Expression and role of matrix metalloproteinases and the laminin-5 γ2-chain in wound healing and cell migration

Emma Pirilä

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Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine and Institute of Dentistry, Faculty of Medicine, Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland and Institute of Dentistry, Faculty of Medicine, University of Oulu, Oulu, Finland

Academic Dissertation

To be publicly discussed with the permission of the Faculty of Veterinary Medicine, University of Helsinki in Auditorium XII at the University of Helsinki, Unioninkatu 34, Helsinki on the 5th of December at noon.

Helsinki 2003
Dedicated to the memory of

my grandparents Enni & Juho Pirilä

and

Professor Päivi Maisi
Pirilä, Emma, Expression and role of matrix metalloproteinases and the laminin-5 γ2-chain in wound healing and cell migration
Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine and Institute of Dentistry, Faculty of Medicine, University of Helsinki and Department of Oral Maxillofacial Diseases, Helsinki University Central Hospital, Helsinki, Finland and Institute of Dentistry, Faculty of Medicine, University of Oulu, Oulu, Finland.

Abstract

Inflammation is the host response to resolve any intrusion or attack to the organism. The host response involves removal of the foreign particles or regeneration of open surfaces, extracellular matrix (ECM) remodelling and cell migration. At the tissue level, inflammation occurs through the co-ordinated action of multiple molecular pathways. The matrix metalloproteinases (MMPs) are endopeptidases which cleave most ECM and basement membrane proteins. MMPs mediate important functions during normal physiological remodelling and development, however, MMP activity is known to be aberrantly upregulated during pathological conditions, including chronic inflammatory diseases and tumor cell invasion. Thus, many attempts have been made to develop synthethic inhibitors against MMP activity. Laminin-5 (Ln-5) is a heterotrimeric glycoprotein part of the hemidesomosomal complex which anchors surface epithelia to the underlying stroma. The Ln-5 γ2-chain can be cleaved by MMP-2 and -14, which induces cell migration.

In this study, the role and expression of MMPs were studied in chronic inflammatory periodontal disease tissue representing a typical disease state, where the hosts ability to resolve the primary inflammation is impaired. In conjunction with this, the effect of inflammatory-related cytokines was studied in cultured human oral mucosal keratinocytes and different MMP inhibitors (MMPIs) were tested. Wound healing is known to be delayed due to estrogen-deprivation, which is a typical state affecting women past menopause. In this study, MMP expression and activity as well as the Ln-5 γ2-chain processing were studied in cutaneous wound healing of estrogen-deficient rats, and the effect of estrogen or MMPI treatment was additionally assessed. Finally, the direct ability of different MMPs to process the Ln-5 γ2-chain and subsequently induce carcinoma cell migration was studied in vitro.

The results demonstrate a role for cytokine regulation of MMP-2 and –9 in human oral mucosal keratinocytes. MMP-2 and –9 activities could be downregulated in vitro by chemically modified tetracyclines as well as in situ by an MMP-2 and –9 specific inhibitor, the CTTHWGFTLC-peptide. Cutaneous wound healing in estrogen-deprived rats was impaired due to significantly reduced collagen deposition and content, instability of the re-epithelialized wound surface, changes in MMP activity and expression as well as aberrant Ln-5 γ2-chain processing. These changes could be reversed by treating the animals with estrogen or a chemically modified tetracycline. Several MMPs in addition to the previously characterized MMP-2 and –14 were found to process the Ln-5 γ2-chain and induce carcinoma cell migration.

In summary, the results demonstrate a role for MMPs and the Ln-5 γ2-chain in tissue degradation and remodelling associated with inflammatory processes and in regulating cell migration. The use of MMPIs could be useful in the treatment of both cancer and chronic inflammatory diseases.
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Helsinki, November 2003

Emma Pirilä
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-PI</td>
<td>α1-proteinase inhibitor</td>
</tr>
<tr>
<td>α2M</td>
<td>α2-macroglobulin</td>
</tr>
<tr>
<td>AP</td>
<td>Adult periodontitis</td>
</tr>
<tr>
<td>APMA</td>
<td>p-aminophenylmercuric acetate</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>C-</td>
<td>Carboxy-</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium-ion</td>
</tr>
<tr>
<td>CMT</td>
<td>Chemically modified tetracycline</td>
</tr>
<tr>
<td>CTT</td>
<td>CTTHWGFTLC-peptide</td>
</tr>
<tr>
<td>EB</td>
<td>Epidermolysis bullosa</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic-acid</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
</tr>
<tr>
<td>FC</td>
<td>Focal contact</td>
</tr>
<tr>
<td>GCF</td>
<td>Gingival crevicular fluid</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma cells</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloride acid</td>
</tr>
<tr>
<td>HD</td>
<td>Hemidesmosome</td>
</tr>
<tr>
<td>HMK</td>
<td>Human oral mucosal keratinocytes</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Inhibitory concentration on 50% of the measured parameter</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LDD</td>
<td>Low-dose doxycycline</td>
</tr>
<tr>
<td>Ln</td>
<td>Laminin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
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<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>N-</td>
<td>Amino-</td>
</tr>
<tr>
<td>n</td>
<td>Nano</td>
</tr>
<tr>
<td>NHK</td>
<td>Normal human keratinocytes</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomized</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Pg</td>
<td>Plasminogen</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-OH-kinase</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>Sulcular epithelium</td>
</tr>
<tr>
<td>TAT-2</td>
<td>Tumor associated trypsinogen-2</td>
</tr>
<tr>
<td>TC</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
</tbody>
</table>
\(X\) any amino acid
\(Y\) any amino acid
\(Zn^{2+}\) Zinc-ion

\(\alpha\) alpha
\(\beta\) beta
\(\gamma\) gamma
\(\mu\) micro [Mu]

Amino acid abbreviations:

<table>
<thead>
<tr>
<th>A</th>
<th>Ala</th>
<th>Alanine</th>
<th>M</th>
<th>Met</th>
<th>Methionine</th>
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<tr>
<td>C</td>
<td>Cys</td>
<td>Cysteine</td>
<td>N</td>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>D</td>
<td>Asp</td>
<td>Aspartate</td>
<td>P</td>
<td>Pro</td>
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<tr>
<td>E</td>
<td>Glu</td>
<td>Glutamate</td>
<td>Q</td>
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<td>F</td>
<td>Phe</td>
<td>Phenylalanine</td>
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<td>Gly</td>
<td>Glycine</td>
<td>S</td>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>H</td>
<td>His</td>
<td>Histidine</td>
<td>T</td>
<td>Thr</td>
<td>Threonine</td>
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<tr>
<td>I</td>
<td>Ile</td>
<td>Isoleucine</td>
<td>V</td>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>K</td>
<td>Lys</td>
<td>Lysine</td>
<td>W</td>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>L</td>
<td>Leu</td>
<td>Leucine</td>
<td>Y</td>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
</tbody>
</table>

(Alberts et al. 1994)
List of original publications


Abstract
Acknowledgements
Abbreviations
List of original publications
Contents
1. Introduction
2. Review of the literature
   2.1. The collagenous extracellular matrix
   2.2. Matrix metalloproteinases
      2.2.1. General characteristics of MMPs
      2.2.2. Collagenases
      2.2.3. Gelatinases
      2.2.4. Stromelysins
      2.2.5. Matrilysins
      2.2.6. MT-MMPs
      2.2.7. The X-files of the MMPs
      2.2.8. Activation mechanisms of MMPs
         2.2.8.1. Cell-surface activation by MMP-14
      2.2.9. Transcriptional regulation of MMPs
      2.2.10. In vivo functions of MMPs
      2.2.11. In vivo localization of MMPs
   2.3. MMP inhibition
      2.3.1. Physiological inhibition of MMPs
      2.3.2. Synthetic MMPIs
         2.3.2.1. Batimastat
         2.3.2.2. Marimastat
         2.3.2.3. Tetracyclines
         2.3.2.4. The chemically modified tetracyclines
         2.3.2.5. The CTTHWGFTLC-peptide
      2.3.3. MMPIs in clinical trials
   2.4. Laminin-5
      2.4.1. Structure of the heterotrimeric Ln-5 molecule
      2.4.2. Ligands for Ln-5
      2.4.3. Processing of the Ln-5 chains
      2.4.4. In vivo functions of Ln-5
   2.5. Cell migration
      2.5.1. Mechanisms of cell migration
      2.5.2. Proteolysis and ECM remodelling during cell migration
      2.5.3. MMPs and tumor cell migration
      2.5.4. Ln-5 in physiological cell migration
      2.5.5. Ln-5 and tumor cell migration
   2.6. Periodontal tissue
   2.7. Skin
      2.7.1. The basement membrane
         2.7.1.1. The hemidesmosomal complex
      2.7.2. Estrogen and skin
         2.7.2.1. Estrogen and skin collagen content
   2.8. General characteristics of inflammation
      2.8.1. Chronic inflammatory periodontal disease
         2.8.1.1. MMPs in chronic periodontal disease
         2.8.1.2. MMP inhibition in periodontal disease
      2.8.2. Cutaneous wound healing
         2.8.2.1. Clotting
2.8.2.2. The inflammatory response................................................................. 47
2.8.2.3. Re-epithelialization................................................................. 47
2.8.2.4. Wound contraction................................................................. 47
2.8.2.5. Growth factors regulating wound healing.................................... 48
  2.8.2.5.1. TGF-β and scarring.......................................................... 48
2.8.2.6. MMPs in wound healing............................................................. 49
2.8.2.7. MMP inhibition in wound healing.............................................. 49
2.8.2.8. Ln-5 in wound healing............................................................ 50
3. Aims of the study.................................................................................... 51
4. Materials and Methods............................................................................ 52
  4.1. Animals................................................................................................. 52
  4.2. Tissue samples.................................................................................. 52
  4.3. Materials............................................................................................. 52
  4.4. Cell lines............................................................................................ 53
  4.5. MMP inhibition of cell proliferation.................................................. 53
  4.6. Cell migration assays.......................................................................... 53
    4.6.1. Radial migration........................................................................... 53
    4.6.2. Scratch assay................................................................................ 53
    4.6.3. Transwell migration...................................................................... 53
  4.7. Collagenase and gelatinase activity assays.......................................... 54
  4.8. Zymography........................................................................................ 54
  4.9. Western immunoblotting..................................................................... 55
  4.10. In situ zymography............................................................................ 55
  4.11. Immunohistochemistry...................................................................... 56
  4.12. In situ hybridization.......................................................................... 56
  4.13. MMP cleavage in vitro...................................................................... 57
  4.14. N-terminal sequencing...................................................................... 57
5. Results...................................................................................................... 58
  5.1. MMP inhibition and cell growth............................................................ 58
  5.2. MMP inhibition and gelatinase production by cells................................ 58
  5.3. MMP inhibition and cell migration......................................................... 58
    5.3.1. Radial migration........................................................................... 58
    5.3.2. Scratch assay................................................................................ 58
    5.3.3. Transwell assay............................................................................ 59
  5.4. Immunolocalization of MMP-2, MMP-9, Ln-5 γ2-chain and CD45-positive cells in inflamed human gingival tissue.......................................................... 59
  5.5. MMP-2 and MMP-9 mRNA expression in inflamed gingival tissue........ 59
  5.6. In situ gelatin zymography................................................................... 59
  5.7. Immunolocalization of MMP-2 and Ln-5 in epithelial in vitro wounds.......................................................... 60
    5.7.1. Controls for in situ hybridization and immunohistochemistry..... 60
  5.8. Effect of estrogen and CMT-8 in rat cutaneous wound healing.................. 60
    5.8.1. Wound collagen content............................................................... 60
    5.8.2. Collagenase and gelatinase activity............................................... 60
    5.8.3. Expression of collagenases in rat day 7 cutaneous wounds............ 61
      5.8.3.1. MMP-8 and MMP-13 protein and mRNA expression................ 61
      5.8.3.2. Western immunoblotting of MMP-8 and MMP-13.............. 61
    5.8.4. Expression of gelatinases and MMP-14 in rat day 7 cutaneous wounds .......................................................... 61
      5.8.4.1. MMP-2............................................................................. 61
      5.8.4.2. MMP-9............................................................................. 62
      5.8.4.3. MMP-14.......................................................................... 62
    5.8.5. Basement membrane..................................................................... 62
      5.8.5.1. Ln-5 γ2-chain expression.................................................... 62
      5.8.5.2. Ln-5 γ2-chain processing................................................... 63
  5.9. Ln-5 γ2-chain and β-casein processing by MMPs in vitro.......................... 63
5.9.1. N-terminal sequences of MMP cleaved Ln-5 γ2-chain fragments

5.9.2. Cell migration over MMP cleaved Ln-5

6. Discussion

6.1. MMP inhibition and HMK cell proliferation

6.2. MMP and Ln-5 γ2-chain expression in HMKs and inflamed gingival tissue

6.3. MMP inhibition in HMKs and inflamed periodontal tissue

6.4. Cutaneous wound healing

6.5. Therapeutic aspects of estrogen and MMPIs in cutaneous wound healing

6.6. Cell migration and Ln-5 γ2-chain processing

6.7. MMP inhibition-last aspects

7. Conclusions

References

Original publications
1. Introduction

An acute inflammation is a host response to resolve tissue injury caused by foreign particles, pathogenic attack or mechanical tissue injury. The inability of the host to resolve an acute inflammation and restore tissue integrity leads to chronic inflammation and host-derived matrix degradation. Inability to resolve inflammation may be due to nutritional factors, disease-related or hormonally influenced. Many of the molecular mechanisms underlying progression from acute to chronic inflammation are unresolved. Cell migration is a critical feature for many physiological processes such as wound healing, development, remodelling of the female reproductive organs, bone formation, angiogenesis and neuronal outgrowth and in pathological processes such as cancer and chronic inflammatory diseases.

Matrix metalloproteinases (MMPs) are a family of Zn\(^{2+}\)-dependent endopeptidases capable of cleaving most extracellular matrix (ECM) and basement membrane (BM) molecules. MMPs mediate important functions during normal tissue remodelling and development but aberrant MMP activity has been reported in several tissue destructive pathological conditions such as cancer and chronic inflammatory diseases. MMP inhibitors (MMPIs) have been developed for treatment of several severe human diseases, however, MMP inhibition is often associated with unwanted side-effects due to interference with normal tissue remodelling processes. In addition, the parameters measuring MMPI efficacy in the present models of clinical trials in cancer are ill-fitting. As an exception, adult periodontal disease has been successfully treated with a pharmaceutical inhibiting MMPs in the USA.

Laminin-5 (Ln-5) is a heterotrimeric glycoprotein part of the hemidesmosome (HD) structure within the BM and linking surface epithelia to the underlying connective tissue. Epithelial cells deposit Ln-5 while migrating and MMP-2 and -14 cleavage of the Ln-5 \(\gamma 2\)-chain induce cell migration. In women past menopause, estrogen deprivation leads to impaired wound healing due to delayed re-epithelialization, reduced skin collagen synthesis and a prolonged inflammatory response. While it is known that elevated MMP expression and activity are associated with estrogen-deprived delayed wound healing, little is known about the effects of estrogen-deprivation on Ln-5 \(\gamma 2\)-chain expression and processing.

Periodontal disease exhibits all typical features of chronic inflammation and resembles wound healing events. When acute inflammatory gingival inflammation is left untreated, it may progress into chronic adult periodontitis (AP), leading to soft and hard tissue destruction and eventually tooth loss. Periodontal disease and delayed wound healing are typically associated with elevated MMP activity, reduced expression of tissue inhibitor of metalloproteinases (TIMPs) and excess degradation of ECM and BM molecules. The present work was conducted to investigate the expression, activity and inhibition of MMPs and the Ln-5 \(\gamma 2\)-chain processing in situations associated with chronic inflammation such as cutaneous wound healing in estrogen-deprived rats, human periodontal disease and cell migration.
2. Review of the literature

2.1. The collagenous extracellular matrix

Tissues within an organism are composed of a cell mixture and the surrounding ECM consisting of insoluble protein fibrils, mainly collagens and elastins, as well as soluble polymers such as glycosaminoglycans, proteoglycans and cell adhesive glycoproteins. Cells bind to the surrounding ECM mainly by transmembrane molecules. ECM supports cell motility within the connective tissues, regulates cell proliferation, shape and function as well as allows nutrients and chemical messengers to freely diffuse (Alberts et al. 1994). Insoluble collagen fibrils make up about 30% of the human body's proteins and approximately half of the human body's collagens are within the bones while most of the remaining collagen content resides within the skin (Myllyharju and Kivirikko 2001). Up to now over 30 different collagen α−chains differing in the primary sequence have been characterized. The sequence of the various α−chains contains a variable number of the classical Gly-X-Y repetitive motifs, which form the so-called collagenous domains and noncollagenous domains of variable length and location. A mature collagen molecule is constituted by a 300 nm helical rod terminated by very short noncollagenous sequences, the telopeptides. During fibrillogenesis collagen molecules self-assemble into fibrils and subsequently aggregate to form a collagen fiber (Aumailley and Gayraud 1998).

The turnover of most adult tissue macromolecules is considered to be very slow. Highly regulated degradation of ECM molecules serves several distinct functions, namely assembly of the ECM, removal of excess components and remodelling of ECM structure. These three processes are the key to ECM synthesis and assembly, the physiological remodelling during growth, differentiation, morphogenesis and wound healing (Basbaum and Werb 1996).

2.2. Matrix metalloproteinases

Diffusible proteinases capable of degrading fibrillar collagen were first identified in 1962 from involuting tadpole tail (Gross and Lapier 1962). From there on, several families and subclasses of these proteinases have been characterized. MMPs are a family of highly conserved endopeptidases dependent on Zn²⁺-ions for activity. Evolution of MMPs may, however, be more ancient than currently realized. The Bacteroides fragilis-bacteria contain an amino acid sequence with some identity to an MMP (Massova et al. 1998). MMPs can collectively cleave most ECM and BM macromolecules (Table 1). At present, 25 vertebrate MMPs and 22 human homologues have been identified and characterized. MMP numbering is usually determined by the order of the discovery, MMP-1 being the first. However, MMP-4, -5 and –6 have been eliminated as a consequence of duplication. MMP nomenclature often includes a characteristic name, for example gelatinase A/MMP-2. MMPs participate in many physiological processes, such as embryonic development, organ morphogenesis, blastocyst implantation, ovulation, nerve growth, cervical dilatation, post-partum uterine involution, mammary development, endometrial cycling, hair follicle cycling, angiogenesis, inflammatory cell function, apoptosis, tooth eruption, bone remodelling and wound healing. To ensure physiologically beneficial MMP activity, regulation takes place at the level of transcription, translation, trafficking of membrane-bound forms (secretion and endocytosis), extracellular binding proteins, shedding, oligomerization, internalization and autolysis, activation and in addition, MMP activity is regulated by their physiological inhibitors, i.e.TIMPs. Regulatory action or induction is mediated by growth factors, hormones, cytokines, cell-matrix, cell-cell interactions and cellular transformation as well as by extrinsic chemical/physical factors. Aberrant MMP regulation has been associated with numerous tissue destructive diseases, including cancer, arthritis, cardiovascular disease, nephritis, neurological disease, breakdown of the blood brain barrier, infectious diseases, periodontal disease, lung diseases, gastric ulceration, eye diseases, corneal ulceration, skin diseases, liver fibrosis, atherogenesis, emphysema and chronic wound healing (Nagase and Woessner 1999).
### TABLE 1. Matrix metalloproteinases

<table>
<thead>
<tr>
<th>Name (specific name)</th>
<th>Size latent/active (kDa)</th>
<th>Substrates</th>
</tr>
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<tbody>
<tr>
<td>MMP-1 (Collagenase-1)</td>
<td>52/41</td>
<td>Types I, II, III, VII, X, XI collagens, gelatin, entactin, aggrecan, tenascin, perlecan, vitronectin, IGFBP-2,3, α1-α1Pi, α2M, proTNF-α.</td>
</tr>
<tr>
<td>MMP-2 (Gelatinase A)</td>
<td>72/62</td>
<td>Types I, III, IV, V, VII, X, XI collagens, gelatin, elastin, fibronectin, laminin, aggrecan, vitronectin, tenascin, decorin, pro-HB-EGF, proL-1Δ, plasminogen, E-cadherin, IGFBP-3, α1-antich, α2M, proTNF-α.</td>
</tr>
<tr>
<td>MMP-3 (Stromelysin-1)</td>
<td>54/43, 28</td>
<td>Types I, IV, V, VI, X, XI collagens, elastin, proteoglycans, laminin, fibronectin, gelatin, fibrin/fibrinogen, α1-antichymotrypsin, α1PI, α2M.</td>
</tr>
<tr>
<td>MMP-7 (Matrilysin)</td>
<td>28/19</td>
<td>Elastin, proteoglycans, laminin, fibronectin, gelatin, types I, III, IV, V, VI, X, XI collagens, fibrin/fibrinogen, α1-antichymotrypsin, α1PI, α2M.</td>
</tr>
<tr>
<td>MMP-8 (Collagenase-2)</td>
<td>75/58, 54/42</td>
<td>Types I, II, III, VII, X collagen, gelatin, entactin, perlecan, vitronectin, decorin, α1-PI, α2M, proTNF-α.</td>
</tr>
<tr>
<td>MMP-9 (Gelatinase B)</td>
<td>92/82</td>
<td>Types I, IV, V, VII, X, XI, XIV, XVII gelatin, elastin, fibronectin, laminin, aggrecan, vitronectin, decorin, proTGF-2RA, plasminogen, α1PI, proTNF-α, α1-antichymotrypsin, α2M, α1PI.</td>
</tr>
<tr>
<td>MMP-10 (Stromelysin-2)</td>
<td>54/43, 24</td>
<td>Types III, IV, V, VI, X, XI, proteoglycans, laminin, fibronectin, gelatin, aggrecan, elastin, fibrin/fibrinogen, α1-antichymotrypsin, α1PI.</td>
</tr>
<tr>
<td>MMP-11 (Stromelysin-3)</td>
<td>55/44</td>
<td>α1PI, IGFBP-1, α2M.</td>
</tr>
<tr>
<td>MMP-12 (Metalloelastase)</td>
<td>54/43, 22</td>
<td>Types I, IV collagen, aggrecan, decorin, gelatin, elastin, fibronectin, fibrin/fibrinogen, laminin, proteoglycan, α1-antichymotrypsin, α2M, α1PI.</td>
</tr>
<tr>
<td>MMP-13 (Collagenase-3)</td>
<td>60/48</td>
<td>Native types I, II, III collagen, gelatin, fibrinogen, tenascin, perlecan, ntidogen, vitronectin, factor XII, α1-antichymotrypsin, α2M, α1PI.</td>
</tr>
<tr>
<td>MMP-14 (Membrane type 1 metalloproteinase)</td>
<td>66/80</td>
<td>Native types I, II, III collagen, gelatin, fibrinogen, tenascin, perlecan, nidogen, vitronectin, factor XII, α1-antichymotrypsin, α2M, α1PI.</td>
</tr>
<tr>
<td>MMP-15 (Membrane type 2 metalloproteinase)</td>
<td>76/61</td>
<td>Laminin, fibronectin, tenascin, nidogen, entactin, gelatin, aggrecan, vitronectin, α1-antichymotrypsin, α2M, α1PI.</td>
</tr>
<tr>
<td>MMP-16 (Membrane type 3 metalloproteinase)</td>
<td>70/56</td>
<td>Gelatin, type III collagen, perlecan, fibronectin, vitronectin, α1-antichymotrypsin, α2M, α1PI.</td>
</tr>
<tr>
<td>MMP-17 (Membrane type 4 metalloproteinase)</td>
<td>71/67</td>
<td>Gelatin, fibrin/fibrinogen, α2M, proTNF-α.</td>
</tr>
<tr>
<td>MMP-18</td>
<td>xCol4</td>
<td>Type I collagen.</td>
</tr>
<tr>
<td>MMP-19 (RASI)</td>
<td>57</td>
<td>Type I and IV collagen, fibronectin, gelatin, tenascin, casein, laminin, entactin, aggrecan, COMP.</td>
</tr>
<tr>
<td>MMP-20 (Enamelysin)</td>
<td>54/43</td>
<td>Amelogenin, casein, gelatin, fibrinogen, type IV, XVIII collagen, laminin, tenascin C, aggrecan, COMP.</td>
</tr>
<tr>
<td>MMP-21</td>
<td>62/49 (human)</td>
<td>Gelatin, casein.</td>
</tr>
<tr>
<td>MMP-22</td>
<td>42/28</td>
<td>Gelatin, casein.</td>
</tr>
<tr>
<td>MMP-23 (CA-MMP)</td>
<td>66</td>
<td>Progelatin, type I collagen, fibronectin, laminin.</td>
</tr>
<tr>
<td>MMP-24 (Membrane type 5 metalloproteinase)</td>
<td>73/64</td>
<td>Gelatin, type IV collagen, fibronectin.</td>
</tr>
<tr>
<td>MMP-25 (Membrane type 6 metalloproteinase)</td>
<td>63/58</td>
<td>Fibrinogen, fibrinogen, gelatin, type IV collagen, α1PI, laminin-1.</td>
</tr>
<tr>
<td>MMP-26 (Matrilysin 2, Endomatis)</td>
<td>29/19</td>
<td>Type II collagen, gelatin, fibronectin, Casein.</td>
</tr>
<tr>
<td>MMP-27</td>
<td>75</td>
<td>Casein.</td>
</tr>
<tr>
<td>MMP-28 (Epiplas)</td>
<td>59/45</td>
<td>Casein.</td>
</tr>
</tbody>
</table>

2.2.1. General characteristics of MMPs

MMPs consist of a single polypeptide, varying between 20-100 kiloDalton (kDa) in size (Figure 1). Upon translation, the full-length MMP is in a prepro-form and the amino (N)-terminal predomain of MMPs contains a hydrophobic leader sequence which targets most of these enzymes to the secretory pathway. Concomitant with secretion, the signal sequence is cleaved off, resulting in, at least usually, an inactive proMMP. The prodomain has a conserved PRCGVPD motif involved in maintaining the latency of the MMPs. The cysteine within this sequence (the so called “cysteine switch”) ligates the catalytic Zn$^{2+}$ to maintain proMMPs in an inactive state. Upon activation, the prodomain is cleaved off or the enzyme undergoes conformational changes to become an active proteinase. The catalytic domain contains a HEXGHXXXHS motif with three His-residues and Glu-residues considered to be the critical catalytic Zn$^{2+}$-binding sites and a conserved methionine, which forms a unique “Met-turn” structure (Nagase and Woessner 1999). The catalytic domain may contain two Zn$^{2+}$-ions of which one has been suggested to be structural (Willenbrock et al. 1995). The catalytic domain dictates cleavage-site specificity through its active site cleft and is linked to the Carboxy (C)-terminal hemopexin domain by a proline-rich linker peptide. The hemopexin-domain has an ellipsoidal disk shape consisting of four antiparallel $\beta$-strands and a $\alpha$-helical central cavity occupied by a Ca$^{2+}$-ion. Membrane type (MT)-MMPs, which are destined to the cell surface, contain an additional single-pass transmembrane domain located at the C-terminus (Nagase and Woessner 1999, Sternlicht and Werb 2001).

2.2.2. Collagenases

The three mammalian collagenases are MMP-1 (collagenase-1), MMP-8 (collagenase-2) and MMP-13 (collagenase-3). The collagenases cleave the $\alpha$-chains of fibrillar collagens I, II and III at the (P$_1$)Gly$^{775}$-(P$_1$)Ile/Leu$^{776}$ site resulting in the generation of N-terminal $\frac{1}{4}$ ($\alpha^A$) and C-terminal $\frac{3}{4}$ fragments ($\alpha^B$), which then rapidly denature at body temperature and are further degraded by gelatinolytic MMPs (Kähari and Saarialho-Kere 1999). MMP-8 cleaves triple helical type I collagen more efficiently than type III collagen (Hasty et al. 1987), MMP-1 prefers type III collagen over type I collagen (Hasty et al. 1987, Mallya et al. 1990), and MMP-13 type II collagen (Knauper et al. 1996c, Krane et al. 1996). In comparison, MMP-13 is a much stronger gelatinase than MMP-1 or –8 (Knäuper et al. 1996a). Collagenases also degrade various other extracellular molecules (Table 1). In all collagenases, a Tyr-Asp-Gly triplet is proposed as essential and specific for collagenase activity and the specific action of collagenases on triple helical collagen is determined by the presence of a 16-amino acid sequence in their C-terminal domain (Hirose et al. 1993). MMP-8 is distinct from the two other collagenases in that it can be stored in the secondary granules of polymorphonuclear (PMN) neutrophils while MMP-1 and –13 requires transcriptional activity and de novo protein synthesis (Hasty et al. 1986, Kähäri and Saarialho-Kere 1999).

MMP-1 is expressed by fibroblasts, endothelial cells, macrophages, hepatocytes, chondrocytes, osteoblasts, tumor cells and migrating epidermal keratinocytes (Pilcher et al. 1998) and its expression is induced in various inflammatory diseases and cancers (Johansson et al. 1997, 2000). Until recently, it was generally accepted that rodents lack a homologue for human MMP-1. However, two MMP-1 homologues have been cloned and characterized in the mouse and are expressed by extra-embryonic tissue trophoblast giant cells (Balbin et al. 2001).

MMP-8 was first cloned from messenger RNA (mRNA) extracted from the peripheral leukocytes of a patient with chronic granulocytic leukemia (Devarajan et al. 1991, Hasty et al. 1990) and is synthesized during the myelocyte stage of neutrophil development (Hasty et al. 1986). PMN neutrophil-stored MMP-8 is released to the ECM upon chemotactic stimulation in vitro or during inflammatory conditions in vivo (Tschesche et al. 1991). MMP-8 has been found in two different forms, of which the neutrophil-derived 75 kDa MMP-8 contains complex N-linked carbohydrates and the ~50 kDa less glycosylated form of MMP-8 is usually detected within all other types of cells (Hasty et al. 1986, Mallya et al. 1990). In cell culture, MMP-8 expression has been detected in mucosal fibroblasts, squamous cell carcinoma (SCC) cells of the tongue (Moilanen et al. 2002),
Figure 1. Structure of MMPs. MMPs typically consist of a predomain, a prodomain including the cysteine switch (C), a hinge region, the catalytic domain including the Zn$^{2+}$ binding active site and a hemopexin domain. The membrane type MMPs also contain a transmembrane domain and MMP-2 and -9 fibronectin domains. Modified from (Vu and Werb 2000).
chondrocytes (Cole et al. 1996), odontoblasts (Palosaari et al. 2000), melanoma cells (Giambemardi et al. 1998), leukemia cells (Kim et al. 2001) and human endothelial cells (Hanemaaijer et al. 1997). MMP-8 has been found to be expressed in vivo by bronchial epithelial cells and macrophages involved in bronchiectasis (Prikk et al. 2001), oral SCCs (Moilanen et al. 2002), chondrocytes in rheumatoid arthritic and osteoarthritic lesions (Chubinskaya et al. 1999), rheumatoid synovial fibroblasts (Hanemaaijer et al. 1997), in human gingival sulcular epithelial cells (Tervahartiala et al. 2000), in cells of human atheroma (Herman et al. 2001) and by plasma cells associated with oral keratocysts (Wahlgren et al. 2001).

MMP-13 was first cloned and identified from human breast carcinoma (Freije et al. 1994) and subsequently cloned from interleukin-1 (IL-1) stimulated chondrocytes (Mitchell et al. 1996). MMP-13 is expressed in culture by skin fibroblasts and keratinocytes (Johansson et al. 2000), leukemia cells (Kim et al. 2001) and in several other cultured carcinoma cells (Giambemardi et al. 1998) as well as by plasma cells in vitro and in vivo (Wahlgren et al. 2001). MMP-13 expression in tissues is generally detected at sites of active remodelling, i.e. fetal bone development and postnatal bone remodelling (Johansson et al. 1997) and in severe inflammations such as osteoarthritic cartilage (Mitchell et al. 1996), rheumatoid synovium (Lindy et al. 1997), macrophages of chronic cutaneous ulcers (Vaalamo et al. 1997), intestinal ulcerations, cancers such as malignant tumours, SCCs, cutaneous basal cell carcinoma and chondrosarcomas (Kähari and Saarialho-Kere 1999).

2.2.3. Gelatinases

The two main gelatinases (also called type IV collagenases) MMP-2 (gelatinase A) and MMP–9 (gelatinase B) are characterized structurally by three repeats of fibronectin-type II domains inserted in the hemopexin domain interacting with collagens and gelatins (Roeb et al. 2002). MMP-2 and -9 are highly efficient in cleaving gelatin along with type IV collagen and several other substrates (Table 1). MMP-2 can also cleave native type I and II collagen to the characteristic αA and αB identical to those generated by collagenases (Aimes and Quigley 1995, Konttinen et al. 1991). MMP-2 was the first identified type IV collagenase and first purified from a malignant murine PMT sarcoma cell line (Salo et al. 1983) and later cloned by (Huhtala et al. 1990). MMP-2 is typically expressed constitutively by cells of mesenchymal origin (van den Steen et al. 2002) and its expression is associated with many different cell types and cancers, such as melanoma and fibrosarcoma (Huhtala et al. 1991) and numerous cultured carcinoma cells (Giambemardi et al. 1998).

MMP-9 is synthesized during late stages of PMN neutrophil development, stored within the tertiary granules and released upon stimulus. MMP-9 may exist both as a monomer or homodimer and as a covalent complex with neutrophil gelatinase B-associated lipocalin in neutrophils (Kjeldsen et al. 1992, van den Steen et al. 2002). In contrast to the constitutively expressed MMP-2, MMP-9 expression in other cell types than PMN neutrophils requires transcriptional activity. MMP-9 is expressed by skin and gingival epithelial cells in vitro and vivo (Salo et al. 1991, 1994), alveolar macrophages, plasma cells and lymphocytes (Di Girolamo et al. 1998, van den Steen et al. 2002) as well as in several cultured carcinoma cell lines (Giambemardi et al. 1998). MMP-9 plays a physiological role in reproduction, growth and development and has been implicated to play a role in tumor invasion and inflammatory diseases such as arthritis, corneal ulcers, Alzheimers disease, skin blistering diseases (Vu et al. 1998), periodontitis (Westerlund et al. 1996), premature ruptures of amniotic membranes, lung diseases, neuroinflammatory diseases, HIV, vascular diseases and various cancers (van den Steen et al. 2002).

2.2.4. Stromelysins

This MMP subclass includes MMP-3 (stromelysin-1) and -10 (stromelysin-2), which can cleave the globular domain, but not the helical type IV collagen in addition to a wide variety of other matrix molecules (Table 1). Stromelysins are structurally characterized by an insertion of an XPPVPTXXV motif in the C-terminal part of their catalytic domain (Hirose et al. 1993, Nagase and Woessner...
MMP-11 (stromelysin-3) and -12 (metalloelastase) are often included in this subgroup although they are structurally different from MMP-3 and -10 (Johansson et al. 2000, Kähari and Saarialho-Kere 1999).

MMP-3 and –10 are expressed by fibroblastic cells and by normal and transformed epithelial cells in culture and in vivo (Giambernardi et al. 1998, Johansson et al. 2000, Kähari and Saarialho-Kere 1999). MMP-11 has not been found to degrade any ECM components; however, MMP-11 cleaves serine proteinase inhibitors and insulin-like growth factor binding protein-1 in vitro. MMP-11 is expressed during wound healing, in fibroblasts, in breast cancer, uterus, placenta, cycling endometrium and involving mammary gland (Luo et al. 2002).

MMP-12 degrades elastin very efficiently and is expressed by macrophages and stromal cells at sites of rapid matrix turnover during murine fetal development, in granulomatous diseases of the intestine and skin (Johansson et al. 2000, Kähari and Saarialho-Kere 1999) and in human alveolar macrophages associated with pulmonary emphysema (Belaouaj et al. 1995). Inspite of macrophage accumulation in MMP-12 deficient mice there was no enlargement of air space due to exposure to cigarette smoke indicating that MMP-12 plays a major role in the destruction of lung tissues (Hautamäki et al. 1997).

2.2.5. Matrilysins

MMP-7 (matrilysin-1) and MMP-26 (endometase/matrilysin-2) are similar in that they both lack the hinge- and hemopexin-domain. MMP-7 was first isolated from a mixed tumor library and has a high affinity for elastin (Table 1). MMP-7 is primarily expressed by cells of epithelial origin and specifically in a lumenal direction. MMP-7 can be stored in the secretory epithelial cells of exocrine glands of the skin, endometrium, gastrointestinal tract and airways as well as in early involuting uterus of rat. MMP-7 is also expressed by malignant epithelial cells in tumors of the gastrointestinal tract, prostate, and breast (Wilson and Matrisian 1996).

MMP-26 was cloned from a human endometrial tumor cDNA library and is expressed in human placenta and uterus as well as in various tumor cells (Uriu and Lopez-Otin 2000). MMP-26 has a unique cysteine switch sequence with a His-residue replacing the common Arg-residue. MMP-26 cleaves gelatin and human plasma α1-proteinase inhibitior (α1-PI; Table 1) (Park et al. 2000).

2.2.6. MT-MMPs

The membrane-type MMPs form a distinct class of MMPs in that they have a membrane-spanning domain near their C-terminus followed by a nine-amino-acid insertion between the pro and catalytic domain which ends in a RXKR furin activation consensus that may be cleaved by furin or furin-like enzymes leading to intracellular activation (Apte et al. 1997). MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP) and MMP-24 (MT5-MMP) are type I transmembrane MMPs with a traditional 24 amino acid-residue transmembrane domain at their C-termini, while MMP-17 (MT4-MMP) and MMP-25 (MT6-MMP) are glycosylphosphatidylinositol-anchored MMPs. The type I transmembrane MMPs can activate MMP-2 (Sternlicht and Werb 2001).

MMP-23 can be considered a specialized member of the MT-MMPs. The human MMP-23 lacks a recognizable signal sequence at its N-terminus and the Cys-switch. MMP-23 is prominently expressed in reproductive tissue as well as in heart, intestine, colon, placenta, lung and pancreas (Velasco et al. 1999). The mouse orthologue for MMP-23, the CA-MMP, is a type II transmembrane and contains unique cysteine-rich, proline-rich and IL-1 type II receptor-like domains (Pei et al. 2000). Collectively, MT-MMPs cleave a wide variety of ECM molecules in vitro (Table 1).

MMP-14 was first identified on the surface of invasive tumor cells and was found to increase invasiveness of cultured carcinoma cells (Sato et al. 1994). MMP-14 has been identified in both a shed soluble and a membrane-associated form in cultured breast cancer cells and fibroblasts (Li et al. 1998). MMP-14 is expressed by skin fibroblasts (Madlener 1998, Okada et al. 1997), in tumor cells of epithelial origin, bronchial epithelial cells (Maisi et al. 2002) and endothelial cells (Silletti et al. 2001).
2.2.7. The X-files of the MMPs

The latest “rookies” to the MMP family have distinct structural and/or functional properties from the other MMP subcategories. These MMPs do not show specific characteristic to any other subclass and thus it can be considered legitimate to assign them to the X-files.

MMP-18 (Table 1) was identified as a novel interstitial collagenase (xColl4) from the *Xenopus laevis* tadpole. This collagenase deserves a special attribute, since collagenase activity was first identified in the Xenopus tadpole tail some 40 years ago, and MMP-18 found in 1996 was the first identifiable amphibian collagenase (Stolow et al. 1996).

MMP-19 (Table 1) was first cloned from a human mammary gland cDNA library and is most similar to the stromelysin class of MMPs in terms of activity. Expression of MMP-19 has been found in placenta, lung, pancreas, ovary, small intestine, spleen, thymus and prostate (Cossins et al. 1996; Pendas et al. 1997).

Human MMP-20 (enamelysin) was first found in odontoblastic cells of the dental papilla and MMP-20 mRNA is typically expressed during tooth development by ameloblasts of the enamel organ (Bartlett et al. 1998), by human tongue SCC cells, in the tooth pulp and in placenta (Väänänen et al. 2001). MMP-20 cleaves amelogenin very efficiently (Table 1).

MMP-28 or epilysin was first cloned from a human testis and keratinocyte cDNA library and is most related to MMP-19. Epilysin is highly expressed in testis and also in lungs, heart, colon, intestine and brain and in addition, in cultured keratinocytes and migrating wound keratinocytes. MMP-28 is unusual in being abundantly expressed in normal adult tissue and cleaves casein in vitro (Lohi et al. 2001).

2.2.8. Activation mechanisms of MMPs

The latency of MMPs is regulated through a cysteine-switch comprised of the unpaired Cys\(^{75}\) sulfhydryl group within the conserved P\(^{71}\)RCGVPD-motif keeping MMPs inactive by binding to the catalytic Zn\(^{2+}\). A key step in MMP activation is the dissociation of Cys from the Zn\(^{2+}\) allowing the interaction of Zn\(^{2+}\) with the H\(_2\)O required for catalysis and concomitant exposure of the active site. Thus, the Zn\(^{2+}\) is viewed as the “switch” that leads to activation. This is attained by proteolytic removal of the prodomain, modification of the sulfhydryl-group or by molecular perturbation. The disengagement of the Cys-Zn\(^{2+}\) bond may also lead to autoactivation of the MMP (Nagase 1997, Van Wart and Birkedal-Hansen 1990).

The activation process of MMPs encompasses three different mechanisms: stepwise activation, activation on the cell-surface and intracellular activation (Figure 2).

Non-proteolytic activation without concomitant loss in molecular weight of most MMP zymogens can be accomplished by SH-reactive agents (iodoacetate, p-aminophenylmercuric acetate (APMA), HOCl, oxidized glutathione, mercurial compounds, anti-rheumatic gold (I) compounds, denaturants (urea, SDS, NaSCN), oxidation and freeze/thaw (Birkedal-Hansen et al. 1993, Murphy et al. 1999, Nagase 1997, Sorsa et al. 1987, Stetler-Stevenson et al. 1989, Van Wart and Birkedal-Hansen 1990). The nonproteolytic activation proceeds in a stepwise manner in which several intermediates are initially generated by an intramolecular reaction. The final cleavage to an active MMP follows a bimolecular reaction (Nagase 1997).

The proteolytic activation of MMPs also proceeds in a stepwise manner. Activator proteinases first attack the proteinase susceptible “bait” region located in the middle of the prodomain. This cleavage induces conformational changes in the prodomain and renders the final activation site readily cleavable by a second proteolysis. The latter reaction is usually catalyzed by an MMP but not by the activator proteinase (Nagase 1997). Many proteinases activate MMPs and many MMPs activate each other. The activation cascade of MMPs is fairly complex (Figure 3). However, most
Figure 2. Activation of MMPs. proMMP activation can proceed through different intermediate forms by chemical and proteinase activation, both eventually rendering the active MMP enzyme. Adapted from (Nabeshima et al. 2002, Nagase 1997).


The fully active MMPs have Phe or Tyr at their N-termini, generated by MMP activation. Activation of MMPs leading to forms with other N-termini usually results in reduced enzymatic activity of the MMP (Nagase 1997). For MMP-9, it has been demonstrated that mere substrate binding to proMMP-9 induces its enzymatic activity without removal of the prodomain. MMP-9 was shown to be enzymatically active despite the fact that only the proforms of the enzyme could be detected in placental tissue extracts (Bannikov et al. 2002). A similar phenomenon for MMP-9 has been detected in human inflammatory gingival crevicular fluid (GCF) (Westerlund et al. 1996).
Figure 3. The activation network of MMPs. The picture illustrates the highly complex activation of MMPs by other MMPs and serine proteinases. MMP activation data is mostly based on in vitro studies, little is known about MMP activation in vivo. Adapted from (Balbin et al. 1998, Cowell et al. 1998, Knäuper et al. 1996b, 1997, Murphy et al. 1999, Sorsa et al. 1997, Van den Steen et al. 2002).

### 2.2.8.1. Cell-surface activation by MMP-14

MMP-2, unlike the other MMPs, has a propeptide generally not susceptible to proteolytic initiation of activation by proteinases (Nagase 1997, Sorsa et al. 1997). However, MMP-2 can be activated by the cell surface-anchored MMP-14, -15, -16 and -24 (Kang et al. 2000, Llano et al. 1999, Sato et al. 1996, 1994, Strongin et al. 1995) and also by the soluble, transmembrane-truncated form of MMP-14 (Will et al. 1996). MMP-14 is a pericellular activator of MMP-2, -8, -9 and -13 (Knäuper et al. 1996c).

MMP-14 activation of MMP-2 occurs through a trimolecular-complex of MMP-2/TIMP-2/MMP-14 which concentrates the components to the cell surface (Atkinson et al. 1995, Cowell et al. 1998, Sato et al. 1996, Strongin et al. 1995). MMP-14, through its catalytic domain, acts as a cell membrane receptor for TIMP-2. Binding of the TIMP-2 N-terminal domain to the MMP-14 catalytic domain mediates the activation of MMP-2 through the TIMP-2 C-terminal domain. Thus, a second MMP-14 molecule is needed for activation of MMP-2 (Zucker et al. 1998). The initial cleavage of MMP-2 destabilizes the structure of the MMP-2 prodomain and autoproteolysis at a second site then releases the rest of the prodomain generating fully active MMP-2 (Cowell et al. 1998, Strongin et al. 1995). In the activation process, the C-terminal hemopexin domain of proMMP-2 binds to the C-terminal end of TIMP-2 (Murphy et al. 1992).

MT-MMPs (and MMP-11) themselves are not activated by typical chemical activators which induce autocatalytic activation of other secreted MMPs, i.e. APMA or SDS (Will et al. 1996). Both the golgi-associated endopeptidase furin as well as plasmin have been suggested to be physiological activators of MMP-14 and other MT-MMPs (Okumura et al. 1997, Yana and Weiss 2000) although furin-mediated activation of MMP-14 is not a prerequisite for MMP-2 activation (Cao et al. 1996).

Some results demonstrate that the MMP-14 prodomain would be required for the binding of TIMP-
2 to the catalytic domain and for the catalytic activity of MMP-14 (Pavlaki et al. 2002). In fact, membrane association alone may expose MMP-14 to furin-independent processing (Yana and Weiss 2000).

### 2.2.9. Transcriptional regulation of MMPs

Many functions of MMPs overlap and therefore the temporal, spatial and tissue-specific expression of MMPs is necessary. The basal expression of several MMPs (such as MMP-1,-3,-7,-8,-9,-10,-12 and -13) in cultured cells is low, and their transcription is induced by a variety of extracellular stimuli. These include growth factors, cytokines, chemical agents, physical stress and oncogenic cellular transformation acting through intracellular cascades involving secondary messengers. MMP expression can subsequently be down-regulated by suppressive factors. MMP expression is also regulated by bacterial endotoxin and other virulence factors, phorbol esters, ECM proteins, cell-membrane associated proteins, cell stress and changes in cell shape (Kähäri and Saarialho-Kere 1999).

In the genome, chromosome 11 contains a cluster of MMP genes, some of the latest MMP family members map to chromosome 1 while MT-MMP genes are spread on several chromosomes. The 5'-flanking regulatory regions of most inducible MMP genes exhibit common features, including a TATA box and an activator protein 1 (AP-1) regulatory elements in the proximal promoter region. Extracellular stimuli activate the nuclear AP-1 transcription factor complex composed of members of the Jun and Fos family, which bind to the AP-1 cis-element and activate transcription of the corresponding MMP gene. The expression of the AP-1 dimer, c-Jun and c-Fos, are induced as a result of the activation of mitogen-activated protein kinases (Kähäri and Saarialho-Kere 1999).

MMP-2 is often constitutively expressed and controlled through activation and to some degree by post-transcriptional mRNA stabilization. The MMP-2 gene is relatively unresponsive to stimulation in cultured cells and lacks both the AP-1 element and the classical TATA box. In addition, it contains several potential SP-1 binding sites not found in collagenase gene promoters, an activator protein-2 binding sequence and an transcriptionally important tumor suppressor p53 binding site as well as heterogenous initiation sites for transcription. Other nuclear factors controlling MMP transcription are the ETS family of oncoproteins binding PEA3 sites, nuclear factor of κB, signal transducers and activators of transcription, p53 and negative regulatory elements such as transforming growth factor-β (TGF-β) inhibitory element (Johansson et al. 2000, Kähari and Saarialho-Kere 1999, Mauviel 1993, Van den Steen et al. 2002). Common bi-allelic single-nucleotide polymorphisms that influence the rate of transcription have also been identified within several MMP gene promoters and are associated with increased susceptibility to various types of cancers (Van den Steen et al. 2002, Ye 2000).

Tumor necrosis factor-α (TNF-α) is an inflammatory response-mediating cytokine which is synthesized as a transmembrane molecule that can bind to its receptor by cell-cell contact. TNF-α is proteolytically processed to a soluble homotrimer (Gearing et al. 1995) typically upregulating many MMPs in different cell types. TNF-α and IL-1β stimulate MMP-9, but not MMP-2 production in human gingival mucosal keratinocytes (Mäkela et al. 1998, Salo et al. 1994). The bacterial lipopolysaccharide (LPS) is a very potent inducer of both MMP-2 and –9 in vitro which may act either directly or indirectly via cytokine stimulation on gene expression (Van den Steen et al. 2002). TGF-β is part of a super-family of growth factors and there are three structurally very similar mammalian isoforms. TGF-β requires activation to a mature form for receptor binding and intracellular signalling. TGF-β1 typically reduces the expression of collagenase genes and activity in cultured cells while simultaneously elevating the expression of TIMPs (Verrecchia and Mauviel 2002). In contrast, TGF-β1 upregulates both MMP-2 and MMP-9 activities in cultured fibroblasts and transiently also MMP-2 mRNA expression (Salo et al. 1991, Van den Steen et al. 2002, Verrecchia and Mauviel 2002). TGF-β induces MMP-2 and –9 production in human gingival mucosal keratinocytes (Salo et al. 1991) and increases MMP-2 and –9 expression in peripheral blood monocytes and in various tumorigenic and non-tumorigenic cell lines (Van den Steen et al. 2002).
MMPs can themselves modulate growth factor and cytokine activity. MMP-3 and -13 cleavage of the heparan sulphate proteoglycan perlecan results in the release of basic fibroblast growth factor (Whitelock et al. 1996) and the cleavage of decorin by MMP-2, -3 and -7 releases TGF-β bound to decorin (Vu and Werb 2000). MMP-2 and -9 directly process TGF-β into an active ligand (Van den Steen et al. 2002, Yu and Stamenkovic 2000). At least MMP-1, -3 and -7 efficiently and MMP-2 and -9 less efficiently, can process proTNF-α into the biologically active form (Gearing et al. 1995). Proteolysis may also downregulate components of mitogenic signaling pathways. Thus, MMPs can regulate the bioavailability and/or activity of growth factors and cytokines by cleavage of both matrix and non-matrix substrates or by mediating receptor turnover (Table 1).

Post-transcriptional mechanisms also modulate MMP expression. Examples are stabilization of MMP-1 and -13 mRNA transcripts by phorbol esters and epidermal growth factor (EGF) as well as stabilization of MMP-2 and -9 mRNA transcripts by TGF-β (Van den Steen et al. 2002). The turnover of MMP-1 mRNA is apparently regulated by AU-rich sequences in the 3′-untranslated region and similar sequences may also regulate the stability of other MMP transcripts (Sternlicht and Werb 2001). MMP expression could also be regulated by alternative splicing. Multiple transcripts have been reported for MMP-8, -11, -13, -16, -17, -20, -25 and -26 (Hu et al. 1999, Luo et al. 2002).

2.2.10. In vivo functions of MMPs

None of the individual MMP-null mice generated to date have had an embryonic lethal phenotype. The most severe phenotype is associated with MMP-14 knock-out mice, which developed a severe skeletal phenotype including dwarfism, delayed membraneous ossification of calvarial bones, osteopenia, severe generalized arthritis, reduced activity of osteogenic cells and fibrosis of soft tissues. MMP-14 deficiency also imparts a severe defect in collagenolytic activity (Holmbeck et al. 1999). Furthermore, MMPs and in particular MMP-14 have been implicated in endothelial cell neovascularization processes through pericellular degradation of the fibrin-rich scaffold both in vitro and in vivo (Hiraoka et al. 1998). In a study with MMP-9 null mice, this gelatinase was demonstrated to play a role in vascularization of hypertrophic cartilage, reproduction, apoptosis of hypertrophic chondrocytes and endochondral ossification during bone formation (Vu et al. 1998).

Although MMPs can cleave virtually all ECM molecules in vitro, much less is known about their actual in vivo substrates. Based on Table 1, one would think that some of the ECM molecules presented there as substrates for MMPs would actually have been proven to be substrates for MMPs in vivo. The literature comes up with very few, one being amelogenin which truly appears to be the in vivo substrate for MMP-20. The other two evidently in vivo MMP substrates are not ECM molecules. In addition, while MMP-9 indirectly mediates tissue destruction in vivo (which has been proposed to be the main function of MMPs from the birth of MMPs) MMP-7 actually acts to protect the host from microbial attack.

The serpin α1-PI has been established as a substrate for MMP-9 in vivo. In studies with mice induced with the subepidermal blistering disease bullous pemphigoid, it was shown that while neutrophil elastase is primarily responsible for the destruction of the BM protein BP180 (type XVII collagen), MMP-9 is required for the inactivation of the α1-PI which act as an plasma inhibitor of neutrophil elastase. Both neutrophil elastase and MMP-9 cleave BP180 in vitro, however, in vivo MMP-9 acts upstream of neutrophil elastase indirectly facilitating blister formation (Liu et al. 2000). The antimicrobial peptide α-defensin has been shown to be a substrate for MMP-7 in vivo. Intestinal Paneth cells secrete procryptidin (the mouse homologue for α-defensin) which is activated into its mature form through cleavage by MMP-7 both in vivo and in vitro. In MMP-7 null mice, only precursor forms of cryptidins were detected. MMP-7 null mice were also more susceptible to exogenous bacterial infection in the intestine giving further evidence that MMP-7 is important in restricting bacterial colonization and access to the intestinal mucosa (Wilson et al. 1999).
2.2.11. In vivo localization of MMPs

Many investigators are interested in detecting the net MMP activity in tissues. However, MMP antibodies used for immunohistochemistry poorly discriminate between the pro- and active forms of MMPs. Excess TIMP activities also interfere with detection of immunoreactive active MMP species. Gel zymography by itself is a very sensitive method for detection of MMP activity, however, homogenization of tissue for this assay excludes localization of the cell type exhibiting activity. In addition, the extraction procedure and sample treatment can activate enzymes or permit contact of enzymes and inhibitors localized in distinct compartments in the intact cells or tissues. \textit{In situ} zymography allows preservation of tissue architecture while detecting actual enzyme activity and importantly, does not require species-specific reagents. This method was first introduced by Galis et al. (Galis et al. 1995).

\textit{In situ} zymography as a method is not quantitative and difficult to standardize. However, valuable information regarding localization of net MMP activity at the tissue level can be outlined by this method. The methodology is also rather simplistic and cheap. In principle, any purified MMP substrate can be used and conjugated to a fluorophore, such as fluorescein or resorufin (Galis et al. 1995). To eliminate false interpretation due to autofluorescence originating from ECM molecules in the tissues, substrates can be conjugated to quenched fluorophores (Sarment et al. 1999). Autoradiographic gelatin emulsions are also used in addition to different kinds of films, referred to as film \textit{in situ} zymography. When using gelatin as a substrate, mostly MMP-2 and –9 activity is detected by both gel and \textit{in situ} zymography. However, MMP-14 also causes focal degradation of gelatin films (d’Ortho et al. 1998).

Thus, the clear drawback of this procedure has been the rather non-specific detection of protease activity since the substrates generally used are casein or gelatin. The use of broad-spectrum MMPIs and serine proteinase inhibitors only allow for conclusions of which enzyme family is the major protease detected (Korostoff et al. 2000). Since MMPs are active at neutral pH, regulating the pH in the methodology allows for detection of either MMPs or serine proteinases. \textit{In situ} zymography has thus been shown to be specific for enzyme activity by incubating samples timely at different temperatures and at different pHs (Berndt et al. 2000, Sarment et al. 1999).

2.3. MMP inhibition

2.3.1. Physiological inhibition of MMPs

MMPs can be self-regulated by their own proteolytic inactivation. Some cleavages inactivate MMPs or generate truncated enzyme species resulting in a concomitant change of action. MMPs are also inhibited and cleared by endogenous inhibitors like \(\alpha_2\)-macroglobulin (\(\alpha_2\)M), the major plasma inhibitor of MMPs. \(\alpha_2\)M functions by a mechanism involving the presentation of a cleavable “bait” region that, once proteolytically cleaved, causes a conformational change that entraps the proteinase, which becomes covalently anchored by transacylation. \(\alpha_2\)M/MMP-complexes are irreversibly cleared by scavenger receptor-mediated endocytosis (Hahn-Dantona et al. 2001).

TIMPs are specific, physiological tissue inhibitors of MMPs. At present, four TIMPs (TIMP1-4) have been identified in vertebrates. Mammalian TIMPs are 20-30 kDa variably glycosylated two-domain molecules. TIMPs play an important role in tissue remodelling due to their abilities to inhibit MMPs by forming 1:1 molar stoichiometric enzyme-inhibitor complexes (Brew et al. 2000). The critical residue involved in MMP inhibition is located around the disulphide bond between Cys\(^1\) and Cys\(^70\) resulting in bidental coordination of the catalytic Zn\(^{2+}\) (Nagase 1997).

TIMP -1, -2 and -4 are secreted in a soluble form, whereas TIMP-3 is sequestered to the ECM. TIMP-1 and -2 inhibit the activity of most MMPs. As an exception, TIMP-1 is a poor inhibitor of MMP-14, -15, -16 and -24 as well as MMP-19. TIMP-3 inhibits the activity of MMP -1, -2, -3, -9 and -13 being most potent in inhibiting MMP-9. TIMP-4 inhibits the activity of MMP-2 and -7 somewhat more potently than that of MMP -1, -3 and -9. TIMP-2 and -3, unlike TIMP-1 are effective inhibitors of the MT-MMPs (Baker et al. 2002, Brew et al. 2000).
TIMPs differ in the types of non-inhibitory complexes they form, mediated by their C-terminal domains. Thus, TIMP-2 binds tightly to the C-terminal domain of proMMP-2, and TIMP-1 to proMMP-9 of which the former is important in the cell-surface activation of proMMP-2 (Baker et al. 2002, Brew et al. 2000). The kinetics of TIMP-MMP interactions indicate that it is a non-competitive, two-step complex formation. In this, an inactive reversible complex (E•I) is formed rapidly which rearranges more slowly to an inactive tight complex (E•I*) (Baker et al. 2002, Brew et al. 2000). The fate of the MMP-TIMP complex is likely to be determined through internalization (Hahn-Dantona et al. 2001). Overexpression of TIMPs1-4 can inhibit invasion of malignant cells in vitro and in vivo (Baker et al. 2002). However, TIMPs also exhibit MMP-independent biological activity and paradoxically both tumour progressive and suppressive activity (Baker et al. 2002, Brew et al. 2000).

2.3.2. Synthetic MMPIs

Since MMPs have been implicated to play a detrimental function in virtually all pathological conditions, great effort has been put into developing inhibitors against the activity of these enzymes. Clinically, synthetic MMPIs may inhibit tumour growth either as cytostats by maintaining small clusters of metastatic cells in a dormant state or by inhibiting tumour-induced angiogenesis (Coussens et al. 2002).

Initially, the task was to design molecules that mimicked the substrate-cleavage site for collagenases in the collagen molecule. The goal was to obtain enzyme inhibition through chelation of the active-site Zn$^{2+}$-ion by incorporating a Zn$^{2+}$-binding group into peptide analogues. In terms of collagenase inhibition a right-hand side hydroxyamate group was identified as one of the best choices for chelating the active site Zn$^{2+}$. The hydroxyamate acts as a bidentate ligand with each oxygen at an optimum distance from the active-site Zn$^{2+}$-ion. The inhibitor binding is reversible. The broad-spectrum hydroxyamate MMPIs, Batimastat (BB-94) and Marimastat (BB-2516) were the first MMPIs to enter clinical trials in the treatment of malignant tumours. The non-peptidomimetic MMPIs include at least tetracyclines and bisphosphonates (Hidalgo and Eckhardt 2001).

2.3.2.1. Batimastat

Batimastat (Figure 4) is well tolerated, but its utility is limited by poor water solubility, which requires intraperitoneal or intrapleural administration. Batimastat inhibits at least MMP-1, -2, -3, -7, -8, -9, -13 and -14 with IC$_{50}$s between 1-20 nM. Batimastat has been extensively studied in preclinical animal models and found to effectively inhibit tumor growth, metastasis, tumor-associated angiogenesis and prolong survival time in several murine models including melanoma, hemangioma, xenograft models of human ovarian carcinoma, colorectal carcinoma, breast and pancreatic cancer (Coussens et al. 2002, Rasmussen and McCann 1997).

2.3.2.2. Marimastat

Marimastat (Figure 4) is water soluble and can be administered orally. Marimastat is a broad-spectrum MMPI with IC$_{50}$s against MMP-1, -2, -3, -7, -8, -9, -12 and -14 of 5, 6, 200, 20, 2, 3, 3 and 1.8 nM, respectively (Coussens et al. 2002, Rasmussen and McCann 1997, Whittaker et al. 1999). Results with Marimastat-treatment in clinical trials are mixed. Marimastat has been tested in more than 400 patients in Phase I/II studies in a number of different solid tumors in the USA. Marimastat treatment significantly reduced all the measured cancer specific antigen rates of rise in a dose-dependent fashion (Rasmussen and McCann 1997). Marimastat has also been tested in combinatorial treatments together with conventional cytotoxic drugs.
2.3.2.3. Tetracyclines

In 1944, the mycologist B.M. Duggar discovered the broad antimicrobial effect of Aureomycin (chlortetracycline hydrochloride). Tetracyclines (TCs; Figure 4) were taken into pharmaceutical use in 1948 and are highly effective against aerobic and anaerobic bacteria, mycoplasma, chlamydia, spirochetes, some protozoa and rickettsiae. The chemically improved compounds doxycycline (1967) and minocycline (1972) are second-generation semisynthetic tetracycline derivatives with enhanced antibacterial activity, rapid intestinal absorption, longer half-life, superior tissue-fluid penetration and reduced toxicity (Rifkin et al. 1993).

In the 1980s, Golub and colleagues showed that TCs inhibit mammalian collagenase activity and production independently from their antimicrobial action (Golub et al. 1998). The TCs can affect the activity and/or expression of MMP-1, -2, -3, -8, -9, -13 and –20 (Golub et al. 1998, Joshi and Miller 1997, Lee et al. 2001, Rifkin et al. 1993, Suomalainen et al. 1992, Väänänen et al. 2001). The ability of the TCs and its chemically modified analogues to inhibit MMPs is based on multiple mechanisms: 1) inhibition of the active MMPs by chelation of the active site cations, 2) interference with the processing of the proenzyme into its active form, 3) reduction of MMP mRNA expression and 4) rendering the MMPs more susceptible to degradation and fragmentation (Rifkin et al. 1993, Smith et al. 1999, Sorsa et al. 1998). TCs are known to inhibit MMPs originating from a variety of cells: neutrophils, keratinocytes, macrophages, osteoblasts, chondrocytes, synovial fibroblasts, T-lymphocytes, HUVECs and from a wide range of tissues: skin, gingiva, cornea, cartilage, and rheumatoid synovium (Hanemaaijer et al. 1997, Rifkin et al. 1993, Smith et al. 1999). Doxycycline and minocycline are more potent inhibitors than the parent compound tetracycline. For doxycycline, MMP-8 has IC$_{50}$ values between 25-40 µM, MMP-1 over 200 µM while MMP-13 is extremely sensitive with IC$_{50}$ values around 2 µM (Greenwald et al. 1998, Sadowski and Steinmeyer 2001, Suomalainen et al. 1992).

Doxycycline inhibits MMP-8 activation and activity in vitro resulting in fragmentation of the enzyme most likely due to chelation of Ca$^{2+}$, which shields MMPs from proteolytic attack. Inhibition by doxycycline can be reversed by high concentrations of Ca$^{2+}$ (Smith et al. 1996). Inhibition of collagenases by doxycycline occurs in a noncompetitive manner through alteration of MMP conformation rather than by interaction with the catalytic Zn$^{2+}$ (Sorsa et al. 1994). Inhibition involves binding between the inhibitor and the enzyme and/or the inhibitor and the enzyme-substrate complex (Smith et al. 1999). The differences in sensitivity of the three different collagenases to TC inhibition is likely due to differences in structure/function including high collagenolytic/gelatinolytic activity, S$_1$ pocket size and broad substrate specificity. TCs have been shown to be effective in the treatment of human periodontal disease, sterile corneal ulceration, rat adjuvant arthritis, dog and guinea pig osteoarthritis, rodent rickets, human rheumatoid and reactive arthritis, diabetic and ovariectomized (OVX) rat osteoporosis, aortic aneurysms and in vitro osteoclast-mediated bone resorption and cartilage degradation system (Golub et al. 1998, Greenwald et al. 1998, Rifkin et al. 1993). TCs can also block nitric oxide synthase activity, impact arachidonic acid metabolism and inhibit protein kinase C mediated transcriptional activity of several MMPs (Golub et al. 1998).

The TCs have also been tested for their ability to inhibit tumor invasion. In cultured highly invasive breast carcinoma cells, doxycycline inhibited the secretion and activity of MMP-2 and -9. In addition, doxycycline inhibited the proliferation of prostate and breast cancer cell lines in vitro, induced apoptosis and decreased the invasion and metastatic potential of highly aggressive breast cancer and melanoma cells. In Phase I evaluation studies with cancer patients, side-effects such as fatigue, confusion, nausea and vomiting were encountered (Hidalgo and Eckhardt 2001).

2.3.2.4. The chemically modified tetracyclines

To identify the site of anticollagenase function, Golub et al. synthesized different chemically modified analogs of tetracycline, the CMTs (Figure 4). The CMTs comprise a group of at least 10 (CMTs 1-10) analogues plus some special modified CMTs that differ in their MMP specificity and potency (Table 2). CMTs are modified so that the dimethylamino group from carbon-4 position (the side-chain required for antimicrobial activity in TCs) is removed. The removal of antimicrobial
Figure 4. Chemical formulas of the MMP inhibitors used in the study. Adapted from (Greenwald et al. 1998, Shapiro et al. 1997, Whittaker et al. 1999).
activity retained and enhanced anticollagenase activity (Golub et al. 1998). In CMTs, the advantages over TCs include lack of gastrointestinal toxicity, fatigue and photosensitivity due to long-term systemic administration, greater plasma concentrations and longer elimination half-life thus requiring less frequent drug administration. While all CMTs lack antimicrobial activity, only CMT-5 lacks any anti-collagenase effect. Apparently, TC/CMT inhibition of the catalytically active MMP involves interaction by the Ca$^{2+}$ and Zn$^{2+}$ binding sites at the carbonyl and hydroxyl groups at C$_{11}$ and C$_{12}$, respectively on ring B at physiological pH (Figure 4). These sites are nitrogenated in CMT-5 and may thus account for the poor inhibitory effect of CMT-5 on MMPs (Greenwald et al. 1998, Rifkin et al. 1993, Sorsa et al. 1994, Sorsa et al. 1998).

The potency of the various TCs as MMP inhibitors appears to be CMT-8>CMT-3>CMT-7>Doxycycline>CMT-1. CMT-1 is more effective than doxycycline, but less effective than the further developed CMTs. CMT-3 is the only compound effective against MMP-1. In addition, CMT-3 and –8 are the most effective collagenase activity inhibitors and CMT-8 is the most effective inhibitor of MMP-13 activity (Greenwald et al. 1998). CMTs-1, -3, -7 and –8 also inhibits oxidative activation, collagenolytic and gelatinolytic activity of proMMP-8 and -9 in vitro (Sorsa et al. 1998). In preclinical studies, CMT-3 inhibited tumor growth, reduced lung and bone metastasis and induced apoptosis. The compound is orally available and well tolerated with side-effects such as photosensitivity and fatigue. CMT-3 reduces the plasma concentrations of MMP-2 and –9 as well as the production of MMP-9 by peripheral blood mononuclear cells (Lokeshwar et al. 2002). CMT-3 has also been shown to inhibit vasculogenic mimicry by aggressive melanoma cells in vitro. CMT-3 decreased both MMP-2 and –9 active and proforms and MMP-1 expression in aggressive melanoma cells (Seftor et al. 2002).

### Table 2. Non-antimicrobial Chemically Modified Tetracyclines

<table>
<thead>
<tr>
<th>Compound</th>
<th>Description</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMT-1</td>
<td>4-dedimethylamino-tetracycline</td>
<td>CMT discovery compound</td>
</tr>
<tr>
<td>CMT-2</td>
<td>tetracyclonitrile</td>
<td>Not absorbed by oral route</td>
</tr>
<tr>
<td>CMT-3</td>
<td>6-demethyl, 6-deoxy, 4-dedimethylamino-tetracycline</td>
<td>Most lipophilic CMT</td>
</tr>
<tr>
<td>CMT-4</td>
<td>7-Chloro, 4-dedimethylamino-tetracycline</td>
<td>Orally bioavailable</td>
</tr>
<tr>
<td>CMT-5</td>
<td>tetracyclinpyrazole</td>
<td>No MMP inhibition but can scavenge free radicals</td>
</tr>
<tr>
<td>CMT-6</td>
<td>5-hydroxy, 4-dedimethylamino-tetracycline</td>
<td>Not absorbed by oral route</td>
</tr>
<tr>
<td>CMT-7</td>
<td>12$\alpha$-deoxy, 4-dedimethylamino-tetracycline</td>
<td>Orally bioavailable</td>
</tr>
<tr>
<td>CMT-8</td>
<td>6$\alpha$-deoxy, 5-hydroxy-4-dedimethylamino-tetracycline</td>
<td>CMT derived from doxycycline</td>
</tr>
<tr>
<td>CMT-9</td>
<td>12$\alpha$,4$\alpha$-anhydro, 4-dedimethylamino-tetracycline</td>
<td></td>
</tr>
<tr>
<td>CMT-10</td>
<td>7-dimethylamino, 4-dedimethylamino-tetracycline</td>
<td>CMT derived from minocycline</td>
</tr>
</tbody>
</table>

Modified from (Greenwald et al. 1998)

### 2.3.2.5. The CTTHWGFTLC-peptide

A synthetic decapeptide, the CTTHWGFTLC (CTT)-peptide was initially isolated from a phage display library in which the HWGF-motif selectively inhibits MMP-2 and -9 activity efficiently (IC$_{50}$ 10 $\mu$M). The CTT-peptide also inhibits migration of HT1080 fibrosarcoma, C8161 melanoma, SKOV-3 ovarian carcinoma and KS1767 Kaposi’s sarcoma cell lines as well as human endothelial cells. In vivo, the CTT-peptide homes to tumors, suppresses tumor formation, targets tumor vasculature and prolongs survival of tumor-bearing mice. The peptide has not been found to be cytotoxic. It has been thought that the Trp-residue in the HWGF-motif may bind to the hydrophobic pocket of the substrate cleft in the gelatinases and that the His-residue may act as a ligand for the catalytic Zn$^{2+}$-ion. The cyclic conformation is also critical for peptide function (Koivunen et al. 1999). In later experiments, CTT-bearing liposomes were found to be effectively taken up and internalized by gelatinase-expressing carcinoma cell lines. In addition, uptake of CTT-liposomes
containing adriamycin, a widely used anti-cancer drug, was significantly enhanced and increased cancer cell-killing activity (Medina et al. 2001).

2.3.3. MMPIs in clinical trials

In early phase I clinical trials prolonged treatment with hydroxyamate-based broad-spectrum MMPIs caused musculoskeletal pain and inflammation resulting from the inhibition of MMP activity involved in normal turnover of connective tissue in tendons and joints, complications not seen in preclinical models. The conditions were reversible and patients were able to continue treatment after a brief pause in drug receiptment. Recently, inhibition of MMPs involved in the release of membrane-bound proTNF-α from its receptor has been implicated in the musculoskeletal pain side effects.

Phase II trials have also turned out to be problematic. MMPIs are cytostatic rather than cytotoxic, and thus conventional measures of drug efficacy such as reduction in tumor size can not be used to monitor drug activity. Instead, reduction in the increasing rate of tumor markers in serum was used to define a biologically active dose of MMPIs although changes in biomarker levels in serum do not necessarily reflect tumor regression.

Phase III trials were initiated in the mid-1990s. MMPIs were often administered at randomly selected doses or at the maximum tolerated dose instead of optimal biological doses. The conducted phase III trials have not been very encouraging (Table 3). In one study patients receiving the MMPI Tanomastat for treatment of pancreatic and small-cell lung cancer showed significantly poorer survival than patients receiving a placebo. Despite the large number of patients accrued in MMPI clinical trials, there remains no clear demonstration that any dosing schedule or compound tested has reached levels sufficient to inhibit target MMP activity within the tumor tissue (Coussens et al. 2002). However, CMT-3 (Metastat) was in a multicenter phase I clinical trial. Disease stabilization in patients with nonepithelial type of cancer for up to 26 months was demonstrated as well as a significant reduction in plasma MMP-2 levels (Rudek et al. 2001). Due to positive outcomes and responses of treating patients with HIV-related Kaposi’s sarcoma in phase I clinical trials with CMT-3, phase II clinical trials are now ongoing.

Table 3. Fate of MMPIs in clinical trials for various cancers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Company</th>
<th>Phase of clinical trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB-94, Batimastat</td>
<td>British Biotechnology Ltd.</td>
<td>Terminated since 1998</td>
</tr>
<tr>
<td>TA-2516, Marimastat</td>
<td>Schering Plough/Tanabe Seiyaku</td>
<td>Terminated for now</td>
</tr>
<tr>
<td>BMS-275291</td>
<td>Bristol-Myers Squibb</td>
<td>Phase II/III recruiting</td>
</tr>
<tr>
<td>AG3340, Prinomastat</td>
<td>Agouron/Hoffman-La Roche</td>
<td>Halted</td>
</tr>
<tr>
<td>BAY12-9566, Tanomastat</td>
<td>Bayer</td>
<td>Halted</td>
</tr>
<tr>
<td>AE-941, Neovastat</td>
<td>Aeterna Laboratories</td>
<td>Phase III ongoing</td>
</tr>
<tr>
<td>MMI270</td>
<td>Novartis</td>
<td>Phasel/II conducted</td>
</tr>
<tr>
<td>CMT-3, Metastat</td>
<td>CollaGenex Pharmaceuticals, Inc.</td>
<td>Phase II</td>
</tr>
</tbody>
</table>

Adapted and modified from (Coussens et al. 2002, Kähäri and Saarialho-Kere 1999).

2.4. Laminin-5

Ln-5 is a non-collagen heterotrimeric glycoprotein constituted by the association of three genetically distinct polypeptides, the α3, β3 and γ2 chains. Ln-5 is a major component of the anchoring filament in the hemidesmosomal complex within the BM (Figure 7,10) mediating important functions in cell adhesion, migration, proliferation, wound healing, homeostasis of skin and development (Colognato and Yurchenco 2000). Ln-5 was discovered in the beginning of the 90's and is also known as kalinin (Rousselle et al. 1991), epiligrin (Carter et al. 1991), nicein (Marinkovich et al. 1993, Vaillly et al. 1994) and ladsin (Miyazaki et al. 1993). Under non-reducing conditions, the intracellular heterotrimeric human Ln-5 molecule is a 460 kDa high-molecular weight precursor form. This form contains an unprocessed 200 kDa α3-chain, a 140 kDa β3-chain
and an unprocessed 155 kDa γ2-chain. Upon secretion, human Ln-5 heterotrimers exist in 440 kDa and 400 kDa forms, resulting from extracellular processing of the α3 and γ2-chains, while the β3-chain remains unprocessed. In the 440 kDa form, the α3-chain is processed to a 165 kDa form and in the 400 kDa form, also the γ2-chain is processed into a 105 kDa form. In cell culture, the α3-chain is infrequently found in a 145 kDa form, while it is abundant in tissues (Marinkovich et al. 1992, Rousselle et al. 1991). The γ2 and α3-chain also exist in different alternative spliced variants (Airenne et al. 2000).

2.4.1. Structure of the heterotrimeric Ln-5 molecule

Ln-5 folds into a rod with two asymmetric globular domains at either end of a long central helical domains (Marinkovich et al. 1993). Ln-5 is currently the only laminin with truncations in all three short arms being a rod bounded by the G domain at one end and by one or two globular domains at the other. The α3-chain contains one small globular domain and three EGF-like modules. The β3-chain contains one globular domain and six EGF-like repeats (Aumailley and Gayraud 1998). In the human Ln-5 γ2-chain, the first N-terminal amino acids form a signal peptide. The N-terminal domain V (residues 28-196) is formed of three and one half cysteine-rich EGF-modules. Domain IV (residues 197-381) contains a single cysteine and forms a globular structure. Domain III (residues 382-608) is formed by the second half of an EGF module and four additional modules. Domain I/II (residues 608-1193) starts with two closely spaced cysteines and contains one cysteine close to the C-terminus. The γ2-chain is a α-helical domain with a heptad repeat structure typical for coiled coil proteins. The γ2-chain lacks the N-terminal globular domain VI present in the other γ-chains, which forms the globular structure at the end of the short arms. There are six putative Asn-linked glyclosylation sites (Kallunki et al. 1992). The N-terminal region of mouse Ln-5 γ2-chain comprises 606 residues including a signal peptide of 21 peptides (Sasaki et al. 2001). The mouse and human Ln-5 γ2-chain are highly conserved (Pyke et al. 1994).

2.4.2. Ligands for Ln-5

Ln-5 binds to integrins α6β1, α6β4 and α3β1 (Carter et al. 1991), syndecan (Utani et al. 2001) and possibly a-dystroglycan presumably through the C-terminal G domain of the α3-chain (Aumailley et al. 2003, Shang et al. 2001). Keratinocyte adhesion to Ln-5 via α3β1 promotes assembly of gap junctions and gap junction intercellular communication integrating individual cells into synchronized colonies (Nguyen et al. 2000b). Ln-5 and α6β4-integrin on the other hand forms a complex interacting with the intermediate filaments forming stable hemidesmosomal adhesion complex that retain cells stationary at the BM (Carter et al. 1991). Studies on functional blocking of α3β1 and α6β4 integrins on migratory cells depositing Ln-5 are very contradictory. Blocking α3- or α6-integrin subunits either increases or decreases normal human keratinocytes (NHKs) motility and adhesion (Goldfinger et al. 1999, O’Toole et al. 1997, Rousselle and Aumailley 1994, Zhang and Kramer 1996). Blocking both α3β1 and α6β4 integrins usually abolishes both adhesion and migration (Goldfinger et al. 1999, Zhang and Kramer 1996). This dispersity may be due to differences in intracellular signalling. α3β1-focal adhesions (FAs) interact with actin-containing stress fibers and mediates HNKs adhesion and spreading on Ln-5 via two pathways, one is phosphoinositide 3OH-kinase (PI3K) dependent and the other pathway is through RhoGTPase activity, a regulator of actin stress fibers. Migration of HNKs on collagen is dependent on α2β1-integrin mediated signalling and is only seen after TGF-β1 treatment. The diverse signalling is thought to be served to regulate communication between cell-substrate and cell-cell adhesions (Decline and Rousselle 2001, Nguyen et al. 2000a, Shang et al. 2001, Zhang and Kramer 1996). Both haptotactic and chemotactic migration of HaCaT and A431 carcinoma cells on Ln-5 was carried out through α3β1 integrin. α6β4 can inhibit α3β1 controlled migration only when it is haptotactic but not chemotactic and this is mediated through the PI3K pathway. If migration is chemotactic, PI3K plays a stimulatory role, if migration is haptotactic, an inhibitory role. This may
partly explain the differential effects of Ln-5 for NHK and tumor cell migration (Hintermann et al. 2001).

2.4.3. Processing of the Ln-5 chains

The heterotrimeric Ln-5 molecule is assembled intracellularly by formation of a $\beta_3\gamma_2$-chain dimer through disulphide bonds. The association of the $\alpha_3$-chain to the dimer is a rate-limiting step in the heterotrimer formation, assembling into the 460 kDa unprocessed form intracellularly. Assembly is necessary for secretion of the complete Ln-5 molecule (Matsui et al. 1995).

The $\alpha_3$ and $\gamma_2$-chain of the 460 kDa intracellular precursor form of Ln-5 synthesized by cultured human keratinocytes undergoes proteolytic processing extracellularly (Marinkovich et al. 1992, Rousselle et al. 1991) (Table 4). The murine counterparts are 190/150 kDa $\alpha_3$-chain, 140 kDa $\beta_3$-chain and 135/100 kDa $\gamma_2$-chain (Baker et al. 1996b, Hormia et al. 1998, Sasaki et al 2001).

The 200 kDa $\alpha_3$-chain shortening to a 165 kDa and 145 kDa protein at the C-terminus is predicted to occur at Gln$^{1337}$-Asp$^{1338}$ in the spacer region between the LG3 and LG4 domain (Amano et al. 2000, Nguyen et al. 2000a, Tsutota et al. 2000) and is the first cleavage of heterotrimeric Ln-5 processing. It has been demonstrated that MMP-2 and -14 cleave the human $\alpha_3$-chain (Veitch et al. 2002) but not the rat Ln-5 $\alpha_3$-chain (Giannelli et al. 1997, Koshikawa et al. 2000). The second cleavage of the $\alpha_3$-chain to the 145 kDa form occurs within domain IIIA. Both these cleavage sites are highly conserved between mouse and human (Amano et al. 2000). Although a recent study demonstrated MMP-14 cleavage of human Ln-5 $\beta_3$-chain in vitro, this processing has not been demonstrated in vivo (Udayakumar et al. 2003).

The human $\gamma_2$-chain is processed into the 105 kDa form after incorporation of Ln-5 to the ECM, resulting in a lag before processing of the 440 kDa Ln-5 into the 400 kDa form (Amano et al. 2000, Gagnoux-Palacios et al. 2001, Marinkovich et al. 1992). The extracellular processing of the Ln-5 $\gamma_2$-chain removes the globular domain IV and the EGF-rich domain V of the short arm. The $\gamma_2$-chain globular domain IV is required for incorporation into the ECM, but not for synthesis or secretion of the $\gamma_2$-chain. The $\gamma_2$-short arm 50/35 kDa fragment, resulting from the processing is deposited to the ECM by NHKs and mouse epidermal Pam212 cells (Gagnoux-Palacios et al. 2001, Sasaki et al. 2001). The YSGD tetrapeptide is the unique physiological cleavage site of the extracellular processing of the $\gamma_2$-chain (Table 4). The cleavage site is within domain III at the beginning of the second EGF-like repeat at Gly$^{434}$-Asp$^{435}$ and is conserved between humans and mice (Amano et al. 2000, Gagnoux-Palacios et al. 2001, Sasaki et al. 2001). The isoenzymes bone morphogenetic protein-1 (BMP-1), mammalian Tolloid, mammalian Tolloid-like 1 and mammalian Tolloid-like 2 can all process the NHK synthesized Ln-5 $\alpha_3$-and $\gamma_2$-chain at the physiological cleavage site (Amano et al. 2000, Veitch et al. 2002). In one study, MMP-14 readily cleaved the human Ln-5 $\gamma_2$-chain into 105 kDa, ~90 kDa and ~70 kDa forms in vitro (Gilles et al. 2001) while others report that MMP-2 and -14 do not process the human Ln-5 $\gamma_2$-chain (Veitch et al. 2002). BMP-1 and MMP-14 converts both rat and mouse 135 kDa Ln-5 $\gamma_2$-chain to the 100 kDa form at the physiological cleavage site, while MMP-2 and -14 can either directly convert the 135 kDa form to an 80 kDa form or through the 100 kDa form (Koshikawa et al. 2000, Sasaki et al. 2001, Schenk et al. 2003, Veitch et al. 2002). MMP-2 cleaves the rat Ln-5 $\gamma_2$-chain in domain III at A$^{586}$-L$^{587}$ (Table 4) resulting in an 80 kDa $\gamma_2$-chain. The cleavage site precedes two closely spaced cysteines in domain III (Cys-Pro-Ala-Cys), which are thought to be involved in joining the three Ln-5 subunits at the centre of the “cross”. This location indicates that the $\gamma_2$-chain remains attached to the heterotrimer (Giannelli et al. 1997). Mouse epidermal Pam212 cells also secrete an ~70 kDa Ln-5 $\gamma_2$-chain which is most likely produced through direct processing of the 135 kDa form (Sasaki et al. 2001). Human Ln-5 $\gamma_2$-chain is cleaved by plasmin (Veitch et al. 2002) while the rat Ln-5 $\gamma_2$-chain is not (Giannelli et al. 1997).
Table 4. Cleavage sites in the Laminin-5 γ2-chain.

<table>
<thead>
<tr>
<th>Laminin-5 γ2-chain</th>
<th>155 kDa-&gt; 105 kDa cleavage site</th>
<th>105 kDa-&gt; 80 kDa cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>YSG434 - D435ENPDIEC1-5</td>
<td>Human not determined</td>
</tr>
<tr>
<td>Rat</td>
<td>YSG413 - D414ENPDIEC7</td>
<td>Rat AAA586-L587TSCPACYN6</td>
</tr>
<tr>
<td>Mouse</td>
<td>YSG434 - D435ENPDIEC8, 9</td>
<td>Mouse not determined</td>
</tr>
</tbody>
</table>


2.4.4. In vivo functions of Ln-5

During mouse development, Ln-5 is prominently expressed in organs of endodermal and ectodermal origin where it is deposited to the BM area. Ln-5 is not found in muscle, neural or endothelial BMs (Carter et al. 1991). In adult human tissues, expression of Ln-5 mRNA is confined to skin, lung and also kidney, thymus, choroid plexus, cerebellum and the brain intermediate zone (Kallunki et al. 1992).

Ln-5 is an absolute necessity for hemidesmosome formation. Mutations in the three Ln-5 genes results in mechanobullous skin blistering disorders, such as junctional epidermolysis bullosa (EB) of which the Herlitz type is lethal and inherited as an autosomal recessive trait. EBs are characterized by detachment of the epidermis from the dermis (Pulkkinen and Uitto 1999). Ln α3-chain null mice exhibited very similar blistering defects in skin and oral mucosa as human Herlitz type of EB patients with junctional epidermal-dermal separation and lethal phenotype. Ameloblasts of the α3-null mice incisors were reduced in size relative to the teeth of wild type mice and enamel deposition was abnormal (Ryan et al. 1999a). In both the developing human and murine teeth, Ln-5 γ2-chain mRNA and protein were detected in dental epithelial cells and along the BMs of the enamel organ. Ln-5 γ2-chain mRNA was expressed in differentiating ameloblasts and later continued in the secretory ameloblasts (Kainulainen et al. 1998, Sahlberg et al. 1998).

2.5. Cell migration

Active cell motility is essential in physiological tissue development and homeostasis, including embryological morphogenesis, wound healing, immune surveillance and inflammation. In the process of migration, cells must make their way through the macromolecules of the ECM through adhesion, change in shape as well as ECM proteolysis. Cells may move in a directed (taxis) or random (kinesis) manner along soluble (chemo) or solid (hapto) attractants. Thus, haptotaxis occurs along gradients of ECM solid substrata. Factors stimulating epithelial cell motility include growth factors, cytokines, ECM fragments and cryptic sites. With respect to motility, the microenvironment regulates epithelial morphogenetic movements critical for organogenesis, tissue formation, remodelling, healing and renewal (Quaranta 2002).

Cell migration is also a means for tumor cell invasion, the hallmark of malignant tumors. Tumor cell metastasis proceeds through neovascularisation and penetration of the BM resulting in intravasation of tumor cells to the blood flow or lymphatic vessels followed by extravasation of tumor cells to new sites and growth of tumors. Migrating tumor cells must cross multiple barriers covering distances up to several hundreds of micrometers (Friedl and Brocker 2000).

The pattern of cell motility is the same for both cancer cells and in normal tissue remodelling. Also normal cells invade tissues locally as can be seen in any morphogenetic migratory movements involving epithelial cells (Quaranta 2002). Sheet migration is a common strategy for epithelia to cover or envelop tissue which occurs in development as well as during wound repair. Epithelial SCC cells express the same MMPs as epithelial keratinocytes during wound healing. In this aspect, tumors have been compared to nonhealing wounds (Quaranta 2002).
Cell adhesion is essential for cell migration and prior to forward movement, cells require a rearrangement of adhesion complexes. When a cell prepares to move, a protrusion or filopod/lamellipod with ruffling spikes is formed for attachment to the substrate. Upon attachment to the substrata, integrins cluster in the cell membrane to ligand-binding sites which induces intracellular signalling and subsequent assembly of cytoskeleton-binding FA complexes (Friedl and Brocker 2000), typically composed of integrins and different laminin-isoforms. FAs provide attachment-dependent outside-in signalling leading to the recruitment and polymerization of actin monomers and the formation of actin cables or bundles (stress fibers) (Ray and Gately 1996). Following substrate binding at the leading edge, a gradient of binding and traction forces is established in the cells resulting in forward movement of the cell in relation to the underlying substrate. In locomotive cells, FAs, then termed focal contacts (FCs), are dynamic structures of smaller size, incompletely assembled, less stable and may also contain molecules not present in FAs (Friedl and Brocker, 2000).

**2.5.2. Proteolysis and ECM remodelling during cell migration**

Cells may move in the three-dimensional ECM without focalized proteolysis simply by changing shape. Focalized pericellular proteolysis mediated by membrane-associated MMPs can help cells to “make the way” for migration by reducing the mechanical force in the matrix. Focalized proteolysis can also be used to release matrix-bound growth factors, chemotactic signals or to decrease adhesion of the cells on the surrounding substrata thus enhancing migration. Pericellular proteolysis gives the action of proteinases a spatially restricted and regulated environment through which extracellular signal transduction can be mediated, concentrates MMPs within a near vicinity of their substrates, limits access of MMP inhibitors and enhances MMP activation (Sternlicht and Werb 2001).

**2.5.3. MMPs and tumor cell migration**

MMPs have been implicated not only in promoting cancer development through removal of the ECM barrier but also in the modulation of signals affecting cellular transformation, tumor growth, angiogenesis and apoptosis. The first indication for a role of MMPs in cancer came from Liotta and his collagues (Liotta et al. 1979) in the 1980s when he and his collagues discovered that a type IV collagenase was involved in melanoma invasion and metastasis through breakdown of BMs. Several studies show that blocking tumor cell migration with both broad-spectrum MMPIs and serine proteinase inhibitors do not fully abolish the ability of tumor cells from migrating (Friedl and Brocker 2000). Proteases may mediate important migratory functions not directly related to the shear ability of a tumor cell to move and invade. In a tumor cell aspect, such functions would be adhesion to any substrata coming in its way, clearance, finding the path of least resistance and directionality. Some MMPs are expressed by the tumor cells, i.e. MMP-7 is expressed by the carcinoma cells in gastrointestinal, breast, lung and in cutaneous carcinomas (Wilson and Matrisian 1996). MMP-8 is expressed by oral SCC tumor cells (Moilanen et al. 2002). MMPs-2, -9 and –14 originate from epithelial cells of various carcinomas (Coussens et al. 2002). MMP-14 is produced by stromal fibroblasts in breast, colon, and head and neck cancers, and in both carcinoma cells and fibroblasts in pancreatic, ovarian, gastric and thyroid cancers (Nabeshima et al. 2002).

Recent research has led to the concept that MMPs in tumor tissues are largely produced by reactive stromal cells recruited to the neoplastic environment (Coussens et al. 2002). MMPs from adjacent stromal cells are often induced and commandeered by the malignant epithelial cells. In addition, inflammatory cells are a prominent feature of many malignant tumours and can be a source of certain MMPs in the peritumoural environment. MMP-1, -2, -3, -9, -11, -12, -13 and –14 are all localized to the stromal compartment of breast cancer tumors and in colon adenocarcinomas (Basset et al. 1997, Lynch and Matrisian 2002). It has also been noted that
increased MMP expression correlates with tumor aggressiveness. MMPs –1, –2, –7, –8, –9, –10 and –11 are expressed and increasingly secreted with tumorigenicity by cultured epithelial and SCC cells (Bachmeier et al. 2000a, Bachmeier et al. 2000b). Increased expression of MMP-2 and collagenases correlates with the invasiveness of malignant melanomas (Kähäri and Saarialho-Kere 1999, Väisänen et al. 1996).

Actual evidence for tissue degradative activity of distinct MMPs in tumour tissues in vivo is limited. A study with MDCK-cells overexpressing various MMPs indicated that MMP-14 provided cells with an invasive phenotype and collagen-degrading ability, while other MMPs did not (Hotary et al. 2000). In general, MMP-overexpressing transgenic mice develop spontaneous hyperproliferative lesions. MMP-7 knock-out mice exhibit reduction in intestinal tumourigenesis and MMP-2 deficient mice show reduced angiogenesis and tumour progression (Kähäri and Saarialho-Kere 1999). With some exceptions, however, there are few indications that the elimination of a single MMP can reduce tumor invasion and metastasis.

Since MMPs participate in angiogenesis, MMPs can also be considered critical for metastasis through the formation of new blood vessels. This is particularly true for the activity of MMP-2, -9 and -14 mediated fibrinolysis, but also for other MMPs that can release or activate growth factors involved in neovascularisation (Hiraoka et al. 1998, McCawley and Matrisian 2000, Vu et al. 1998). MMPs can also regulate angiogenesis by generating anti-angiogenic peptides. MMPs –2, –3, –7, –9 and –12 generate angiostatin in vitro by cleaving plasminogen (Pg) and MMP-7 and –9 in vivo. Angiostatin inhibits basic fibroblast growth factor-induced endothelial cell proliferation and tube formation in vitro (Cornelius et al. 1998). Endostatin is a C-terminal fragment of type XVIII collagen with antiangiogenic activity. MMP-3, -7, -9, -13 and –20 can generate endostatin-like fragments from type XVIII collagen in vitro. Recombinant endostatin efficiently blocks angiogenesis and suppresses primary tumor and metastasis growth in animal models (Nyberg et al. 2003, Sternlicht and Werb 2001). The hemopexin domain of MMP-2, released after autolysis in human tumors can also inhibit angiogenesis, cell proliferation and migration (Bello et al. 2001).

MMP-8 expression was associated with non-metastatic breast carcinoma cell lines both in vitro and in vivo. Knock-down of MMP-8 expression by antisense perturbation resulted in an increase in the invasive potential of the non-invasive breast carcinoma cell line (Agarwal et al. 2003). MMPs may also cleave apoptotic signalling molecules and affect immunoevasion (Lynch and Matrisian 2002, Sheu et al. 2001). Proteolytic exposure of a cryptic site in the type IV collagen molecule by at least MMP-2 can regulate angiogenesis and tumor growth in vivo (Xu et al. 2001). MMP-9 associates with the hyaluronan receptor CD44 on invasive carcinoma cells and promotes tumor invasion and angiogenesis (Yu and Stamenkovic 2000) while MMP-14 can cleave CD44 and induce carcinoma cell migration (Kajita et al. 2001). MMP-14 and MMP-2 are typically localized at the leading front of invading cultured carcinoma cells for efficient pericellular proteolysis and MMP-2 activation (Nabeshima et al. 2002, Nakahara et al. 1997).

### 2.5.4. Ln-5 in physiological cell migration

Ln-5 mediates adhesion of many different cell lines and blocking the Ln α3-chain effectively inhibits adhesion of NHKs (Rouselle and Aumailley 1994, Rousselle et al. 1991). NHKs deposit Ln-5 to the ECM, however, NHKs are usually stationary but can be induced to migrate by TGF-β1, which decreases the adhesion of NHKs on human Ln-5. TGF-β1-treated migrating NHKs and MCF-10A cells deposit the 200 kDa unprocessed α3-chain (Decline and Rousselle 2001, Nguyen et al. 2000b, Zhang and Kramer 1996). Plasmin-processing of the α3-chain has been found to induce nucleation of HDs, at least in carcinoma cells (Goldfinger et al. 1999, Goldfinger et al. 1998, Veitch et al. 2002). The excised 40 kDa fragment resulting from α3-chain processing does not exhibit any significant cell scattering-activity (Tsubota et al. 2000). Domain III of the γ2-chain is involved in mediating cell migration. In transgenic mice, potential involvement of the γ2-chain in cell migration was shown to be related with a 613 bp upstream region flanking the LAMC2-gene and containing a GATAA box, as well as AP-1 and Sp1 binding sites (Salo et al. 1999).
2.5.5. Ln-5 and tumor cell migration

Ln-5 was initially found to have cell-scattering effects when purified from human gastric carcinoma cells and rat NBT carcinoma cells and when applied to both normal and carcinoma cell lines it stimulated cell migration and disruption of intercellular connections. The Ln-5-like molecule induced actin cytoskeleton reorganization and FC formation in the tumor cells (Miyazaki et al. 1993). It was also demonstrated that addition of soluble Ln-5 to culture media induces rapid adhesion and spreading of HaCat and pancreatic carcinoma cell lines (Baker et al. 1996a). Cell migration on rat Ln-5 is induced by MMP-2 and -14-mediated cleavage of the γ2-chain while MCF10 cell migration on Ln-5 can be induced by exogenous addition of MMP-2 (Giannelli et al. 1997, Koshikawa et al. 2000). Constitutive HT1080 and BRL cell migration on Ln-5 is mediated by Ln-5 cleavage through endogenously produced MMP-2 and −14 (Koshikawa et al. 2000). Very aggressive melanoma cells secrete both the 100 and 85 kDa Ln-5 γ2-chains along with MMP-2, -9 and −14 (Seftor et al. 2002). Recently, a study with human prostate cancer cell lines showed that the human Ln-5 β3-chain was cleaved by MMP-14 and induced carcinoma cell migration (Udayakumar et al. 2003). Also hepatoma, breast and colon carcinoma cell migrations are induced on Ln-5 via the MMP cleavage mechanism (Koshikawa et al. 2000). In vivo, the 80 kDa Ln-5 γ2x-chain is present in rodent tissues undergoing remodelling and in mouse carcinomas but not in quiescent tissues (Giannelli et al. 1997, Giannelli et al. 1999). MMP-2, -14 and Ln-5 are co-localized in breast and colon carcinoma tissues in vivo (Koshikawa et al. 2000). EGF induces migration and expression of both MMP-14 and Ln-5 in the cell periphery of the outgrowth (Gilles et al. 2001) and cleavage of the Ln-5 γ2-chain liberates a fragment with EGF-family of ligand homology (Schenk et al. 2003). Endogenously produced MMP-2 activated TGF-β1, subsequently stimulating noninvasive hepatocellular carcinoma cells (HCC) cells to migrate on Ln-5 and with a concomitant upregulation of α3β1-integrin (Giannelli et al. 2001, Giannelli et al. 2002). In vivo, the loss of BM continuity has been associated with increasing malignancy of carcinomas, based mostly on morphologic data. BMs are not only degraded, but also synthesized in carcinomas (Lohi 2001). The Ln-5 γ2-chain protein and mRNA is expressed in colon adenocarcinomas, ductal mammary carcinomas, melanomas, ameloblastomas, pancreatic carcinomas, prostate carcinomas, SCCs and most types of lung carcinomas by the cancer cells and in most cases in the budding tumor cells confined at the invasive front (Fukushima et al. 1998, Hao et al. 1996, Kainulainen et al., 1998, Määttä et al. 1999, Pyke et al. 1994, 1995, Tani et al. 1997). Ln-5 is neoexpressed in thyroid tumors although Ln-5 is totally lacking in the normal adult as well as fetal thyroid tissue (Lohi et al. 1998). A peculiar observation is that gastric carcinoma cells secrete monomeric Ln-5 γ2-chain (Koshikawa et al. 1999). Ln-5 has also been suggested to be a marker for malignant transformation of tumors in cervical carcinomas (Skyldberg et al. 1999). The significance of the Ln-5 γ2-chain expression for the malignant behaviour of tumors is at present unknown. Ln-5 γ2-chain expression does not seem to have a major influence on tumour clinical behaviour (Määttä et al. 1999).

2.6. Periodontal tissue

The healthy periodontium, providing support to maintain teeth in adequate function, is composed of four principal components: the gingiva, periodontal ligament, alveolar bone and cementum (Figure 5). The gingiva is histologically composed of the stratified squamous keratinizing gingival epithelium which covers the underlying connective tissue. The gingival epithelium can be divided into three different types based on their location and composition: the outer oral epithelium, the sulcular epithelium (SE) lining the gingival sulcus extending from the tip of the gingival crest to the coronal most portion of the junctional epithelium. The SE and oral gingival epithelium are very similar in cellular structure and composition. The junctional epithelium extends from the base of the gingival sulcus to an arbitrary point close to the alveolar bone crest and forms the tissue attachment of the gingiva to tooth structures. The junctional epithelium is associated with two BM-like structures, the internal basal lamina involved in the dento-epithelial complex and the external basal lamina facing the underlying connective tissue (Bartold et al. 2000). The internal basal
lamina is unique in that it lacks typical structural molecules for BMs, the major in human tissues thus being Ln-5. Since Ln-5 is unable to self-polymerize, it has been suggested that Ln-5 may bind directly to the tooth enamel surface (Hormia et al. 1998, Oksanen et al. 2001). The main macromolecule in the gingival connective tissue is type I collagen. The gingival connective tissue serves primarily to protect the root surface and alveolar bone from the external oral environment and additionally, provides support of epithelial tissues and the teeth within their alveolar housing (Bartold et al. 2000).

Figure 5. Structure of the tooth and the surrounding periodontal tissue. Picture reprinted with permission from Taina Tervahartiala from her doctoral thesis, printed in Helsinki 2003.

2.7. Skin

Skin is the largest organ of the body comprising some 16% of the body weight (Figure 6). Skin covers the body surface being the primary defence against microbial invasion, dehydration and mechanical, chemical, osmotic, thermal and photic damage. Skin consists of two main layers, the keratinizing stratified squamous surface epithelium or epidermis and the subjacent collagen-rich connective tissue layer providing support and nourishment, the corium or dermis (Figure 6). The BM structure separates the epidermis from the underlying dermis. Beneath the dermis is a looser connective tissue layer, the superficial fascia, or hypodermis, which in many places is largely transformed into subcutaneous adipose tissue. The hypodermis is loosely connected to an underlying deep fascia, aponeurosis, or peristeum (Bloom and Fawcett 1968).
Figure 6. Cutaneous wound healing. Upon injury of intact skin, a clot forms to prevent further blood flow and is further covered by a scab. Neutrophils are the first inflammatory cells to arrive to the wounded site, which occurs within hours. Within 3-7 days, keratinocytes migrate resealing the denuded surface, fibroblasts migrate to the wound site forming the granulation tissue and neutrophils are replaced by macrophages. Wound contraction with extensive connective tissue remodelling occurs for several weeks after injury.
2.7.1. The basement membrane

The BM is a specialized extracellular structure, whose main function is to act as a selective and physical barrier between cells and the underlying connective tissue (Figure 7). The BM also provides a base for tissue repair and acts as a dynamic link between the attached epithelial cells and the underlying connective tissue, mediating signals for cellular movement, polarization, proliferation and differentiation. The integrity of BMs is indeed important as a barrier to the spread of tumor cells. The 80 nm thick BM or basal lamina consists of two distinct layers: a 40 nm electron-lucent layer (lamina lucida or rara) adjacent to the basal plasma membrane of the cells that rest on the lamina and a 40 nm electron-dense layer (lamina densa) just below (Figure 7). The basal lamina underlying the epidermis of the skin is made up of the HDs and FAs (Ray and Gately 1996).

2.7.1.1. The hemidesmosomal complex

The HDs are highly specialized junctional complexes attaching epithelial cells to the underlying BM in stratified and other complex epithelia such as the skin, the cornea, parts of the gastrointestinal and respiratory tract, oral mucosa, mammary gland and the amnion (Ray and Gately 1996). Ultrastructurally, HDs are small electron dense domains of the plasma membrane on the ventral surface of basal keratinocytes in human skin. Their most conspicuous component is a tripartite cytoplasmic plaque to which bundles of intermediate filaments are attached. HDs are associated with a sub-basal dense plate in the lamina lucida and are connected via fine thread-like anchoring filaments to the lamina densa. The cross-banded anchoring fibrils project into the upper regions of the papillary dermis where they loop back and reinsert into the lamina densa. These structures constitute the functional unit called the hemidesmosomal adhesion complex (Borradori and Sonnenberg 1999).

The cytoplasmic constituents include at least bullous pemphigoid antigen 230 and plectin belonging to the plakin family. The transmembrane proteins of HDs are α6β4 integrin and bullous pemphigoid antigen 180 (type XVII collagen). The ectodomain of bullous pemphigoid antigen 180 spans the lamina lucida and a flexible tail intertwines within the lamina densa. α6β4 integrin is localized at the basal pole of the basal epithelial cells where it coordinates a structural linkage between intermediate filaments and the ECM portion of the BM (Borradori and Sonnenberg 1999).

The association of Ln-5 with α6β4 integrin is crucial for the nucleation of HD assembly and for the maintenance of stable adhesion (Baker et al. 1996a). The lamina densa separates the connective tissue from the BM structure. The structures in the lamina lucida include the anchoring filaments which are thin, threadlike structures connecting HDs to the lamina densa. These layers are composed of type IV and VII collagen, Ln-5, Ln-6 (α3β1γ1), Ln-7 (α3β2γ1), Ln-8 (α4β1γ1), Ln-10 (α5β1γ1), nidogen, perlecan, fibulin −1 and −2. The anchoring fibrils extend from the lower portion of the lamina densa to the underlying mesenchyme and are almost exclusively composed of type VII collagen (Burgeson and Christiano 1997). The truncated Ln-5 γ2-chain lacks the domain VI containing the high-affinity nidogen binding site found in the Ln γ1-chain. Thus, Ln-5 is not directly connected to the type IV collagen network and perlecan. The Ln-5 through its β3-chain forms a covalently linked complex with the α3-chain of Ln-6, containing nidogen-binding sites (Amano et al. 2000, Borradori and Sonnenberg 1999). However, Sasaki et al. demonstrated that the N-terminal region of the Ln-5 γ2-chain contains two binding sites each for fibulin-2 and heparin and additional binding sites for fibulin-1 and nidogen-1 (Sasaki et al. 2001). A model predicts that while monomeric Ln-5 is concentrated below the hemidesmosomal plaque linking the α6β4-integrin (and probably bullous pemphigoid antigen 180) to type VII collagen, the Ln-5-6-7 complex (but not the Ln-5 monomer) is participating in the stabilization of the BM in the interhemidesmosomal space (Aumailley and Gayraud 1998, Borradori and Sonnenberg 1999, Rousselle et al. 1997)
2.7.2. Estrogen and the skin

Estrogen is a reproductive hormone mediating essential biological processes in the body. In the developed world, due to longer life span, women spend more than one-third of their life-time postmenopausally in a state of profound estrogen deprivation. Hill has estimated that by 2030 the world population of postmenopausal women will be of the order of 1.2 billion (Hill 1996).

Menopause is marked by a sharp change in hormonal balance and a decline in estrogen proceeds to reduced progesterone levels and permanent amenorrhea. The menopause is known to have deleterious effects on bone mass, increase the risk of cardiovascular disease as well as breast and endometrial cancers (Hill 1996). However, the effects of estrogen-deprivation on skin have been less well documented. The skin is a target for several hormones, including estrogen, and most cells within the skin contain estrogen receptors (ERs). A higher ER content has been found in women than in men. Estrogen increases the mitotic rate of epidermal cells and enhances vascularization. Estrogens have been extensively used in hormone replacement therapy (HRT) and birth control (Ashcroft et al. 2003).

2.7.2.1. Estrogen and skin collagen content

Type I collagen comprises about 80% of dermal collagen and plays a major role in providing tensile strength to skin. Skin thickness is proportional to the collagen content (Shah and Maibach 2001). Premenopausal women were found to deposit more collagen in acute test wounds than age-
matched men (Jorgensen et al. 2002). In postmenopausal women, skin collagen content and thickness declines exponentially after 2-4 years into the menopause (Affinito et al. 1999). In addition, BM type IV collagen decreases with age while BM thickness increases (Vázquez et al. 1996). Studies addressing the relationship between skin collagen content, skin thickness and chronological age are controversial. Apparently, the reduction in collagen content correlates with years from the menopause rather than chronological age (Affinito et al. 1999, Brincat et al. 1985, 1987a, 1987b). However, in postmenopausal women treated with HRT the age-related decrease is reversed. Skin thickness increases within 6 months of HRT and then seems to plateau (Brincat et al. 1985, 1987a, 1987b). However, there are also contradictory results, since one year treatment with systemic estrogen had no effect on skin collagen in postmenopausal women (Haapasaari et al. 1997). In addition, topical estrogen treatment increases type I and III collagen synthesis and content in human skin (Shah and Maibach 2001).

### 2.8. General characteristics of inflammation

Inflammations share several similar characteristics although distinct features exist in respect to the disease etiology and the involved tissue. Wound healing can be considered a specialized form of inflammation. The difference between acute and chronic inflammation is not clearly distinguishable (Figure 8). In general, an acute inflammation becomes chronic when a prolonged inflammatory response resides in the affected tissue. Recovery from acute inflammation involves elimination of the factor (bacteria, foreign bodies etc.) causing the inflammatory response, formation of an intermediate tissue, removal of necrotic debris, recruitment of cells to the site of injury (fibroblasts and endothelial cells), neoangiogenesis, epithelial cell migration (if present), as well as regeneration and remodelling of the tissue. Inflammation *per se* is necessary to protect the host from infection, but persistent inflammation can also cause disease and tissue destruction. Acute inflammation may proceed to chronic inflammation if the cause of the initial injury is not completely eliminated but may also develop in the absence of acute inflammation. Causes of chronic inflammation can be infections, remnants of dead organisms, foreign bodies, metabolic products, immune reactions, chemical and physical factors/irritants or other disease-related factors (Trowbridge & Emling 1989).

In most inflammations, neutrophils are the first line of defence and within a few days, neutrophils are replaced by macrophages and lymphocytes. Neutrophils and macrophages release proteases, pro-inflammatory cytokines and engage in essential functions including phagocytosis of debris, microbial agents and degraded matrix components (Trowbridge & Emling 1989). MMP expression by inflammatory cells is spatially and temporally restricted, however, the battery of MMPs which can be expressed by inflammatory cells contributing to the inflammatory response in tissues is impressive: monocytes express MMP-1, -7, -8 and -9; macrophages MMP-1, -2, -3, -8, -9, -10, -12 and -13; PMN neutrophils store MMP-8 and MMP-25 in their secondary granules as well as MMP-9 in the tertiary granules and also MMP-19 and MMP-25 are localized to neutrophils; plasma cells express at least MMP-2, -8, -9 and –13. Thus, the inflammatory cells have an impressive capacity for MMP secretion at the site of inflammation (Barrick et al. 1999, Prikk et al. 2001, Wahlgren et al. 2001). The MMPs within neutrophils can be secreted to ECM after stimulus with various cytokines, of which IL-8 is one of the most potent. Since neutrophils do not synthesize TIMPs, neutrophil degranulation is a powerful ECM degradative pool (Van den Steen et al. 2002).
Figure 8. Typical features and differences in acute and chronic inflammation. Adapted from (Nwomeh et al. 1998b).

2.8.1. Chronic inflammatory periodontal disease

Adult periodontitis is characterized by chronic periodontal inflammation and irreversible attachment loss associated with alveolar bone destruction leading to tooth loss. The host response is responsible for the matrix degradation (Trowbridge and Emling 1989, Van Dyke and Serhan 2003). The initial phase of chronic periodontal disease, gingivitis, is the most common chronic inflammatory disease in humans (Williams et al. 1992). Gingivitis and periodontitis are the result of an induction of host inflammatory responses to the accumulation of periodontopathogens and their products (i.e. LPS, leukotoxin, proteolytic enzymes) on tooth surfaces adjacent gingival tissues. Initially, gingivitis represents a generalized acute inflammatory response but if the inflammatory response contained within the gingivitis lesion spreads to the deeper periodontal tissues and alveolar bone is lost, the resultant lesion is termed periodontitis. The progression from gingivitis to periodontitis is unclear and people can have gingivitis which never progress into periodontitis (Bartold et al. 2000, Van Dyke and Serhan 2003). Typically, the gingival pocket epithelium proliferates into the underlying connective tissue in the form of a network of finger-like projections coinciding with ECM degradation and loss of tooth attachment. Subsequent to the initial inflammatory response, connective tissue destruction occurs within 3-4 days after plaque accumulation. As the inflammatory process develops, alveolar bone loss results in tooth mobility and, ultimately, tooth loss if the disease is left untreated and continues to progress (Bartold et al. 2000, Van Dyke and Serhan 2003).
2.8.1.1. MMPs in chronic periodontal disease

In periodontitis, the degradation of collagen fibers of the periodontal ligament and alveolar bone resorption are the most profound manifestations of the uncontrolled soft and hard tissue degradation. High levels of proteinase activity in periodontal tissue and GCF correlates with disease severity (Van Dyke and Serhan 2003).

In inflamed periodontal tissue, gingival fibroblasts express MMP-1, -2, -3, -8, -11 and -14 while gingival keratinocytes express MMP-1, -2, -3, -7, -8, -9, -10 and -13. Macrophages and endothelial cells express MMP-1, -2, -3 and -9. GCF typically contains IL-1α, IL-1β, TNF-α, IL-6, IL-8 and IFN-γ (Birkedal-Hansen et al. 1993, Van Dyke and Serhan 2003). Active MMP-8 and -13 are significantly elevated in GCF from patients with AP, in comparison to GCF from healthy subjects. MMP-8 levels also correlate well with clinical status of the patients (Ingman et al. 1996, Mäntyla et al. 2003, Sorsa et al. 1988, Tervahartiala et al. 2000). MMP-2 and -14 mRNA are expressed by fibroblast-type cells in both healthy and diseased gingival tissue, although the cells expressing MMP-2 and -14 tend to localize closer to the site of inflammation in AP (Dahan et al. 2001, Tervahartiala et al. 2000). MMP-2 mRNA expression is increased in SE of AP patients when compared to clinically healthy gingiva (Tervahartiala et al. 2000). While proMMP-2 and -9 are present in both healthy and inflamed gingival tissue extracts, active MMP-2 is seen only in inflamed samples and total MMP-9 protein and mRNA are elevated in patients with periodontal disease (Korostoff et al. 2000, Westerlund et al. 1996). In AP patients, TIMP-1 mRNA and protein levels are usually unchanged in both GCF and at the tissue level (Ingman et al. 1996).

2.8.1.2. MMP inhibition in periodontal disease

TCs have been successfully used in the treatment of human inflammatory periodontal disease. The non-antimicrobial effect of the TCs promotes connective tissue attachment, inhibits MMPs and osteoclast function. Due to side-effects of TCs, the low-dose doxycycline (LDD) therapy was proposed. By administering specially-formulated capsules each containing as little as 20% (20 mg) as opposed to commercially available (100 mg) doxycycline capsules, the side-effects could be overcome. More importantly, the treatment significantly inhibited the pathologically-excessive collagenase activity in the gingival tissue and GCF of AP patients, significantly decreased periodontitis-associated bone loss as well as MMP-8 and -13 levels in GCF (Golub et al. 1997, Rifkin et al. 1993). Currently, the only approved MMP-inhibition based pharmaceutical on the market is LDD (trade name Periostat) for treatment of human inflammatory periodontal disease in the USA. Also CMT-1, -3, and –8 effectively inhibited the elevated gelatinase activity, reduced bone loss and reduced the proinflammatory cytokine levels associated with LPS-induced periodontitis in rats (Ramamurthy et al. 2002) and CMT-1 inhibited also collagenase activity in the gingiva of diabetic rats (Yu et al. 1993).

2.8.2. Cutaneous wound healing

Wound healing is an acute inflammation which is very commonly referred to as a “pathological event” (Figure 6). Wound healing can be regarded to be pathological if one considers any trauma pathological, if the wound is of substantial depth or size, or if there is chronicity associated with the wound. Nutritional deficiencies, chronic diseases, adrenal steroids, diabetes and estrogen-deficiency affect wound healing adversely. Wound healing is, however, a physiological inflammation in that it is necessary for the organism to reseal any openings in the surfaces of the body. It is highly unlikely that any human will live her entire life without gaining any minor skin injury. In this regard, it is more accurate to say that living organisms have a well developed “physiological” means to deal with torn surfaces. Wounds are divided into acute and chronic based on healing speed and wound quality Chronic wounds are etiologically different but they share one or more characteristics: repeated trauma, bacterial infection and local tissue ischemia (Chen et al. 1999).
2.8.2.1. Clotting

When the skin is injured, a blood clot consisting of thrombocytes, neutrophils and monocytes embedded in fibrin fibers is formed. The function of the fibrin clot is to provide a temporary shield and cover the wound and also to act as a provisional matrix for cell attachment, migration, wound contraction and re-epithelialization during the tissue repair process. The clot also serves as a reservoir of cytokines and growth factors that are released as activated platelets degranulate (Martin 1997).

2.8.2.2. The inflammatory response

Neutrophils normally begin arriving at the wound site within minutes of injury (Martin 1997, Yamaguchi and Yoshikawa 2001). Immunohistochemical staining for monocytes/macrophages and B lymphocytes have shown that there is a delayed inflammatory response in aged mice when compared to young animals (Ashcroft et al. 1997b). There is also an age-related increase in pro-inflammatory cytokines such as TNF-α by neutrophils (Ashcroft et al. 1999). In this regard, elevated TNF-α, IL-1, -6 and -8 levels have been associated with chronic wounds (Chen et al. 1999, Trengove et al. 1999). Estrogen has been shown to enhance the oxidative metabolism of activated neutrophils during phagocytosis and also to down-regulate the macrophage migration inhibitory factor, a pro-inflammatory cytokine produced by monocytes, T-lymphocytes, endothelial cells and keratinocytes. This leads to reduced inflammation, enhanced matrix deposition and accelerated wound healing. These effects depend upon a predominant ER-α response. Neutrophils and ER-positive cells are more numerous in untreated wounds from elderly humans than in subjects with estrogen-treated wounds. Estrogen also modulates the inflammatory response not only by inhibiting neutrophil chemotaxis to the wound but also by altering the expression of neutrophil adhesion molecules such as L-selectin, thereby dampening the migration of cells from the vasculature into the injured tissue (Ashcroft et al. 1999).

2.8.2.3. Re-epithelialization

Re-epithelialization involves the formation of a provisional wound bed matrix, the migration of epidermal keratinocytes from the cut edge of the wound, the proliferation of keratinocytes feeding the advancing and migrating epithelial tongue, the stratification and differentiation of new epithelium and the restoration of BM integrity. The keratinocytes forming the cut edge of the wound begin to migrate within 24-48 hours. The keratinocytes migrate from the edge of the wound under a provisional matrix of fibrin and fibronectin and over or through the viable dermis (Martin 1997, Yamaguchi and Yoshikawa 2001). Initially, keratinocytes form FCs with the fibrillar collagen, wherafter cell protrusion is favoured by gaps and pathways of least resistance. The gaps may be provided by collagenolytic activity. Several studies with postmenopausal women and OVX animals show that estrogen-deprivation leads to delayed re-epithelialization and reduced type I and type IV collagen deposition (Ashcroft et al. 1997a, 1997b). However, women receiving systemic HRT for only 3 months show a marked increase in the rate of reepithelialization and collagen deposition almost comparable to that of premenopausal women (Ashcroft et al. 1997a, 1999). Studies with topical estrogen treatment of both elderly human subjects as well as OVX animals show accelerated wound healing (Ashcroft et al. 1997a).

2.8.2.4. Wound contraction

Wound repair involves phenotypic change of fibroblasts from quiescent to proliferating cells, and subsequently to migratory mediated through different chemotactic signals, and then to stationary matrix producing and contractile cells. Wound contraction is accomplished by myofibroblasts that contain α-smooth muscle actin and mediate contractile forces produced by granulation tissue in wounds. Granulation tissue is composed of a fibronectin-rich scaffold, fibroblasts, new blood vessels and increasing amounts of collagen types I/III. During granulation tissue formation,
fibroblasts are stimulated by TGF-β to deposit new ECM proteins. The apoptosis of myofibroblasts and vascular cells finally mediates the transition from a granulation tissue into a scar. The final phase is that of remodelling in which collagen is synthesized, degraded and reorganized (Yamaguchi and Yoshikawa 2001). Studies on the effects of estrogen on fibroblast infiltration, granulation tissue formation, collagen deposition and angiogenesis are very controversial. It is known though, that estrogen modulates the expression of cytokines such as IL-1, expressed by macrophages and fibroblasts, known to stimulate formation of granulation tissue. Aging is also associated with reduced capillary growth and delayed angiogenesis (Schnaper et al. 1996). In a rat model of ovariectomy, it was shown that wound contraction was delayed 4 months post-OVX but not 2 weeks post-OVX compared to Sham-operates (Calvin et al. 1998).

2.8.2.5. Growth factors regulating wound healing

Growth factors enhancing keratinocyte migration are EGF, TGF-α, keratinocyte growth factor (KGF), TGF-β1, hepatocyte growth factor, insulin like growth factor-1 and insulin. The levels and function of many of these growth factors are regulated by pro-inflammatory cytokines such as IL-1 and TNF-α during cutaneous injury. These growth factors act as both mitogenic as well as promigratory factors on the epidermis to drive wound closure. EGF induces lamellipodial extension and the assembly of FA complexes as part of the crawling response of tissue culture fibroblasts and epithelial cells (Martin 1997). KGF protein expression was enhanced in cutaneous wounds of estrogen-treated OVX rats when compared to untreated controls (Ramamurthy et al. 2003). Wound fluids from chronic wounds inhibited the proliferation of cultured cells while that from acute wounds stimulated growth. MMPs within the fluid from chronic wounds degrade exogenously added EGF significantly more than fluid from acute wounds in vitro (Trengove et al. 1999).

2.8.2.5.1. TGF-β and scarring

The two extremities of life have one thing in common, reduced or negligible scarring following wound healing. Until late fetal stages, there is generally no sign of a connective tissue scar where a wound has healed: the repair is perfect. In addition, oral mucosal wound healing is characterized by less scarring and accelerated wound closure.

Scar tissue is composed of excessive accumulation of disorderly arranged collagens, mostly type I and III, proteoglycans and persistent myofibroblasts. During the course of wound healing, type I collagen mRNA expression starts within 24 hours after wounding, being prominent in fibroblasts up to 21. Collagen α1(I) chain expressing fibroblasts are initially localized to the deeper layers of granulation tissue but at later stages (day 8 and forward) they are mainly detected beneath the newly reconstituted epidermis (Scharffetter et al. 1989). ECM accumulation in fibrotic conditions is accompanied by elevated mRNA steady-state levels of fibrillar collagens (Verrecchia and Mauviel 2002). In the embryo, TGF-β1 is expressed transiently and at low levels after injury, but at the adult wound site it is present at high levels for the duration of healing and beyond. Adult gingival fibroblasts located in the papillary connective tissue share many properties with fetal fibroblasts (Martin 1997). TGF-β1 induces the synthesis and inhibits the degradation of ECM, stimulates the formation of granulation tissue and collagen deposition. TGF-β also acts as a negative regulator of epithelial cell proliferation, but in contrast, stimulates the proliferation of mesenchymal cells. TNF-α inhibits collagen transcription and suppresses TGF-β stimulation of collagen mRNA expression (Jacinto et al. 2001). Wound TGF-β1 levels are decreased at day 7 of wound healing in elderly women when compared to young women or women receiving HRT. Cell culture studies indicate that estrogen is the major reproductive hormone involved in dermal fibroblast production and/or secretion of TGF-β1. Thus, TGF-β1 is hormonally modulated during wound healing (Ashcroft et al. 1997a). Interestingly, lack of estrogen and TGF-β1 improves scarring, which is superior in older subjects in terms of color, texture and contour when compared to hypertrophic scarring in young women (Ashcroft et al. 1997a, 1997b).
2.8.2.6. MMPs in wound healing

MMPs are crucial for wound healing (Ågren et al. 2001, Lund et al. 1999). However, most studies demonstrate that MMP expression and activity are elevated in chronic wounds when compared to acute wounds (Saarialho-Kere 1998, Trengove et al. 1999). Usually MMP activity decreases consistently in patients with chronic wounds when progress from nonhealing to healing occurs. MMP-1 is prominently expressed in leading edge migrating keratinocytes in both acute and chronic human wounds (Ashcroft et al. 1997c, Inoue et al. 1995, Pilcher et al. 1999, 1998; Saarialho-Kere 1998). In human cutaneous wound healing, extensive research has been conducted upon the function of MMP-1 in the process of leading edge keratinocytes. When migrating keratinocytes come into contact with fibrillar collagen, α2β1 integrin rapidly triggers MMP-1 expression, which is sustained by subsequent crosstalk with activated EGF receptor in an autocrine manner. MMP-1 then cleaves fibrillar collagen I leaving behind denatured collagen, i.e. gelatin. It has been hypothesized, that in partially degraded collagen, an RGD site is exposed, recognized by integrin αvβ3 leading to upregulation of MMP-2, which then cleaves the gelatin (Pilcher et al. 1997, 1998, 1999).

Both in acute human open dermal wounds and in chronic nonhealing ulcers, the elevated level of MMP-8 in wound exudates and tissue extracts is a hundred fold higher than MMP-1 levels. MMP-8 was primarily present in the proform in acute wound fluids and in its active form in chronic wound fluids (Nwomeh et al. 1998a, 1999). At the initial phases of wound healing, large amounts of damaged type I collagen is present and MMP-8 has a high affinity for type I collagen (Armstrong and Jude 2002).

MMP-13 is abundantly expressed by activated fibroblasts as well as macrophage-like cells in chronic wounds distinct from areas of stromal cells expressing MMP-1. In contrast, MMP-13 mRNA expression is usually low or undetectable in acute human wounds (Saarialho-Kere 1998, Vaalamo et al. 1997). Since the mouse has only just recently been found to have two MMP-1 genes, future studies remain to show the expression and function of murine MMP-1 in wound healing (Balbin et al. 2001). Most murine studies have analysed the expression of MMP-13 during cutaneous wound healing and it appears that this MMP does mimick the expression of human MMP-1 at least to some extent. In acute murine wounds, MMP-13 is expressed by the leading edge keratinocytes and has also been detected in macrophage-like cells in the granulation tissue directly beneath the scab (Lund et al. 1999, Madlener 1998, Okada et al. 1997).

MMP-9 expression is usually confined to leading edge keratinocytes of human burning wounds, oral mucosal wounds and in the epidermis of various blistering diseases (Ashcroft et al. 1997c, Oikarinen et al. 1993, Salo et al. 1994) while it has not been detected in the keratinocytes of human cutaneous chronic wounds (Saarialho-Kere et al. 1993). In addition, MMP-9 is usually expressed by neutrophils and macrophage-like cells in the wound granulation tissue in human acute, chronic and blistering wounds (Ashcroft et al. 1997c, Oikarinen et al. 1993, Salo et al. 1994, Saarialho-Kere et al. 1993). In acute murine dermal wounds, MMP-9 is prominently expressed in migrating keratinocytes (Lund et al. 1999, Madlener 1998, Okada et al. 1997) and MMP-9 has also been detected in distinct cells of the mouse granulation tissue (Madlener 1998). MMP-2 is predominantly expressed by fibroblasts within the granulation tissue and basal expression is readily elevated upon tissue injury in the dermis of acute, burn and chronic wounds as well as in various blistering diseases (Oikarinen et al. 1993, Salo et al. 1994). MMP-2 protein has also been detected in the leading epidermal wound edge (Ashcroft et al. 1997c, Oikarinen et al. 1993, Salo et al. 1994). Gelatinolytic activity in chronic wound fluids is significantly elevated when compared to acute wounds. In addition, the ratio of MMP-9 to TIMP-1 is aberrant in chronic wound fluids. There is also a shift from more latent to active forms of gelatinases in the wound fluid from chronic wounds (Bullen et al. 1995, Trengove et al. 1999). In healing murine wounds, MMP-2 and -14 mRNA expression co-localizes and is detected mostly in fibroblasts of the wound granulation tissue juxtaposed to the proliferative epithelial cell layer (Lund et al. 1999, Madlener 1998, Okada et al., 1997).

TIMP-1 is usually expressed in epidermis and in the stroma of acute cutaneous wounds, however, TIMP-1 is usually undetectable in chronic wound tissue and wound fluids (Bullen et al. 1995,
Nwomeh et al. 1999, Vaalamo et al. 1997). In mice, TIMP-1 was not detected in epithelia, in contrast to human acute and chronic wounds (Madlener 1998). Also diminished levels of α1-PI and α2M in chronic wounds have been reported (Yager et al. 1996). MMP-2, MMP-9 and neutrophil elastase levels were found to be increased at late stages of wound healing in elderly persons (Ashcroft et al. 1997c).

2.8.2.7. MMP inhibition in wound healing

Most studies on acute wound healing combined with the experimental use of MMPIs have resulted in delayed re-epithelialization. GM6001-treatment, however, was found to increase incisional wound strength without affecting collagen content. By using an experimental model for sponge granulomas, GM6001 reduced the inflammatory response and decreased type I collagen content. GM6001 upregulated MMP activity, reduced wound fluid TNF-α levels and increased total TGF-β1 levels in the sponge granulomas (Witte et al. 1998). In contrast, GM6001-treatment resulted in delayed re-epithelialization of human suction blisters and in murine acute cutaneous wounds (Ågren et al. 2001, Lund et al. 1999). When Pg-deficient mice were additionally treated with GM6001, the acute wounds did not heal at any time point due to a reduction in the ability of the leading edge keratinocytes to degrade fibrin and subsequently make their way through the fibrin clot (Lund et al. 1999). In human suction blisters, GM6001 treatment resulted in an upregulation of stromal MMP-1 and -2 mRNA expression and in epithelial MMP-9 expression, while in the Pg-deficient mice, MMP-9 expression was also detected in the macrophage-like cells. MMP-2 was detected in the dermo-epidermal junction and GM6001-treatment prevented activation of MMP-2. In the GM6001-treated Pg-deficient mice, MMP-2 expression changed so that MMP-2 mRNA was also detected in the leading edge keratinocytes. Overall MMP expression was upregulated in GM6001-treated Pg-deficient mice, a pattern not seen in Pg-deficient animals without GM6001 treatment (Lund et al. 1999).

2.8.2.8. Ln-5 in wound healing

Within 8 hours of injury to the epidermis or the oral mucosa in both human and mouse and prior to migration, a precursor form of Ln-5 is synthesized and deposited into the BM by keratinocytes at the wound edge (Kainulainen et al. 1998, Larjava et al. 1993, Nguyen et al. 2000b, Pyke et al. 1994). Early deposition contrasts with the delayed expression of other BM components, such as type VII collagen and type IV collagen, which are missing under the migrating keratinocytes. Keratinocyte attachment and spreading on collagen via the α2β1 integrin is associated with deposition of Ln-5 concurrent with adhesion. The γ2-chain short arm (domain III) is the ligand for the α2β1 integrin making it likely that the α2β1 binds the 140 kDa γ2-chain precursor form (Decline and Rousselle 2001). Ln-5 expression is upregulated by TGF-β in cultured oral mucosal keratinocytes while TNF-α had no effect on Ln-5 expression in epidermal keratinocytes (Kainulainen et al. 1998).
3. Aims of the study

1. Human periodontal disease exhibits all the typical features of chronic inflammation. The advantage of periodontitis as a model for chronic tissue destructive inflammation is easy and usually non-invasive sample access. MMPs are known to be elevated in inflammatory diseases and an MMPI has successfully been used in the treatment of adjunctive AP. Therefore, we assessed the effect of two inflammatory cytokines, TGF-β1 and TNF-α on MMP-2 and MMP-9 expression in cultured human oral mucosal keratinocytes. We further studied the in vivo localization of gelatinolytic activity and the expression of gelatinases MMP-2, MMP-9 and the Ln-5 γ2-chain in inflamed human gingival tissue. Concomitantly, we investigated the effect of different synthetic MMPIs on MMP-2 and MMP-9 expression and activity in cultured oral mucosal keratinocytes as well as on in situ gelatinolytic activity.

2. Reproductive hormones such as estrogen are known to influence wound healing. MMPs are elevated in wound tissue from women who are past menopause but no studies have investigated the effect of estrogen on MMP-8 expression during wound healing. Estrogen is known to modulate the Ln-5 γ2-chain but no studies have previously investigated the effect of estrogen on Ln-5 γ2-chain expression and processing during wound healing. The mechanisms by which MMPIs impact wound healing are poorly understood and generally tested on acute wound healing. We therefore investigated the effect of an MMPI and estrogen on cutaneous wound healing in OVX rats by studying re-epithelialization, collagen content, MMP expression and the Ln-5 γ2-chain processing.

3. MMP-2 and -14 are known to cleave the Ln-5 γ2-chain and induce cell migration. Migrating keratinocytes deposit Ln-5 while migrating on many substrata, such as collagen during wound healing. Carcinoma cells synthesize Ln-5 and may use it for spreading and invasion. Thus, we investigated the MMP expression, Ln-5 deposition and effect of synthetic MMPIs on migration of cultured human oral mucosal keratinocytes. We also assessed if other MMPs in addition to the previously characterized MMP-2 and -14 could cleave the Ln-5 γ2-chain and induce carcinoma cell migration in addition to testing the effect of MMPIs on cell migration over Ln-5.
4. Materials and Methods

4.1. Animals (III & IV)

Rat wound tissue biopsies were obtained from Professor Nungavarm S Ramamurthy, Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, USA. Animal experiments were conducted with the appropriate IACUC approvals granted to Dr. Nungavarm Ramamurthy. Wound tissues were excised from six months old adult female Sprague-Dawley rats which were either Sham operated or bilaterally ovariectomized. 120 days later the rats were anesthetized with a mixture of Ketamine and Xylazine. Eight full thickness skin wounds were made in the dorsal thorax (4 on each side) using a 6 mm diameter circular biopsy punch and the wounds were allowed to heal by secondary intention. Wound biopsies were standardized and white petrolatum was applied to the wounds as previously described (Eckles et al. 1990). The Sham rats were divided into two groups: placebo and CMT-8. OVX rats were divided into three groups: placebo, CMT-8 and estrogen. There were six rats in each group except for the estrogen group which had 4 rats. The CMT-8 (15 mg/kg body weight) was administered to the rats by oral gavage in 1 ml of 2% carboxymethyl cellulose. Placebo rats received only 1 ml of 2% carboxymethylcellulose. Estrogen, 100 µg/ml was dissolved in corn oil and 10 µg was administered daily by subcutaneous injection. On day 7 after creating the wounds, the animals were anesthetized, blood samples were collected and the skin containing four wounds was excised for histological analysis. Only the granulation tissue was removed from three other wounds for biochemical analysis using a 3 mm disposable biopsy punch. The wound closure and amount of granulation tissue were measured as previously described (Ramamurthy et al. 1999).

4.2. Tissue samples (I & II)

Inflamed buccal oral mucosa from AP patients (n=5) and clinically healthy gingival tissue specimens were obtained from patients prior to any therapy, following the patients informed consent. The samples were snap-frozen in liquid nitrogen. The collection of human tissue samples for the studies was approved by the ethical committee of the Institute of Dentistry, University of Helsinki, Finland.

4.3. Materials (I-V)

Ln-5 was purified from serum-free DME of 804G cells as described (Koshikawa et al. 2000, Koshikawa et al. 1992). Non-functional Ln-5 mouse mAb TR-1 (Plopper et al. 1996) was chemically conjugated to protein A-Sepharose 4B as described (Koshikawa et al. 1992). Concentrated culture media was passed over the TR-1 antibody column and absorbed Ln-5 was eluted with 0.05% trifluoroacetic acid, pH 2.5. APMA, an optimal organomercurical activator of latent proMMPs (Van Wart and Birkedal-Hansen 1990) was purchased from Sigma Chemicals, St. Louis, MO. The broad spectrum MMP-inhibitors, BB-2516 (Marimastat) and BB-94 (Batimastat) were kindly provided by British Bio-Technology, Ltd., Oxford, England. LW-1 (3,4-dihydro-1-oxo-1,2,3-benzotriazine-3-(3-tetrahydrofuranyl)carbonate, LW-2 (1,2-dihydro-3,6-dioxo-2-phenyl-pyridazine-1-methylcarbonate), LW-3 (3,4-dihydro-1-oxo-1,2,3-benzotriazine-3-(2-methoxy)ethylcarbonate), LW-4 (1,2-dihydro-2-ethoxy carbonyl-1-oxo-isochinolin-5-phenyl) ethylcarbonate and LW-5 (1H-phthalazinone-2-(4-methoxy-phenyl)carbonate) were kindly provided by Dr. R. Klause, Drug Research Department, Luipold Pharma, Munich, Germany. All the CMTs (Figure 4) were kindly provided by CollaGenex Pharmaceuticals Inc., Newtown, Pennsylvania. Recombinant TGF-β and TNF-α were obtained from R&D Systems, Abingdon, United Kingdom.
4.4. Cell lines (I & V)

Human oral mucosal keratinocytes (HMKs) were obtained from surgical gingival biopsy and cultured as described (Salo et al. 1994). The HMKs were found to be spontaneously immortalized and its passages 13-18 were used in the experiments. The chromosome number of the cells was examined and found to be around the hypertriploid range (70-76). Primary cultures of normal human skin keratinocytes were obtained during mastectomy for breast cancer (supplied by Dr. Juha Peltonen, University of Turku, Turku, Finland). Cells were maintained in specialized keratinocyte medium (KGM, Clonetics Corp., San Diego, CA).

A549 is a human lung carcinoma and MCF-7 is a human breast adenocarcinoma cell line. These cell lines were maintained in DME (Gibco BRL) with 10% fetal calf serum (v/v) (Irvine Scientific), penicillin, streptomycin at 37°C in 5% CO₂/95% air.

4.5. MMP inhibition of cell proliferation (I)

The effect of MMP inhibitors was analyzed as described (Kueng et al. 1989). 20,000 keratinocytes were seeded into 96-well plates (Nunclon, Denmark) and allowed to attach for 24 hours at 37°C. The cells were then incubated with 200 nM to 50 μM concentrations of CMTs, LWs or Batimastat. Cells were treated with 100 ng/ml recombinant TNF-α to stimulate MMP-9 production. The cells were fixed with 4% (v/v) formaldehyde in phosphate-buffered saline (PBS) containing 5% (w/v) sucrose and stained with 0.1% crystal violet in boric acid (pH 6.0). After destaining with 10% acetic acid, the absorbance was measured with a Multiscan MS plate reader (Version 4.0, Labsystems, Helsinki, Finland) at 595 nm. Cell numbers obtained were used to equalize the quantity of medium MMP levels analyzed by zymography. The effect of TGF-β on keratinocyte MMPs was analyzed by zymography of both medium and cell-associated fractions. HMKs were cultured into 80% confluence and then 10 ng/ml TGF-β1 was added for 48 hours. Culture medium was collected and the cells were extracted at 4°C for 10 min in PBS containing 2% Triton X-100 (Sigma).

4.6. Cell migration assays

4.6.1. Radial migration (I)

Radial migration was measured by seeding 50,000 cells into cylinders placed in uncoated 24-well culture plates and allowed to attach overnight. The cylinders were removed and non-adherent cells were washed away and adherent cells were allowed to migrate out from the cell disk for 4 days in the presence or absence of inhibitors at 37°C. The culture medium was collected for zymography and the cells were fixed with 4% (v/v) formaldehyde in PBS containing 5% (w/v) sucrose and stained with 0.1% crystal violet in boric acid (pH 6.0). The amount of migration was measured by densitometric scanning of the area covered by the cells.

4.6.2. Scratch assay (I)

Confluent keratinocyte cultures were seeded onto uncoated coverslips and a scratch “wound” was made with a pipette tip. Cells were then allowed to migrate in the presence or absence of 10 ng/ml TGF-β1 and 1 μM batimastat for 20 hours. The cells were then fixed as described above and cell migration was measured under the microscope by counting the cells moved into the “wounded” space.

4.6.3. Transwell assay (V)

Cell migration was performed in Transwell chambers (Corning Star) as previously described (Koshikawa et al. 2000). Filters were coated with 750 ng/ml Ln-5 or 1 μg/ml type IV collagen overnight at 4°C and thereafter blocked with 5% milk powder. DME including 1% Hepes, penicillin,
streptomycin, glutamine (w/v) and 0.1% BSA (w/v) was added to the lower chamber. MMP-3, -8, -13, -14, or –20 (Table 5) was added to the lower chamber at a final concentration of 10 nM. Cells were resuspended in DME (see above) and 100 µl was seeded to the upper chamber. Cell migration assays were carried out in the presence or absence of 500 nM, 1 µM Batimastat, 100 µM CTT-peptide or 100 µM unrelated control peptide CERGGLETSC. After 16 h of incubation, the cells that had migrated onto the lower surface of the filters were stained with 0.5% crystal violet/20% methanol and counted.

Table 5. MMPs used in the study.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Type and Source</th>
<th>Ratio (enz:sub)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>human gingival fibroblast derived</td>
<td>1:10</td>
<td>(Westerlund et al. 1996)</td>
</tr>
<tr>
<td>MMP-3</td>
<td>human recombinant</td>
<td>1:10</td>
<td>Sigma Chemicals, St.Louis, MO</td>
</tr>
<tr>
<td>MMP-3</td>
<td>synovial fibroblast derived</td>
<td>1:5</td>
<td>(Sorsa et al. 1994)</td>
</tr>
<tr>
<td>MMP-7</td>
<td>human recombinant</td>
<td>1:10</td>
<td>Chemicon International Inc., Temecula, CA</td>
</tr>
<tr>
<td>MMP-8</td>
<td>human recombinant</td>
<td>1:20</td>
<td>Chemicon International Inc., Temecula, CA</td>
</tr>
<tr>
<td>MMP-8</td>
<td>human neutrophil derived</td>
<td>1:10</td>
<td>(Konttinen et al. 1991)</td>
</tr>
<tr>
<td>MMP-12</td>
<td>human recombinant</td>
<td>1:30</td>
<td>Elastin Products Company Inc., Owensville, MO</td>
</tr>
<tr>
<td>MMP-13</td>
<td>human recombinant</td>
<td>1:5</td>
<td>Invitek GmbH, Germany</td>
</tr>
<tr>
<td>MMP-13</td>
<td>synovial fibroblast</td>
<td>1:5</td>
<td>(Lindy et al. 1997)</td>
</tr>
<tr>
<td>MMP-14</td>
<td>human recombinant</td>
<td>1:10</td>
<td>Invitek GmbH, Germany</td>
</tr>
<tr>
<td>MMP-20</td>
<td>human recombinant</td>
<td>1:20</td>
<td>Dr.John Bartlett, Harvard Forsyth Dental Center, Boston, MA</td>
</tr>
</tbody>
</table>

4.7. Collagenase and gelatinase activity assays (III & IV)

For hydroxyproline (a specific iminoacid marker of collagen) analysis, the samples were hydrolysed in 6 M hydrochloride acid (HCl) at 106°C for 24 hours and then assayed by Stegmenn's colorimetric assay. For collagenase activity assay, wound tissues from each group were pooled and extracted in collagenase buffer (pH 7.4) containing 5 M urea and precipitated with 65% ammonium sulphate. Collagenase and gelatinase activities in the pooled extracts were measured as described (Golub et al. 1994) using radiolabeled collagen or gelatin as substrates.

4.8. Zymography (I,IV,V)

Zymography was performed as previously described (Mäkelä et al. 1994, Ryan et al. 1996). A549 serum-free culture media was collected and spun down at 800 rpm to remove cells. The supernatant was concentrated using ammonium sulphate at 80% saturation. The protein pellet was reconstituted in 300 µl of 20 mM Tris-HCl, pH 7.5; 0.1 M NaCl-buffer. Media was dialyzed against the same buffer and prepared for zymography. Wound tissue extracts and culture media were analyzed by separating proteins according to size by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/ml 2-methoxy-2,4-diphenyl-3(2H)-furanone-labeled gelatin (O'Grady et al. 1984) or unlabeled gelatin. The lysis of gelatin was monitored by long-wave UV light or by staining the gel with with 0.1% Serva Blue R (Serva Feinbiochemica, GmbH, Heidelberg, Germany) and subsequent destaining with 20% methanol, 10% acetic acid.
4.9. Western immunoblotting (I-V)

Proteins were characterized by separation with SDS-PAGE under non-reducing or reducing conditions. Conditioned media from the HMK culture was shaked overnight at 4°C with gelatin-sepharose-4B (Pharmacia, Sweden) to bind MMP-9. MMP-9 was released from the pellet by heating the samples at 90°C for 3 min with Laemmlli buffer (Laemmlli 1970). Wound tissue extracts, culture media or protein samples were equally treated with Laemmlli buffer (with or without reducing agents) and the proteins were separated according to size by 8% or 11% SDS-PAGE. The proteins were then electrotransferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) in Blotting-buffer (25 mM Tris-HCl, pH 8.3; 192 mM Glycine, 20% methanol). Non-specific binding was blocked with 5% milk powder (Valio, Finland) or 3% gelatin. The membranes were incubated with primary antibodies (Table 6) overnight and subsequently with secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase. Proteins were detected by incubating the membranes with nitroblue tetrazolium (Sigma, St.Louis, MO) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO), diaminobenzidine tetrahydrochloride solution or by the enhanced chemiluminesence technique (Amersham Pharmacia Biotech Inc, NJ, USA). When the enhanced chemiluminesence technique was used, the membranes were exposed to Hyperfilm-ECL autoradiography films. The intensities of immunoreacted proteins were semiquantitated with a Bio-Rad Model GS-700 Imaging Densitometer using the Molecular Analyst™ program.

Table 6. Antibodies used in the study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>mouse anti-human</td>
<td>1:1000</td>
<td>Chemicon International, Temecula, CA</td>
</tr>
<tr>
<td>MMP-2</td>
<td>rabbit anti-human</td>
<td>1:200</td>
<td>Henning Birkedal-Hansen, University of Alabama</td>
</tr>
<tr>
<td>MMP-3</td>
<td>rabbit anti-human</td>
<td>1:1000</td>
<td>Chemicon International, Temecula, CA</td>
</tr>
<tr>
<td>MMP-9</td>
<td>rabbit anti-human</td>
<td>2 µg/ml</td>
<td>Oncogene Science Inc., Cambridge, MA</td>
</tr>
<tr>
<td>MMP-9</td>
<td>rabbit anti-human</td>
<td>1:300-1500</td>
<td>(Kjeldsen et al. 1992, Westerlund et al. 1996)</td>
</tr>
<tr>
<td>MMP-13</td>
<td>mouse anti-human</td>
<td>1:500</td>
<td>Oncogene Research Products, Cambridge, MA</td>
</tr>
<tr>
<td>MMP-13</td>
<td>rabbit anti-human</td>
<td>1:500</td>
<td>(Freije et al. 1994)</td>
</tr>
<tr>
<td>Type I Collagen</td>
<td>mouse anti-human</td>
<td>1:1500</td>
<td>Sigma, St Louis, MO</td>
</tr>
<tr>
<td>MMP-14</td>
<td>rabbit anti-human</td>
<td>1:100-500</td>
<td>Biogenesis Ltd., Poole, UK</td>
</tr>
<tr>
<td>MMP-20</td>
<td>mouse anti-human</td>
<td>1:1000</td>
<td>(Väänänen et al. 2001)</td>
</tr>
<tr>
<td>CD-45</td>
<td>mouse anti-human</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology Inc., USA</td>
</tr>
<tr>
<td>Laminin-5</td>
<td>rabbit anti-human</td>
<td></td>
<td>GB3 commercial</td>
</tr>
</tbody>
</table>

Antibodies have been verified to crossreact with murine tissue antigens as previously described ¹(Ramamurthy et al. 1999, Ryan et al. 1999b) or by the ²manufacturer. ⁴

4.10. In situ zymography (II)

Untreated frozen tissue sections were thawed and warmed to room temperature. Sections were covered with either in situ zymography (ISZ)-buffer (50 mM Tris-HCl, pH 7.4; 1 mM CalCl₂) alone or with ISZ-buffer containing 0.5 mM APMA. All samples were incubated for 30 min at 37°C after which the samples were covered with ISZ-buffer alone or ISZ-buffer containing one of the following proteinase activity modifiers: 0.5 mM APMA, 0.5 mM Pefabloc (Boehringer Mannheim, GmbH,
Germany), 100 µM CTT-peptide, 100 µM CERGGLETSC-peptide, 100 µM GACFSIAHECGA-peptide or 10 mM Ethylenediamine-tetraacetic acid (EDTA). Incubation was carried out for 30 min at 37°C after which the buffers were discarded. All samples were covered with a 1:1 mixture of 1% (w/v) low melting temperature agarose (Sigma, St. Louis, MO) in ISZ-buffer and 1 mg/ml Oregon Green-conjugated gelatin (Molecular Probes, Inc., Eugene, OR). Samples were covered with the mixture alone or with the mixture containing one of the following: 0.5 mM PefablocSC Plus, 100 µM CTT-peptide, 100 µM CERGGLETSC-peptide, 100 µM GACFSIAHECGA-peptide or 10 mM EDTA. The mixture was covered with a coverslip and allowed to gel at room temperature and then the samples were incubated in a humid chamber in the dark at 37°C for up to 96 hours. Gelatinolytic activity was evidenced as dark areas in the otherwise uniform fluorescence substrate layer.

4.11. Immunohistochemistry (I-IV)

Tissue sections were thawed or deparaffinized and rehydrated. The Vectastain ABC Elite Kits (Vector Labs, Burlingame, CA) were used for immunostaining as described. HMKs were seeded in KGM medium on uncoated coverslips for 3 days. Cells were fixed in 4% formaldehyde and 5% sucrose in Ca²⁺ and Mg²⁺-containing PBS (PBS+) for 30 min. Sections were pretreated with 0.4% pepsin (paraffin sections only) and endogenous peroxidase activity was blocked by incubation in 0.6% H₂O₂ in methanol. Cultured cells were permeabilized with 0.5% Triton X-100 in PBS for 4 min. Samples were blocked with goat/horse normal serum in 2% bovine serum albumin and incubated with antibodies overnight at +4°C. Following incubation with antibodies, samples were incubated with biotinylated secondary antibody and subsequently with Avidin-Biotin enzyme-complex or a rhodamine-conjugated secondary antibody (Boehringer Mannheim, Indianapolis, USA). Sections were stained using 3-amino-9-ethylcarbazole as chromogen and counterstained with Mayer’s hematoxylin (Merck KGaA, Darmstadt, Germany). Samples processed for dual immunostaining were incubated with a second primary antibody and stained with SG-color (Vector Labs). Controls for immunostaining included omitting antibodies from the staining sequence or incubation with nonrelevant immunoglobulins. The intensity of immunoreactivity was detected by connecting a Kappa CF 15/4-camera to a Leica DMRB microscope and a Kappa MCUII-multicontrol unit from the camera to the computer. The software used for analysis was Leica Qwin.

4.12. In situ hybridization (I-IV)

cDNAs presented in Table 7 were used as templates for in vitro transcription with a riboprobe transcription kit (Boehringer Mannheim GmbH, Germany) according to the manufacturers instructions. The sense and antisense cRNA transcripts were labelled with digoxigenin-11-UTP. In situ hybridization was performed principally as previously described (Tervahartiala et al. 2000). For in situ hybridization, solutions were treated with 0.1% diethylpyrocarbonate. Samples were fixed in 4% paraformaldehyde, proteolyzed in 0.2 M HCl at room temperature and in 0.1-0.5 µg/ml Proteinase K (Finnzymes, Helsinki, Finland) at 37°C. The reaction was stopped by 100 mM glycine (in PBS) and acetylation was done with 0.25%-0.5% acetic anhydride (in 100 mM triethanolamine). Samples were dehydrated in a graded alcohol series prior to prehybridization. Hybridization buffer containing 400-800 ng/ml digoxigenin-labeled antisense or sense probe was applied to the sections and incubated under stringent conditions at 55-58°C overnight. The hybridization buffer contained 10 mM dithiothreitol, 250 µg/ml yeast total-RNA (Boehringer Mannheim GmbH), 250 µg/ml salmon sperm DNA (Sigma), 50% deionized formamide, 4 x SSC, 10 (w/v) dextran sulphate, 2 x Denhardt’s solution (Sigma). Washing was done under stringent conditions as previously described (Tervahartiala et al. 2000). The digoxigenin-label was detected by incubating samples with alkaline phosphatase-conjugated anti-digoxigenin Fₐb-fragments (diluted 1:100) and by using Fast Red tablets as chromogen. The samples were counterstained with Mayer’s hematoxylin (Merck KGaA, Darmstadt, Germany).
Table 7. cDNA used for cRNA probe synthesis for *in situ* hybridization.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Accession number</th>
<th>Fragment</th>
<th>Length (base pairs)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin-5 γ2-chain</td>
<td>NM_005562</td>
<td><em>PstI-EcoRI</em></td>
<td>845</td>
<td>(Kallunki et al. 1992)</td>
</tr>
<tr>
<td>MMP-2</td>
<td>NM_004530</td>
<td><em>SacI-SacI</em></td>
<td>635</td>
<td>(Huhtala et al. 1990)</td>
</tr>
<tr>
<td>MMP-8</td>
<td>NM_002424</td>
<td><em>SphI</em></td>
<td>95</td>
<td>(Hasty et al. 1990)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>NM_004994</td>
<td><em>HindIII-EcoRI</em></td>
<td>574</td>
<td>(Huhtala et al. 1991)</td>
</tr>
<tr>
<td>MMP-13</td>
<td>NM_002427</td>
<td><em>BamHI-HindIII</em></td>
<td>729</td>
<td>(Freije et al. 1994)</td>
</tr>
</tbody>
</table>

Footnote: The probes have been tested by Northern hybridization for specificity to human sequences and by sequencing. The cDNA has been analyzed for homology between human and murine cDNAs.

4.13. MMP cleavage *in vitro* (II & V)

MMPs (Table 5) were incubated with 52 µM β-casein or 0.1-0.4 µg Ln-5 γ2-chain at indicated enzyme:substrate ratios (Table 5) for different time intervals at 37°C. Some enzymes were activated with 0.5 mM APMA prior to incubation with the substrate. Enzyme activity was inhibited with 50-200 µM CTT-peptide, CERGGLETSC-peptide, GACFSIAHECGA-peptide, 10 mM EDTA or 10-50 µM Marimastat. The reactions were stopped by boiling in Laemmli buffer (with or without reducing agent) and the cleavage of β-casein was evidenced by separating fragments according to size with 11% SDS-PAGE, visualized with 0.1% Serva Blue R and destained with 20% methanol, 10% acetic acid. Fragmentation of the 21 kDa β-casein band was regarded as enzyme activity. Cleavage of the Ln-5 γ2-chain molecule was detected by Western immunoblotting using a Ln-5 γ2-chain specific antibody (Table 6).

4.14. N-terminal sequencing (V)

3-4 µg Ln-5 γ2-chain was incubated with MMP-3, -8, -12, -13, -14 and –20 at 37°C in the presence or absence of 1 mM APMA. After incubations, fragments were separated by 8% SDS-PAGE and proteins transferred onto ProBlott™ PVDF membrane (Applied Biosystems, Foster City, CA). The membranes were treated for sequencing as described (Mozdzanowski et al. 1992). The N-terminal protein sequences were determined by a 492 Procise™ protein sequencer (Applied Biosystems).
5. Results

5.1. MMP inhibition and cell growth (I)

None of the LWs nor Batimastat affected the cell number during the 2-day exposure as compared with control cultures. CMT-3 and -8 at a concentration of 20 µM reduced the cell number by about 50% (P<0.001; Student’s paired t-test) as compared with the control cells. At 20 µM, CMT-1 reduced the cell numbers by about 20% (P < 0.001). CMT-5 had no effect on keratinocyte growth. The results were similar in cultures in the absence or presence of TNF-α.

5.2. MMP inhibition and gelatinase production by cells (I)

The main gelatinolytic proteinase secreted by the HMKs was MMP-2 and only minimal amounts of MMP-9 could be detected in the medium of 48 – and 96-hours cultures. MMP-2 production was not markedly influenced by any of the LWs. TNFα-induced MMP-9 synthesis was inhibited dose-dependently by the CMTs at 50 µM, inhibition was 61% for LW-1 and 79% for LW-2. The CMTs inhibited MMP-2 production dose-dependently. The inhibitory effect of CMT-1 was evident only at 20 µM (26%) and 50 µM (64%), CMT-3 and CMT-8 inhibited MMP-2 production already at 10 µM (60-70%). CMT-5 did not have any effects on MMP-2 or MMP-9 secretion. TNFα-induced MMP-9 was inhibited only at the highest concentration (50 µM) of CMT-1, -3 and -8. CMT-3 at 10 and 20 µM increased MMP-9 levels in medium of TNFα-treated keratinocytes. Batimastat had minimal effects on production of the two keratinocyte gelatinases at the concentrations studied (3-300 µM).

5.3. MMP inhibition and cell migration

5.3.1. Radial migration (I)

Addition of TNF-α to the culture medium induced MMP-9 expression by 15-fold while it had no effect on constitutive MMP-2 expression. The migration of TNFα -treated cells was not different from that of the control cells. Similar results were obtained using both oral mucosal and skin keratinocytes. Using the radial migration method, CMT-3 and -8 at concentrations that inhibited MMP-2 secretion but either increased or had no effect on MMP-9 had marked inhibitory effect (up to 90%) on HMK cell migration. CMT-1, inhibiting MMP-2 less efficiently, reduced cell migration correspondingly to a lesser extent, even though the compound had about the same effect on cell growth as CMT-3 and -8. CMT-5 had no effect on MMP-2 expression and also no effect on cell migration. Batimastat inhibited migration by about 60% at 1 µM. LW-1 and -2 had little or no effect on MMP-2 production and also had negligible effect on cell migration. The migration experiments were repeated using NHKs and the results were very similar to those with the HMKs.

5.3.2. Scratch assay (I)

Inhibitors were also tested on cell migration using the “scratch” wound healing in vitro assay with confluent keratinocytes. These cultures were exposed to TGF-β for 20 hours in the presence or absence of 1 µM Batimastat. TGF-β increased secreted MMP-2 levels by twofold. In 2% Triton-X 100 extracts of the cells that represent both the cell membrane-associated and cytoplasmic enzyme pools, MMP-2 levels were also increased by about twofold. In addition to proforms of the enzyme, active forms of MMP-2 representing about 30% of the total secreted enzyme could be detected in the culture medium of TGFβ-treated keratinocytes. TGF-β increased medium MMP-9 levels by fivefold while its activity could not be detected in the cell extracts. Eight times more cells migrated in the TGFβ-treated cultures, while in control cultures only a few cells migrated into the wounded space. Batimastat fully inhibited the TGFβ-induced epithelial cell migration.
5.3.3. Transwell assay (V)

Batimastat effectively inhibited the migration of MCF-7 cells over Ln-5 substrate. 100 µM CTT-peptide effectively reduced A549 cell migration over Ln-5 and type IV collagen, without fully abolishing it. Migration of A549 cells incubated with 100 µM non-relevant CERGGLETSC-peptide was comparable to migration of PBS-treated A549 cells. A549 cells plated on filters lacking Ln-5 did not show any migration. The A549 lung carcinoma cells were found to endogenously produce MMP-2, -3, -8, -13, -14 and -20 as detected by Western immunoblot.

5.4. Immunolocalization of MMP-2, MMP-9, Ln-5 γ2-chain and CD45-positive cells in inflamed human gingival tissue (II)

Intense MMP-9 immunoreactivity was detected in clearly defined areas of the inflamed human gingival connective tissue, directly beneath the SE. Extracellular MMP-9 immunoreactivity could occasionally be detected within the SE of inflamed human gingiva. In the deeper layers of inflamed gingival connective tissue associated with less infiltration of inflammatory cells, only sporadically distributed, small patches of MMP-9 immunoreactivity could be detected. Gingival connective tissue areas with strong MMP-9 immunoreactivity were otherwise predominantly associated with heavy infiltration by inflammatory cells. Immunostaining with CD45 antibody showed that MMP-9 immunoreactivity colocalized with areas infiltrated by CD45-positive cells. Similar to MMP-9 immunoreactivity, CD45-positive cells were confined to the gingival connective tissue area directly beneath the SE and only sporadically distributed CD45 immunoreactive cells could be detected in the deeper layers of the connective tissue. In inflamed human gingival specimens, MMP-2 immunoreactivity was confined to the BM region just beneath the SE in areas where inflammatory cells were abundant. In unaffected areas lacking inflammatory cells, MMP-2 immunoreactivity was hardly detectable. MMP-2 immunoreactivity was also detected in gingival fibroblasts. Ln-5 γ2-chain immunoreactivity was detected in the BM region and occasionally in basal SE cells of the inflamed gingival tissue. MMP-2 and Ln-5 γ2-chain were found to colocalize in the BM area. In unaffected areas of the gingival connective tissue, no CD45, MMP-2 or -9 immunoreactivity could be detected.

5.5. MMP-2 and MMP-9 mRNA expression in inflamed human gingival tissue (I & II)

MMP-2 mRNA expression was clearly induced in severely inflamed human gingival tissue in all four patients examined within the area of inflamed pocket epithelium showing local invasion into the connective tissue stroma. The expression was observed in both basal and suprabasal cells. MMP-2 was less intense in SE of gingival specimens that showed a weaker inflammatory reaction. In epithelium of weakly inflamed gingival epithelium that showed no growth into the subepithelial connective tissue, MMP-2 was weakly induced, predominantly in the suprabasal cells. In addition to being expressed in the SE, MMP-2 mRNA was noted in some fibroblast-like cells. MMP-9 mRNA was confined to inflammatory cells located beneath the SE in inflamed gingival tissue. A few epithelial cells were also found to express MMP-9 mRNA in the inflamed area.

5.6. In situ gelatin zymography (II)

By in situ zymography, gelatinolytic activity was predominantly detected in the connective tissue directly beneath SE in both APMA-activated and native tissue samples of inflamed human gingiva. Negligible gelatinolytic activity was detected in the SE. Gelatinolytic activity increased temporally being readily detectable on day 2 and clearly increasing up to day 3. In samples incubated with 100 µM CTT-peptide the intensity of gelatinolytic activity was clearly reduced or totally abolished in both native and APMA-treated inflamed human gingival tissue. Weak gelatinolytic activity could be detected in clinically healthy gingival tissue and this was completely inhibited by 100 µM CTT-peptide. In the deeper layers of inflamed human gingival tissue, associated with less inflammatory cell infiltration, only sporadically distributed patches of gelatinolytic activity could be detected even after several days of incubation and 100 µM CTT-peptide effectively inhibited all gelatinolytic
activity. Also EDTA abolished all gelatinolytic activity in both native and APMA-treated samples. Samples incubated with 100 µM of the unrelated GACFSIAHECGA- and CERGGLETSC-peptides showed no inhibitory effect on gelatinolytic activity. Only minimal reduction of gelatinolytic activity present in inflamed human gingival tissue specimens incubated with 0.5 mM of the serine proteinase inhibitor Pefabloc was observed.

5.7. Immunolocalization of MMP-2 and Ln-5 in epithelial in vitro wounds (I)

Immunostaining demonstrated that MMP-2 was localized in specific streaks resembling ECM contactlike structures at the ventral surface of the keratinocytes. These structures were present only in the migrating cells which were more numerous in TGFβ3-treated cultures than in control cultures. MMP-9 staining was diffuse, with no specific localization in the migrating or stationary cells. Ln-5 was present under the migrating as well as stationary keratinocytes throughout the cultures.

5.7.1. Controls for in situ hybridization and immunohistochemistry (I-IV)

Incubating samples for immunohistochemistry with nonrelevant IgG or omitting the primary antibody resulted in no specific staining. Incubating samples for in situ hybridization with corresponding sense probes resulted in no specific hybridization signal.

5.8. Effect of estrogen and CMT-8 in rat cutaneous wound healing (III & IV)

Histologic examination of the wounds showed re-epithelialization over the healing dermis in all experimental groups 7 days postwounding. Only a minimal delay in re-epithelialization could be detected (<10%) in some of the untreated OVX animals with a concomitant decrease in the amount of granulation tissue. Newly re-epithelialized wounds from OVX animals showed detachment of epidermis from the underlying dermis in the epidermal-dermal junction. In OVX animals treated with CMT-8, epidermal attachment was almost comparable to Sham-operated rats. Skin samples and sections from all experimental groups were prepared in the same manner, suggesting that the ruptures in the epidermal-dermal junctions were due to physical trauma rather than to mechanical stress during sample preparation.

5.8.1. Wound collagen content (III)

The wound collagen content in the 120 days post OVX rats was significantly reduced by 50% (p < 0.05) compared to Sham and CMT-8 treated Sham as detected by hydroxyproline assay. Treating the rats with CMT-8 or estrogen significantly increased the type I collagen deposition (p < 0.05) in the wound tissue comparable to that of Sham operated animals. Type I collagen immunoreactivity was prominent in the wound bed between the two leading epithelial fronts of the regenerating day 7 wounds in all samples and groups examined. Intense type I collagen immunoreactivity was also seen in endothelial layers. In all groups, type I collagen immunoreactivity was also found in the granulation tissue beneath the newly organized epidermis juxtaposed to the intact dermis. Within the wound area, the intensity of immunoreactivity for type I collagen appeared the same in both Sham and OVX rats. CMT-8 treatment increased type I collagen in OVX rats, however, estrogen had the most profound effect on type I collagen by increasing it within the wound area. Deposition of type I collagen in the re-epithelialized area was clearly less in the OVX group, compared to the CMT-8 treated OVX group.

5.8.2. Collagenase and gelatinase activity (III & IV)

Only APMA-activated samples demonstrated collagenolytic activity from day 7 cutaneous wound extracts. CMT-8 treatment of Sham rats decreased collagenolytic activity in comparison to Sham.
Collagenolytic activity in OVX wound samples was decreased compared to Sham while CMT-8 and estrogen treatment decreased collagenolytic activity further in the day 7 wound extracts. Using radiolabeled gelatinase assay, both total (+APMA) and active gelatinase activity were highest in the Sham group when compared with the other groups in the day 7 wound tissue extracts. OVX reduced gelatinase activity and both Sham-operates treated with CMT-8 and OVX rats demonstrated even lower gelatinase activity. Estrogen-treatment reduced active gelatinase in comparison to OVX rats. Gelatin zymography from wound tissue extracts demonstrated the presence of both pro-and active forms of MMP-9 and proforms of MMP-2. Gelatin zymography showed similar results for gelatinolytic activity as detected by the functional assay although CMT-8 and estrogen treatment of OVX rats more markedly decreased gelatinolytic enzymes in the zymograms. Western immunoblotting for MMP-2 and -9 showed similar results.

5.8.3. Expression of collagenases in rat day 7 cutaneous wounds (III)

5.8.3.1. MMP-8 and MMP-13 protein and mRNA expression

MMP-8 immunoreactivity was localized to the suprabasal layers of the proliferating wound epithelium in all groups examined. In Sham and CMT-8 treated OVX day 7 wounds, MMP-8 immunoreactivity was also detected in inflammatory cells, including PMN neutrophils within the granulation tissue beneath the proliferating wound epithelium and near the intact dermis. MMP-8 mRNA was expressed by cells in the proliferating and migratory wound epithelia, in dermal fibroblasts and inflammatory cells in both Sham and OVX rats. Less intense expression was detected in the wound epithelium of CMT-8 treated rats. MMP-13 mRNA was confined to basal cells in the migratory and proliferative epithelium and decreased progressively away from the wound edge, being absent in the intact epidermis. Expression intensity was similar in all groups examined. However, MMP-13 mRNA expressing cells appeared more numerous in estrogen-treated OVX and in these wounds, MMP-13 mRNA was also detected in monocyte/macrophage-like cells. MMP-13 protein within the wound area was confined to dermal fibroblasts, inflammatory and pericyte-like cells in the granulation tissue in front or directly beneath the leading edge epithelial front. The immunohistochemical staining was very similar within all groups, except in the OVX wounds, where MMP-13 was almost undetectable. In Sham and CMT-8 treated OVX-rats, MMP-13-positive inflammatory cells had an appearance resembling either plasma cells or PMN-type cells, while other cells were more pericyte-like. In the re-epithelialized area of day 7 wounds, MMP-13 was detected in fibroblast-and pericyte-like cells as well as in inflammatory cells within the granulation tissue in both OVX and CMT-8 treated OVX rats. MMP-13 immunoreactive cells appeared more numerous in the CMT-8 treated OVX group compared to OVX.

5.8.3.2. Western immunoblotting of MMP-8 and MMP-13 (III)

Pro-and active forms of MMP-8 in skin wound extracts were reduced in both CMT-8 treated Sham and OVX rats when compared to Sham. MMP-8 pro-and active forms were further reduced in estrogen-and CMT-8 treated OVX wounds when compared to both OVX and Sham ones. Active and complexed forms but negligible levels of proMMP-13 were detected in skin wound extracts from all groups examined. Active forms of MMP-13 were reduced in both CMT-8 treated Sham and OVX rats when compared to Sham ones.

5.8.4. Expression of gelatinases and MMP-14 in rat day 7 cutaneous wounds (IV)

5.8.4.1. MMP-2

Immunoreactive MMP-2 protein expression was prominent in both wound keratinocytes of the regenerating epidermal edge and in the intact epidermis within all experimental groups. MMP-2 immunoreactivity was also present in fibroblasts, endothelial cells as well as in inflammatory cells
in the deeper layers of the granulation tissue. By double immunostaining MMP-2 was found to colocalize with immunoreactive MMP-8 protein in keratinocytes of the leading epidermal edge. MMP-2 mRNA was confined to basal cells of the regenerating epidermal edge and decreased progressively away from the wound edge, being absent in the intact epidermis in all groups examined.

5.8.4.2. MMP-9

MMP-9 protein expression was mainly localized to inflammatory cells and fibroblasts within the wound stroma. Weak MMP-9 protein expression was detected in basal keratinocytes within the leading edge of the regenerating epidermis. Most prominent expression was detected in Sham rats, where immunoreactive MMP-9 protein was confined to fibroblasts and inflammatory cells located in the wound bed in front of the leading epidermal edge and in the subepithelial layer juxtaposed to the intact dermis. No clear differences in intensity or number of cells expressing MMP-2 or MMP-9 expression could be detected by histological techniques between the different treatment groups.

5.8.4.3. MMP-14

MMP-14 protein expression was detected in a few keratinocytes within the leading edge of the regenerating epidermis in all experimental groups. Additionally, immunoreactivity for MMP-14 was sporadically located to the BM region extending to a length of approximately three to five basal wound keratinocytes. Immunoreactivity for MMP-14 was very prominent and abundant in fibroblasts and occasional inflammatory cells located in the granulation tissue. Most prominent immunoreactivity for MMP-14 was detected in the subepithelial layer directly beneath the BM. When the wound area was studied, no differences in MMP-14 immunoreactivity between the different experimental groups could be detected which was in line with Western immunoblot results for MMP-14. In the re-epithelialized area, MMP-14 immunoreactivity was most prominent in the area of epidermal-dermal splits present in untreated OVX wounds. In contrast, both MMP-14 protein and epidermal-dermal splits were clearly reduced in the CMT-8 treated rats.

5.8.5. Basement membrane

5.8.5.1. Ln-5 γ2-chain expression (IV)

By in situ hybridization, Ln-5 γ2-chain mRNA expression was localized to keratinocytes in the leading edge of regenerating wound epidermis in day 7 wounds with less intense expression in OVX rats as compared to Sham. Ln-5 γ2-chain immunoreactivity was localized to the cytoplasm of basal wound keratinocytes and to the BM area in all experimental groups. The distribution of Ln-5 γ2-chain immunoreactive protein in the BM area of OVX rats was clearly more diffuse and discontinuous when compared to Sham. In wounds from CMT-8 or estrogen-treated animals, Ln-5 γ2-chain immunoreactivity appeared more prominent compared with both Sham and OVX rats. When the newly re-epithelialized area in day 7 wounds was studied, Ln-5 γ2-chain immunoreactivity was clearly more prominent in appearance within the BM area of CMT-8 treated rats when compared to OVX rats. Detachment of the epidermis colocalized with diffuse and discontinuous expression of Ln-5 γ2-chain immunoreactivity in OVX rat wounds. Ln-5 γ2-chain immunoreactivity appeared to be present on both the epidermal and the dermal side of the split, although most abundantly on the epidermal side. Semi-quantitative data on Ln-5 γ2-chain immunoreactive protein intensity in the BM area and the basal keratinocytes showed that OVX rats had reduced Ln-5 γ2-chain total immunoreactivity when compared to Sham rats. CMT-8 or estrogen-treated OVX rats exhibited higher levels of the Ln-5 γ2-chain immunoreactivity in comparison to both Sham and OVX rats.
5.8.5.2. Ln-5 γ2-chain processing (IV)

In tissue extracts from day 7 wounds, weak immunoreactivity for the unprocessed 135 kDa Ln-5 γ2-chain form was detected. Immunoreactivity for the processed, mature 100 kDa form of the Ln-5 γ2-chain was detected in both Sham and CMT-8 treated Sham, but was reduced in OVX rats and treating OVX rats with CMT-8 or estrogen further decreased the immunoreactivity. The 80 kDa Ln-5 γ2x-chain was readily detected in Sham as well as CMT-8 or estrogen-treated OVX rats, but was almost absent in CMT-8 treated Sham and totally absent in OVX.

5.9. Ln-5 γ2-chain and β-casein processing by MMPs *in vitro* (II & V)

Human recombinant autoactivated MMP-2 proteolytically processed the Ln-5 γ2-chain *in vitro* from the 100 kDa form to the 80 kDa Ln-5 γ2x-chain. 100 µM of CTT-peptide fully inhibited this conversion. The efficacy of CTT-peptide inhibition was also confirmed by autoactivated MMP-2 and APMA-activated MMP-9 cleavage of β-casein *in vitro*. The CTT-peptide dose-dependently inhibited the cleavage of the 21 kDa β-casein molecule by both gelatinases with an apparent IC50 of 25 µM. The unrelated GACFSIAHECGA or CERGGLETSC-peptides did not have any effect on MMP-2 and –9 activity.

The Ln-5 γ2-chain was cleaved by MMP-3, -8, -12, -13, -14 and -20 at indicated enzyme to substrate ratios (Table 5) as detected by Western immunoblotting with specific anti-Ln-5 γ2-chain antibody (Table 6). MMP-7 did not cleave the Ln-5 γ2-chain. Without MMPs, Ln-5 γ2-chain was detected as the 135 kDa and 100 kDa forms. MMPs increased the Ln-5 γ2-chain processing dose-dependently. Within 1 hour, MMP-20 processed the 140 kDa and 100 kDa forms of the Ln-5 γ2-chain into 80 kDa and 66 kDa forms. After 48 h of incubation with MMP-20, Ln-5 γ2-chain was fully converted into a 66 kDa form as well as to low molecular weight 30-40 kDa forms. Both collagenases MMP-8 and MMP-13 generated an 80 kDa Ln-5 γ2x-fragment within 1 h of incubation and after 3 h the 66 kDa form was clearly detectable. After 24 h of incubation, the 140 kDa and 100 kDa forms of the Ln-5 γ2-chain were almost fully processed into the 80 and 66 kDa forms and the 80 kDa Ln-5 γ2x-chain was continuously further processed to the 66 kDa form. MMP-13 further generated 20-40 kDa low molecular weight Ln-5 γ2-fragments. MMP-2, -3, -12 and –14 processed the Ln-5 γ2-chain solely into an 80 kDa form.

Ln-5 γ2-chain was incubated with MMP-8, -13 and -20 in the presence or absence of 50 µM Marimastat which fully inhibited MMP processing of the Ln-5 γ2-chain.

5.9.1. N-terminal sequences of MMP cleaved Ln-5 γ2-chain fragments (V)

The N-termini of MMP-3, -12, -13 and –20 processed 80 kDa Ln-5 γ2x-fragment were LTSCPACYNQ and the cleavage occurred between Ala586 and Leu587 (Table 8). The 80 kDa Ln-5 γ2x-fragment produced by MMP-8 was one amino acid longer than the 80 kDa form produced by MMP-3, -12, -13 or –20 being TSCPACYNQV and the cleavage occurred between Leu587 and Thr588. The 100 kDa forms of Ln-5 γ2-chain were sequenced after MMP-12 or -14 treatment and the N-terminal sequences of both these polypeptides were almost identical, DENPDIE(A/E)(S/A)D, the cleavage site in both being Gly513-Asp515. A schematic diagram of MMP-mediated cleavages of the Ln-5 γ2-chain is presented in Figure 9.

<table>
<thead>
<tr>
<th>MMP</th>
<th>Size (kDa)</th>
<th>N-terminus</th>
<th>Cleavage site in the Ln-5 γ2-chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-3</td>
<td>80</td>
<td>LTSCPACYNQ</td>
<td>Ala&lt;sup&gt;586&lt;/sup&gt; – Leu&lt;sup&gt;587&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-8</td>
<td>80</td>
<td>TSCPACYNQV</td>
<td>Leu&lt;sup&gt;587&lt;/sup&gt; – Thr&lt;sup&gt;588&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-12</td>
<td>80</td>
<td>LTSCPACYNQ</td>
<td>Ala&lt;sup&gt;586&lt;/sup&gt; – Leu&lt;sup&gt;587&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>DENPDIEASD</td>
<td>Gly&lt;sup&gt;413&lt;/sup&gt; – Asp&lt;sup&gt;414&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-13</td>
<td>80</td>
<td>LTSCPACYNQ</td>
<td>Ala&lt;sup&gt;586&lt;/sup&gt; – Leu&lt;sup&gt;587&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-14</td>
<td>100</td>
<td>DENPDIEEAD</td>
<td>Gly&lt;sup&gt;413&lt;/sup&gt; – Asp&lt;sup&gt;414&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-20</td>
<td>80</td>
<td>LTSCPACYNQ</td>
<td>Ala&lt;sup&gt;586&lt;/sup&gt; – Leu&lt;sup&gt;587&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The amino acid cysteine (C) was not recognized by automatic microsequencing and was deduced from cDNA.

5.9.2. Cell migration over MMP cleaved Ln-5

Added MMP-3, -13, -14 or -20 at final concentrations of 10 nM significantly induced migration of MCF-7 breast carcinoma cells over Ln-5 substrate. Of the five MMPs tested, MMP-14 was most effective in inducing migration of MCF cells over Ln-5 substrate and also MMP-3, -13 and -20 clearly induced migration of MCF-7 cells in relation to PBS-treated controls, while MMP-8 did not. MCF-7 cells do not synthesize any MMPs in detectable amounts (Giambernardi et al. 1998). MCF-7 cells plated on filters lacking Ln-5 did not show any migration.

Figure 9. The structure of the heterotrimeric Ln-5 molecule. The three chains (α3, β3 and γ2) are connected by disulphide bonding. MMP cleavage sites in the Ln-5 γ2-chain are indicated with arrows. Arabic letters represent the different domains within the laminin chains.
6. Discussion

6.1. MMP inhibition and HMK cell proliferation

MMP inhibitors can have multiple effects on cells, affecting MMP expression, secretion, activation and catalytic competence as well as cell proliferation and migration. HMK cell growth was reduced most by CMT-3 and -8 and less by CMT-1 while CMT-5 did not have any effect. These results are in line with a study with human and rat prostate cancer cell lines, where all CMTs (except CMT-5) and doxycycline were found cytotoxic (Lokeshwar et al. 2002). Batimastat or the LWs on the other hand, did not show any cell proliferation inhibition effects. Batimastat is designed to chelate the Zn$^{2+}$-binding groups in MMPs and thus may not interfere with other molecules crucial for cell viability. CMTs bind Ca$^{2+}$ and are thus likely to affect intracellular Ca$^{2+}$-levels resulting in cytotoxicity also independent of MMP inhibition. In this aspect, CMTs have been shown to interfere with Ca$^{2+}$-receptors in bone and interfere with the osteoclast function (Greenwald et al. 1998). Cell proliferative inhibition was independent of TNF-$\alpha$ treatment indicating that the CMTs would act more direct on cellular mitogenic mechanisms.

6.2. MMP and Ln-5 $\gamma$2-chain expression in HMKs and inflamed gingival tissue

MMP-2 was the primary gelatinase secreted by the HMKs while MMP-9, but not MMP-2 synthesis was induced by TNF-$\alpha$ in line with previous results (Mäkelä et al. 1998, Salo et al. 1994). MMP-2 was instead induced by TGF-$\beta$ treatment and more importantly, MMP-2 was activated. MMP-9 production was increased upon TGF-$\beta$ treatment but activation did not increase. Both MMP-2 and MMP-9 expression have been previously found to increase upon TGF-$\beta$ treatment of oral mucosal keratinocytes (Salo et al. 1994). MMP-2 expression by the cultured HMKs could be confirmed in vivo in human gingival tissue epithelial cells by both immunohistochemistry and in situ hybridization. MMP-2 expression was most dramatic in inflamed human gingival samples. This indicates that while oral mucosal keratinocytes may express baseline levels of MMP-2, expression is induced upon stimulus such as inflammatory cytokines and in response to LPS, which is a product of periopathogenic bacteria in gingival inflammation (Van den Steen et al. 2002).

Interestingly, it has been shown that periodontal pathogens such as *Porphyromonas Gingivalis* inhibit migration of HMKs on Ln-5 through proteolysis of FC proteins. This may impair keratinocyte motility and subsequently the wound healing process associated with periodontitis (Hintermann et al. 2002). In addition, when cultured human gingival fibroblasts were incubated with periopathogenic bacteria this resulted in an increase in MMP-2 expression (Dahan et al. 2001) and MMP-2 has been found to dampen inflammation by cleaving monocyte chemoattractant protein-3 (McQuibban et al. 2000). Thus, MMP-2 could be involved in host defense during inflammation. Protrusions of SE into the gingival connective tissue is typically associated with periodontal inflammation which could be associated with MMP-2 action and possible cleavage of the Ln-5 $\gamma$2-chain in the BM area subsequently inducing the benign invasive phenotype of the SE cells. We detected MMP-9 mRNA and protein expression mainly in inflammatory cells (CD45-positive cells) of inflamed human gingival tissue with minimal expression in basal SE cells in line with previously published work (Westerlund et al. 1996). Since MMP-9 expression is induced by TNF-$\alpha$ in both oral mucosal and skin keratinocyte cultures, the stromal cells may orchestrate the expression of MMP-9 in different tissues in vivo. In this context, TGF-$\beta$ has been found to induce MMP-9 expression in human skin explants and in the presence of TNF-$\alpha$, proMMP-9 was proteolytically activated. No similar effect was found with isolated fibroblasts or keratinocytes (Han et al. 2001). In comparison, MMP-9 is prominently expressed in migrating skin wound keratinocytes, while MMP-2 is associated with more constitutive expression (Okada et al. 1997). Thus, both gelatinases are most likely subject to tissue specific regulation.

By in situ gelatin zymography, gelatinolytic activity was detected in the stroma beneath the SE in inflamed human periodontal tissue in line with previous reports (Korostoff et al. 2000). By using the MMP-2 and -9 specific inhibitor, the CTT-peptide, we could demonstrate that most gelatinolytic activity in inflamed human periodontal tissue was accounted for by MMP-2 and -9. The CTT-
peptide may thus be very useful in the qualitative analysis of in situ gelatin zymography. In situ zymography also demonstrated that MMP-2 and -9 mediated gelatin degradation in inflamed human periodontal tissue was clearly increased when compared to periodontal tissue of healthy humans. This is in line with previous reports (Korostoff et al. 2000, Westerlund et al. 1996). Ln-5 γ2-chain was confined to the BM region of inflamed gingival SE, in line with previous reports (Hormia et al. 1998, Uitto et al. 1998). Intracellular Ln-5 and MMP-13 expression in inflamed human gingiva has been found to correlate and be co-expressed in cells by immunohistochemistry (Uitto et al. 1998). In our study, we found that MMP-2 and the Ln-5 γ2-chain co-localized in the BM region of inflamed human gingiva. MMP-2 has previously been shown to cleave the Ln-5 γ2-chain (Giannelli et al. 1997) and we show that MMP-13 cleaves the Ln-5 γ2-chain within the same site. While immunohistochemistry showed that MMP-9 appears to be the main gelatinase in the stromal compartment associated with CD-45 positive inflammatory cells, MMP-2 may instead be associated with actions of the gingival keratinocytes.

6.3. MMP inhibition in HMKs and inflamed periodontal tissue

Batimastat and CMTs are effective inhibitors of MMP-2 and -9 activity in vitro (Coussens et al. 2002, Hidalgo and Eckhardt 2001, Rasmussen and McCann 1997, Rifkin et al. 1993). Batimastat did not affect synthesis of the two gelatinases further confirming that Batimastat mostly acts on MMP activity, not transcription. MMP-2 production was reduced by the CMTs, CMT-3 and –8 being most effective, while TNFα-induced MMP-9 synthesis increased after addition of 10-20 µM CMT-3. Previous studies with both melanoma and prostate cancer cell lines demonstrated effective downregulation of both MMP-2 and -9 expression and secretion in culture media (Lokeshwar et al. 2002, Seftor et al. 2002). However, MMP inhibitors have also been found to increase MMP synthesis during acute wound healing (Ågren et al. 2001, Lund et al. 1999). Thus, non-tumorigenic cells most likely respond differentially to MMPIs than aggressive tumor cells or cells associated with chronic inflammation. In addition, intracellular Ca2+-levels affect MMP expression differentially through secondary messengers, which is probably cell type specific. TC can prevent the proteolytic cleavage of membrane-bound proTNF-α into its active, soluble form in human LPS-treated monocytes without affecting TNF-α synthesis (Ramamurthy et al. 2002). This indicates that TC-based MMP inhibition affect not only MMPs but also cytokines involved in MMP induction and repression cascades.

Our results are the first to show that the CTT-peptide effectively inhibits in vivo gelatinolytic activity associated with chronic inflammation. The CTT-peptide has previously been shown to inhibit tumor cell migration, MMP-2 and -9 activity in vitro and angiogenesis (Koivunen et al. 1999). LDD (Periostat) has been successfully used in the treatment of periodontal disease in the USA and inhibits both collagenases and gelatinases effectively (Golub et al. 1998). Also the CMTs are potent inhibitors of tissue destruction and collagenase activity in experimental periodontitis (Ramamurthy et al. 2002, Yu et al. 1993). Taking that the CTT-peptide inhibits MMP-2 and -9 with IC50 of 10 µM, it may well be of potential use in the future treatment of chronic inflammation. In addition, both doxycycline and CMTs are cytotoxic, whereas the CTT-peptide is not. Batimastat is not cytotoxic, however, this broad-spectrum MMPi causes unwanted side-effects. Although many chronic inflammatory diseases have been associated with elevated MMP activity, there are still problems analyzing the efficacy of MMP inhibition in vivo. These problems include sampling relevant tissues and biologic fluids (e.g. rheumatoid synovium and the adjacent synovial fluid) to demonstrate MMP inhibition and subsequent ECM protection at the lesion site. In this regard, periodontal disease offers the advantages of accessible tissue and adjacent easily-sampled non-invasive biologic fluids; i.e. GCF, mouthrinse or saliva and well documented clinical parameters of disease severity (Golub et al. 1997, Mäntylä et al. 2003). This may, at least in part, explain why LDD is currently the only MMP-inhibition based drug on the market, being targeted for adjunctive treatment for periodontal disease, acne and roseacea.
6.4. Cutaneous wound healing

Wound healing and estrogen-deficiency results in many of the features associated with chronic inflammation. In our study, we did not find any differences in re-epithelialization rates in contrast to some previous studies (Ashcroft et al. 1997a, 1997b, 1999). However, in these studies, animals only 18 days post-OVX were used. In the animal model for estrogen-deficiency used herein, rats were kept ovariectomized 120 days before wounding. It has been shown that in women, chronological age is not the major factor influencing the impact of estrogen-deficiency in skin, rather time after menopause (Brincat et al. 1985, 1987a, 1987b). Women are not truly hypoestrogenic until 2 years post menopause. Many of the animal models for estrogen-deprivation present contradictory results. This is due to the use of different species and gender of animals, various types of administered hormones, routes of administration, dosage and frequencies of the hormone, duration of estrogen-deprivation, diversity of methods assessing wound healing and a wide variety of tissues and cell culture in which the healing process is studied (Calvin 2000). To use an animal model for investigating estrogen-deficiency and therapies for clinical use on human, eventually this field needs a more standardized animal model.

In our study, wound collagen content was significantly decreased in OVX animals and treatment with CMT-8 or estrogen significantly increased wound tissue collagen content. These results are in line with previous human studies (Ashcroft et al. 1997a, 1997b, 1999). A decrease in wound collagen content in OVX animals could imply aberrant collagen turnover as seen in diabetic rats and concomitantly reduced wound strength and quality. Estrogen has been shown to dampen the inflammatory response and neutrophil chemotaxis thus leading to decreased collagen degradation (Ashcroft et al. 1999). Our study is the first to demonstrate the effect of CMTs on cutaneous wound healing associated with estrogen-deprivation. CMT-1 has previously been shown to inhibit body weight loss associated with a reduction in skin collagen in diabetic rats without affecting hyperglycemia. CMT-1 stimulated collagen protein synthesis in diabetic animals through increased steady-state levels of pro α1(I) mRNA collagen, however, CMT-1 did not increase mRNA levels in non-diabetic rats (Craig et al. 1998, Yu et al. 1993).

The wounds from the OVX animals showed one thing in common, abnormal attachment of the epidermis to the dermis. This was clearly seen in the areas where re-epithelialization was complete. Junctional detachment of the epidermis from the dermis is associated with several blistering diseases, where BM molecules such as Ln-5 are targeted or depleted. Wounds from estrogen or CMT-8 treated animals showed a similar epidermal-dermal junction as seen in Sham operates. In this context, anti-inflammatory properties of TCs have been useful in the treatment of specific cutaneous diseases, including EB and bullous pemphigoid (Loo et al. 2001). Immunostaining for Ln-5 γ2-chain in the wounds demonstrated a more discontinuous staining and less Ln-5 γ2-chain mRNA in the BM area in OVX rats when compared to the other treatment groups. In this regard, skin BM type IV collagen decreases with age (Vazquez et al. 1996). Thus, while estrogen-deficiency eventually leads to wound closure, delayed wound healing may result from either reduced attachment of the newly formed epidermis to the underlying dermis decreasing wound strength and/or aberrant collagen turnover resulting in delayed granulation tissue turnover and concomitant wound contraction. In line with this, wound contraction in rats 4 months post OVX was delayed as compared to Sham operates (Calvin et al. 1998). Overall, macroscopic wound healing was enhanced in treated OVX animals in comparison to untreated OVX animals.

One interesting observation in our study was the MMP-8 mRNA expression in the wound keratinocytes. MMP-8 has previously only been found in dermal fibroblasts and infiltrating neutrophils (Fisher et al. 2001, Hanemaaijer et al. 1997). MMP-8 expression has been detected in keratinocytes by RT-PCR (Bachmeier et al. 2000b), however, no study thus far has presented with any in situ hybridization data from wound tissues. In other studies, MMP-8 mRNA expression has been found in plasma cells of oral cysts (Wahlgren et al. 2001), bronchial epithelial cells (Prik et al. 2001), oral SCC cells (Moilanen et al. 2002) and gingival SE cells (Tervahartila et al. 2000). However, we could not detect MMP-8 protein other than in a few scattered keratinocytes of the suprabasal epithelial layer in the wound. It thus appears that studies investigating the transcriptional control and especially the stability of the MMP-8 mRNA transcript would be
necessary. Indeed, also other investigators have noted that MMP RNA and protein profiles correlate poorly during murine wound healing which has been attributed to differences in stability and degradation rates between mRNA and protein (Wall et al. 2002).

In our study, MMP-8 was also immunohistochemically detected within inflammatory cells in the granulation tissue. Overall, these results point to a possibility that MMP-8 could participate in collagen turnover and clearance during murine wound healing. We found active forms of MMP-8 in wound tissue extracts from all experimental groups. In humans, MMP-8 levels have been found to be severalfold higher than MMP-1 in wound fluids from both acute and chronic wounds. MMP-8 is also significantly elevated in chronic wound fluids when compared to acute wound fluids (Nwomeh et al. 1999, Nwomeh et al. 1998a). MMP-8 has also been found to cleave tissue factor pathway inhibitor involved in regulating coagulation cascades upon vascular injury (Cunningham et al. 2002). Increased MMP-8 activity may thus also lead to coagulation disorders in chronic wounds. In this regard, we found that CMT-8 decreased MMP-8 mRNA expression in the wound keratinocytes when compared to OVX. Also Western immunoblotting demonstrated that CMT-8 and estrogen-treatment reduced both pro-and active forms of MMP-8. Recently, MMP-8 expression was found to be under hormonal control. Male MMP-8 knock-out mice was more susceptible to cancer than female mice (Balbin et al. 2003).

If ignoring the fact that rodents possess two MMP-1 genes and old-fashionably referring to MMP-13 as the murine orthologue for human MMP-1, our results are in line with previous studies (Lund et al. 1999, Madlener 1998, Okada et al. 1997). MMP-13 has been suggested to be the main collagenase responsible for collagen degradation in murine keratinocytes similar to MMP-1 in humans (Netzel-Arnett et al. 2002). We also found active forms of MMP-13 in wound tissue extracts evidencing that MMP-13 participate in matrix turnover during murine wound healing. MMP-9 protein was only detected at low levels and mostly in inflammatory cells in day 7 wounds. This may be due to the fact that others have reported MMP-9 expression during acute murine wound healing to be maximal at day 5 and thereafter it ceases rapidly (Lund et al. 1999, Madlener 1998, Okada et al. 1997).

Interestingly, MMP-2 mRNA and protein was detected in the leading edge keratinocytes within all groups in addition to fibroblasts. MMP-2 mRNA expression has been mostly reported to be confined to dermal fibroblasts (Lund et al. 1999, Madlener 1998, Oikarinen et al. 1993, Okada et al. 1997, Salo et al. 1994). Some reports exist on MMP-2 protein expression in wound epidermal cells (Oikarinen et al. 1993, Salo et al. 1994), normal skin epidermal cells (Dumas et al. 1999), and cultured skin keratinocytes secreting MMP-2 (Bachmeier et al. 2000a, Kobayashi et al. 1998). However, MMP-2 expression was confined to the basal keratinocytes of intact skin in elderly persons (Ashcroft et al. 1997c). We also detected MMP-2 expression in SE cells of inflamed human periodontal tissue (Giambernardi et al. 1998) and bronchial epithelial cells (Maioli et al. 2002). In addition, in the wound scratch assay presented herein, HMKs expressed mainly MMP-2 and expression was induced after TGF-β treatment.

In the rat wounds, MMP-2 mRNA was expressed with a similar intensity in all groups examined. In a study with diabetic mice and in line with our data on gelatinolytic activity for OVX rats, diabetic mice actually showed lower levels of proMMP-2 in wound tissue extracts during early wound healing stages (Wall et al. 2002). Since our and other studies (Giannelli et al. 1997) demonstrate that MMP-2 is directly involved in inducing cell migration reduced MMP-2 production could be associated with delayed re-epithelialization in certain situations. Estrogen was found to increase MMP-2 production in suction blister fluids from patients receiving systemic estrogen therapy (Haapasaaari et al. 1997).

Abundant MMP-2 expression in fibroblasts is in line with previous studies (Lund et al. 1999, Madlener 1998, Okada et al. 1997). We did, however, also find MMP-14 immunoreactivity associated with a few basal keratinocytes and in the BM region. A recent study demonstrates that primary murine keratinocytes indeed synthesize MMP-14 in line with our findings (Netzel-Arnett et al. 2002). Most intense MMP-14 immunoreactivity was found in wounds from the OVX animals confined to the BM area with epidermal-dermal detachment. MMP-14 is capable of cleaving the BM component Ln-5 γ2-chain and was co-localized with the Ln-5 γ2-chain in the BM area. This may indicate that MMP-14 is associated with degradation of BM molecules in the OVX animals. In
line with this, Ln-5 γ2-chain staining in the BM area correlated well with the junctional epidermo-
dermal split, being weakest in the OVX rats. Western immunoblot data presented interesting results; the 80 kDa Ln-5 γ2x-chain was clearly present in wound tissue extracts from Sham-operates, but was lacking in OVX wound tissue extracts. Interestingly, both CMT-8 and estrogen-treatment resulted in the neoexistence of the Ln-5 γ2x-chain. However, treating Sham-operates with CMT-8 also nearly fully abolished the Ln-5 γ2x-chain. It therefore appears that while treatment of estrogen-deprived wounds was beneficial in terms of Ln-5 γ2-chain processing, treating Sham operates with CMT-8 may not be. Our study is novel in presenting the effect of CMTs and estrogen on Ln-5 γ2-chain expression and processing in cutaneous wound healing. Ln-5 deposition is critical for wound keratinocytes and Ln-5 neosynthesis starts within hours of wounding (Larjava et al. 1993, Nguyen et al. 2000b). Upon re-epithelialization, hemidesmosomal Ln-5 is critical for the integrity of the epidermal-dermal junction and stability of the regenerated skin. Clearly, the impact of estrogen and MMPIs on Ln-5 γ2-chain with focus on wound healing and inflammation deserves further studies in the future.

6.5. Therapeutic aspects of estrogen and MMPIs in cutaneous wound healing

Chronic wound therapy lacks specific treatment, in large part due to the lack of knowledge of the molecular abnormalities within the wound that prevent it from healing (Chen et al. 1999). Acute wound healing is a precisely defined orchestra of spatially and temporally regulated protease activity. The use of an MMPI for the treatment of acute wound healing is not necessary, since it may interfere with the tightly controlled proteolytic events and Ca2+-levels essential for wound healing. Upon damage, the leading edge keratinocytes become transiently leaky to Ca2+ leading to AP-1 activation in cells of the wound margin (Jacinto et al. 2001). AP-1 is involved in the induction of several MMPs and Ln-5 expression and possibly cell migration on Ln-5 (Kährä and Saarialho-Kere 1999, Olsen et al. 2000, Salo et al. 1999). Interference with the intracellular Ca2+-balance with an MMPI may thus be detrimental for acute wound healing. However, chronic wound healing has been associated with increased Ca2+-levels (Sank et al. 1989) which may further add to the benefits of using MMPIs for chronic wound healing. In this regard, CMT-1 was found to normalize collagen-synthesis in diabetic rats without affecting collagen-synthesis in non-diabetic rats (Craig et al. 1998, Yu et al. 1993).

Estrogen has been shown to improve acute wound healing in terms of re-epithelialization and scarring (Ashcroft et al. 1997a). HRT for women past menopause can reverse changes associated with skin fragility and delayed wound healing, however, HRT is also associated with an increased risk for breast cancer. Thus, the use of locally applied estrogen for acute wound treatment and most importantly for treatment of chronic wounds could be beneficial. In a chronic wound, not only MMPs are upregulated, TIMP activity is either unchanged or reduced which leads to uncontrolled MMP activity. At present there are no studies addressing the effect of estrogen on TIMP expression in cutaneous wounds or skin cells. Estrogen do modulate TIMP production in other cell types and CMT-3 decreases TIMP-1 and –2 production in prostate cancer cell lines (Lokeshwar et al. 2002).

The complex pattern of MMP expression and activity in chronic inflammations and wound healing may be the result of an altered balance in matrix deposition and degradation. Impaired ECM turnover or misdirected ECM deposition may result in excessive MMP activity leading to uncontrolled matrix degradation (Stetler-Stevenson 1996). Thus, MMPs may be either upregulated or downregulated in chronic inflammations depending on signals mediated by the ECM. We have shown that the use of an MMPI for the treatment of wounds associated with chronicity can improve wound healing and wound integrity.

6.6. Cell migration and Ln-5 γ2-chain processing

The Ln-5 γ2-chain was processed by MMP-3, -8, -12, -13 and -20 in addition to the previously described MMP-2 and -14 (Giannelli et al. 1997, Koshikawa et al. 2000). Cleavage of the Ln-5 γ2-chain resulted in the formation of the 80 kDa Ln-5 γ2x-chain. N-terminal sequencing of the 80 kDa
Ln-5 γ2x-chain revealed that the cleavage site for MMP-3, -12, -13, and -20 was identical to the previously described cleavage site for MMP-2 (Giannelli et al. 1997). However, the cleavage site for MMP-8 in the Ln-5 γ2-chain differed by one amino acid. The cleavage by MMP-12 and -14 resulting in processing of the 135 kDa Ln-5 γ2-chain to the 100 kDa form was identical to the previously described physiological cleavage site (Amano et al. 2000, Gagnoux-Palacios et al. 2001, Schenk et al. 2003, Veitch et al. 2002) and this cleavage site is conserved within humans and rodents (Sasaki et al. 2001). MMP-14 cleaves the human Ln-5 γ2-chain in vitro generating fragments of roughly 90 kDa and 70 kDa (Gilles et al. 2001). These results contrast with another study where MMP-2 and -14 did not cleave the human Ln-5 γ2-chain (Veitch et al. 2002). However, in the latter study, immunoprecipitated Ln-5 was used as a substrate in contrast to detecting the native Ln-5 γ2-chain in humans and rodents is not conserved, the cleavage sites contain amino acids of the same polarity in both the human and rodent Ln-5 γ2-chain (personal communication with Dr. Vito Quaranta). Human Ln-5 γ2-chain is also more resistant to MMP proteolysis, another reason why cleaved Ln-5 γ2-chain is more seldom detected in media from cultured human cells or tissues (personal communication with Dr. Naohiko Koshikawa). In addition, MMPs show divergence in the cleavage sites within various substrates (Netzel-Arnett et al. 1993). Thus, it appears that conformational restrictions within biological molecules determine the access of MMPs to cleave a certain molecule.

Interestingly, while MMP-3, -13, -14 and -20 induced MCF-7 cell migration, MMP-8 did not. In one study, the expression of MMP-8 was associated with a non-invasive phenotype of breast carcinoma cells while perturbing MMP-8 expression led to a more invasive phenotype (Agarwal et al. 2003). Further studies will confirm how critical the one amino acid difference in reality is. MCF-7 cells express negligible levels of MMPs (Giambernardi et al. 1998) and so MCF-7 cell migration is only induced after exogenous addition of MMPs. However, it has been shown that constitutive HT1080 and BRL cell migration on Ln-5 is mediated by endogenously produced MMP-2 and -14 cleavage of the Ln-5 γ2-chain (Koshikawa et al. 2000). In addition, A549 lung carcinoma cells used in this study migrated on Ln-5 and type IV collagen and were found to secrete MMP-2, -3, -13, -14 and -20 all of which also efficiently induced MCF-7 cell migration on Ln-5.

The domainIII EGF-like fragment released by MMP-2 cleavage of the Ln-5 γ2-chain from 100 kDa to 80 kDa exhibits promigratory properties, binds to the cell surface EGF receptor and increases MMP-2 expression (Schenk et al. 2003). In line with this, also selective EGF-like repeats in the Tenascin C molecule can bind and activate the EGF receptor (Swindle et al. 2001). The Ln-5 γ2-chain domainIII/EGF-like fragment could thus potentially act in a paracrine manner binding to many neighbouring cells and induce migration of a larger cell cluster which can be seen in certain types of cancer. Highly invasive melanoma cells for example are typically migrating as larger clusters and produce MMP-2 and the Ln-5 γ2x-chain is present in the culture media (Seftor et al. 2002).

Why are so many MMPs processing the Ln-5 γ2-chain? It makes sense in away. MMP expression is often spatially and temporally restricted. For any mechanism to be used in the whole organism, several pathways must exist. One excellent example is the co-localization of MMP-20 and Ln-5 in ameloblasts of the developing tooth (Bartlett et al. 1998). Ln-5 is also the major component of the internal BM facing the tooth enamel surface in adult tooth (Hormia et al. 1998). Patients with junctional EB have hypoplastic defects in their enamel, which may be caused by detachment of the ameloblast layer from the enamel surface (Kainulainen et al. 1998). We and others have also demonstrated co-localization of MMP-2 and MMP-13 with Ln-5 γ2-chain in inflamed periodontal tissues (Uitto et al. 1998) and MMP-14 and Ln-5 γ2-chain in the BM area within cutaneous wounds. Our study with HMKs demonstrated that TGF-β-induced HMK cell migration was dependent on MMP-2 expression, secretion and activity along with Ln-5 deposition, in line with previous studies (Kainulainen et al. 1998). In summary, all the MMPs that have been demonstrated here to cleave the Ln-5 γ2-chain are present during remodelling processes and wound healing as well as in cancers (see Review of the literature).

Careful interpretations should always be made when comparing results between cell lines, which differ in tumorigenicity, integrin expression, MMP expression and the capability of forming HDs.
The Ln-5 γ2x-chain is rarely found in culture media of non-migrating NHKs (Gagnoux-Palacios et al. 2001, Gilles et al. 2001). In a study with nonmigratory HCC cells on Ln-5, MMP-2 activated the latent TGF-β1 secreted by the HCC cells which stimulated α3-integrin expression subsequently modulating cell migration over Ln-5. This emphasizes that cells that do not secrete MMP-2 could potentially become invasive or activated because of MMP-expression by surrounding stromal cells (Giannelli et al. 2001, Giannelli et al. 2002). In contrast, the cleaved 80 kDa Ln-5 γ2x-chain is clearly detected in culture media of highly aggressive melanoma cells correlating with their ability to invade (Seftor et al. 2002). While not many studies have addressed the Ln-5 processing in vivo yet, it is known that in quiescent human tissues, the Ln-5 γ2-chain is mostly present in the 105 kDa processed form and the Ln α3-chain is always present in its processed form (Marinkovich et al. 1992). MMP-2 was found to modulate the processing of the Ln-5 γ2-chain to the Ln-5 γ2x-chain in mammary tissue from sexually mature, pregnant or involuting rats but not in sexually immature rats. When sexually immature rats were injected with estrogen, MMP-2 expression was induced and the Ln-5 γ2-chain processed (Giannelli et al. 1999). In our study, the Ln-5 γ2x-chain was clearly detected in the wound tissue extracts, except for the OVX group. Thus, the further processing of the Ln-5 γ2-chain is apparently associated with remodelling, migration or pathological situations in vivo and should be studied under such conditions.

Normal cell migration differs from tumor cell invasion only in terms of regulation. Any epithelial cell making its way through the connective tissue during normal remodelling uses the same mechanisms as an invading tumor cell. The tumor cell just don’t have a brake anymore. In addition, it makes sure that the brakes can’t be fixed by producing molecules that accelerate its way through the matrix. In normal cells, Ln-5 is assembled intracellulary and secreted as a heterotrimer without continuously ongoing synthesis. In contrast, carcinoma cells synthesize and secrete monomeric Ln-5 γ2-chain, which is detected in the leading edge of tumors (Koshikawa et al. 1999, Olsen et al. 2000). Hepatocyte growth factor and TGF-β1 synergistically upregulate the Ln-5 γ2-chain expression but not Ln α3 or β3 chain expression in cultured carcinoma cells (Olsen et al. 2000). The Ln-5 γ2-chain expressed by tumor cells may not be fully identical in form and/or function to the form synthesized by normal cells. CD44, fibronectin and tenascin are found in tumor tissues in alternatively spliced forms most likely promoting tumor growth and invasion (Stetler-Stevenson 1996). In addition, MT1-MMP and Ln-5 γ2-chain expression in the invasive front of colon carcinomas is upregulated by the nuclear β−catenin/T-cell factor-4 transcription complex (Hlubek et al. 2001, Takahashi et al. 2002). Thus, the Ln-5 γ2-chain appears to be one of those molecules which carcinoma cells may use for accelerating their way through matrix.

However, if carcinoma cells can secrete the monomeric γ2-chain, when and how is this monomer processed? One possibility is that Ln-5 and its processing is associated with FCs during migration. It would make sense in that the migratory phenotype in cells is associated with the formation and disassociation of FCs which would include Ln-5 γ2-chain deposition and processing. If so, Ln-5 would also be perfectly located for focalized proteolytic processing by the MMPs generating a promigratory Ln-5 γ2-fragment and inducing migration. In our study with HMKs, MMP-2 and Ln-5 were detected in specific streaks resembling ECM contactlike structures. It also makes sense due to the observation that Ln-5 is neosynthesized by regenerating epidermal cells residing on a partially intact BM, such as in suction blisters (Kainulainen et al. 1998), where Ln-5 is available and still the epidermal keratinocytes produce and deposit novel Ln-5 for some purpose. Furthermore, tumor cells may have changed their ability to take advantage of the promigratory role of the Ln-5 γ2-fragment and exclusively synthesize the Ln-5 γ2-chain in the FCs of invading cells, excluding the stop signal which may reside in the ability of the cells coming from behind to form stable adhesion complexes, i.e. HDs. Indeed, highly invasive tumor cells do not generally form HDs and Ln-5 γ2-chain synthesis is mostly associated with the invasive front cells in tumors (Hao et al. 1996).

Strikingly, blocking the Ln-5 α3-chain does not block HCC cell adhesion to either uncleaved or MMP-2-cleaved Ln-5 but instead efficiently blocks cell migration on MMP-2 cleaved Ln-5 γ2-chain (Giannelli et al. 2001, Giannelli et al. 1997). In this regard, it makes even more sense that tumor cells would synthesize monomeric γ2-chain, getting rid of one possible control mechanism, i.e. the
α3-chain. In this context, the Ln α3-chain is known to be the rate-limiting factor in Ln-5 assembly and secretion. Thus, the spatially and timely directed processing of the Ln-5 α3-chain and γ2-chain by different proteinases could be one means by which cells control migration. Based on results presented herein and in other studies (Schenk et al. 2003), MMP cleavage of the Ln-5 γ2-chain results in a promigratory capable of inducing migration (A schematic summary is presented in Figure 10).

6.7. MMP inhibition-last aspects

MMPI development for clinical use in treatment of human cancers and chronic inflammation should by no means be buried just because of an initial setback. As shown in this study and by many others (Hidalgo and Eckhardt 2001), MMPIs have potential in both cancer and chronic inflammatory diseases treatment. Many of the MMP-knockouts have demonstrated resistance to several diseases, for example to endotoxin-induced septic shock and to experimental bullous pemphigoid in MMP-9 deficient mice (Van den Steen et al. 2002), which should be enough to attract the pharmacological development of MMPIs. However, to develop effective, target-specific MMPIs, the MMPs being expressed and activated in a certain type of cancer at certain stages or in a particular chronic inflammatory environment must be carefully determined. There are many new, exciting techniques emerging for this purpose (Lopez-Otin and Overall 2002). In addition, we must also determine the true function of MMPs in pathological conditions. MT1-MMP activity was found to reduce tumor cell migration due to the fact that excess MT1-MMP activity degraded the substrate critical for tumor cell migration (Deryugina et al. 2003). However, MT1-MMP activity also enhances tumor cell proliferation in 3D type I collagen gels in comparison to MT1-MMP deficient tumor cells and this can be inhibited by MMPIs (Hotary et al. 2003). Thus, the true meaning of MMP activity in different disease states in vivo must be resolved.

There are several issues that need to be resolved before MMPIs can be considered for more extensive use in humans. A major problem is the time of drug administration; based on both animal models and human clinical trials, an MMPI is most likely to have full effect when administered during the early phases of the disease. In addition, dose-evaluation and modes to evaluate dose-efficacy must be developed. The effect of an MMPI can indeed vary tremendously depending on the time of administration. As known for CMTs, application of the drug during activation of MMPs in vitro drops IC50s dramatically in comparison to adding the drug after activation (Golub et al. 1998). The goal must be the definition of a dose that is suitable for continuous oral administration and results in sustained plasma concentrations exceeding the inhibitory concentrations for MMPs in vitro with tolerable toxicity. Critically, the development of MMPIs lacks endpoint assessments for the efficacy of the compound in modulating the activity of its target MMP in vivo and the relationship between target modulation and clinical response. Many investigators point out the pressing need to develop novel clinical trials which include the use of parameters of tumor progression instead of response rate as the principal endpoint, randomized instead of single-arm phase II trials and randomized discontinuation trials (Hidalgo and Eckhardt 2001). Interestingly, CMT-3 can be monitored by analysis of changes in MMP-9 plasma levels, a measurement that does appear to correlate with efficacy (Hidalgo and Eckhardt 2001). MMPIs could also possibly be analyzed in other tissues, such as circulating blood cells, oral, lung and tear fluids (Holopainen et al. 2003, Mäntylä et al. 2003, Prikk et al. 2001, Sorsa et al. 1999). There is also a pressing need to develop and validate markers of tumor progression, a task that might best be approached by radiologists and imaging scientists (Bremer et al. 2001).
Figure 10. Schematic illustration of a possible role for Ln-5 in inducing cell migration. In a quiescent cell, the Ln-5 molecule (with a processed α3-chain and γ2-chain) is part of the anchoring filaments in the BM hemidesmosomal complex. A signal triggering the cell to move results in neoexpression of Ln-5 (containing an unprocessed α3-chain) and MMP activation. MMPs cleave the Ln-5 γ2-chain generating a cryptic domainIII fragment which binds to the EGF-receptor of the cell (and possibly to neighbouring cells) inducing intracellular signalling. Subsequently, the cell is triggered to form filopodia or invadopodia (in the case of a cancer cell) and migrate. Concomitantly, MMP-2 (and most likely other MMPs) synthesis is induced. The figure is drawn based on results in the present study and from (Koshikawa et al. 2000, Schenk et al. 2003).
Animal models must be more standardized and developed to mimic the human disease more precisely (Coussens et al. 2002). A “Wild West” mentality in the use of animal models will only result in contradictory and confusing results with information not very valuable for the assessment of a human disease. Malignant cells are genetically highly unstable so therapies targeting these cells can result in modifications resulting in resistance to cancer therapy. MMP activity in cancers usually originates from both tumor and stroma and importantly, stromal cells are much less likely to develop drug resistance (McCawley and Matrisian 2000). MMPs and Ln-5 could exhibit potential use in diagnostics as prognostic markers of cancers and chronic inflammatory diseases (Skyldberg et al. 1999, Väisänen et al. 1996). Indeed, one such diagnostic point-of-care test for monitoring MMP-8 in the GCF of chronic periodontal patients has been developed (Mäntylä et al. 2003, Sorsa et al. 1999). Also new, exciting aspects of MMP inhibition have been presented that involve a noncatalytic targeting of MMPs. These include malignant growth arrest by the MMP-2 hemopexin domain generated through autolysis, development of substrate-targeted inhibitors and MMP-activable cytotoxics (Bello et al. 2001, Overall and Lopez-Otin 2002). In the development of MMPIs, one must also emphasize that proteolytic activity may result in anti-angiogenic peptides, activation of anti-tumor molecules and that, in fact, upregulated MMP activity may result in the excess degradation of ECM molecules and subsequent reduction in migration of tumor cells. The same goes for any chronic inflammation of course, and the same problems reside there.

Of the MMPIs used in the studies presented herein, two are being developed for the use in clinical treatment of humans. We used CMT-8, which exhibits very similar MMP-inhibitory efficacy as the CMT-3. Thus, the more lipophilic CMT-3 (Metastat) is currently in clinical trials for treatment for various cancers, one being human immunodeficiency virus-related Kaposi’s sarcoma. This is particularly interesting since human immunodeficiency virus-1 protease inhibitors were found to regress Kaposi’s sarcoma tumor formation and inhibit MMP-2 activation (Sgadari et al. 2002). While most MMPIs are more or less cytostatic, not cytotoxic, CMT-3 exhibits both features, making it an interesting candidate for cancer treatment. In this aspect, an interesting target for cytotoxic MMPIs could be diseases associated with fibrosis. A study with experimental fibrosis in rats demonstrate that doxycycline attenuated neovascularisation, inhibited early fibrous-tissue formation and regression (Lamparter et al. 2002). In addition, since TGF-β1, which is involved in excess scar tissue formation, is activated by MMPs (at least MMP-2), then there certainly should be a pharmacological interest in the development of certain types of MMPIs for fibrotic diseases. The CTT-peptide is also being developed for future clinical trials for the treatment of cancer. However, as expected with any peptide, it can be easily degraded in vivo, restricting its oral availability. Attempts are made to “shield” or envelope the peptide for delivery to its target where it can be liberated. The CTT-peptide has also been used for the delivery of anti-cancer drugs to tumors, based on its ability to home to tumors expressing MMP-2 and MMP-9 (Medina et al. 2001). Thus, the CTT-peptide is not only a potential anti-cancer compound per se due to its gelatinase inhibitory activity, but can also be used as a tool for more targeted delivery of other anti-cancer drugs as well as gene delivery. In this way, the development of more specific MMP-inhibitors may also indirectly have benefits in treatment of cancer patients.

Clearly, the function of MMPs is only now emerging. While little is known about the true in vivo function of MMPs, it is becoming evident that MMPs are not mere matrix degrading enzymes. MMPs participate in virtually all extracellular events, from cleavage and remodelling of large macromolecules, activation and inhibition of growth factors and cytokines, unmasking of cryptic sites within macromolecules involved in cell migration and proliferation, generation of antiangiogenic peptides, tumor suppressing and supporting activities, inflammatory responses and development. With this in mind, the development of future MMPIs poses a true but beneficial challenge.
7. Conclusions

1. Both HMKs and murine skin keratinocytes express MMP-2. TGF-β induced HMK cell migration was dependent on MMP-2 production, activity and Ln-5 deposition while TNF-α induced MMP-9 played a minor role. This indicates a role for MMP-2 in keratinocyte migration during both periodontal and cutaneous wound healing as well as in the host defense in inflammations.

2. This study is novel in presenting in vivo expression of MMP-2 in HMKs. In inflamed gingival tissue, MMP-2 and Ln-5 localizes to the BM region of SE while MMP-9 expression is associated with the inflammatory cells. In vivo gelatinolytic activity was localized to the stroma directly beneath the SE. These results indicate a role for MMP-2 in keratinocyte migration and proliferation during inflammation. Inhibition with the CTT-peptide demonstrates that most gelatinolytic activity in inflamed periodontal tissue in vivo is mediated by MMP-2 and MMP-9. The use of the CTT-peptide improves the specificity and qualitative analysis of in situ gelatin zymography. LDD (Periostat) is currently used for treatment of periodontal disease in the USA. Thus, the CTT-peptide, being a more specific and non-cytotoxic MMPI, could be of potential use in the therapy for chronic inflammatory diseases.

3. This study is novel in investigating the effects of CMTs on wound healing associated with estrogen-deprivation and on Ln-5 γ2-chain processing. Estrogen-deprivation affected wound healing in OVX rats adversely in terms of collagen content, wound strength and Ln-5 γ2-chain processing and expression. CMT-8 or estrogen treatment increased collagen deposition, wound strength and normalized Ln-5 γ2-chain processing and expression. MMP activity and expression was down-regulated by both OVX and further by treating animals with estrogen or CMT-8. This study is also novel in demonstrating MMP-8 expression in murine skin wound keratinocytes. The role for MMP-8 could be type I collagen degradation, similar to MMP-1 activity in human cutaneous wound healing. Contradictory results from different studies on the effects of estrogen on skin clearly show that a more valid, standardized animal model should be established for future studies. While HRT most likely improve skin quality and wound healing in postmenopausal women, the present study and other related work also indicate that local estrogen application could be a means to treat impaired wound healing. CMT-8 treatment showed similar effect as estrogen and thus, the related compound CMT-3 which is being developed for cancer treatment, could be of potential use in the treatment of chronic wounds. The CMTs tested on HMKs in culture were found to inhibit cell proliferation and thus, the use of CMTs should be considered especially in the treatment of fibrotic lesions.

4. This study is novel in presenting evidence that several other MMPs in addition to the previously described MMP-2 and –14 can cleave the Ln-5 γ2-chain and induce carcinoma cell migration. This is presumably mediated through liberation of a promigratory EGF-like fragment. Ln-5 γ2-chain processing may be important for many biological processes including development, wound healing and remodelling. This also further demonstrates that multiple MMPs can potentially induce tumor cell invasion through the processing of the Ln-5 γ2-chain, which is usually neoexpressed at the invasive front of tumors and deposited by migrating cells. Of the MMPs tested, N-terminal sequences showed that the Ln-5 γ2-chain contain multiple cleavage sites for different MMPs. Thus, MMPs may regulate other cellular processes in addition to migration through the Ln-5 γ2-chain.

5. The present study shows that MMPs are expressed and activated at sites and conditions typically involved in remodelling and cell migration. MMPs play multiple roles in both physiological and pathological conditions. In this study, we have presented novel evidence for the use of different MMPIs in the treatment of both inflammatory processes as well as carcinoma cell migration. In summary, while the development of MMPIs should take new directions toward more precise methods to evaluate efficacy, the role of MMPs in pathological conditions demonstrate that the MMPIs are still attractive for drug development.
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