was down-regulated in a dose-dependent manner by TGF-β1 in UT-SCC-42B cells (IV). This result is in line with the findings that TGF-β1 also down-regulates MMP-8 production in osteoarthritic chondrocytes at the mRNA level (Shlopov et al. 1999) and in human odontoblasts and dental pulp cells at the mRNA and protein levels (Palosaari et al. 2000). TGF-β1 has been shown to inhibit the proliferation of dysplastic and metastatic cells established from the oral cavity and, in contrast, to induce the invasive potential of the most malignant cells (Hsu et al. 2002). It has been shown that IL-1β and TNF-α modulate MMP-8 expression cell line-dependently. They either up-regulate or have no effect on MMP-8 expression (Cole et al. 1996, Hanemaaijer et al. 1997, Abe et al. 2001, Stremme et al. 2003, Li de et al. 2003, Stadlmann et al. 2003). Phorbol 12-myristate 13 acetate (PMA) up-regulated and partially activated MMP-8 in UT-SCC-42B cells at the protein level. This result agrees with Shlopov et al. (1999) and Hanemaaijer et al. (1998), who reported PMA-mediated MMP-8 induction of chondrocytes and endothelial cells in osteoarthritis. αvβ6 integrin, which up-regulated MMP-9 and down-regulated MMP-13, did not modulate MMP-8 mRNA or protein expression in OSCC cells (III).

Recently, Owen et al. (2004) demonstrated that MMP-8 is membrane-bound in activated PMN cells. Binding to the PMN cell membrane seems to protect MMP-8 from its inhibitors, TIMPs, and maintains its catalytic stability. We showed that MMP-8 is a cell-surface associated protein in OSCCs, too. We found a granular pattern of intracellular immunoreactivity for MMP-8 and immunoreactivity in cell membrane in confocal microscopic analysis. Previously, Bachmeier et al. (2000) found that MMP-8 localizes intracytoplasmically in a granular pattern in skin keratinocytes, but not in cell membrane. Furthermore, they did not find MMP-8 in cell extracts by Western blotting. Our Western blotting revealed approximately 90 and 110 kDa MMP-8 species in cell membrane extracts, which correspond well to the molecular weights of membrane-bound MMP-8 in activated PMN cells (Owen et al. 2004), although we did not find the 30 kDa form that was present in PMN cells. The receptor/ligand for MMP-8 in the cell surface is unknown. It has been shown that MT1-MMP can active MMP-8 in vitro (Holopainen et al. 2003), but it is not known if MT1-MMP is a cell membrane receptor for MMP-8 in vivo.

There are contradictory suggestions about the role of MMP-8 in cancers. Elevated serum levels of MMP-8 have been found in HNSCCs and benign and malignant adrenal
tumours (Kolomecki et al. 2001, Kuropkat et al. 2002). In HNSCCs, elevated levels correlate with the TNM status of the tumours but not with their histopathological grading (Kuropkat et al. 2002). In peripheral blood, MMP-8 expression in mononuclear cells and granulocytes increased significantly in chronic myeloid leukemia patients analyzed by cDNA microarray assay (Bruchova et al. 2002). In ovarian cancer, the intensity of MMP-8 immunostaining correlated with tumour grade, tumour stage and poor prognosis (Stadlmann et al. 2003). However, in human breast cancer, no relationship was seen between the MMP-8 protein levels and either tumour size or metastasis. MMP-8 correlated inversely with estrogen and progesterone receptor levels. (Duffy et al. 1995.) Recent findings indicate, interestingly, that MMP-8 may even have a gender-specific protective role in certain cancers. Male mice that lack the MMP-8 gene (MMP-8-/-) have more skin tumours than female MMP-8-/- or wild-type mice. Moreover, skin tumours are more aggressive and undifferentiated in MMP-8-/- male mice than in MMP-8-/- female or wild-type mice. Interestingly, ovarian estrogen protects against skin tumourigenesis in female MMP-8-/- mice. (Balbín et al. 2003.) Moreover, metastatic breast cancer cells manipulated to up-regulate MMP-8 have a decreased metastatic capacity in athymic mice compared to breast cancer cells whose MMP-8 production is down-regulated. (Montel et al. 2004.)

These recent findings have, however, been obtained with cultured cells and mouse assays. Our study (V) is the first with human samples, and these results are in line with the suggested gender-specific protective role of MMP-8 in epithelial cancers. Our results demonstrated that MMP-8 expression in tongue SCC samples was associated with improved cause-specific survival in females, but not in males (V). MMP-8 production was independent of the stage or grade of the tumour. The difference in cause-specific survival between females and males supports the interaction of MMP-8 and estrogen in protection against cancer growth. MMP-8 may, for instance, contribute to the modulation of the host’s immune response during carcinogenesis. Balbin et al. (2003) suggest that the absence of MMP-8 influences the early stages of tumour development in male mice. The discrepancy between the possible roles of MMP-8 in variable cancers may reflect different functions of MMP-8 in different cancers. Our study focused exclusively on mobile tongue SCCs. Based on our results, we can speculate that one reason for the failures in clinical cancer trials using broad-spectrum MMP inhibitors (which are also effective against MMP-8) might be the inhibition of the unknown protective effect of MMP-8 in cancer.
The presence and activity of various matrix metalloproteinases in oral carcinomas play important roles in tumour growth and spreading. This study demonstrated that TAT-2 can activate proMMPs in a panel of tumour cells derived from otolaryngeal cancers. TAT-2 transfection led to an efficient activation of proMMP-9 in only one (HSC-3) of the three transfected cell lines, and it had no effect on proMMP-2 activation. TAT-2-transfected HSC-3 cells intravasated more efficiently in a chicken chorionallantoic membrane assay compared to untransfected cells. Specific tumour-associated trypsin inhibitor, TATI, diminished TAT-2-induced proMMP-9 activation and intravasation. Also, a specific MMP-9 inhibitor, CTT peptide, decreased the intravasation of TAT-2-transfected HSC-3 cells, indicating the important role of active MMP-9 in the tongue cancer intravasation process.

TAT-2 also converted collagenases (MMP-1, -8 and -13) and stromelysin-1 (MMP-3) into 10-20 kDa smaller forms in vitro. Collagenases induced collagenolytic activities during TAT-2 treatment, and the TAT-2-specific inhibitor TATI diminished MMP conversion and activities. However, in TAT-2-transfected HSC-3 cells, the conversion of collagenases or stromelysin-1 was similar to that seen in non-transfected cells, indicating that in vitro conversion may not always occur at the cellular level. TAT-2 was demonstrated to degrade native fibrillar type I collagen in vitro, which may induce the proteolytic degradation of ECM surrounding the carcinoma cell islands. By immunohistological stainings, we could demonstrate the production of TAT-2 in OSCC may participate in the initial activation cascade of MMPs and the degradation of collagen fibers leading to enhanced modulation of carcinoma ECM.

Integrin $\alpha_v\beta_6$ is associated with malignant transformation of OSCCs. However, although $\alpha_v\beta_6$ transfection induces the production of OSCC-associated MMP-9, we found, surprisingly, that the amounts of MMP-13 mRNA and protein were clearly reduced in $\alpha_v\beta_6$ cells compared to controls. Integrin $\alpha_v\beta_6$ down-regulated MMP-13 production in both ligand-dependent and -independent manners similarly to MMP-9 up-regulation. Integrin $\alpha_v\beta_6$ transfection did not change the MMP-1, -8 or TIMP-1 expression levels. Interestingly, we could not see any difference in the collagenolytic activities or invasion properties of OSCCs by assessing the type I collagen of $\beta_v$-
transfected and control cells, although the amounts of MMP-13 differed clearly in these two cell lines. Our results on the role of MMP-13 in OSCC carcinogenesis differ from the previous publications, and further studies are necessary to evaluate the importance of $\alpha_v\beta_6$ integrin-dependent regulation of MMP-13 in oral carcinomas.

We found a low level of MMP-8 mRNA and protein in various established head and neck carcinoma, tumour and dermal fibroblast cell lines. MMP-8 protein was also identified in oral cancer sections in carcinoma, PMN and plasma cells as well as in tumour fibroblasts. MMP-8 transcripts were located in carcinoma cells. The overall level of MMP-8 expression was low in all HNSCC sections. However, by MMP-8 immunohistochemistry of mobile tongue SCC sections, we demonstrated that the presence of MMP-8 was associated with improved cause-specific survival in females, but not in males, suggesting an interesting gender-specific protective role of MMP-8 in tongue cancer. Further studies with larger materials and SCCs from various locations should be done before the possible diagnostic or therapeutic value of this finding can be evaluated.
References


