Department of Dermatology
University of Helsinki
Finland

Matrix metalloproteinases in human wounds and epithelial cancer in the skin and oral mucosa

ULLA IMPOLA

Academic dissertation
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Supervised by:
Professor Ulpu Saarialho-Kere, M.D., Ph.D.
Department of Dermatology
Helsinki University Central Hospital
Helsinki, Finland
and Department of Dermatology,
Karolinska Institutet, Stockholm Söder Hospital
Stockholm, Sweden

Reviewed by:
Docent Klaus Elenius, M.D., Ph.D.
Department of Medical Biochemistry and Molecular Biology,
University of Turku
Turku, Finland
and
Professor Ilkka Harvima M.D., Ph.D.
Department of Dermatology
University of Kuopio, Kuopio University Hospital
Kuopio, Finland

Opponent:
Professor Tuula Salo DDS, PhD
Institute of Dentistry, Faculty of Medicine
University of Oulu
Oulu, Finland

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Yliopistopaino
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To my family

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LIST OF ORIGINAL PUBLICATIONS

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I Mirastschijski U, Impola U, Jahkola T, Karlsmark T, Ågren MS, Saarialho-Kere U. Ectopic localization of matrix metalloproteinase-9 in chronic cutaneous wounds. Human Pathology 2002; 33:355-64.

II Mirastschijski U, Impola U, Karsdal MA, Saarialho-Kere U, Ågren M. Matrix metalloproteinase inhibitor BB-3103 unlike the serine proteinase inhibitor aprotinin abrogates epidermal healing of human skin wounds ex vivo. Journal of Investigative Dermatology 2002; 118:55-64.

III Impola U, Jeskanen L, Ravanti L, Syrjänen S, Baldursson B, Kähäri VM, Saarialho-Kere U. Expression of MMP-7 and MMP–13 and loss of MMP-19 and p16 are associated with malignant progression in chronic wounds. Submitted

IV Impola U, Toriseva M, Suomela S, Jeskanen L, Hieta N, Jahkola T, Grenman R, Kähäri VM, Saarialho-Kere U. Matrix metalloproteinase-19 is expressed by proliferating epithelium but dissappears with neoplastic dedifferentiation. International Journal of Cancer 2003; 103:709-16.

V Impola U, Uitto VJ, Hietanen J, Häkkinen L, Zhang L, Larjava H, Isaka K, Saarialho-Kere U. Differential expression of matrilysin-1 (MMP-7), 92 kDa gelatinase (MMP-9) and metalloelastase (MMP-12) in oral verrucous and squamous cell cancer. Journal of Pathology 2004; 202: 14-22.

ABBREVIATIONS

BCC	basal cell carcinoma
BM	basement membrane
bFGF	basic fibroblast growth factor
cDNA	complementary DNA
COMP	cartilage oligomeric matrix protein
ECM	extracellular matrix
EGF	epidermal growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony stimulating factor
HNSCC	head and neck squamous cell carcinoma
HA	hyaluronic acid (hyaluronan)
HD	hemidesmosome
HGF	hepatocyte growth factor
HME	human macrophage metalloelastase
IGF	insulin-like growth factor
IL-1β	interleukin-1β
KA	keratoacanthoma
KGF	keratinocyte growth factor
Ln-5	laminin-5
MMP	matrix metalloproteinase
NF	neutrophil, (polymorphonuclear cell)
RT-PCR	reverse transcriptase polymerase chain reaction
PMN	polymorphonuclear cell
TIMP	tissue inhibitor of metalloproteinases
TGF-α	transforming growth factor -α
TGF-β	transforming growth factor -β
PMA	phorbol myristate acetate
TNF-α	tumor necrosis factor -α
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel
VC	verrucous carcinoma
VH	verrucous hyperplasia

Ulla Impola, Matrix metalloproteinases in human wounds and cancer in the skin and oral mucosa. Department of Dermatology, Helsinki University Hospital and University of Helsinki

ABSTRACT

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes that are collectively able to degrade all components of the extracellular matrix (ECM). MMPs influence cell dissociation, death and division, angiogenesis, and the bioavailability of growth factors and cytokines, events needed in wound healing and tumor invasion. Normal cutaneous re-epithelialization involves many MMPs, including MMP-1, -9, and -10 in migratory keratinocytes and MMP-3, -19 and -28 in the proliferating zone. However, many of these same MMPs as well as various others, such as MMPs-1, -2, -7, -9, -13, -14 and -26, have been implicated for various cancers.

I investigated the role of MMPs, especially MMP-9 and MMP-19, in wound healing and in epithelial cancers. I studied the possible mechanisms for delayed epithelialization as well as malignant transformation in chronic wounds. I investigated the expression profile of MMPs in the biology and tumorigenesis of pre-malignant lesions in the skin and oral mucosa, and whether the expression patterns of certain MMPs could serve as molecular markers to predict carcinogenesis. Principal methods used were in situ hybridisation, immunohistochemistry, Taqman RT-PCR, Northern, Western and enzyme activity analysis and zymography.

My results show that in acute wounds epithelial cells expressed MMP-9, whereas in chronic wounds, it is expressed by inflammatory stromal cells. The absence of intact ECM molecules due to degradation by inflammatory cell-derived MMP-9 in chronic non-healing wounds may deprive wound edge keratinocytes of proper cell-matrix interactions needed for induction of migration. MMP-2 and MMP-14 are expressed only by stromal cells in all wound types in vivo. Inhibition of MMPs with BB-3103 blocked epithelialization completely in an ex vivo model, whereas aprotinin did not affect it significantly, indicating the requirement for one or more MMPs in epidermal wound healing. This is the first study to show keratinocyte expression of MMP-19. In wounds it is expressed by non-migrating keratinocytes at the hyperproliferating areas but the expression totally dissappeared with malignant transformation. MMP-19 mRNA expression was induced by TNF-α and phorbol myristate acetate (PMA) in primary keratinocytes. MMP-7 is expressed by malignantly transformed epithelium of the skin while it is absent from chronic wounds and keratoacanthomas (KA). Epithelial MMP-13 expression is strong in squamous cell carcinoma (SCC), absent in chronic wounds, but present in KAs. Oral verrucous carcinomas (VC) were devoid of epithelial MMP-3, -7, -9, -12 and -13 expression compared to oral SCCs.

Based on my results I suggest that the epithelial expression of MMPs-7, -12, and –13 and the loss of MMP-19 could aid in making the differential diagnosis between well-differentiated SCC and non-malignant chronic wound or KAs. The invasiveness of oral cancers may be dependent on their MMP expression profile. This phenomenon may thus serve as a prognostic marker in oral SCC. MMP-7, -9, -12, and -13 may be good targets for intervention therapy at the early stages of oral cancer.

I conclude, that several MMPs are needed in wound re-epithelialization, and their expression pattern may promote chronic phenotype as well as malignant transformation. In skin and oral cancers, MMPs might be used as prognostic markers in diagnosing pre-malignant changes from aggressive tumors.

1. INTRODUCTION

Matrix metalloproteinases (MMPs) constitute a family of 23 human zinc-dependent proteolytic enzymes. They take part in the degradation of extracellular matrix (ECM) and basement membranes (BM) during cell migration, angiogenesis and proteolytic activation of growth factors. All these events are needed in fetal development and in normal tissue remodelling as well as in epidermal wound healing, inflammation and tumor invasion. According to their substrate specifity and structure, MMPs can be divided into six subgroups: interstitial collagenases, stromelysins, matrilysins, type IV collagenases, membrane-type MMPs and other MMPs. In normal cell environment, specific tissue inhibitors of metalloproteinases, TIMPs, strictly regulate MMP activity. Under some circumstances MMP activation is also regulated by integrins, cell adhesion receptors mediating migration and invasion.

In human skin, MMPs are mainly produced by keratinocytes, fibroblasts, macrophages and endothelial cells. Normal cutaneous re-epithelialization involves many MMPs, including MMP-1, -9, and -10 in migratory keratinocytes and MMP-3, -19 and –28 in the proliferating zone of the wound area. In chronic wounds, the MMP expression pattern deviates from acute wound repair. How exactly the MMP expression profile changes during tumorigenesis of chronic wounds is poorly understood.

Tumor growth depends critically on the ability of the tumor cells and the surrounding stromal cells to produce these same proteolytic enzymes, receptors mediating cell adhesion, cytokines and certain ECM proteins. Many MMPs such as MMP-1, -2, -7, -9, -13, and -14 have been implicated in cancer invasion. The role of the novel MMPs (from –19 upwards) in skin biology has not been well elucidated.

The aim of the present work was to examine the role of matrix metalloproteinases, especially MMP-9 and MMP-19, in wound healing and in epithelial cancers. We wanted to study the mechanisms associated with delayed epithelialization as well as the malignant transformation of chronic wounds. We also wanted to investigate the possible role of MMPs in the biology and tumorigenesis of pre-malignant lesions, and if the expression patterns of certain MMPs could serve as molecular markers to predict carcinogenesis.

2.REVIEW OF THE LITERATURE

2.1. Structure of the skin and oral mucosa

Skin is the largest human organ. Its primary function is to produce a semi-permeable outer layer of the body that protects from microbes, dehydration, and radiation and also from mechanical, chemical and thermal forces. Skin can be divided into the upper epithelial layer, *epidermis*, the underlying connective tissue, *dermis*, and a subcutaneous layer (see Alberts, 1994). Like skin, the oral mucosa acts as an important mechanical barrier that prevents local or systemic invasion by microorganisms.

Epidermis and dermis

The upper layer of the skin, the epidermis, consists of *stratified squamous epithelium*. In addition to keratinocyte layers, the epidermis contains pigment-producing melanocytes, antigen producing Langerhans cells and Merkel cells which function as mechanoreceptors. During normal epidermal differentiation, four different types of keratinocytes can be distinguished in the epithelium: the basal, squamous, granular, and cornified (see Tomic-Canic, 1998). Basal cells are characterized by their contact with the intact basement membrane, mitotic activity and their undifferentiated phenotype. Basal cells are responsible for epidermal regeneration. During differentiation basal cells move from the basal zone upwards through the spinous and granular layers to the cornified layer of the skin. During their differentiation, keratinocytes change their shape, loose their nuclei, and get filled with keratin filaments and keratohyalin. Terminally differentiated keratinocytes, corneocytes, contain mostly keratin, but remnants of organelles, melanin and membrane profiles are found (Eckert et al., 1989). Keratin proteins comprise the predominant cytoskeletal component of the epidermis. If the cells of stratum corneum contain remnants of nuclei, the epithelium is said to be parakeratinized.

The *dermis* is the connective tissue of the skin and protects the body from mechanical injury. The dermis is divided into subepithelial papillary dermis of loose connective tissue and reticular layer, which lie on an adipose tissue. In the dermis, fibroblasts, endothelial cells, macrophages and mast cells are all embedded in the ECM. Also cells derived from blood vessels: neutrophils, eosinophils, monocytes and lymphocytes are present as well as in other types of ECM (see Alberts et al., 1994). In the skin the dermis also contains hair follicles, sebaceous and sweat glands as well as sensory nerves and blood vessels. The dermis consists

mostly of collagens type I and III, but also thin elastic fibers are found throughout the dermis between collagen bundles.

Oral mucosa

The morphology of oral mucosa is not appreciably different from that of skin. Oral mucosa differs from skin in that it has superficial mucus, which acts as a lubricant as well as a protective coating. Oral mucosa is mostly composed of stratified squamous epithelium. Keratinized epithelium is found in the hard palate, dorsum of the tongue, and gingiva. Non-keratinized epithelium is found on the lining mucosa (floor of the mouth, ventrolateral surface of the tongue, soft palate complex, labial vestibule, and buccal mucosa). In oral mucosa, the surface layer is keratinized *stratum corneum* with no remnants of the nuclei (orthokeratinization). If the cells in this surface layer contain remnants of nuclei, the epithelium is called parakeratinized.

2.1.1. Extracellular matrix of the connective tissue

Extracellular matrix determines the tissue's physical properties and has an essential role in cellular communication. ECM provides support for the surrounding cells but it also has a role in regulating cell shape, proliferation, adhesion, migration and differentiation (Engvall 1995, Timpl, 1996). ECM of the dermal connective tissue is composed of fibrous proteins, embedded in a gel-like matrix composed of glycosaminoglycans (*hyaluronan, chondroitin and dermatan sulfate, heparan sulfate, and keratan sulfate*) and of two types of fibrous proteins: structural (*collagens and elastin*) and adhesive (*laminins, fibronectin, nidogens* etc.). Proteoglycans (*syndecan, versican, aggrecan, decorin*) and glycosaminoglycans, which are secreted locally mainly by fibroblasts, form a well-designed protein network and a hydrated environment where rapid diffusion of nutrients, metabolites and hormones is possible. Connective tissue component of oral mucosa consists of two layers: papillary layer and dense fibrous layer. Between the oral mucosa and the underlying tissues there may be an intermediate layer of connective tissue, a so-called submucosa (Moss-Sajentijn and Klyvert, 1980). In the connective tissue of oral mucosa fibroblasts provide the collagen network for lamina propria and submucosa. ECM of oral mucosa is composed of same proteins as that of dermal connective tissue.

Fibronectin

Fibronectin, which is one of the ECM glycoproteins synthesized by many types of cells, like keratinocytes, fibroblasts and macrophages, participates in cell-matrix adhesion by its affinity to collagens and glycosaminoglycans. It also interacts with cell surface integrins. The attachment of cells to fibronectin plays an important role during developmental processes as embryonic cells migrate on or through this protein. During wound healing keratinocytes use the same kind of migration pattern for re-epithelialization. Migrating keratinocytes in human wounds express two major receptors that are able to bind fibronectin, namely $\alpha 5\beta 1$ and $\alpha v\beta 6$. Wounding induces expression of these receptors. Malignantly transformed cells tend to lack fibronectin production (Yamada, 1991; Ruoslahti, 1999; Amstrong et al., 2000).

Collagens

Collagens are the main ECM components and also the most abundant proteins in the human body. Collagens are synthesized and secreted as procollagens, with large N- and C-terminal propeptides, which are later proteolytically processed to their mature forms. Collagens are triple-helical molecules composed of three α-chains with series of Gly-X-Y triplet sequences. The family of collagens is divided into subclasses on the basis of their structure. The main collagens found in connective tissue are fibrillar collagens (types I, II, III, V and XI), of which the types I and III are the main collagens in skin. Type I collagen gives strength and type III flexibility to the tissues (Mauch and Krieg, 1993). Non-fibrillar collagens form sheet-like structures and connect ECM components to collagen fibrils (Prockop and Kivirikko, 1995; Aumailley and Gayraud, 1998). These non-fibrillar collagens include groups of *network-forming collagens* (type IV, VIII and X), *fibril associated collagens* (types IX, XII, XIV, XVI and XIX), collagens that have a transmembrane domain (types XIII and XVII), *multiplexins* (types XV and XVIII), which have multiple triple- helical domains and interruptions and orphans (types VI and VII), (Prockop and Kivirikko, 1995; Pihlajaniemi and Rehn, 1995).

2.1.2. Basement membrane

Epidermis, as well as all other epithelial sheets and tubes, is separated from the underlying or surrounding tissue by a basal lamina, which is composed of three different layers: *lamina lucida, lamina densa* and *lamina fibroreticularis* (Yurchenco and Schittny, 1990). This composition of three layers, also called basement membrane (BM), is a special structure of ECM. BM provides tissue compartmentalization by acting as a barrier to cell penetration and filtration (Timpl, 1996). BM supports the epidermis and by its ligands, it interacts with cellular

receptors and modulates cell shape, gene expression, cell migration and proliferation as well as the programmed cell death, apoptosis. Typical constituents of BM are highly cross-linked type IV collagen and laminins, perlecan, entactin, calcium-binding and adhesive proteins (fibulins, agrin, cadherins, etc.). Type IV collagen and laminin-1 form a network connected by nidogen (Dziadek et al., 1995), which also binds several other components, like proteoglycans and glycoproteins (see Figure 1.).

Epithelial cells in stratified epithelia attach to the BM via hemidesmosomes, which are a multiprotein junctional complexes in the BM. Hemidesmosomes consist of three classes of proteins: cytoplasmic plaque proteins, transmembrane proteins and BM associated proteins. Plaque proteins, *plectin* (McLean et al., 1996), *bullous pemphigoid antigen 1* (BPAG) (Stanley et al., 1988) and *intermediate filament associated protein* act as linkers of the cytoskeleton. The transmembrane proteins, *α6β4 integrin* and *type XVII collagen* (BPAG 2) (Stepp et al., 1990; Sonnenberg et al., 1991; Giudice et al., 1992) connect the cell interior to the ECM. Finally, BM associated protein laminin-5 is linked to the type VII collagen (Rousselle et al., 1991 and 1997) as well as to the α6β4 integrin forming the HD and maintaining the stable adhesion (Borradori and Sonnenberg 1999).

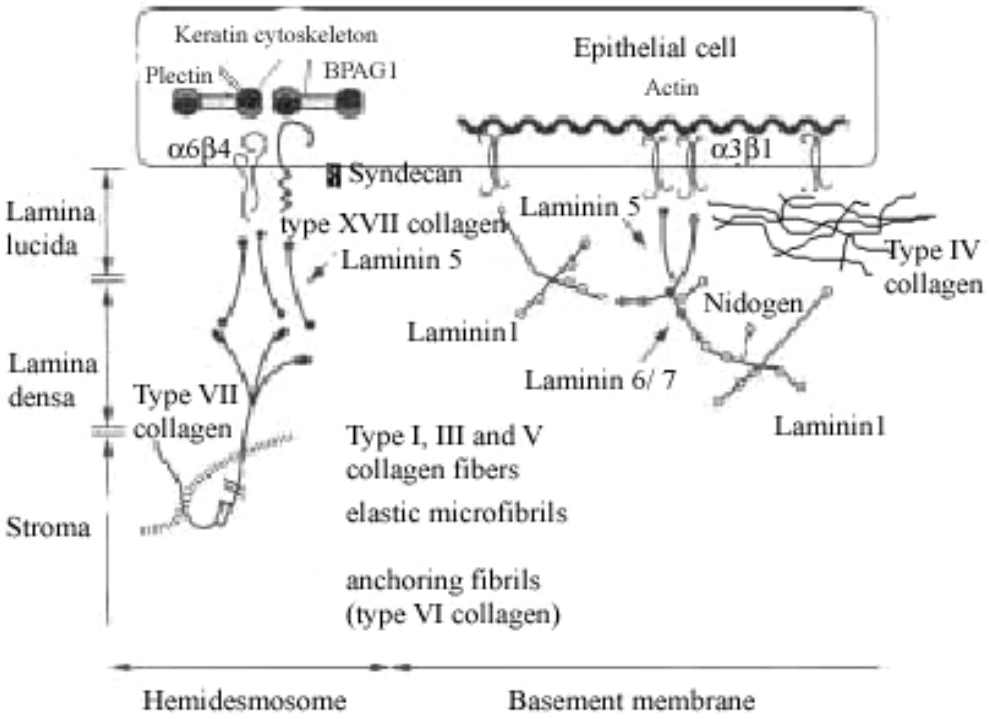

Figure 1. Some of the BM network proteins and the structure of hemidesmosome (modified from Burgeson and Christiano, 1997).

Laminins

Laminins *(Ln)* are cross- or T-shaped heterotrimeric proteins consisting of α, β and γ chains. Laminin-5 (also called kalinin, epiligrin and nicein), a member of this family, is composed of genetically distinct polypeptides, the α3, β3 and γ2 chains, which are assembled in a coiled coil structure. Ln-5 is mainly deposited in BM promoting static adhesion and hemidesmosome formation in the BM structure (Giannelli and Antonaci, 2001). It mediates cell proliferation, wound healing and homeostasis of skin. It also stimulates cell migration and invasion after having been specifically cleaved by MMPs such as MMP-2 or MT1-MMP (Koshikawa et al., 2000; Giannelli et al., 1997). The γ2 chain of laminin-5 is expressed in the cytoplasm of epithelial human cancer cells at the advancing edge of tumors (Giannelli and Antonaci, 2001) and has been shown to be a good marker for cancer invasion in various tumor types (Lohi, 2001). Laminin-5 binds to integrins α6β4, α3β1 and α6β1 (Carter et al., 1991). Together with α6β4, Ln-5 interacts with intermediate filaments and forms a hemidesmosome adhesion complex, which plays a crucial role in the keratinocyte-BM structure.

2.1.3. Cell-cell and cell-matrix interactions and principles of cell migration

The cell, as a living organism, needs signals from the environment as well as attachment to a certain matrix to survive. Cell adhesion molecules glue cells together forming multicellular organisms and tissues and giving cell the ability to communicate with the surroundings. Cell adhesion molecules are situated on the surfaces of cells and bind to ECM molecules, receptors or on to the other cells. Integrins and E-cadherin are the most important cell adhesion molecules expressed by stratified squamous epithelium. Altered expression of these molecules is characteristic for tumor cells as has been found in skin and oral carcinomas (Thomas and Speight, 2001a).

Cadherins

Cell-cell contacts are made with specific adherens junctions and desmosomes, mediated by transmembrane glycoproteins, cadherins. These are a large family of calcium-dependent, "cell adhesion molecules". In addition to their structural role, cadherins have a number of important functions in the control of cell growth and differentiation. Desmosomes are composed of two types of cadherins, desmocollins and desmogleins (Huber, 2003), whereas epithelial adherens junctions contain usually only E-cadherin, which is linked to actin filaments by catenins. The

desmosomal cadherins are linked to the keratin cytoskeleton via several cytoplasmic plaque proteins, including desmoplakin and plakoglobin (gamma-catenin).

Integrins

Integrins form a large family of heterodimeric cell surface receptors involved in cell-cell and cell-matrix adhesion and communication. Their participation in normal tissue remodelling, development, immune response and maintenance of tissue integrity, is well elucidated. They also play a significant role in pathological conditions, especially in chronic inflammation, cancer invasion and metastasis (see Ivaska and Heino, 2000). Integrins are known to have a essential role in cell migration, during wound healing and cancer invasion (Heino, 1996), not only by mediating the cell movement but also by regulating the expression of MMPs (see Ivaska and Heino, 2000). It has been shown that $\alpha 1$ integrin knock out mice have defects in collagen synthesis as well as in matrix metalloproteinase expression. $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are the most important collagen receptors. $\alpha 2\beta 1$ is an important collagen receptor of platelets and epithelial cells (Zutter and Santoro, 1990). Keratinocyte migration requires $\alpha 2\beta 1$ integrin-mediated interaction with Ln-5 chain (Decline and Rousselle, 2001). Up-regulation of another epithelial integrin, $\alpha v\beta 6$, which binds fibronectin and tenascin (Sheppard et al., 1990), has been found in oral cancer, suggesting that it may play an active role in disease progression (Thomas and Speight, 2001b and 2002).

Cell migration

Cell migration is an essential phenomenon during embryonic development (Zagris, 2001). The capacity of cell movement may be activated by wounding, inflammation or by malignant transformation. Cell migration requires cell adhesion and rearrangement of the adhesion molecule complexes. When a cell starts to migrate, it forms a filopod/ lamellipod like structure at the leading edge of the cell and uses these spikes to attach to the underlying substrate. At the same time, integrins cluster to the cell membrane at the ligand binding sites inducing intracellular cell signalling and assembly of focal adhesion complexes (Friedl and Brocker, 2000). Cell movement results from the adhesive traction by focal contacts and actin filament contraction. The direction of cell migration may be affected by chemo (soluble) or hapto (solid) attractants, like cytokines and growth factors, matrix fragments or cryptic sites. Proteolytic enzymes and their inhibitors modulate cell migration by degrading the restricting ECM and thus clearing the way for cell movement. They also remodel ECM components better suitable for

migration. MMPs regulate the bioavailability of growth factors, cytokines and chemokines that participate in the guidance of cell motility. During tumor cell invasion, the cell migration proceeds via penetration of the BM to the underlying stromal tissue.

2.2. PROTEOLYTIC REMODELLING OF THE EXTRACELLULAR MATRIX

Precise degradation of the connective tissue is essential in many developmental and reparative processes, such as reproduction, fetal development, wound healing and angiogenesis. The proteinases comprise both exopeptidases and endopeptidases (proteinases). These are divided into groups based on their catalytic group at their active site: serine/threonine, cysteine, aspartic or metallo (Woessner,1998). Metalloproteinases are further divided into several superfamilies, one of which is *metzincins*. They bind zinc at the catalytic site and have a conserved "Met-turn" motif and conserved structural topology. Metzincins consists of four groups: serralysins, matrixins, astacins and adamalysins (see Stöcker et al., 1995; Bergers and Coussens, 2000).

Serine proteinases contain a serine residue in their catalytic site. The group of serine proteinases include chymotrypsin, chymase, trypsin, plasminogen activators (PAs), plasmin, enteropeptidase, neutrophil elastase, cathepsin G and furin proteinases. Tissue-type PA as well as the urokinase type PA both convert the plasma protein, plasminogen to active plasmin, which has wide substrate specificity and is able to activate several latent metalloproteinases. Serine proteinases are inhibited by serpins (Silverman et al., 2001).

2.2.1. Matrix Metalloproteinases

Matrix metalloproteinases (MMP) are zinc-dependent proteolytic enzymes, which take part in proteolytic degradation of the ECM and BM during morphogenesis, cell migration and angiogenesis. Together they are able to degrade practically all ECM proteins (see Table 1., page 23). The basic structure of MMPs consists of a catalytic domain and additional amount of variable inserts depending on the specific MMP (see Figure 2.). These variable inserts include the signal peptide, propeptide, furin-cleavage site insert, fibronectin like repeats, hinge region, hemopexin domain, and membrane insertion extension. MMPs are generally divided into six subgroups: interstitial collagenases (MMP-1, -8 and MMP-13), stromelysins (MMP-3, -10, -11 and MMP-12), matrilysins (MMP-7 and MMP-26), type IV collagenases (MMP-2 and MMP-9), membrane-type MMPs (MMP-14, -15, -16, -17, -24 and MMP-25) and others (MMP-19, -23 and MMP-28) (Nagase and Woessner, 1999; Uria et al., 2000; Lohi et al., 2001). At least eight of the known human MMP genes (MMP-1, MMP-3, MMP-7, MMP-8, MMP-10, MMP-12,

MMP-13, and MMP-20) are clustered in chromosome 11 at 11q21–23 (Shapiro, 1998). Other known MMP genes are scattered along chromosomes 1, 8, 12, 14, 16, 20, and 22.

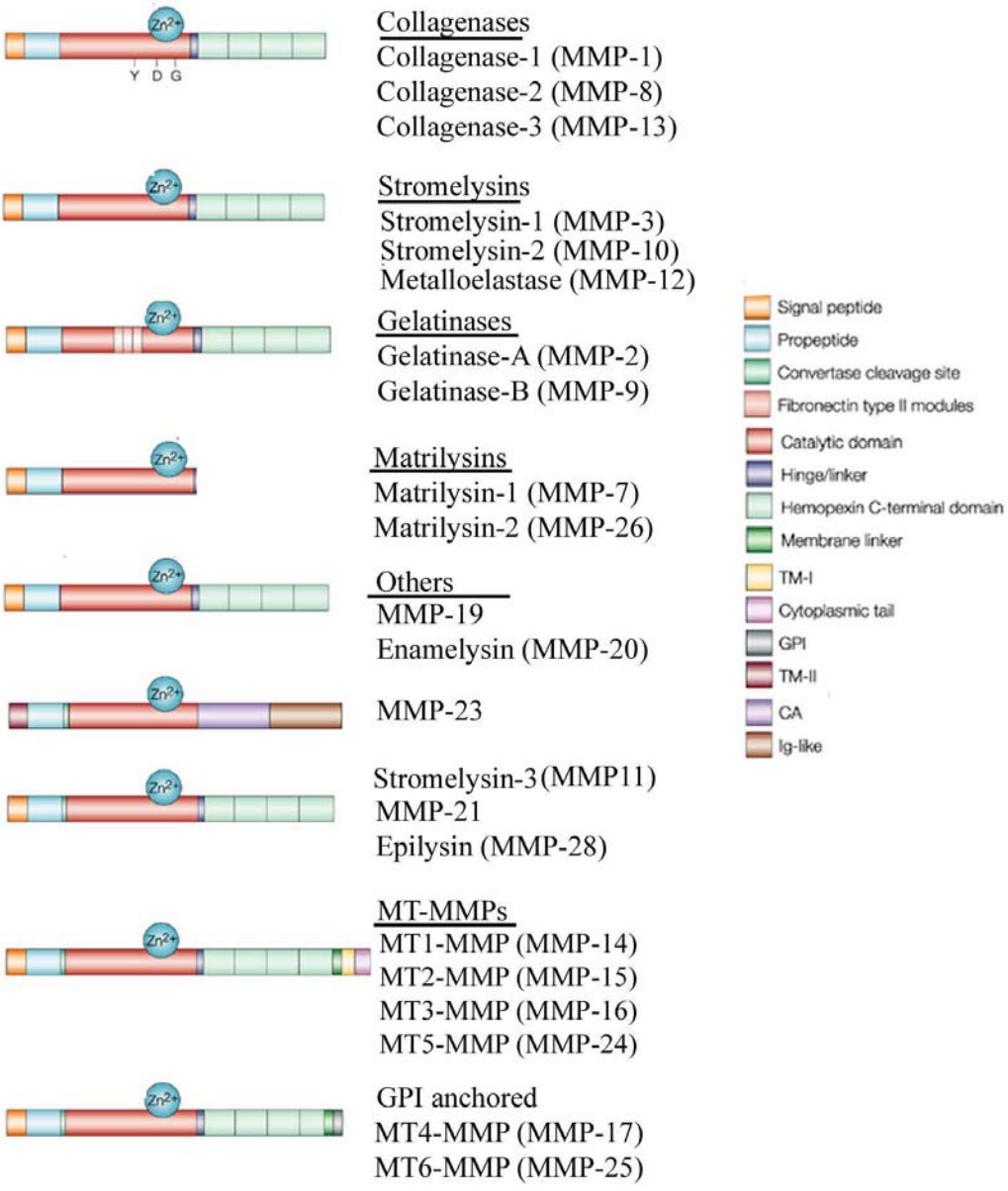

Figure 2. Structure and subclasses of vertebrate MMPs. (Modified from Overall and Lopéz-Otín, 2002). Abbreviations TM, transmembrane domain; CA, cysteine array; GPI, glycosyl phosphatidylinositol-anchor; Ig, immunoglobulin like

2.2.1.1. Collagenases

Collagenases are able to degrade native fibrillar interstitial collagen, which otherwise is very resistant to proteolytic degradation. Collagen is cleaved at a specific site producing ¾ N-terminal and ¼ C-terminal fragments, which denature spontaneously to gelatin at 37^{o} C. These fragments are further degraded by other MMPs, e.g. gelatinases. Three collagenases are known:

collagenase-1 (interstitial collagenase-1, MMP-1) (Goldberg et al., 1986), collagenase –2 (neutrophil collagenase, MMP-8) (Hasty et al., 1990), and collagenase-3 (MMP-13) (Freije et al., 1994).

Collagenase-1 (MMP-1) was the first MMP found from the metamorphosing tadpole (Gross and Lapière, 1962). It was also the first vertebrate collagenase purified and determined by cDNA cloning (Stricklin et al., 1977; Goldberg, 1986). MMP-1 is expressed in vivo in various physiological and pathological situations, such as in embryonic development, in wound repair and in malignant tumors (McGowan et al., 1994; Saarialho-Kere, 1998; Stetler-Stevenson et al., 1993). In cultured cells, expression of MMP-1 is detected in keratinocytes, fibroblasts, endothelial cells, macrophages, monocytes, hepatocytes, chondrocytes and osteoblasts (Birkedal-Hansen et al., 1993; Stetler-Stevenson et al., 1993). Proteolytic activation of human skin MMP-1 procollagenase results in removal of 81 amino acid residues from the amino-terminal portion of the proenzyme (Goldberg at al., 1986). Active MMP-1 is able to cleave collagen types I-III, VII, VIII and X, agrecan, serpins and α2macroglobulin.

Collagenase-2 (MMP-8) was first described in 1960s and later purified and cloned (Hasty et al., 1987 and 1990). MMP-8 is synthesized in polymorphonuclear leukocytes during their maturation in bone marrow and stored in specific intracellular granules, out of which it is secreted to the cell environment in response to an external stimuli (Hasty et al., 1986, 1987 and 1990). MMP-8 is also detected in chondrocytes as well as in mononuclear fibroblast-like cells in rheumatoid synovial membrane, in gingival fibroblasts, and bronchial epithelial cells (Cole et al., 1996; Hanemaaijer et al., 1997; Abe et al., 2001; Prikk et al., 2001). As active collagenase -1 is specialized to degrade type III collagen, MMP-8 prefers types I and II (Welgus et al., 1981), and in fact, MMP-8 is believed to be the initiator for type I collagen degradation. It is also able to degrade aggrecan (Fosang et al., 1996).

Collagenase –3 (MMP-13) was originally cloned from human breast tumor cDNA library. It has a 86% homology with a rodent collagenase and only 50% with human MMP-1 (Freije et al., 1994). MMP-13 has a key role in the MMP activation cascade and appears to be critical in bone metabolism and homeostasis. MMP-13 is predicted to have an important role in tumor invasion and metastasis due to its wide substrate specificity and its upregulated expression in cancer cells (Leeman et al., 2002). MMP-13 expression is seen in skin cancers (Airola et al., 1997; Airola et al., 1999) and adenovirus-mediated gene delivery of its inhibitor, TIMP-3, inhibits invasion and

induces apoptosis in melanoma cells (Ahonen et al., 1998). The latent form of MMP-13 is activated by stromelysin-1 and -2, MMP-2, MT1-MMP and MT2-MMP, trypsin and plasmin. MMP-13 has a large variety of substrates (types I-IV, IX, X, XIV collagens, fibronectin, aggrecan, gelatin, large tenascin C, fibrillin, osteonectin, and serine proteinase inhibitor) (Knäuper et al., 1996 and 1997; Mitchell et al., 1996; Ashworth et al., 1999) (see also Table 1 on page 23).

2.2.1.2. Stromelysins and stromelysin-like MMPs

The stromelysin subfamily consists of stromelysin-1 (MMP-3) and –2 (MMP-10), which are structurally related. Stromelysin-3 (MMP-11) and human macrophage metalloelastase (MMP-12) are often included in a group of stromelysin-like MMPs.

Stromelysins-1 and –2 are expressed by keratinocytes in culture and in vivo and in fibroblasts in culture (Birkedal-Hansen et al., 1993, Windsor et al., 1993; Saarialho-Kere, 1998). MMP-10 is upregulated by cytokines during normal wound repair (Rechardt et al., 2000). It is also able to activate other MMPs, such as MMP-1, MMP-8 and MMP-9 (Nagase, 1998; Nakamura et al., 1998). Stromelysins -1 and -2 digest type IV, IX and XI collagens, tenascin, vitronectin, perlecan, versican, laminin, elastin and IL-1β (Nagase, 1998).

Stromelysin -3 (MMP-11) was cloned from invasive breast cancer tissue (Basset et al., 1990). In the skin, MMP-11 is expressed by fibroblasts in BCCs and SCCs (Thewes et al., 1999) and its high expression level correlates with increased tumor aggressiveness in breast cancer (Basset et al., 1993). MMP-11 diverges from MMP-3 and -10 in amino acid sequence and in enzymatic activity. MMP-11 can degrade serine proteinase inhibitors and α1-proteinase inhibitor, but not any ECM components (Pei et al., 1994).

Human macrophage metalloelastase (HME, MMP-12) is often included in a group of stromelysin-like MMPs. It was initially found in alveolar macrophages of cigarette smokers (Shapiro et al., 1993). The expression of MMP-12 is also found in vivo in macrophages in granulomatous skin diseases, solar elastosis, in intestinal ulcerations and inflammation (Vaalamo et al., 1998; Salmela et al., 2001; Chung et al., 2002) as well as in tumor cells in SCC of vulva (Kerkelä et al., 2000) and in skin cancers (Kerkelä et al., 2000). MMP-12 is the most efficient degrader of elastin, but it is also able to cleave type IV collagen, fibronectin, laminin-1, gelatin, vitronectin, entactin and a variety of ECM proteoglycans and glycosaminoglycans

(Chandler et al., 1996; Gronski et al., 1997; Hiller et al., 2000). MMP-12 is able to cleave plasminogen into angiostatin thus preventing tumor growth by inhibiting angiogenesis (Dong et al., 1997; Cornelius et al., 1998).

2.2.1.3. Matrilysins

Matrilysin-1 (MMP-7) and *endometase* (MMP-26) are the smallest MMPs. They lack the hinge region and hemopexin domains, which restricts their substrate specificity (Park et al., 2000). MMP-7 was originally identified as the small putative uterine metalloproteinase (PUMP) (Mueller et al., 1988). It is produced by sweat and salivary glands, airway ciliated cells, and by the ductal or glandular epithelium of breast, liver, pancreas and urogenital tissues (Rodgers et al., 1994; Saarialho-Kere et al., 1995; Wilson et al., 1995). MMP-7 is upregulated in injured epithelium in the intestine (Saarialho-Kere et al., 1996) and airways (Dunsmore et al., 1998), and has thus been implicated in epithelial repair. Also in tumors of epithelial origin, like breast, skin, stomach and colon carcinomas, MMP-7 is overexpressed (Basset et al., 1990, Karelina et al., 1994; Newell et al., 1994; McDonnell et al., 1991). MMP-7 is able to inhibit tumor angiogenesis by generating angiostatin (Patterson and Sang, 1997); this was is proven in vivo (Pozzi et al., 2000). Furthermore, MMP-7 functions in mucosal immunity by regulating the level of antimicrobial peptides (Parks et al., 2001).

Matrilysin-2 (Endometase, MMP-26) is a fairly recently discovered MMP. It was cloned from fetal (de Coignac et al., 2000), placenta (Uria and Lopez-Otin, 2000), and human endometrium cDNA libraries (Isaka et al., 2003). It is expressed in uterus and in placenta and also various tumor cell lines as well as in carcinomas of the lung, prostate and breast by RT-PCR (Marchenko et al., 2001). MMP-26 is able to degrade fibrinogen, fibronectin, type IV collagen, gelatin, vitronectin and α1-proteinase inhibitor (Park et al., 2000; Uria and Lopez-Otin, 2000; Marchenko et al., 2001).

2.2.1.4. Type IV collagenases

Type IV collagenases, also called *gelatinases*, consist of two enzymes, MMP-2 (72-kDa gelatinase or gelatinase A) and MMP-9 (92-kDa gelatinase or gelatinase B). MMP-2 is expressed by various cell types e.g. dermal fibroblasts, keratinocytes, and endothelial cells (Birkedal-Hansen, 1993). MMP-9 is produced by polymorphonuclear leukocytes, macrophages and cultured keratinocytes, osteoclasts, invading trophoblasts and by many types of transformed cells (Hibbs et al, 1987; Birkedal-Hansen, 1993; Zeigler et al., 1996). MMP-9 expression is

enhanced by TGF-β, TNF-α or IL-1β in cultured mucosal keratinocytes (Salo et al., 1994). TNF-α mediated activation of pro-MMP-9 is associated with down-regulation of TIMP-1 in human skin (Han et al., 2002). In addition to gelatin, MMP-2 and MMP-9 degrade type IV, V, VII and X collagens, laminins, elastin, fibronectin and vitronectin (for review see Ravanti and Kähäri, 2000). MMP-2 and MT1-MMP (MMP-14) are capable of cleavaging Ln-5, resulting in a fragment that promotes cell migration of normal breast epithelial cells and tumor cells (Giannelli et al., 1997; Koshikawa et al., 2000).

2.2.1.5. Membrane-type MMPs

Membrane-type MMPs (MT-MMPs) constitute a subclass of recently identified matrix metalloproteinases. In addition to the highly conserved MMP functional domains, the MT-MMPs have additional insertion sequences. MT-MMPs are membrane associated and a number of these have cytoplasmic domains which may be important in cellular signaling. The first MT1-MMP (MMP-14) was found on the surface of invasive tumor cells in 1994 (Sato et al., 1994) and nowdays six members are reported (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 and MMP-25) (Takino et al., 1995; Will and Hintzman, 1995; Puente et al., 1996; Llano et al., 1999; Pei, 1999). MT-MMPs are localized at the cell surface. Their localization facilitates their role in cell-matrix interactions and activation of other MMPs. For instance active MMP-14 is auxiliary to TIMP-2 for the full activation of MMP-2 (Kinoshita et al., 1998). The expression of MT1-MMP is not detected in normal epithelial cells, but can be seen in transformed epithelial carcinoma cells. In addition it is shown that MT1-, MT2- and MT3-MMPs are able to degrade cell surface tissue transglutaminase at the leading edge of invasive cancer cells (Belkin et al., 2001). Based on these findings, it is believed that MT-MMPs are central mediators of surface proteolytic events that regulate cancer cell adhesion, motility, metastasis and angiogenesis.

2.2.1.6. Other MMPs

MMP-19 is one of the recently cloned members of the MMP family. It differs from the others by its unique chromosomal location (12q14) (Pendas et al., 1997). MMP-19 lacks several structural features known to be present in other MMP subclasses, including Asp, Tyr, and Gly residues close to the zinc-binding site, the fibronectin-like and the transmembrane domains as well as the furin-activation sequence, based on which it cannot be included into any of the known subclasses (Pendas et al., 1997). In vitro MMP-19 is able to degrade many important

BM components such as type IV collagen, laminin-1, nidogen and fibronectin as well as tenascin-C isoform, aggrecan, type I gelatin and cartilage oligomeric matrix protein COMP (Stracke et al., 2000a) but does not activate any other latent MMPs (Stracke et al., 2000b). MMP-19 was originally detected in placenta, lung, pancreas, liver, ovary, spleen and intestine by Northern Analysis (Pendas et al., 1997). Independently, it was isolated as an autoantigen from the inflamed synovium of a patient suffering from rheumatoid arthritis (Sedlacek et al., 1998). MMP-19 has been described in smooth muscle cells of the tunica media of large blood vessels and those of normal skin and uterine ligaments as well as in endothelial cells of acutely inflamed synovial capillaries (Kolb et al., 1999). It is also detected on the surface of activated peripheral blood mononuclear cells, TH1 lymphocytes and Jurkat T lymphoma cells (Sedlacek et al., 1998). MMP-19 has been suggested to play a role in matrix remodeling processes and in the pathogenesis of rheumatoid arthritis (Sedlacek et al., 1998; Konttinen et al.,1999). Because of its expression in normal tissues it is possible that MMP-19 is important in normal tissue remodelling or activation of secreted and membrane bound proteins, like growth factors.

Enamelysin (MMP-20) expression is restricted to ameloblasts and odontoblasts of developing teeth (Llano et al., 1997). Its´ substrates include the major component of the enamel matrix, amelogenin, and aggrecan and COMP (Stracke et al., 2000a). MMP-20 is apparently important in tooth enamel formation and is expressed by human tongue carcinoma cells (Väänänen et al., 2001).

MMP-21 was found from human placenta cDNA (Ahokas et al., 2002). A 2.5 kb messenger RNA was observed in fetal liver by Northern analysis and by RT-PCR, MMP-21 is expressed in various human fetal and adult tissues as well as in cancer cell lines. MMP-21 protein can also be detected in several cancer types, such as ovarian and colon carcinomas by immunohistochemical staining. It has been suggested that MMP-21 functions in embryogenesis and tumor progression (Marchenko et al., 2003; Ahokas et al., 2003).

MMP-23 was cloned from an ovary cDNA library (Velasco et al., 1999) and it is mainly expressed in ovary, testis and prostate, indicating its possible role in reproductive processes. MMP-23 has a unique structure and its biological function is not known (Pei et al., 2000).

MMP-27 has been identified on the basis of EST sequences. The function of this enzyme is not known (Yang and Kurkinen, 1998).

MMP-28 (epilysin), is another novel MMP, recently cloned from the testis and keratinocyte cDNA libraries (Lohi et al., 2001). It is a 59 kDa protein and is structurally mostly related to MMP-19. It is found in lung and in a variety of carcinomas (Marchenko and Strongin, 2001). Epilysin expression is also associated with cell proliferation during epithelial repair, while migrating keratinocytes do not express MMP-28 (Saarialho-Kere et al., 2002).

Table 1. MMPs and their substrates.

Enzyme	Substrates
Collagenase-1 (MMP-1)	Col I, II, III, VII, VIII, X, aggrecan, entactin/nidogen, MBP, serpins, α2M, perlecan, vitronectin, tenascin, fibrinogen, TNF precursor, IGFBP
Collagenase-2 (MMP-8)	Col I, II, III, aggrecan, serpins, α2M, fibrinogen
Collagenase-3 (MMP-13)	Col I, II, III, IV, IX, X, XIV, aggrecan, fibrillin, fibronectin, gelatin, Ln-1, large tenascin C, osteonectin, serpins, PAI, fibrinogen
Gelatinase-A (MMP-2)	Col, I, IV, V, VII, X, gelatin, fibronectin, tenascin, fibrillin, osteonectin, entactin, aggrecan, vitronectin, decorin, MBP, decorin, plasminogen, α2M, Ln-5, IGFBP, TNF precursor, pro-TGF-β, α1PI
Gelatinase-B (MMP-9)	Col I, IV, V, VII, XI, XIV, gelatin, elastin, fibrillin, osteonectin, aggrecan, fibronectin, vitronectin, decorin, MBP, α2M, TNF precursor, IGFBP, plasminogen, pro-TGF-β, α1PI
Stromelysin-1 (MMP-3)	Col III, IV, V, VII, IX, X, elastin, fibronectin, fibrillin, fibrinogen, gelatin, aggrecan, Ln-1, nidogen, vitronectin, osteonectin, decorin, tenascin, α1PI, TNF precursor, MBP, E-cadherin, IGFBP, plasminogen, osteopontin
Stromelysin-2 (MMP-10)	Col III; IV, V, IX, X, elastin, fibronectin, gelatin, aggrecan, Ln-1, nidogen
Stromelysin-3 (MMP-11)	α1PI, IGFBP
Metalloelastase (MMP-12)	Elastin, col IV, fibronectin, Ln-1, gelatin, vitronectin, entactin, proteoglycan, heparan and chondroitin sulfates, TNF precursor, plasminogen, fibrillin, fibrinogen, α1PI
Matrilysin (MMP-7)	Col IV, elastin, fibronectin, Ln-1, entactin, tenascin, osteonectin, aggrecan, vitronectin, MBP, decorin, versican, α1PI, osteopontin, E-cadherin, plasminogen, β4 integrin, α-prodefensin, Fas ligand, pro-TNF-α
Matrilysin-2 (MMP-26)	Col IV, gelatin, fibronectin, fibrin, α1PI, β-casein, TACE-substrate
MT1-MMP (MMP-14)	Col I, II, III, gelatin, fibronectin, Ln-1, vitronectin, aggrecan, tenascin, nidogen, perlecan, fibrinogen/fibrin, fibrillin, α1PI, α2M, LN-5, CD44, tTG
MT2-MMP (MMP-15)	Fibronectin, LN-1, gelatin, aggrecan, tenascin, nidogen, perlecan, vitronectin, tTG
MT3-MMP (MMP-16)	Col III, fibronectin, gelatin, laminin, aggrecan, casein, vitronectin, α2M, α1PI, tTG
MT4-MMP (MMP-17)	Gelatin, TNF-α precursor, fibrillin, fibronectin
MT5-MMP (MMP-24)	ND
MMP-19	Col IV, gelatin, LN-1, nidogen, tenascin, fibronectin, aggrecan, fibrinogen, COMP
Enamelysin (MMP-20)	Amelogenin, aggrecan, COMP
MMP-21	ND
MMP-23	gelatin
MMP-27	ND
MMP-28	casein

ND = not determined (Modified from Kerkelä and Saarialho-Kere, 2003; Lohi et al., 2001)

2.2.2. Regulation of MMPs

In normal tissue the secretion and activity of MMPs is very low, but their production and release are rapidly induced when tissue remodelling is needed (Nagase and Woessner, 1999). Generally MMPs are not expressed in normal skin but are upregulated in inflamed or diseased skin. Regulation of MMPs occurs at many levels, including transcription, modulation of mRNA half-life, secretion, localization, zymogen activation and inhibition of the proteolytic activity.

2.2.2.1. Transcriptional regulation of MMP genes

Control at the level of transcription is the major level of MMP regulation (Fini et al., 1998). Various effectors including growth factors and cytokines (EGF, TNF-α, IL-1β, bFGF, PDGF, IL-6 and TGF-β), chemical agents, physical stress, oncogenic cellular transformation as well as cell-cell and cell matrix interactions regulate MMP gene expression (see Nagase and Woessner, 1999). Extracellular stimuli affect MMP expression via signal transduction pathways that lead to AP-1 (activator protein-1) binding site activation. AP-1 site is in the genes of MMPs-1, -3, -7, -8, -9, -10, -12 and MMP-13. MMP-1, -3 and MMP-9 have also another AP-1 site, but the role of this site is not clear. The expression of AP-1 transcription factors are induced by mitogen activated proteine kinase (MAPK) pathways, i.e. extracellular signal-regulated kinase (ERK1, 2), stress activated proteinase kinase/Jun N-terminal kinase (SAPK/ JNK) and p38. ERK 1, 2 pathway plays a crucial role in growth factor induced mitogenesis, differentiation and cellular transformation, but it can also be induced by stress stimuli. SAPK/JNK and p38 are merely activated by cytokines and stress such as UV-light (Karin et al., 1997). MMP-2, MMP-11, MMP-28 and MT1-MMP genes do not have AP-1 site.

TGF-β inhibitory elements (TIE) are described in some MMP gene promoters (MMPs-1, -7 and -13), but their role is not clear. TGF-β and TNF-α might stimulate MMP-1 expression as shown in cultured keratinocytes (Clark et al., 1995; Mauviel et al., 1996; Johansson et al., 1997). MMP-7 can be both up- and downregulated by TGF-β (Wilson and Matrisian, 1998). In rats, TGF-β downregulates gene expression of MMP-3 (Kerr et al., 1990). MMP-10 is responsive for EGF, KGF, TNF-α, TGF-β1 and TGF-α, as well as for phorbol ester in keratinocytes (Windsor et al., 1993; Madlener et al., 1996). MMP-12 is upregulated by IL-1, TNF-α, M-CSF, VEGF and PDGF-BB and inhibited by TGF-β (Feinberg et al., 2000). MMP-13 is induced by TPA, IL-1β and TGF-β in human fibroblasts (Uria et al., 1997, 1998).

2.2.2.2. Activation of proMMPs

Most proMMPs are secreted from cells and activated extracellularly, but some of them are stored in and released from intracellular granules (MMP-8, MMP-9). ProMMPs secreted as inactive zymogens can be activated by proteinases or by nonproteolytic agents (Nagase, 1997). In vitro the activation can occur by plasmin, trypsin, furin, kallikrein, chymase, mast cell tryptase as well as by other, such as bacterial proteinases. Activation of proMMPs by plasmin is a relevant pathway in vivo. Plasmin is generated from plasminogen by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Plasmin has been reported to activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10, and proMMP-13 (Lijnen, 2001). ProMMP-1 can also be activated by MMP-3, MMP-7 or by MMP-10 (Imai et al., 1995; Suzuki et al., 1990).

Many MMPS are activated by other MMPs (see Figure 3.). ProMMP-2 is activated by MT-MMPs (Strongin et al, 1995), including MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP and MT6-MMP (see Visse and Nagase, 2003). MT4-MMP does not activate proMMP-2 (English et al., 2000). MMP-2 activation with active MT1-MMP needs TIMP-2, but MT2-MMP is independent of it (Morrison et al., 2001). During MMP-2 activation, MT1-MMP forms dimers or multimers on the cell surface through interaction of the hemopexin domains and then binds TIMP-2. ProMMP-2 binds to the C-terminal domain of TIMP-2 through its hemopexin domain. The second, active, MT1-MMP then cleaves proMMP-2, thereby partly activating it. The MMP-2 dissociates from the membrane and is fully activated by intermolecular processing. Thus TIMP-2 enhances the activation of MMP-2 at low levels, but inhibits it at high levels. ProMMP-11 possesses a furin recognition sequence and it is activated intracellularly by furin (Pei and Weiss, 1995). MT-MMPs (Sternlicht and Werb, 2001; Woessner and Nagase, 2000), MMP-21, MMP-23, and MMP-28 (Marchenko and Strongin, 2001; Lohi et al., 2001; Ahokas et al., 2002) have a similar basic motif in the propeptide. ProMMP-13 can be activated by MMP-3, MMP-10, MT2-MMP as well as by MT1-MMP (Knäuper et al., 1996; d´Ortho et al., 1997; Murphy et al., 1999) and MMP-13 itself is able to activate gelatinases (Knäuper et al., 1997). MMP-26 is an exception, because it is auto- activated (Marchenko et al., 2003).

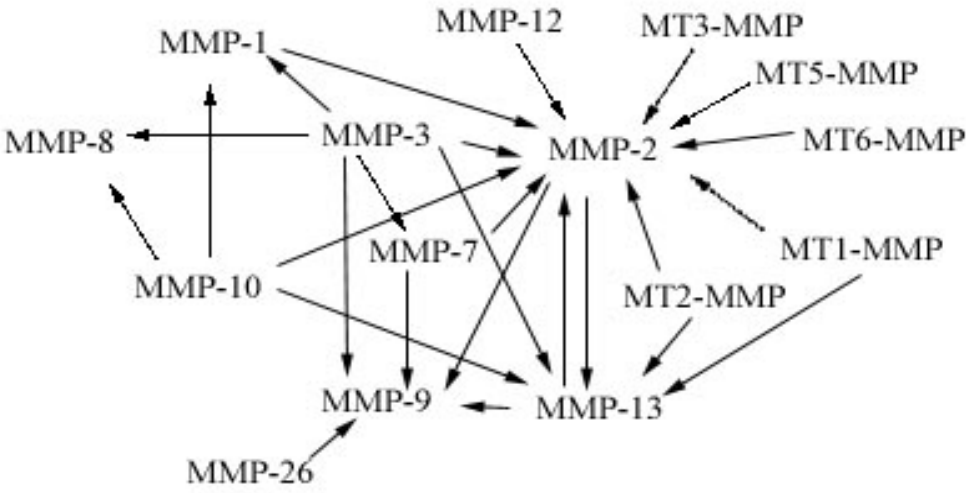

Figure 3. Summary of the activation cascades of MMPs. Arrows indicate the activation (see text for details) (see Ravanti and Kähäri, 2000).

2.2.2.3. Inhibition of MMPs

Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are a family of secretory proteins that are able to inhibit MMP activity through non-covalent binding of active forms of MMPs in the extracellular space in 1:1 molar stoichiometry. Four TIMPs have been identified in vertebrates, namely TIMP-1, -2, -3 and -4 (Edwards et al., 1996; Gomez et al., 1997). Their expression is regulated during development and tissue remodelling (Brew et al., 2000). TIMPs are expressed in various tissues and by many cell types (Gomez et al., 1997). TIMP-1, TIMP-2 and TIMP-4 stay in secreted form in ECM whereas TIMP-3 is associated with it (Leco et al., 1994). All TIMPs are capable of inhibiting all MMPs, with the following exceptions: TIMP-1, which is not able to inhibit MT1-MMP and TIMP-3 , which is a better inhibitor for ADAM-17 (TACE) and aggrecaases (ADAMTS-4 and -5) than for MMPs (see Visse and Nagase, 2003). TIMPs have N- and C-terminal domains and the MMP inhibition occurs through folding the N-domain and binding it to the active site of MMP. TIMPs play an important role in many biological processes, including fetal development, angiogenesis and cancer. An imbalance between TIMP and MMP activities is believed to result in excessive degradation of matrix components in tumor invasion, but the balance between various TIMPs may also be a critical factor in determining the degradative potential of cells in normal and pathological conditions.

Overexpression of different TIMPs can inhibit invasion of malignant cells in vivo and in vitro. Therefore, adenovirus-mediated gene delivery of TIMP-1, -2 and -3 into malignant cells may be a potent way of inhibiting tumor invasion (Ahonen et al., 1998). However, the lack of effective methods for gene delivery has limited the clinical utility of this approach.

Other MMP inhibitors

Non-specific plasma *α-macroglobulins* are expressed in many human tissues and are an example of endopeptidase inhibitors that are able to inhibit MMPs as well as most other proteinases (serine, cysteine-, and aspartate proteinases). In addition, *serine proteinase inhibitors* (plasminogen activator inhibitor and α1-antitrypsin) are able to regulate proteinase activity in tissue. *Chelating compounds, like biphosphonates and tetracyclins* are able to inhibit MMP activity (Woessner, 1999; Hidalgo and Eckhardt 2001a) by binding to the zinc ion at the catalytic site. Tetracyclin derivatives are able to block both synthesis and activation of MMPs, whereas biphosphonates inhibit only the enzymatic activity of MMPs. *TGF-β, glucocorticoid hormones and retinoids* are common downregulators of the transcription of MMPs (Egeblad and Werb, 2002). Also the inhibition of the activity of different protein kinases (MAPKs) may serve as a potent way of inhibiting MMP expression (see Overall and Lopez-Otin, 2002). Reversion-indicing cysteine -rich protein with kazal motifs (RECK), is a membrane-anchored inhibitor of MMPs. It was recently characterized for its role in development, tissue homeostasis, and tumor angiogenesis.The RECK gene is widely expressed in normal human tissues but it is downregulated in tumor cell lines and oncogenically transformed fibroblasts. RECK suppresses tumor invasion and angiogenesis by regulating matrix-metalloproteinases (MMP-2, MMP-9 and MT1-MMP). Restored RECK expression in cancer cell lines results in a strong suppression of invasion, metastasis, and tumor angiogenesis (Weaver, 2002; Noda et al., 2003).

Synthetic MMP inhibitors

In recent years, many chemical inhibitors for MMPs, like broad-spectrum MMP inhibitors GM 6001 and its hydrophilic form BB-3103, have been synthesized in order to use them as therapeutic agents in the treatment for various cancers or in evaluating the biological significance of MMPs in normal processes such as wound repair. MMP inhibition may occur in several ways; 1) directly by transfecting cells with antisense oligonucleotides or by targeting cells with mRNA ribozymes, 2) by inhibiting interaction between MMPs and other proteins, 3)

by developing cytotoxic agents that are activated by MMPs, or by 4) blocking MMP activity with peptidomimetic or non-peptidomimetic inhibitors, tetracyclins or biphosphonates or with some other chemical compounds such as shark cartilage extract or acetylsalicylic acid (Egeblad and Werb, 2002). Results are not very promising, as many of the trials have been ended because of serious side effects or the lack of efficacy. Some inhibitors have even shown to stimulate disease progression (see Coussens and Werb, 2002). Based on all results, it is learned that MMPs are part of so complex biological cascades with cytokines and growth factors, that the role of individual MMPs should be very carefully studied in vivo before trials with wide spectrum MMP inhibitors are started (Overall and Lopez-Otin, 2003; Matrisian et al., 2003). This is specially warranted so that we do not disturb wound epithelialization mediated by MMPs.

The *hydroxyamate inhibitor* Batimastat and its analogue Marimastat were the first inhibitors studied in detail. *Batimastat* is able to inhibit MMPs-1, -2, -3, -7, -8, -9, -13 and -14. It has been studied in animal models and its possible role in inhibition of tumor growth, metastasis as well as in tumor associated angiogenesis has been reported (Rasmussen and McCann, 1997; Coussens et al., 2002). *Marimastat* inhibits MMPs-1, -2, -3, -7, -8, -9, -13 and –14 (Rasmussen and McCann, 1997; Whittaker et al., 1999; Coussens and Werb, 2002). Phase III clinical trials using Marimastat, Batimastat alone or in combination with chemotherapy in patients with advanced cancers have been disappointing and have been terminated. The *non-peptidomimetic MMP inhibitors*, such as BAY 12-9566 and AG3340, were synthesized on the basis of the conformation of the MMP active site in an attempt to improve the oral bioavailability, pharmaceutical properties and specificity. Clinical trials are still ongoing with several other MMPIs, including Prinomastat (halted), shark cartilage extract Neovastat (Phase III), Metastat (Phase II), BMS-275291 (Phase II/III) and MMI270 (Phase II) (Hidalgo and Echardt, 2001b; Vihinen and Kähäri, 2002; Coussens and Werb, 2002; Egeblad and Werb, 2002).

2.3. CUTANEOUS WOUND HEALING

2.3.1. General

Wound healing is a complex process where several controlled events, like inflammation, formulation of granulation tissue, angiogenesis, matrix formation and remodelling, partly overlap in time. During wound healing, several molecules that are not normally present in adult tissue are found in the wound site. *Clot formation* takes place right after the injury. Fibrin clot is composed of thrombocytes, neutrophils and monocytes, which are embedded in a provisional

matrix. Clot functions as a reservoir of cytokines and growth factors (Martin 1997). Many growth factors and cytokines, which are mainly produced by platelets and injured cells, attract inflammatory cells to the wound area. Compared to adult wounds, fetal wounds are characterized by the absence of clot formation and inflammatory response (Larjava et al., 1993). During *re-epithelialization* keratinocytes start migrating into the defect within 3-6 h. Migration is presumably initiated by exposure to various growth factors and cytokines, which are released by the damage. Epithelial cells from the edges of the defected epithelium change their hemidesmosomal connections, detach from the BM, contact type I collagen in the wound bed, change their expression of BM components and start migration. Attachment to type I collagen is mediated by integrin $\alpha 2\beta 1$. Migrating keratinocytes deposit Ln-5 and synthesize Ln-1, type IV and VII collagens, fibronectin as well as several integrins, e.g. $\alpha 5\beta 1$ and $\alpha 6\beta 4$, into the provisional matrix, which is mainly composed of fibronectin, fibrin and vitronectin (Larjava et al., 1993). Cutaneous keratinocytes move quickly underneath this provisional matrix across the injured epidermis. Few hours later, the keratinocyte proliferation is increased distal from the migrating edge. At the same time, neutrophils invade to the wound site being responsible for the *inflammatory response* (Martin 1997; Yamaguchi and Yoshikawa, 2001). Mature BM is re-established by keratinocytes after the completion of re-epithelialization (Martin 1997; Jacinto, 2001). Cross-talk between keratinocytes and fibroblasts is crucial during the re-organization of BM as wound fibroblasts synthesize a significant portion of BM components. *Granulation tissue formation* is characterized by neovascularization and fibroplasia. Fibroblasts are also responsible for the wound contraction, which is carried out by specialized myofibroblasts that contain α-smooth muscle actin. Granulation tissue, which is composed of HA, fibrin, fibronectin, type I and III collagens, is remodelled into a new connective tissue. In mature connective tissue, majority of type III collagen is replaced with type I collagen, and HA. Fibrin and plasma fibronectin are degraded and proteoglycans deposited (Clark 1995; Gailit and Clark, 1994).

2.3.2. Wound healing in oral mucosa

In general, wound healing in oral mucosa is similar to cutaneous wound repair (Larjava et al., 1993). The difference is, that in dermal wounds keratinocytes are believed to migrate under the clot in contact with the dermal matrix and type I collagen, whereas in small gingival wounds, keratinocytes migrate through the fibrin-fibronectin matrix, without collagen contact (Larjava et al., 1993; Häkkinen et al. 2000). Based on animal studies it seems that wounds in oral mucosa

heal faster and with less scarring than in skin (see Häkkinen et al., 2000). In fact, oral wound healing resembles fetal wound healing. Adult gingival fibroblasts, located in the papillary connective tissue, share many properties with fetal fibroblasts and therefore differ from those of dermal origin. There are several factors in saliva, like growth factors, ions such as magnesium and calcium as well as redox activity reducing agents, that favor gingival wound healing (Häkkinen et al., 2000), but also considerable amounts of bacteria reducing the healing capacity (see Häkkinen et al., 2000). Lubrication of oral mucosa by saliva is by itself beneficial for wound closure.

2.3.3. Chronic wounds

In chronic wounds highly controlled co-operation of various growth factors, cytokines and proteolytic enzymes and their inhibitors is disturbed and healing does not proceed and the re-epithelialization does not occur within the normal biological time range of 2-3 weeks. Decubitus ulcers, venous leg ulcers, rheumatic, and diabetic ulcers are common examples of these poorly healing wounds. The prevalence of chronic leg ulcer patients in Sweden is 2% (Nelzen et al., 1996) and in Finland there are ca 45000-10000 venous leg ulcer patients (Malanin et al., 1990). The usual cause of these types of ulcers is chronic venous insuffiency (80-90%) followed by arterial disease and diabetic neuropathy (Baker et al., 1992).

Chronic ulcers are characterized by chronic inflammation with elevated numbers of macrophages, plasma cells, PMNs and B-lymphocytes (Loots et al., 1998). Chronic wounds have high number of tryptase-positive mast cells and they are associated with delayed wound healing and epithelialization (Huttunen et al. 2000). Although the underlying pathogenesis is well-defined, the molecular mechanisms for impaired epithelialization are still unknown. Several factors causing poor healing have been proposed: 1) Failure of wound margin keratinocytes to move over the chronic wound bed although they proliferate normally (Adair, 1977; Falanga et al., 1994; Andriessen et al., 1995) indicating the possible lack of proper matrix components for adhesion or improper signals to the cell. 2) Excessive proteolysis may interfere with proper wound healing by degrading provisional matrix components that are needed for the cell migration or by inactivating growth factors (Grinnell et al., 1992). 3) Hypoxia in the wound area may interfere with normal healing by causing fibrosis (Ferguson and Leigh, 1998). 4) Fibrin deposition surrounding the numerous dermal blood vessels may inhibit normal transportation of growth factors, oxygen and proteins to the wound area (Herrick et al., 1992; Hickley et al., 1995). It has been shown that wound fibroblasts proliferate at a slower rate and

are morphologically distinct from normal fibroblasts. This can be stimulated by growth factors bFGF, EGF, and IL-1β. Trengove et al. (2000) showed significantly higher concentrations of pro-inflammatory cytokines IL-1, IL-6 and TNF-α in wound fluid from nonhealing compared to healing leg ulcers. It has been shown that the balance between MMPs and their inhibitors TIMPs is disturbed in chronic wounds versus acute wounds (Falanga et al., 1994; Vaalamo et al., 1996; Ferguson and Leigh, 1998).

2.3.4. The role of proteinases in epithelial wound repair

Proteinases are involved in several processes during wound healing. Serine proteinases, such as urokinase-plasminogen activator (uPA) and plasmin, and matrix metalloproteinases (MMPs) are the main proteinases implicated in epidermal repair (Rømer et al., 1996; Ravanti and Kähäri, 2000). Proteinases primarily 1) facilitate keratinocyte and fibroblast migration by remodeling ECM proteins, especially by fibrin and fibronectin degradation. In addition, proteinases 2) modulate intracellular signaling, secretion and bioactivation of cytokines and growth factors important for epidermal healing (Gak et al, 1992; Ito et al., 1996; Gallea-Robache et al., 1997; Imai et al., 1997; Wakita et al, 1997). Proteinases are also needed in 3) the removal of devitalized tissue, 4) in angiogenesis, 5) in remodelling of the BM zone as well as 6) in control of the cell proliferation and inflammation in the wound area.

Plasmin degrades fibrin matrices in the provisional normal matrix and also activates latent MMPs. Plasminogen activation has been shown to be essential in wound healing (Lund et al., 1999). Migrating keratinocytes express uPA in an *in vitro* wound model (Morioka et al., 1987). Analogously, uPa mRNA expression is found both in chronic and in acute wounds at the wound edge (Vaalamo et al., 1996).

In wounds, various MMPs are expressed by keratinocytes, fibroblasts, endothelial cells and by inflammatory cells; neutrophils and macrophages, in particular. Migrating keratinocytes, in early wounds, express MMP-1, MMP-9, MMP-10 (Saarialho-Kere, 1993; Salo et al., 1994; Vaalamo et al., 1996), as well as TIMP-1. However, the non-migratory proliferating cells at the wound margin express MMP-3 and TIMP-1 and -3. Difference in the MMP expression is probably due to different roles of these keratinocytes; migrating keratinocytes degrade provisional matrix forming a pathway for migration, whereas keratinocytes distal to the wound edge detach from the BM in order to permit motility. In the wound area MMPs-1, -2, -3, -13 and MMP-14 are produced by dermal cells (Ravanti and Kähäri 2000).

MMP-1 is induced in basal epidermal cells (keratinocytes), in response to injury, as the cells remove the BM and contact type I collagen in the underlying dermis (Saarialho-Kere, 1993). Only basal keratinocytes in contact with dermal type I collagen express MMP-1, and this inductive response is specifically controlled by the collagen-binding integrin α2β1, which also directs secretion of the enzyme to the points of cell–matrix contact (Pilcher et al., 1997). MMP-1 is also abundantly expressed by various types of stromal cells (macrophages, fibroblasts and endothelial cells) in the granulation tissue (Stricklin et al., 1993). MMP-1 mRNA and protein is also detected at the epithelial edge of chronic venous and decubitus ulcers (Saarialho-Kere et al., 1992, 1993). MMP-13 is expressed only in chronic wounds by stromal fibroblasts at the bottom of dermal granulation tissue (Vaalamo et al., 1997), whereas acute dermal wounds lack this enzyme. MMP-13 is expressed by fibroblasts in acute gingival wounds, suggesting a role as an effective remodulator of collagenous granulation tissue resulting in scarless wound healing (Ravanti et al., 1999).

Gelatinases have previously been shown to be differentially expressed and regulated during wound healing. MMP-2 is expressed by fibroblasts in both resting and healing human oral mucosa and skin (Oikarinen et al., 1993; Salo et al., 1994), but it is absent from the wounded epithelium, nor it is expressed by the dermal cells of the wound margin (Saarialho-Kere et al., 1993; Salo et al., 1994). MMP-2 is believed to participate in the long-term remodelling of the dermis. MMP-9 is detected in basal and suprabasal keratinocytes in nonwounded oral mucosa (Salo et al., 1994), however, it is not found in normal skin (Pyke et al., 1992). MMP-9 expression by basal keratinocytes is induced in human suction blisters (Oikarinen et al., 1993) as well as in granulation tissue of oral wounds (Salo et al., 1994). Induction of MMP-9 after injury is rapid, reaching the highest activity at 4-5 d after injury, but is later declined (Tarlton et al., 1997). Based on this finding, MMP-9 is thought to play a role in re-epithelialization. Eleveted amounts of gelatinases are found in chronic wound fluids, indicating that excess of these enzymes may disturb wound healing (Wysocki et al., 1993; Bullen et al., 1995). Especially, elevated MMP-1 and MMP-8 activity, possibly derived from inflammatory cells, is often associated with the non-healing state of chronic wounds (Nwomeh et al., 1999; Trengove et al., 1999; Ågren et al., 2000).

MMP-3 mRNA is expressed in chronic and in 2 d samples of normally re-epithelializing wounds by basal keratinocytes adjacent to, but distal from the migrating front as well as by the stromal cells (Saarialho-Kere et al., 1994; Vaalamo et al.,1996). MMP-10 is produced only by

keratinocytes at the migrating front and seems to co-localize with MMP-1 and uPA expressing cells (Vaalamo et al., 1996). Thus, MMP-10 appears to be critical for the migration, whereas MMP-3 could play a role in the remodelling of newly formed BM and wound matrix. MMP-11 expression has been shown in late stages of cutaneous wound healing in areas of inflammatory fibrosis (Wolf et al., 1992).

MMP-12 is expressed by stromal macrophages in acute dermal wounds and in chronic ulcers (Vaalamo et al., 1999). MT1-MMP expression is induced in dermal wounds and its role as an activator of cell surface proMMP-2 is assumed (Okada et al., 1997; Madlener et al., 1998). Of the MMPs expressed in human dermal wounds *in vivo*, MMP-3, -9 and MMP-12 may play a role in controlling the new blood vessel formation as they all are able to convert plasminogen to angiostatin, which is a potent anti-angiogenic factor (Patterson and Sang, 1997; Dong et al., 1997; Lijnen et al., 1998).

2.3.5. MMP inhibition in wound healing

Inhibition of MMPs has generally been shown to delay acute wound re-epithelialization in most experimental studies. However, Batimastat and Marimastat seemed to have no effect on acute wound healing, but only in sutured surgigal wounds (Pilcher et al., 1999). GM6001 (Ilomastat) treatment, was shown to increase wound strenght without affecting collagen content in rats (Witte et al., 1998). GM6001 has been shown to reduce inflammatory response, decrease type I collagen gene expression and upregulate MMP activity. It also reduced TNF-α, but induced TGF-β wound fluid levels in a sponge granulomas model (Witte et al., 1998). In human suction blisters and in acute cutaneous wounds, re-epithelialization was delayed with GM 6001 treatment (Ågren et al., 2001; Lund et al., 1999). In human suction blisters, GM6001 upregulated stromal MMP-1 and MMP-2 expression and epithelial MMP-9 expression. In plasminogen deficient mice, GM6001 treatment re-localized the MMP-2 expression from wound stroma into the leading edge of migrating keratinocytes (Lund et al., 1999) and overall MMP expression was upregulated.

2.3.6. Growth factors and cytokines and their regulation by MMPs in wound healing

Platelet derived growth factor (PDGF) is released from the degranulating platelets, and was the first growth factor shown to be chemotactic for cells migrating into wound, such as neutrophils, monocytes and fibroblasts. PDGF enhances fibroblast proliferation as well as their ECM production. PDGF stimulates fibroblasts to contract collagen matrices, affecting wound

contraction, and induces myofibroblast transformation (see Werner and Grose, 2003). Also several other growth factors and cytokines participate in wound healing (Table 2). They do that by inducing macrophage (e.g. TGF-β, IL-6, IL-8, IL-10) and neutrophil infiltration (e.g.TGF-β, IL-10), re-epithelialization (e.g. FGFs, TGF-α, EGF, IGFs, IL-6, GM-CSF), angiogenesis (e.g. VEGFs, FGF, HGF, GM-CSF), matrix deposition (e.g. FGF, IGF, TGF- β), fibroblasia (PDGF, TGF- β, IGFs) and scarring (e.g. TGF-β, IGF-1, IL-6 and IL-10) (see Werner and Grose 2003). Various growth factors and cytokines (EGF, TNF-α, IL-1β, bFGF, PDGF, IL-6 and TGF-β), which participate in wound healing, affect also MMP expression and activation or are substrates for them (see also chapter Transcriptional regulation of MMPs).

Table 2. Cytokines and growth factors which are substrates for MMPs and their role in wound repair.

Cytokine/Growth factor targeted	MMP	Source of the growth factor	Major effects in wound
Epidermal growth factors EGFs	MMP-3 and	Platelets	Epidermal and mesenchymal regeneration
Epidermal growth factor EGF and Heparin binding EGF-like growth factor	MMP -7	Macrophages	Pleiotrophic cell motility and proliferation
Fibroblast growth factors (FGFS)	MMP-1 and	Macrophages,	Wound vascularization and fibroblast
Basic FGF (b-FGF)	MMP-3	endothelial cells	proliferation
Transforming growth factors TGF-β	MMPs-2, -3,	Platelets and	Fibrosis and increased tensile strength
	-7 and	macrophages	Epidermal cell motility, chemotaxis of
	MMP-9		macrophages and fibroblasts, ECM synthesis and remodelling
Other cytokines	MMP-9	Epidermal cells,	Angiogenesis and increased vascular
Vascular endothelial growth factor (VEGF)		macrophages	permeability
Tumor necrosis factor alpha (TNF-α)	MMPs -1, -	Neutrophils,	Pleiotropic expression of growth factors,
	2, -3, -7, -9,	keratinocytes,	inflammatory phase in wound healing
	-12, -14 and	Langerhans cells,	
	-17	mast cells	
Interleukin-1 (IL-1)	MMP-2, -3,	Neutrophils,	As TNF-α
	and - 9	Fibroblasts	Re-epithelialization and granulation tissue
Insulin like growth factor-1 (IGF-1)	MMP-1, -2,-	epidermal cells	formation
	3, -9 and -11		

(Modified from Ravanti and Kähäri, 2000; Boss and Kapsenberg, 1993; Feiken et al., 1995; Hojilla et al., 2003) 2003).

2.4. CANCER

2.4.1. Tumorigenesis and invasion

Cancer may be thought as a disease where abnormalities in genes result in gain-of- function oncogenes or loss-of-function tumor-suppressor genes. Together with other inducers these mutations cause failure to regulate proliferation, differentiation, cell death, and expression of many cell-type-specific functions properly, and result in an altered phenotype of cell and eventually cancer. Cell adhesion provides a physical restriction that inhibits cell division when cell is in contact to the neighbouring cell, a process known as cell contact inhibition. The loss of cell adhesion is a key feature of transformation. Even if abnormal cell proliferation is necessary for tumorigenesis, proliferation of cells is not a cause of tumor development in itself. In fact, tumor-stroma interactions, namely communication between matrix components, fibroblasts, endothelial and inflammatory cells (Iozzo, 1995) are in central role for tumorigenesis. In a way, tumors may be viewed as wounds that do not heal (Wernert, 1997), because as in wound healing, inflammation may arise in malignancies as part of the normal host response. Inflammatory cells, like macrophages, neutrophils and mast cells, of a developing neoplasm facilitate genomic instability, promote angiogenesis and produce chemokines and cytokines that induce or inhibit MMP transcription or activation and can influence tumor development and its microenvironment (Coussens and Werb, 2001, 2002).

In order to metastasize, cancer cell must be able to detach from the primary tumor, penetrate BM and degrade its way through ECM. Invasion of malignant epithelial cells requires altered interaction between cells and the surrounding matrix. Integrins are known to be essential in cell migration during wound healing and cancer invasion (Heino, 1996), not only by mediating the cell movement but also by regulating the expression of MMPs (see Ivaska and Heino, 2000). MMPs are typically present at the invasive front promoting metastases, but MMP levels can be elevated already in the early stages, mediating the ECM and BM degradation and establishing the microenvironment that promotes tumor growth (Overal and Lopez-Otin, 2002). Metastasis may occur via lymphatic vessels and then malignant cell must survive the circulation and be able to extravasate, and proliferate as a secondary colony in a new environment, respond to growth factors, induce angiogenesis and evade host defences (Ellis and Fidler, 1996; McCawley and Matrisian, 2000).

2.4.2. Pre-malignant and malignant lesions of the skin and oral mucosa

Keratoacanthoma (KA) is a common benign squamous neoplasm most likely derived from hair follicle cells on sun exposed skin (Schwartz, 1994). They have a rapid growth phase for the first 4-8 weeks and a possible spontaneous self-induced regression after 3-6 months (Strieth et al., 2002). Although KAs are benign, fully developed tumors have a capacity to proliferate that overlaps considerably with that of conventional, well-differentiated squamous cell carcinoma (SCC) (LeBoit, 2002). Differential diagnosis between KAs and well-differentiated SCCs, based on clinical and histomorphological data, is problematic and there are many reports on lesions classified as KAs that have metastasized (Clausen et al., 2002). One malignancy-associated criterion is enhanced angiogenesis with increased microvessel density (Strieth et al., 2002). Furthermore, differential expression of adhesion molecules, such as E-cadherin, VCAM, ICAM and syndecan-1, has recently been reported to distinguish KA from SCC (Papadavid et al., 2001; Mukunyadzi et al., 2002; Melendez et al., 2003). Staining pattern for p53 resembles that of grade I SCCs, but they frequently show chromosomal aberrations that differ from those found in SCC (Clausen et al., 2002). Due to the lack of clear diagnostic and prognostic criteria to distinguish between KA and SCC, all KAs are currently surgically excised (see Strieth et al., 2002).

Basal cell carcinoma (BCC) is the most common malignancy in Caucasian people. The BCC incidence of Europe, USA and Australia increases by 3-6 % each year (Armstrong and Kricker, 1995). Exposure to ultraviolet radiation is the main causative factor in the pathogenesis of BCC (Kricker et al., 1994). Also ionizing radiation, chemical carcinogens (e.g. arsenic), and possibly infection with human papillomaviruses, have been associated with BCC development. BCCs are typically locally destructive but have a very limited potential to metastasize. However, people who have this condition are at high risk of developing further BCC and other malignancies (Ramsey, 2001). BCC is considered to arise from multipotential cells within the basal layer of the epidermis or follicular structures and can develop in both a hereditary and sporadic fashion. The histological features of BCC include normal basal keratinocyte resembling cells forming tumor islands, which are surrounded by fibrous stroma and peritumoral cystic space. Different kinds of growth patterns divide BCCs into subtypes such as superficial, nodular, and sclerosing types (Ramsey, 2001).

Squamous cell carcinoma (SCC) is a malignant tumor of keratinocytes of the spinous layer of the epidermis. SCC of the skin is the second most common type of skin cancer (Bernstain et al.,

1996), accounting for twenty percent of all cutaneous malignancies and frequently arising on the sun-exposed skin of the middle-aged and elderly (Johnson et al., 1992). The primary cause of cutaneous SCC is cumulative lifetime sun exposure (especially UVB). Five to ten percent of SCCs arise from sun induced precancerous lesions, actinic keratoses. Immune suppression, chronic inflammation, ionizing radiation and human papillomavirus (HPV) infection may lead to the development of SCC. SCC is highly invasive and invasion occurs through degradation of BM and ECM. There are currently no reliable prognostic tissue or serum markers in routine use to predict whether SCC has metastasized at the time of diagnosis (Helliwell et al., 2001) The tendency to metastasize lymphatically seems to be independent of the primary tumor size, pathological grading or other parameters (Werner et al., 2002). The prognostic risk factors include, diameter, depth of invasion, histologic differentiation, rapid growth, anatomic site, immune suppression, and etiology, so that tumors arising from scars and chronic ulcers tend to be aggressive (Johnson et al., 1992).

Squamous cell carcinoma of the oral cavity (SCC), a tumor of oral stratified squamous epithelium, is the sixth most common cancer worldwide (Silverman et al., 1998) and is similar to SCC of other organs. It occurs most commonly in middle aged and older individuals. Oral cancer can be divided into three categories: carcinomas of oral cavity, carcinomas of lip vermillion and carcinomas arising in oropharynx (Neville and Day, 2002). The common carcinogens causing oral SCC are tobacco and alcohol, but lip tumors are also associated with chronic sun exposure. Patient who are both heavy drinkers and heavy smokers have hundred times greater risk for developing an oral malignancy (see Neville and Day, 2002). The five-year survival rate is still about 50 %, although advances in surgery, chemotherapy and radiation as well as in diagnostics have taken place (Silverman 2001).

Verrucous carcinoma (VC) of the oral cavity (also called oral florid papillomatosis), is a rare low grade variant of oral SCC. This type of tumor invades the underlying connective tissue, and is associated with destruction of collagenous matrix. VC is slow-growing, well-differentiated and causes metastases very late (Neville and Day, 2002). However, some lesions that arise from proliferative verrucous leukoplakia may undergo dedifferentiation into an aggressive oral SCC (Hansen et al. 1985). *Verrucous hyperplasia* (VH) often preceeds VC in smokers. Clinical appearances of VC and VH are the same and thus the diagnosis practically requires recurrent biopsies. VH, however, does not histologically extend below the BM level of the surrounding normal epithelium (Neville and Day, 2002).

2.4.3. Malignant transformation of chronic wounds

In chronic leg ulcers, the risk of squamous cell cancer (SCC) is increased (Baldursson et al., 1993, 1995, 1999). Wound carcinogenesis is unpredictable and often undiagnosed for long periods of time, so that the duration of ulcer before the diagnosis of cancer can be 25 years (Baldursson et al., 1995). The molecular inducers of this type of tumorigenesis are not known, but the long duration of increased cell division in and around the ulcer is thought to be one possibility (Mekkes et al., 2003). Proliferation in chronically inflamed tissue, predisposes humans to carcinoma of the skin and other organs (Coussens and Werb, 2002). The common cause of cutaneous SCC, ultraviolet radiation seems to be an unlikely explanation, because of the assumed limited exposure of ulcer sites to UV (see Baldursson et al., 2000a). Furthermore, human papilloma virus seems not to be the cause of SCC in venous leg ulcers (Baldursson et al., 2000a).

2.4.4. MMPs in tumors

MMPs can regulate the tumor microenvironment and their expression and activation is increased in almost all human cancers compared with normal tissue. MMPs are expressed in tumors by tumor cells but even more often by surrounding stromal cells and inflammatory cells. There is no single MMP consistently overexpressed in every tumor type, or a consistent pattern of MMP expression across a variety of human cancers. At least MMP-1, -2, -3, -7, -9, -10, -11, -13 and -14 are frequently overexpressed in many human tumors (see Kerkelä and Saarialho-Kere, 2003).

The increased MMP expression in tumors is most likely due to transcriptional changes which result from activation of oncogenes or loss of tumor-suppressors (see Egeblad and Werb, 2002). The initial observation of the importance of MMPs in cancer biology was that the ability of tumor cells to invade the surrounding tissue correlated with increased MMP levels and many MMP family members have been isolated from tumor cell lines (Liotta et al., 1980). In addition to MMPs´ ability to degrade ECM, initiating the development of metastasis, their role in tumorigenesis is much more complex. MMPs promote both primary tumor growth and metastatis by activating growth factors, by inactivating growth-factor binding proteins, by cleaving receptors involved in cell adhesion, unmasking cryptic sites of interaction, and by acting on ECM components or other proteins to uncover hidden biologic activities, which can affect cell proliferation, migration and angiogenesis. MMPs are thereby involved in creating an environment suitable for tumor progression or by releasing mitogenic

molecules from matrix that are important in peritumoral ECM (Stetler-Stevenson and Yu, 2001; Overall and Lopez-Otin, 2002).

MMPs regulate proliferation and apoptosis

MMPs generate growth-promoting signals by different ways. First, MMPs promote cancer cell proliferation by releasing cell-membrane bound precursors of some growth-factors, like TGF-α. Then, peptide growth-factors that are sequestered by ECM proteins, like IGF, become bioavailable after MMP degradation. In addition, MMPs regulate proliferative signals indirectly through integrins but may also reduce cancer cell growth by activating TGF-β (see Egeblad and Werb, 2002). MMPs regulate apoptosis e.g. by generating pro-apoptotic molecules like FAS ligand, a trans-membrane stimulator of the death receptor FAS or TNF-α. MMP-3 regulates apoptosis possibly by degrading laminin. MMP-7 releases membrane bound FAS ligand (see Egeblad and Werb, 2002). MMP-7 can also inhibit apoptosis by cleaving pro-heparin-binding epidermal growth factor –like growth factor (HB-EGF) to generate its mature form. This mature HB-EGF promotes cell survival by stimulating ERBB4 receptor tyrosine kinase. MMP-11 inhibits cancer-cell apoptosis by releasing IGF (see Egeblad and Werb, 2002).

MMPs regulate angiogenesis

Angiogenesis is important for tumorigenesis, because for continuous growth, tumors require nutrient and oxygen supply. Blood vessel growth is stimulated by e.g. VEGF or bFGF and inhibited by e.g. thrombospondin-1, angiostatin and endostatin. Tumor´s ability to promote angiogenesis is one of the early events in the transition of a tumor from the pre-neoplastic stage to neoplastic phenotype (Hanahan et al., 1996). Studies of various human cancers have also shown a correlation between an increased number of tumor blood vessels and poor prognosis (Weidner, 1995). MMPs participate in tumor angiogenesis by allowing endothelial cell invasion through BMs to form new blood vessels by cleaving type I collagen (Seandel et al., 2001). They also regulate endothelial cell attachment, proliferation, migration and growth, either directly or by the release of growth factors (Foda and Zucker, 2001). The direct role of some MMPs in angiogenesis is known. MMP-2 participates in angiogenesis by cleaving type IV collagen. MMP-9 acts by increasing the bioavailability of VEGF and MMP-14 might have a role in fibrin degradation around the new vessels. Synthetic and endogenous MMP inhibitors reduce angiogenic responses in animal models, indicating the essential role of MMPs (Stetler-Stevenson, 1999). Paradoxically, MMPs are also responsible for generating the potent

angiogenesis inhibitors like angiostatin and endostatin. Angiostatin is an NH_2-terminal fragment of plasminogen and it effectively inhibits endothelial cell proliferation. MMPs-2, -3, -7, -9 and -12 are all capable of cleaving plasminogen to angiostatin, but MMP-12 is the most potent one (Patterson et al., 1997; Cornelius et al., 1998; Egeblad and Werb, 2002). Endostatin, which is a C-terminal fragment of the basement membrane collagen XVIII, is probably produced by MMP-3, -9, -12, -13 and -20 (Hanahan et al., 1996; see Hojilla et al., 2003). Endothelial cells can produce at least MMP-1, -2, -9, -19 and MT1-MMP, but then again, inflammatory cells and fibroblasts express many MMPs and also contribute to angiogenic phenotype.

MMPs regulate invasion and metastasizing

Invasion of malignant epithelial cells requires altered interaction between cells and the surrounding matrix. During metastasis cells have to pass through the BM and other ECM components in order to invade the surrounding stroma and then enter the blood vessels or lymphatics, extravasate and eventually establish new colonies. A direct correlation between MMP expression and the invasive phenotype of human tumors has been detected in many tissues including oral squamous cell cancers (see Stetler-Stevenson, 2001; Nelson et al., 2000). Generally, the expression of high levels of multiple MMP family members correlates positively with tumor aggressiveness, including increased invasive capacity, metastasis and poor patient survival. Invasion is facilitated by inducing cell migration. Ln-5 cleavage by MMP-2 and MMP-14 reveals a cryptic site of this molecule and induces cell motility (Giannelli et al., 1997, Koshikawa et al., 2000). The ability of MMPs to process cell adhesion molecules, e.g., cadherins contributes to the initiation of epithelial-to-mesenchymal transition (EMT), which is a key differentiation event during cancer progression (Birchmeier et al., 1996). MMP-3 and MMP-7 trigger EMT by cleaving E-cadherin (Noe et al., 2001). The released fragment of E-cadherin promotes tumor cell invasion. During invasion MMP-2, -9 and -14 are localized to specialized cell membrane invadopodia on migrating cells. From there, they communicate with the surrounding tissue, MMP-2 by binding to integrins or other MMPs and MMP-9 by binding to CD44 (see Egeblad and Werb, 2002).

2.4.5. MMP gene targeting in mice showing effects on wound healing and skin cancer

Evidence for the critical role of MMPs are provided with transgenic and knock-out mice. Knock-out mice for MMPs-2, -3, -7, -8, -9, -11, -12 and -14 as well as for TIMPs-1, -2 and -3 have been introduced (Egeblad and Werb, 2002). In plasminogen-deficient mice, Rømer et al.

(1996) demonstrated impaired healing of wounds although epithelialization was not totally blocked. Using the same animal wound model, Lund et al. (1999) were able to completely block healing by systemic administration of the synthetic MMP inhibitor GM 6001. MMP-9 - deficient mice have defects in remodeling of ECM at the epithelial BM zone, in particular, due to failure to effectively remove the fibrin(ogen) provisional matrix (Mohan et al., 2002).
Evidence for the critical role of MMPs also in tumorigenesis has been provided with transgenic and knock-out mice. Mice overexpressing MMP-1 or MMP-7 are characterized by hyperproliferative skin disease and increased cancer susceptibility (see Egeblad and Werb, 2002). Overexpression of MMP-3 and MMP-7 in transgenic mice results in enhanced tumorigenesis in a breast cancer model (Sternlicht, 1999; Rudolph-Owen et al., 1998) Furthermore, MMP-deficient mice (MMP-2, -9 and -14) demonstrate defective angiogenesis (Itoh et al., 1998; Vu et al., 1998). However, there are very few studies on transgenic mice models indicating effects on skin carcinogenesis (see Table 3).

Table 3. MMP gene targeting in mice showing effects on skin carcinogenesis or wound healing.

MMP gene	Phenotype	Reference
Overexpression of MMP-1	Enhanced tumor formation in skin with chemical inducers	D´Armiento et al., 1995
Lack of MMP-8	Increased incidence of skin tumors in male mice	Balbin et al., 2003
Lack of MMP-2	Reduced tumor progression	Wilson et al., 1997
Lack of MMP-7	Reduced tumor progression Inability to repair mucosal epithelial wounds	Itoh et al., 1998 Dunsmore et al., 1998
Lack of MMP-9	Reduced keratinocyte proliferation, decreased incidence of invasive tumors	Coussens et al., 2000
Lack of MMP-3 (stromelysin-1)	Impaired wound contraction Less chemically induced tumors and reduced tumor cell implantation	Bullard et al., 1999 Masson et al., 1998
Overexpression of TIMP-1	Delayed wound healing, retarded migration of epithelial cells	Salonurmi et al., 2003

3. AIMS OF THE STUDY

The principal aim of this study was to examine the role of matrix metalloproteinases in wound healing and in epithelial cancers as well as in malignant transformation of chronic wounds. We aimed to study the underlying mechanisms for delayed epithelialization in chronic wounds. We wanted to investigate if the expression patterns of certain MMPs could serve as molecular markers to predict tumorigenesis of pre-malignant lesions, and on the other hand, the invasiveness of tumors.

In order to develop targeted MMP inhibitors for therapy, the MMPs been expressed in various types and stages of cancer or in chronic wound must be carefully identified. The specific aims of the study were as follows:

I) To investigate the role of MMPs on acute and chronic wound healing by combining enzyme activity assays, immunohistochemistry and in situ hybridization techniques.

II) To study the influence of MMPs and serine proteinases in epidermal wound healing ex vivo using a synthetic broad-spectrum MMP inhibitor (BB3103) or a serine proteinase antagonist (aprotinin).

III) To investigate whether the pattern of epithelial MMP expression is associated with development of SCC from pseudoepitheliomatous hyperplasia of chronic wounds. Keratoacanthomas were studied in parallel as a model of rapidly growing, but still benign hyperproliferative tumors, resembling SCCs.

IV) To study whether the recently discovered MMP-19 is induced in the epithelium during remodeling associated with either wound repair or cancer invasion.

V) To investigate whether the pattern of MMP, $\alpha v\beta 6$ integrin or laminin-5 expression contributes to the differences in the biological behavior of oral SCC and VC. Particular emphasis was on the newly discovered MMP-19 and -26 that have not previously been studied in oral cancer.

4. MATERIALS AND METHODS

4.1. Tissue samples

All studies were approved by the Ethics Committees (KF 01-072/94; TIA 4014; LU 508-99). Informed consent was obtained from participating individuals for all procedures.

Chronic wounds (I-IV)

Formalin-fixed, paraffin-embedded archival specimens were obtained from the Departments of Dermatopathology and Plastic Surgery, Helsinki University Central Hospital and University of Turku. For publications I and III all together 64 different *chronic ulcers* (venous, decubitus, rheumathoid arthritis or diabetic origin) were included. Biopsies from chronic ulcers included ulcer edge and base and surrounding intact skin.

Acute wounds (I-IV)

For adequate comparison of acute and chronic wound healing, 3 *standardized wound types* in humans were studied: [1)] *partial-thickness* wounds (n=6), 0.4 mm in depth, [2)] *full-thickness* wounds (n=12), 8-mm in depth and [3)] *suction blisters* with intact roofs (n=4) (Vaalamo et al., 1999). In addition, clinically *well-granulating ulcers* (n=6) obtained from the Department of Plastic Surgery, Helsinki University Central Hospital, were used in the study.

Full thicknes wounds were made on the anterior thigh of venous leg ulcer patients undergoing a pinch graftting procedure (Vaalamo et al., 1996). The pinch graft donor area were excised with a knife daily on ds 1 to 6 and on d 9 and fixed in 10% formalin (Vaalamo et al., 1999). Suction blisters were induced on the abdominal skin using Dermovac® blistering device (Kiistala, 1968). Biopsies from excised skin on the d-0 dermatome wounds and 6- mm punch biopsies of wound margin on post-wounding day 1 to 7 included surrounding intact skin. Each biopsy was cut into half, one half was fixed in 10% formalin for 24 h and the other half was frozen at -80°C. Variations over the time were also investigated by taking wound margin biopsy specimens after the first and the last sampling of wound fluid.

Ex vivo human skin wound models (I-II)

In publication I, 5- mm biopsies (n=23) of normal skin from different parts of the body, were cultured in 35-mm dishes in DMEM (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 5 % fetal bovine serum and 1% penicillin/streptomycin (Rechardt et al, 2000). At 24, 48 and 72 h the tissue samples were fixed in 10% formalin and prosessed for

paraffin embedding.

The *ex vivo* human skin wound model used in publication II, was originally developed by Kratz et al. (1994). Skin was donated by healthy female patients under 30 y old, undergoing elective mammary reduction plasty. Circular wounds were made using a sterile 3-mm trephine within 4 h after removal. The individual wounds (0,5 mm in depth) were excised using a sterile 6-mm trephine with the wound in the center. Each specimen was incubated submerged in 1 ml of complete culture medium (DMEM, 4.5 mg glucose/ml, 2 mM L-glutamine, 100 μg penicillin/ml, 100 U streptomycin/ml and 10% heat-inactivated mycoplasma-screened fetal calf serum (FCS, Gibco)) in a 24-well plate at 37°C in a humidified atmosphere of 5% CO_2/air. The calcium concentration was determined to 1.8 mM and the medium was exchanged every other day. To study cell proliferation, 5-bromo-2'-deoxyuridine (BrdU; Sigma) was added at 10 μM 24 h prior to termination d 7, at which time medium was replaced with 1 ml 10% buffered formalin per well.

Conditioning of skin specimens with the wound site (II)

Separate skin explants, excised from the same donor, were conditioned in parallel to the ones described above. At days 0 and 7, the explants were conditioned for 10 h in serum-free DMEM alone, or serum-free DMEM supplemented with 10 μM BB-3103, 10 or 100 μg/ml aprotinin. The water-soluble, broad spectrum and hydroxamic acid-based synthetic peptide MMP inhibitor BB-3103 (M_r 475.6 Da; British Biotech Pharmaceuticals Ltd., Oxford, UK) was used at 10 μM. IC_{50}-values indicating the MMP inhibitory profile of BB-3103 were 2 nM for MMP-1, 10 nM for MMP-2, 30 nM for MMP-3, 20 nM for MMP-7, 7 nM for MMP-9, 4 nM for MMP-13 and the IC_{50} for TACE (TNF-α converting enzyme) was 800 nM as provided by the manufacturer. The complete culture medium served as controls in the BB-3103 series. Cytotoxicity of BB-3103 on human epidermal keratinocytes was assessed using the MTT assay essentially as described by Newby et al. (2000) using a kit from Roche (Cat. no. 1465007).One part of 1.4 mg aprotinin per ml saline or 0.14 mg aprotinin per ml saline was mixed with 13 parts of complete culture medium and complete culture medium diluted with corresponding volume of saline served as control to the aprotinin-treated groups.

After conditioning, the media were collected and skin samples were incubated for a further 30 min at ambient temperature with Complete™ (1697498; Boehringer Mannheim GmbH, Mannheim, Germany) proteinase inhibitor cocktail containing 1 mM ethylenediaminetetraacetic acid and supplemented with 1 μM pepstatin (253286; Boehringer Mannheim) to inhibit serine,

cysteine, aspartic and metalloproteinases present in the tissue. The conditioned media and explants were stored at -70°C until analyzed.

Pre-malignant lesions and skin cancers (III-IV)

Skin samples included benign lesions of KAs (n=12) and premalignant tumors of solar keratosis (n=3). Biopsies from the following malignant epidermal were collected: BCC (n = 16), Bowen´s disease (n=4), SCCs (n=24, grades I-IV) and SCCs arisen in chronic wounds (n=9) (Baldursson et al., 1999). All samples were obtained from the Universities of Helsinki and Turku. Diagnoses were made by two experienced pathologists (LJ and SS).

Oral tissues (V)

Formalin-fixed, paraffin embedded samples of oral SCC (n=15), VC (n=15), gingival hyperplasia (n=13), and VH (n= 16) were obtained from the University of Helsinki, Faculty of Dentistry and from the University of British Columbia, Department of Oral Biological and Medical Sciences, Vancouver, Canada. The diagnoses were made by two experienced oral pathologists (JH and LZ).

4.2. Immunohistochemistry (I-V)

Immunostaining of the sections was performed by the avidin-biotin-peroxidase complex technique using Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA), Zymed Kit (Zymed Laboratories Inc., San Francisco, CA) or DAKO Kit (DAKO, A/S, Glostrup, Denmark). Samples were deparaffinized, dehydrated and endogenous peroxidase was blocked with 0,3-0,6% hydrogen peroxidase. Normal serum was used to block non-specific staining. Sections were pre-treated with trypsin (10 mg/ml) or micro-waving in citrate buffer (pH 6.0). Primary antibodies (see Table 4.) were incubated in humidified atmosphere for 1-3 h at 37^{o}C or overnight at 4^{o}C. Diaminobenzidine was used as chromogen and Harris hematoxylin as the counterstain, as described in detail (Saarialho-Kere et al, 1993). Controls were performed with mouse immunoglobulins or with rabbit preimmune serum. The Apoptaq in situ apoptosis detection kit (Oncor Inc., Gaithersburg, MD), a TUNEL technique, was used to detect fragmented DNA of apoptotic cells as described elsewhere (Airola et al., 1997).

Table 4. Antibodies used in immunohistochemistry.

Antibody	Source	Dilution /pre-treatment
MMP-2	IM33L,Calbiochem, Cambridge,MA	1:200 /trypsin
MMP-7	IM40L, Calbiochem	1:50
MMP-8	IM38L, Oncogene, Cambridge, MA	1:10 / trypsin
MMP-9	GE-213, Diabor, Oulu, Finland	1:300 /trypsin
MMP-13	IM64L, Oncogene	1:40
MMP-14	IM42L,Oncogene	1:40
MMP-19 (IV)	RDI-MMP-19abR, Flanders, NJ and	1:70
MMP-19 (III-V)	PC374, Oncogene	1:40
MMP-26 (V)	A gift from Prof. Keichi Isaka (Isaka et al., 2003)	1:200
γ2 chain of LN-5	A gift from Prof. Karl Tryggvason (Pyke et al, 1994).	1:600
$p16^{INK4}$	G175-405; BD Biosciences	1:350
E-cadherin	HECD-1, Zymed, San Francisco, CA	1:60-1:100
Selectin-1	CD138, Clone B-B4, Oxford Biotechnology, JE	1:400
Col IV	M785, Dako, Glostrup, Denmark	1:75 /trypsin
TNF-α	Rockland, Gilbertsville, PA	1:100
Ki67	A047, Dako	1:200
p63	Clone 4A4, Neomarkers, Fermont, CA	1:200
CD31	PECAM-1, Clone JC/70A; Dako	1:10
BrdU	M 744; Dako	1:100

4.3. RNA probes (I, III-V)

The production and specificity of the antisense and sense human MMP-1, -3, -10, -12, –13, and 14 probes have been described previously (Rechardt et al, 2000; Kerkelä et al.,2001; Saarialho-Kere et al., 1993; Vaalamo et al., 1998; Pender et al, 2000). As a control for nonspecific hybridization, sections were hybridized with ^{35}S-labeled sense RNA from a bovine tropoelastin cDNA. The validity of this probe as a negative control has been confirmed by Northern (Prosser et al, 1989) and by in situ hybridization (Saarialho-Kere et al, 1992). A 2.6 kb cDNA containing the entire coding region for human β6 integrin was subcloned into pCDNAIneo plasmid (Weinacker et al., 1994). The construct was linearized with PvuII and a 450 bp antisense probe was obtained using sp6 polymerase. Linearization was done with SspI and transcription with T7 gave a sense probe of equal length. The probe was sequenced.

4.4. In situ hybridisation (I, III-V)

In situ hybridization was performed on 5-µm sections as described in detail (Prosser et al., 1989). Sections were pre-treated with 1 µg/ml of proteinase K and washed in 0,1 M triethanolamine containing 0,24 % acetic acid. Sections were hybridized with ^{35}S-labeled probes (3×10^4 cpm/ µl of hybridization buffer) at 50-55°C for at least 18 h in a humidified chamber. Slides were then washed under stringent conditions. RNAse A treatment was used to wash out the unhybridised probe. After 20-50 d of autoradiography, the photographic emulsion was

developed, and the slides were stained with hematoxylin and eosin. Samples previously positive for the studied mRNAs were used as positive controls. No signal was detected with the sense probes. The slides were analysed independently by two investigators.

4.5. Cell cultures (IV)

Primary human keratinocytes were isolated from normal adult skin obtained from reductive mammoplasties (Rechardt et al., 2000). Subcutaneous fat and deep dermis were removed, and the remaining tissuewas incubated overnight at 0.25% trypsin in solution A (Gibco BRL, Life Technologies). Following the incubation, keratinocytes were scraped off from the epidermis with a scalpel and suspended in Keratinocyte Growth Medium (KGM, Gibco), supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 μg/ml bovine pituitary extract (BPE) (supplied by the vendor), and containing 2% decalcified fetal calf serum (FCS). Keratinocytes were maintained in KGM supplemented with EGF and BPE, and passages 1 to 5 were used in the experiments. For immunostaining, primary keratinocytes (in both low and high Ca^{+2} KGM) were also cultured on Laboratory-Tek chamber slides (plastic or type I collagen coated) and immunostained using MMP-19 and Ki-67 antibodies as described for tissue samples.

HaCaT cells, an immortalized non-tumorigenic human adult epidermal keratinocyte cell line (Boukamp et al., 1988) and *A5 cells*, a ras-transformed tumorigenic HaCaT- derived cell line (Boukamp et al., 1990) were kindly provided by Prof. Norbert Fusenig (Deutsche Krebsforschungszentrum, Heidelberg, Germany). HaCaT and A5 cells were cultured in DMEM containing 10% FCS. *UT-SCC-7 cell line*, which forms squamous cell carcinomas in SCID mice (Ahonen et al., 2002) was established from metastasis of a cutaneous SCC at the time of operation in the Turku University Central Hospital (Servomaa et al., 1996) Cell lines were cultured in DMEM supplemented with 6 mM glutamine, non-essential amino acids and 10% fetal calf serum (FCS). The SCC cells were examined in subcultures 5 to 10 and were homogenous by visual inspection

4.6. Cytokines and growth factors (IV)

To study the regulation of MMP-19 expression primary keratinocytes were plated on 24-well tissue culture plates. 70-80% confluent cells were repeatedly washed with PBS and incubated overnight in KGM without supplements or FCS. After this, keratinocytes were treated with different cytokines or growth factors for 24 h (see Table 5.). All treatments were done in KGM

and in KGM with 1.8mM Ca^{2+}, without supplements or FCS. After 24 h total RNA was extracted from the cells. Untreated cells were used as a control.

Table 5. Cytokines and growth factors used in the study

Cytokine/ Growth factor	Source	Concentration
TNF-α	Sigma, St Louis, MO	10 ng/ml
IL-1β	Roche Molecular Biochemicals, Mannheim, Germany	5 ng/ml
TGF-β1	Sigma	1-5 ng/ml
EGF	Sigma	10 ng/ml
KGF	Sigma	10 ng/ml
HGF	Sigma	10 ng/ml
VEGF	R&D Systems, Minneapolis, MN	10 ng/ml
IGF-1	R&D Systems	100 ng/ml
IL-10	R&D Systems	10 ng/ml
IFN-γ	Promega, WI, USA	1 ng/ml
bFGF	Sigma	10 ng/ml
PMA	Sigma	10 ng/ml

4.7. Quantitative reverse transcription (RT)-polymerase chain reaction (PCR) (IV)

Total cellular RNA from the keratinocytes was extracted by using RNeasy miniprep-kit (Qiagen, Chatsworth, CA) according to the manufacturers´s instructions. RNA was then reverse transcribed to cDNA with TaqMan® Reverse Transcription Reagents (Applied Biosystems) and used as a template in PCR reaction. Real time quantitative PCR reactions were performed with the ABI PRISM® 7700 Sequence Detector System (Applied Biosystems) (Rechard et al., 2000). *PCR primers* and *MGB probe* for MMP-19 were designed using the computer program Primer Express (Applied Biosystems). Primers used for amplification were: forward 5´-GCTTCCTACTCCCCATGACAGT-3´, and reverse 5´-GGCTTCTGTAGGTACCCATATTGT -3´. The fluorogenic MGB probe (CCCGTGGACTACCTG) contained a reporter dye (FAM) covalently attached at the 5'end and a quencher dye (TAMRA) covalently attached at the 3'end. PCR amplifications were performed in a total volume of 20 µl, containing 5µl cDNA sample, 10 µl TaqMan® Universal PCR Master Mix (Applied Biosystems), 200 nM of each primer and 200 nM of fluorogenic probe. Pre-developed TaqMan® assay reagents for endogenous control human GAPDH labeled with VIC™ reporter dye (Applied Biosystems) were used for amplification of control gene. PCR was started with 2 min at 50°C and the initial 10 min denaturing temperature was 94°C, followed by a total of 40 cycles of 15 s of denaturing and 1 min of annealing and elongation at 60°C. The same reactions were done by conventional PCR with primers without fluorogenic probes. PCR products were analysed in 1% low melting point agarose gel in the presence of 5 ng/ml ethidium bromide under UV light.

4.8. Northern analysis (IV)

Total cellular RNA was isolated from cell cultures using the single step method. Aliquots of total RNA (6-20 μg) were fractionated on a 0.8 % agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane (Zeta-Probe®, Bio-Rad Laboratories, Richmond CA) and analyzed by Northern blot hybridization using cDNA probes. The probes were labeled with [α-^{32}P] dCTP by random priming using the RediprimeTM II Random Prime Labelling System kit (Amersham, UK). For hybridizations, a 1.5 kb MMP-19 cDNA (Pendas et al., 1997), a 2.0 kb human MMP-1 cDNA (Goldberg et al., 1986) and a 1.3 kb rat GAPDH cDNA were used as probes. [^{32}P]-cDNA/mRNA hybrids were visualized by autoradiography and quantitated by scanning densitometry.

4.9. Western analysis (II, IV)

Western analysis was used to assay the presence of MMP- 1, -2 and -14 protein in tissue extracts and the production of MMP-19 protein by primary epidermal keratinocytes. Tissue extracts were mixed with equal volume of 2 × sample buffer containing 50 mM 2-mercaptoethanol. The mixture was boiled for 5 min, fractioned by a 7.5% SDS-PAGE, and electroblotted onto nitrocellulose membranes (Bio-Rad). The cell culture medium was collected after 24 h of incubation, concentrated 15-fold with Amicon Ultra Centricon (30,000 MWCO; Millipore Corporation, MA), separated on 8.5% SDS-PAGE and transferred to a Hybond ECL filter (Amersham). After blocking all membranes in PBS containing 5% milk and 0.1% Tween-20, membranes were incubated overnight with mouse monoclonal antibodies against MMP-1 (1:5000; MAB1346; Chemicon), MMP-2 (1:1000; MAB3308; Chemicon), or against MMP-14 (1:1000; AB815; Chemicon) or with a monoclonal antibody against human MMP-19 protein (a kind gift from Dr. Carlos López-Otín, University of Oviedo, Oviedo, Spain) in a final concentration of 2 μg/ml for 1h at room temperature. The specific binding of primary antibodies was detected with peroxidase-conjugated secondary antibody diluted 1:1000 and visualized with ECL^{TM} (Amersham Pharmacia Biotech, Buckinghamshire, UK). Antibody against MMP-1 (AB806, Chemicon) was used as a control for MMP-19 .

4.10. Samples of wound fluids (I)

Wound fluids were collected from the dermatome wounds and from venous leg ulcers. Sterile hydrophobic polyurethane foam disks were applied to the wound and covered by a polyurethane film dressing (Tegaderm®, 3M). 2h after application, the collecting system was removed, wound cleansed with sterile saline (0.9% NaCl) and a new collecting system was applied and left on the wound for another 24 h at 1, 2, 3, 4, 5, 6 and 7 days postoperatively. In the case of the venous leg ulcer, compression bandages were applied during the whole collection period. Collection of wound fluid was performed at inclusion and then after 1, 2 and 3 weeks. The ulcer was treated locally with an inert, non-adhesive and absorbing dressing between the sampling periods. Wound fluids were filtered (0.45 μm) and kept at -80°C until analyzed by zymography.

4.11. Extraction of MMPs (I, II)

Skin samples (n=34) were thawed, weighed and shaken in 20/40 volumes of buffer containing 10 mM cacodylate-HCl pH 6.0, 1 M NaCl, 0.01% (vol/vol) Triton X-100, 1 μM $ZnCl_2$, 0.2 mg NaN_3 per ml and 1 μl of proteinase inhibitor cocktail (539134; Calbiochem®) per ml for 18 h at 4°C (Eeckhout et al., 1986). In a complementary experiment, extraction was carried out in the buffer devoid of the proteinase inhibitor cocktail. Wound tissue extracts were centrifuged, sterile filtered, and kept in supernatant at 4°C until analyzed by zymography within 24 h. Freezing of extracts was avoided due to activation of MMPs by freeze-thaw-cycles (unpublished data). Because the extraction method of Eeckhout et al. (1986) was originally optimized for bone tissues, the MMP levels of the extracts were compared with those extracted by a standard method on some wounded skin samples (Ågren et al., 1998). Tissues were homogenized in 20 volumes of 0.25% Triton X-100 and 10 mM $CaCl_2$ for 1 min on ice with Polytron® PT 1200 Cl (4962300; Buch & Holm, Herlev, Denmark) tissue grinder and centrifuged (Ågren et al., 1998). Protein contents were determined according to a modified Lowry assay (DC Protein Assay, 500 0112; Bio-Rad Laboratories, Hercules, CA). Standard curves were run with bovine serum albumin (Bio-Rad) (0.008-1.0 mg/ml) and absorbances read at 650 nm.

4.12. MMP activity analyses (I)

For quantitative analyses of MMP activities in tissue extracts, ELISA-type assay kits from Amersham Pharmacia Biotech were used for MMP-2 (RPN2631) and for MMP-9 (RPN2630). Specific antibodies were pre-coated onto a microtitre plate capture MMPs. Active MMP present activates the pro-detection enzyme, enabling it to cleave a chromogenic peptide

substrateresulting in p-nitroanilide detection molecule. Endogenous tissue MMP activities were measured without prior activation, total MMP activities were measured for MMP-2 after activation with 0.5 mM aminophenylmercuric acetate (APMA) for 1.5 h and for MMP-9 with 1 mM APMA for 2.5 h according to manufacturer's instructions. Standard curves with human MMP-2 (0.19-12 ng/ml) and MMP-9 (0.125-16 ng/ml) were run simultaneously. Absorbances were read at 405 nm.

4.13. Zymographic analyses of wound fluids, conditioned media and tissue extracts (I, II)

Latent and active forms of MMP-2 and MMP-9 were determined using 0.5 mg/ml gelatin (G-8150; Sigma) of a 7.5% or 10% SDS-PAGE. Thirty µl of tissue extracts, wound fluids or conditioned media were mixed with 10 µl of 4 × sample buffer (0.0625 M Tris-HCl pH 7.4, 2% (w/v) SDS, 10% (v/v) glycerol and 0.04 % (w/v) bromophenol blue. Aliquots of 25 µl were applied to each lane. A human MMP-2 (50 ng/µl)/MMP-9 (50 ng/µl) standard (19101670; Chemicon International Inc.,) was run in a parallel lane. After electrophoresis at 25 mA constant current, the gels were washed 3 times in 2.5% Triton X-100 and incubated in a buffer composed of 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 50 mM Tris-HCl pH 7.4, 0.1% Triton X-100 and 0.003 M NaN_3 for 18 h at 37°C in a closed container with continuous shaking. Zymograms were stained in 0.25% Coomassie Brilliant Blue R-250, 10% acetic acid and 45% methanol for 30 min and destained for 30 min in 20% acetic acid, 20% methanol, 17% ethanol and 0.6% diethylether (Kleiner and Stetler-Stevenson, 1994). Gels were mounted, dried and scanned. The intensity of the gelatin degradation bands was determined with a densitometer (Eagle Eye 2, Strategene Eagle Sight, Strategene Ltd., Cambridge, UK) and expressed in kpixels/inch^2.

To study if residual BB-3103 in samples *per se* interfered with detection of MMP activity by zymography, BB-3103 at 10 µM was added to d-7 conditioned media from control-treated wounds. Conditioned media with or without BB-3103 were then incubated for 30 min at room temperature followed by zymographic analysis. To visualize MMP activation by APMA treatment in activity assays, tissue extracts were incubated either with 1% DMSO alone or with 0.5 mM APMA in 1% DMSO for 1.5 h (MMP-2) and 1 mM APMA for 2.5 h (MMP-9) at 37°C and zymography was performed.

4.14. Cytokine and growth factor analyses of conditioned media (II)

Sandwich enzyme-linked immunosorbent (ELISA) assays were used to determine human tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1ß), epidermal growth factor (EGF), hepatocyte

growth factor (HGF), keratinocyte growth factor (KGF) and tumor growth factor-β1 (TGF-ß1) in incubation or conditioned media. The high sensitivity TNF-α kit (RPN 2788) from Amersham Pharmacia Biotech employed an amplification reagent for enhancement of binding of TNF-α in incubation media. Quantikine® ELISA kits from R&D Systems Inc. (Minneapolis) were used to determine human EGF (DEG00), human KGF (DKG00), human HGF (DHG00), human IL-1β (DLB50) and human TGF-β1 (DB100) in conditioned media according to the manufacturer's instructions. Prior to assaying TGF-β1, the samples were treated with 0.17 N HCl for 10 min at room temperature and then neutralized with 0.17 NaOH/0.07 N Hepes. To check for interference with the different ELISA assay kits, representative conditioned media from all groups and the proteinase inhibitors BB-3103 at 10 μM and aprotinin at 100 μg/ml alone were added to standard antigens diluted appropriately in separate wells and run in parallel to the samples.

5. RESULTS AND DISCUSSION

Controlled spatial and temporal expression of MMPs is crucial for normal wound healing. MMPs have also been implicated at all stages of tumorigenesis. In order to further understand their role in normal and aberrant wound healing as well as in human epithelial cancers, we concentrated on studying the MMP expression *in vivo*. In situ hybridisation and immunohistochemistry were used to investigate localizations of MMPs in tissues. In order to discover novel prognostic markers for carcinogenesis of chronic wounds, we compared the MMP profile of SCCs arisen in chronic wounds to that of non-malignant chronic venous ulcers. KAs were studied in parallel as a model of rapidly growing, but benign hyperproliferative tumors, resembling SCCs. We investigated the markers that could predict tumorigenesis in cutaneous and oral premalignant lesions, like actinic keratoses and verrucous hyperplasias. Therefore MMP expression pattern in BCCs and SCCs as well as in VCs and SCCs of oral tissues were studied. Transcriptional studies (Northern blot, Taqman PCR), and enzyme activity analysess (zymography, ELISA, Western blot analysis) were used to measure the expression levels and activities of MMPs in tissues and ulcer fluids and in cell extracts.

5.1. MMP-9 is differentially expressed in human acute and chronic wounds *in vivo* (I, II)

The localization of MMP-9 protein was quite different between acute and chronic wounds, although no differencies in the overall enzyme activity was observed in the two wound types. In acute wounds, MMP-9 was predominantly expressed by migrating keratinocytes in the advancing epithelium, whereas in chronic ulcers, MMP-9 expression was abundant in macrophages and neutrophils of the wound bed (I, Fig. 3. and 4.). The epithelial edge bordering chronic wounds was only rarely positive. Interestingly, this contrasts the findings with MMP-1, and stromelysins (MMP-3 and MMP-10), which are usually detected at the epithelial edges of chronic wounds (Saarialho-Kere, 1998) (see summary in Figure 4, p 54.). Expression of MMP-9 in keratinocytes bordering acute wound is in agreement with data obtained in oral mucosal wounds and rat and mouse wound models (Salo et al., 1994; Okada et al., 1997; Madlener et al., 1998; Lund et al., 1999). Lack of MMP-9 in keratinocytes bordering chronic wounds could indicate their non-migratory phenotype, although MMP-9 knock-out mice do not demonstrate any delay in wound healing, possibly due to compensation by other MMPs or serine proteinases (Lund et al., 1999). Results with mice are not necessarily directly adjustable to humans as there are differencies in MMP expression when comparing mice to men, different animal species to each other and also wounds of different tissues in the same species as well as gender

differencies in mice (Fini et al., 1996; Okada et al., 1997; Madlener et al., 1998; Balbin et al., 2003). In wounded HaCaT keratinocyte cultures MMP-9 expression does not correlate with migration (Mäkelä et al., 1999) and indeed it could play a role in cleaving type IV collagen during BM remodelling associated with wound healing. This is further substantiated by the fact that MMP-9 is upregulated during maturation of the epidermis in suction blister models (Oikarinen et al., 1993), in which BM remains essentially intact during epithelialization. Thomas et al., (2001b) found that MMP-9 is upregulated through cell contact with fibronectin via the αvβ6 integrin, which is abundantly expressed in the chronic wound epithelium (Häkkinen et al., 2004). Fibronectin, however, seems to be deficient in chronic wounds (Herrick et al.,1992) so it can not be an MMP-9 inducing ligand in this context. We hypothesize that ECM degradation by inflammatory cell-derived MMP-9 in chronic ulcers deprives wound edge keratinocytes of stimulatory cell-matrix protein interactions that are a prerequisite for epithelial MMP expression and migration (Ågren et al., 2000). Alternatively, MMP-9 in wound edge keratinocytes could remodel the deposited BM components in healing wounds (Yi et al., 2001; Daniels et al., 2003). Fini et al. (1996) attributed the failure of epithelium to cover corneal defects due to excessive degradation of BM molecules by MMP-9. MMP-9 -deficient mice reveal defects in remodeling of ECM at the epithelial BM zone and in particular, a failure to effectively remove the fibrin(ogen) provisional matrix (Mohan et al., 2002). They also indicated that MMP-9 acts to inhibit the rate of wound closure due to control of cell proliferation and delay of the inflammatory response.

In this study, the overall MMP-9 enzyme activities did not differ between acute and chronic wounds, although latent MMP-9 increased in chronic wound tissues and fluids (I, Fig. 1. and 2.), confirming results of other previous studies on wound fluids of chronic ulcers (Wysocki et al, 1993; Yager et al, 1996; Bullen et al, 1995; Trengove et al, 1999). MMP-9 upregulated already 24 h post-wounding in epithelial cells in normally healing wounds and it was found up till 9 d in the epidermis of wounds that were epithelialized. This is in accordance with previous studies, which have shown that well healing wounds express maximal levels of MMP-9 at 24 h, followed by a significant decline by 48 h (Tarlton et al., 1997; Ågren et al., 1998). As a conclusion, chronic non-healing wounds are not caused by excessive MMP-9 activity in the ulcer.

5.2. MMP-2 and MMP-14 are expressed by stromal cells in acute and chronic wounds (I, II)

MMP-2 was expressed in stromal fibroblasts, endothelial cells and under the BM zone in stromal connective tissue. Protein was detected both in acute and chronic wounds. No MMP-2 protein was found in the epithelium of wounds (see summary in Figure 4, p 54.). Mäkelä et al. (1999) presented *in vitro* evidence that keratinocyte-derived MMP-2 has a crucial role in keratinocyte migration and mobility at least in gingival keratinocytes. Although no epithelial MMP-2 was found here, epithelial cells could express MMP-2 earlier than 24 h post-wounding as there was occasionally a band-like immunostaining at the BM zone under the epithelium of the wound edge (I, Fig.5.). Ln-5 was detected in the same area, so the active MMP-2 might funtion in the cleavage of Ln-5.

Endogenous activity of MMP-2 enzyme was determined in normal skin, and in wound margin biopsies of acute dermatome wounds on postwounding days 1 and 7 as well as in biobsies of chronic wounds. Activity increased from d 1 to d 7 in acute wounds and was higher than in normal skin, but MMP-2 levels in chronic wounds did not differ significantly from those of acute wounds (I, Fig. 1. and 2.). Our findings are in contrast with the report by Ashcroft et al. (1997) localizing MMP-2 in epithelium, particularly in older subjects. The qualities of antibodies used or the use of paraffin versus frozen tissue may explain this discrepancy.

In acute wounds and in *ex vivo* explants MMP-14 (MT1-MMP) expression was detected in fibroblasts, but epithelial cells were negative. The same pattern was observed in chronic wounds and well-granulating ulcers, where abundant expression of MMP-14 protein was detected in fibroblasts of the wound bed by immunohistochemistry and mRNA by in situ hybridization. Epithelial cells were always negative. *In vitro* studies have related migration of breast epithelial cells to MMP-14 expression (Gianelli et al., 1997; Koshikawa et al., 2000), whereas other groups have not detected MMP-14 in normal epithelial cells. However it has been shown in transformed epithelial carcinoma cells (Seiki, 1999). Migration of some epithelial cell lines was attributed to the expression of MMP-14 (Koshikawa et al., 2000). Here, I could not detect MMP-14 protein in epithelium of acute or chronic wounds, nor in our *ex vivo* model. Moreover, MMP-14 levels were similar in BB-3103-treated wounds and controls. These findings suggest that MMP-14 is not important for epithelialization, verifying earlier in vivo results (Okada et al., 1997). However, stromal MMP-14 co-localized with MMP-2 in fibroblasts, a phenomenon also found in rodent wounds (Okada et al., 1997).

5.3. Matrix metalloproteinase inhibitor BB-3103 blocks epithelialization in human skin ex vivo wound model indicating the need for MMP activity in wound healing (II)

Several MMPs have been shown to be upregulated in migrating epithelial cells (Saarialho-Kere, 2002). Their importance in wound repair is further substantiated by the fact that MMP inhibitors, such as GM 6001 and chemically modified tetracyclines, retard epithelialization (Lund et al., 1999). We investigated the epithelial migration with or without treatment with proteinase inhibitor BB-3103 in an ex vivo skin wound model. Because of the previously reported importance of the plasminogen system on epithelialization (Rømer et al., 1996; Lund et al., 1999), skin explants containing a wounded site were also incubated with the serine proteinase inhibitor aprotinin.

Inhibition of MMPs with BB-3103 blocked epithelialization completely, whereas aprotinin did not have any significant effect, indicating the requirement for one or more MMPs in epidermal wound healing (II, Fig. 1.and 2.). As expected, the broad-spectrum MMP inhibitor prevented activation of both MMP-2 and MMP-9 as probably many other MMPs. Aprotinin blocked enzyme activation of MMP-9, but not MMP-2 (II, Fig.6.), a result which might be explained by the fact that the majority of MMPs, except for MMP-2, are directly activated by plasmin *in vitro* (Carmeliet et al., 1997; Creemers et al., 1998; Murphy et al., 1999).

Different experimental models could explain the discrepancy between our findings and the *in vivo* results in mice of Lund et al. (1999). Our interest was to elucidate the effect of serine proteinases specifically in the epithelialization of wounded sites in skin explants free from influences of fibrin and inflammatory cells. For example, the effect of aprotinin on clot formation would make interpretation difficult. In our *ex vivo* excisional wound model, keratinocytes move over a collagenous matrix to cover the epidermal defect, whereas *in vivo* migration occurs mainly over a fibrin-rich provisional matrix in full-thickness incisional wounds. Supporting our findings, Ando and Jensen (1996), demonstrated that movement of human keratinocytes was unrelated to the proteolytic activities of the uPA/plasminogen system. In addition, aprotinin at 100 µg/ml did not influence the random motility or dispersion of single human keratinocytes (Ando and Jansen., 1996; McCawley et al., 1998). No difference between uPA expression at the leading edge of keratinocytes in well-healing wounds and chronic leg ulcers has been detected (Vaalamo et al., 1996). In earlier *in vitro* wound healing studies, aprotinin has been described to inhibit contraction of collagen type I lattices populated with human fibroblasts or endothelial cells (Pins et al., 2000; Davis et al., 2001).

Our immunohistochemical studies on these *ex vivo* samples showed that MMP-9, but not MMP-2, was found in migrating keratinocytes (II, Fig.4). In another human *ex vivo* study, migrating bronchial epithelial cells were shown to be MMP-9 immunopositive (Legrand et al., 1999). Keratinocyte-derived MMP-9 is preferentially bound to the cell surface, but not to the ECM molecules deposited by human mucosal keratinocytes *in vitro*, implying that there is no functional role for MMP-9 (Mäkelä et al., 1998). Migration of human bronchial epithelium was associated with degradation of type IV collagen by active MMP-9 (Legrand et al., 1999). In the skin explants studied here, keratinocytes at the wounded site synthesized new matrix molecules including laminins, but not type IV collagen (Jansson et al., 1996). Specific cleavage of Ln-5 by active MMP-2 has been found to induce migration of normal human breast epithelial cells, while active MMP-9 did not infer a migratory phenotype in a transmembrane assay.

In summary, the presence of active MMP-2 in *ex vivo* wound healing model and its absence in the "non-healing" ex vivo wounds as well as the wounds treated with the MMP inhibitor BB-3103 indicate that MMP-2 is important for epithelialization. Aprotinin, which inhibits plasmin and many other serine proteinases and MMP-9 activation, influenced neither keratinocyte migration nor MMP-2 activation in fibrin-deficient skin wound healing, suggesting that serine proteinases and MMP-9 *per se* may not be crucial for epithelialization of skin wounds. However, aprotinin does not inhibit mast cell serine proteinases, tryptase and chymase (Harvima et al., 1988; Lohi et al., 1992; Kivinen et al., 2001 and 2003), enzymes which are released during skin organ cultures and which can activate MMPs.

MMP inhibitors are cytostatic (cells are viable, but do not proliferate), not cytotoxic, and we confirmed that the abolished epithelialization was not due to cytotoxicity of BB-3101, nor was there differencies in apoptosis between BB-3103 and control treated groups. As growth factors regulate both MMPs and wound healing (Sato et al., 1995; Zeigler et al., 1996; McCawley et al., 1998), we examined possible secondary effects of the proteinase inhibitors on important cytokines and growth factors. An issue was that the effects of the MMP inhibitor on the epithelialization in the wound healing model were secondary to decreased release of pro-inflammatory cytokines, because hydroxamate inhibitors like BB-3103 decrease shedding of membrane-anchored cytokines like TNF-α but not of interleukins (Barker et al., 1991; Gallea-Robache et al., 1997). BB-3103 and aprotinin reduced TNF-α secretion, but did not appreciably influence the levels of the immunodetectivity of other regulators of MMPs and

epithelialization, such as IL-1β, HGF, KGF or TGF-β1. These results suggest that TNF-α was not a decisive factor for epidermal healing in our model. Later it has been shown that TNF-α mediated activation of pro-MMP-9 is associated with down-regulation of TIMP-1 in human skin (Han et al., 2002).

The levels of immunoreactive HGF, KGF and TGF-β1 in conditioned media were similar in the different treatment groups. However, the ELISA analyses did not discriminate between inactive and bioactive growth factors. For example, aprotinin inhibits bioactivation of HGF and its mitogenic effect on epithelial cells (Gak et al., 1992). Aprotinin treatment at 100 µg/ml also attenuated epithelial proliferation. It is unclear whether the reduced proliferation was due to reduced level of bioactive HGF or to a direct inhibitory effect of aprotinin on keratinocyte proliferation. In contrast, proliferation tended to increase in BB-3103-treated explants. This could be explained by displacement of TIMPs by the synthetic inhibitor resulting in more unbound, mitogenic TIMPs (Buisson-Legendre et al., 2000). Our observations suggest that epithelial migration rather than proliferation is the major closure mechanism of the *ex vivo* wound models.

Use of MMP inhibitors in the treatment of chronic wounds is not well studied. GM6001 is able to increase wound strenght in rat incisions (Witte et al., 1998), and in porcrine acute wounds it impairs re-epithelialization, indicating the substantial role of MMPs in normal wound repair (Ågren, 1999). Therapeutic trials for non-healing ulcers, based on growth-factors, e.g., methods using injection of GM-CSF or adenoviral-mediated overexpression of PDGF in the wound area, have not been promising (see Ravanti and Kähäri, 2000).

5.4. MMP-19 is expressed by hyperproliferative epithelium but disappears with neoplastic dedifferentiation (III, IV, V)

Unlike several other MMPs, such as MMP-1, -9 and –10 (Parks et al., 1999; Rechardt et al., 2000), MMP-19 protein is not expressed by migrating keratinocytes in acute and chronic wounds. However, expression was detected in keratinocytes distal to the migrating edge in areas immunopositive for the proliferation marker Ki67 (IV, Fig.1.). In human chronic wounds, regions that seemed to be acanthotic or hyperproliferative, had abundant expression of MMP-19 protein (see summary in Figure 4.). Epithelial MMP-19 protein colocalized with abnormally faint or absent staining of type IV collagen (IV, Fig.1.). MMP-19 was also induced in the

hyperplastic epithelium bordering malignant wounds and it was widely expressed by keratinocytes at the basal epidermal layer of KAs (III, Fig. 3.). MMP-19 was induced in actinic keratoses and Bowen's disease as well as in hyperplastic epithelium bordering SCC and BCC, when the polarity and density of keratinocytes was disturbed, as assessed histologically and by abnormal E-cadherin staining (IV, Fig 2.). However, MMP-19 was downregulated *in vivo* when keratinocytes become malignant and it disappears during neoplastic transformation (III, IV,V). While this study was in progress MMP-19 was shown to be expressed in the epithelium of normal mammary glands and benign mammary tumours (Djonov et al., 2002). In agreement with our data, Djonov et al. concluded that MMP-19 expression may act in a "protective" manner. These findings suggest that MMP-19 does not take part in the degradation of BM and ECM to induce tumor spread, but rather in normal remodeling or restructuring of the BM induced after microchanges in the BM proteins, such as those detected in wounds and Bowen's disease. This is reasonable, as physiological substrates of MMP-19 are type IV collagen, laminin-1 and nidogen.

MMP-19 has been shown to be coexpressed with type IV collagen in tunica media and with integrin αvβ3 and VEGF-R2 in endothelial cells (Kolb et al., 1999). In my study it located in the area, where BM was at least partly destructed when stained with type IV collagen antibodies (IV, Fig.1.,2.). The presence of MMP-19 in p63-positive epithelium suggests that it is restricted in vivo to areas of keratinocytes with high proliferative potential and is absent from cells undergoing terminal differentiation (Parsa et al., 1999). Quite recently, Sadowski et al., (2003a) also showed that MMP-19 expression correlates with cytokeratin-14, which represents a marker for undifferentiated basal keratinocytes.

The expression of different MMPs in keratinocytes is generally regulated by extracellular signals, such as growth factors or cytokines and by signal transduction pathways, including those activated by phorbol myristate acetate (PMA). We wanted to investigate MMP-19 mRNA expression in primary keratinocytes as MMP-19 mRNA expression is upregulated by phorbol ester (TPA=PMA), EGF and bFGF (Kolb et al., 1999) in endothelial and smooth muscle cells. When we investigated MMP-19 mRNA expression in epidermal keratinocyte-derived cell lines representing different phases of carcinogenesis and varying tumorigenic potentials, our in vivo results were further substantiated. MMP-19 mRNA was detected by Northern analysis only in primary keratinocytes after stimulation with PMA, but was not evident in non-tumorigenic HaCaT cells (Boukamp et al., 1988), in ras-transformed A5 cells,

or in cutaneous SCC metastasis-derived UT-SCC-7 cells (Ahonen et al., 2002) (see Materials and Methods, Cell cultures). Although immortalized HaCaT cells are non-tumorigenic, their MMP expression profile differs from primary keratinocytes,,e.g. MMP-12 and –13 that are upregulated in the course of epithelial transformation, are expressed by HaCaT cells but not by primary keratinocytes (Johansson et al., 1997; Kerkelä et al., 2000).

When detected with real time quantitative PCR, basal MMP-19 mRNA levels in unstimulated keratinocytes were quite low and only PMA and TNF-α were able to stimulate it in primary keratinocytes (IV, Fig.5,A). TNF-α was shown to induce MMP-19 expression also in fibroblsts (Hieta et al., 2003). Treatment with TNF-α, inhibits proliferation and promotes differentiation, cytostasis or cell cycle arrest in cultured keratinocytes (Vieira et al., 1996; Pillai et al., 1989; Symington et al.,1989). TNF-α also induces adhesion molecules and apoptosis of keratinocytes (Uchi et al., 2000). In our samples the localization of MMP-19 did not histologically correlate with apoptosis, but TNF-α protein was expressed in the keratinocytes adjacent to MMP-19 positive cells. The fact that TNF-α induces adhesion molecules and that it stimulates MMP-19 could suggest that MMP-19 is a reconstructive enzyme trying to repair destructed BM. Caution is needed when extrapolating results from cell culture to tissue in vivo, since based on transgenic mouse studies many cytokines have different effects on keratinocytes *in vivo* and in culture (Turksen et al., 1992).

MMP-19 expression shown in wound bed macrophages (Hieta et al., 2003) and blood mononuclear cells and myeloid cells (Mauch et al., 2002) suggests that MMP-19 might have an effect on cell adhesion or it might contribute to the distinct migration capabilities of blood-derived cells (Mauch, 2003). Recently, MMP-19 was also associated with the regulation of IGF-mediated proliferation, migration and adhesion. Sadowski et al., (2003b) showed that MMP-19 degrades insulin growth factor binding protein -3 (IGFBP-3) in HaCaT cell lines inducing the release of IGF, which then exerts mitogenic effects through IGF-IR, thereby maintaining the proliferation of basal keratinocytes. They concluded that MMP-19 expression is down-regulated by E-cadherin mediated cell-cell contacts. These findings are in accordance with ours as we detected low levels of E-cadherin staining in MMP-19 positive areas.

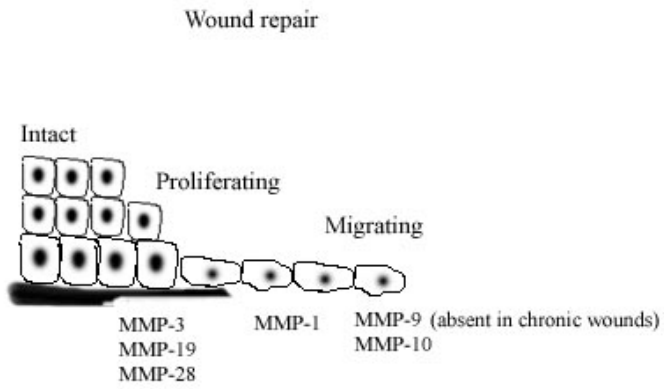

Figure 4. Summary of MMP expression in wound repair.

5.5. Expression profile of MMPs associates with malignant transformation of chronic wounds and KAs as well as invasiveness of oral cancers (III, V)

Tumor growth and cell invasion critically depend on the neoplastic proliferation together with the ability of the tumor cells to produce proteolytic enzymes, receptors mediating cell adhesion, and certain ECM proteins (Egeblad and Werb, 2002; Mueller and Fusenig, 2002). The presence of specific MMPs in cancer tissue can be used as a prognostic marker to predict tumor invasiveness (Vihinen and Kähäri, 2002). Therefore, we wanted to investigate the expression pattern of MMPs and their possible role as diagnostic indicators in development of SCC from pseudoepitheliomatous hyperplasia of chronic wounds. KAs were studied as a model of rapidly growing, benign hyperproliferative tumors, resembling SCCs. In order to discover novel prognostic markers for invasiveness of oral cancers, we analyzed oral SCC, VC and verrucous hyperplasias for several MMPs that have been rarely or never investigated in oral cancer, namely MMP-7, -10, -12, -13, –19, and -26 (Werner et al., 2002). MMP-3 and –9 expression was also studied as they both have been implicated in the invasiveness of oral SCCs (Kusukawa et al., 1995; O-Charoenrat et al., 2001).

MMP-7 is expressed only by invasive keratinocytes both in skin and oral mucosa, but is absent from chronic wound epithelium

MMP-7 is required for healing of airway wounds, but it is not expressed during skin wound repair (Parks et al., 2001). In all wound SCC samples, MMP-7 protein and mRNA were expressed by the invasive epithelial keratinocytes, whereas the epithelium was negative in all acute and chronic wounds (III; Fig.1.). All KA samples were negative for MMP-7 mRNA

supporting their non-malignant behavior. The same pattern of expression was seen in oral tissue, as MMP-7 expression was common in oral SCC, but absent in VC (V, Fig. 1.). All these findings are in agreement with previous reports, that MMP-7 expression correlates with aggressive phenotype of many cancers (Nelson et al., 2000). Furthermore, Van Kempen et al. (2002) also showed in a transgenic mouse model that MMP-7 is only derived from carcinoma. Because MMP-7 is able to degrade fibronectin, tenascin and β4 integrin, proteins that have crucial roles in cell adhesion and migration during tumorigenesis, MMP-7 plays apparently an important part in tumor development (see Kerkelä and Saarialho-Kere, 2003). Mature MMP-7 is also believed to be involved in early tumor development, as it is able to positively or negatively regulate apoptosis via shedding of FasL (see Hojilla et al., 2003). Furthermore, both MMP-7 and -3 contribute to the initiation of epithelial-to-mesenchymal transition by cleavage of E-cadherin (Noe et al., 2001).

Epithelial expression of MMP-12 may predict cancer in epithelial tissue

MMP-12 is expressed both by macrophages and transformed keratinocytes in cutaneous SCC (Kerkelä *et al*, 2000) and it has been implicated in matrix degradation and macrophage migration in many pathological conditions (Kerkelä et al., 2003). MMP-12 mRNA expression in tumor cells correlates with aggressive histology of skin cancer and poor prognosis in vulvar SCC, while MMP-12 positive macrophages may function in host-response and inhibit tumor growth (Kerkelä et al., 2002). MMP-12 also generates angiostatin, thus having a potential to inhibit tumor angiogenesis. In this study, MMP-12 mRNA was seen in macrophages but also in cancer cells in a subset of epithelial SCCs. In KA samples and in non-malignant chronic wounds, it was expressed only by macrophages (III, Fig.1). Similarly, in VH samples, MMP-12 mRNA was expressed by macrophages and this may indicate normal host-response effect. VC samples were negative for MMP-12 (V, Fig.3.). As MMP-12 mRNA expression in tumor cells correlates with aggressive histology, and while MMP-12 positive macrophages may function in host-response and inhibit tumor growth in some cancer types (Kerkelä et al., 2002), epithelial expression of MMP-12 in chronic wounds and in VHs should raise cancer suspicion. The level of MMP-12 is higher in histologically more aggressive and poorly differentiated tumor types (grades II and III), suggesting a possible role in invasion process (Kerkelä et al., 2000). In vulvar SCCs, cancer cell-derived MMP-12 correlates with aggressiveness and dedifferentiation of the tumors, while macrophage-derived MMP-12 is shown to be most abundant in grade I tumors, thus predicting less aggressive behaviour of the tumors (Kerkelä et al., 2002). Larger

cohorts of patients with KAs and VHs are needed to predict whether macrophage-derived MMP-12 would serve as a marker of good prognosis.

MMP-13 expression is upregulated in cancer

MMP-13 expression correlates with tumor invasion and grade of carcinomas of the larynx (Cazorla *et al*, 1998), skin (Ala-Aho et al, 2002) and head and neck (Johansson, et al, 1997; Kusukawa et al, 1996). MMP-13 expression has been shown to be a good marker for malignant transformation in cutaneous keratinocytes (Airola et al., 1997; Ala-aho et al., 2002). In oral mucosal epithelium, MMP-13 can also be induced during chronic inflammation (Uitto et al., 1998). Epithelial cells of VH and VC were negative for MMP-13 by immunohistochemistry. MMP-13 was expressed by malignant keratinocytes in SCCs, and the amount of positive cells increased as the tumor became more invasive (V, Fig.1.). Our results also showed that epithelial MMP-13 expression was strong in malignant wounds, absent in chronic wounds, but seen in the epithelium of KAs (III, Fig.2.). This expression pattern indicates, that MMP-13 might be turned on by inflammation as seen in oral mucosal epithelium during chronic inflammation (Uitto *et al*, 1998). Thus, in SCCs arisen from chronic wounds, it aids in obtaining the correct diagnosis, but does not assist in distinguishing KAs from grade I SCCs.

MMP-8 is not detected in epithelial cells of chronic ulcers or cancers arisen in them

MMP-8, which was originally thought to be expressed only by PMNs has been detected in benign and malignant keratinocytes in vitro (Prikk, et al., 2001). By ELISA analysis, it is detected in wound tissue and its overexpression and activation might have a role in the development of nonhealing chronic ulcers (Nwomeh et al., 1999). However, my results show that its role is not associated with the keratinocyte migration, but that it localizes to stromal neutrophils. In contrast to the results of Pirilä et al. (2001) obtained in mice, we were not able to detect MMP-8 in migrating cells of seven acute human wounds (Impola and Saarialho-Kere, unpublished observations). This discrepancy may be explained by differencies in MMP expression in different species. While this study was in progress, Balbin et al (2003) showed in a transgenic mouse model that MMP-8 might have protective functions in cancer due to processing of inflammatory mediators which contribute to the host antitumor defense system. Our SCC samples arisen from chronic wounds, did not show epithelial MMP-8 expression, nor was it detected in KAs (III, Fig.2.). This slightly deviates from the findings of Moilanen et al. (2003), who found relatively low MMP-8 expression sporadically in cancer cell islands of

oral SCCs. We must, however, bear in mind that our collection of wound SCCs was limited and represented well-differentiated tumors. Thus, we can not exclude the presence of MMP-8 in more dedifferentiated (grade III-V SCCs), Generally, MMP-8 expression is not useful for distinguishing chronic wounds or KAs from SCCs at early stage.

MMP-9 has an important role in cancer, but is not a marker of malignancy in chronic ulcers

MMP-9 protein was present in the epithelium of both malignant and non-malignant chronic wounds (III, Fig.2.). This indicates that MMP-9 operates normally in cell migration and BM remodeling, and its epithelial expression cannot be used as a marker of malignancy in chronic ulcers. MMP-9 positive epithelial cells were found also in KAs, although the number of positive cells was much smaller than in invading cancers (III, Fig. 2.). Gelatinases (MMP-2 and MMP-9) are expressed at elevated level in oral SCCs (Sutinen et al., 1998). In VCs, MMP-2 was expressed only by few stromal fibroblasts, but the tumor itself was negative (V, Fig.1.). In SCCs, invasive cancer cell nests were surrounded by a great number of MMP-2 positive stromal cells. MMP-9 was detected in stroma already in VC and, in addition to neutrophils and macrophages, it was expressed by invasive SCC cells. It has previously been shown that MMP-9 is overexpressed in human cancers (see Kerkelä and Saarialho-Kere, 2003) and its expression correlates with invasion and poor prognosis in SCC (Kupferman et al., 2000; Van Kempen et al., 2002). MMP-9 decreases cancer cell apoptosis, regulates angiogenesis, and influences immune response to cancer (Egeblad and Werb, 2002). For instance, infiltration by mast cells and activation of MMP-9 coincides with the 'angiogenic switch' in premalignant lesions during squamous epithelial carcinogenesis (Coussens et al., 1999). MMP-9 promotes tumor angiogenesis in other mouse tumor models as well (Bergers et al., 2000; Yu and Stamenkovic, 2000). MMP-9 -null mice develop fewer and less differentiated cancers than wild-type mice. The number of cancer colonies formed in the lungs is reduced by the downregulation of MMP-9 and in MMP-9 -null mice (see Egeblad and Werb, 2002).

MMP-3 epithelial expression is detected both in wounds and in cancer

MMP-3 (stromelysin-1) is not expressed by keratinocytes in normal skin, but MMP-3 expression is associated with oncogenesis of keratinocytes (De Angelis et al., 2002) and is detected in SCC and in keratinocytes after wounding (Saarialho-Kere et al., 1994). Expression of MMP-3 in tumors takes place at invasive tumor margins and correlates with tumor size, thickness and mode of invasion (Prosser et al., 1989) as well as with metastasis (Kusukawa et

al., 1996). In our study of chronic wounds, MMP-3 was expressed by proliferating keratinocytes distal to the wound edge. In oral and skin SCCs, MMP-3 was expressed mostly by stromal fibroblasts but occasionally also by invasive cells (V, Fig.2.). However, the enzyme was detected in some VCs and KAs, in which it was produced only by fibroblasts. It is typical for many MMPs, that their stromal signal is evident surrounding low-grade tumors, whereas aggressive tumors show epithelial MMP expression.

MMP-10 is probably induced by inflammation

The role of stromelysin-2 (MMP-10) in cancer progression is unclear. Recent data suggest that malignant transformation per se is not enough to induce MMP-10 (Kerkelä et al., 2001; De Angelis et al., 2002). At least in skin cancer, MMP-10 is probably not involved in the invasion of malignant cells, but may be induced by cytokines and growth factors functioning in ulcerative phenomena and inflammatory matrix remodeling associated with skin tumors (Saarialho-Kere, 1994; Kerkelä et al., 2001). In accordance with this hypothesis, MMP-10 mRNA was detected in the epithelial cells of 3/12 KAs, which were all characterized by prominent inflammation (III, Fig. 2.). In oral samples, MMP-10 was expressed by epithelial cancer cells both in VCs and SCCs (V, Fig. 2.). The number of positive cells was, however, much higher in SCCs, in which the subepithelial inflammation was usually more severe. One of the two VC samples that expressed MMP-10 was strongly infected by yeast. Thus, it is possible that microbes modify MMP-10 expression also in oral cancer.

Role of MMP-26 in SCCs is not yet known

MMP-26 is a fairly novel member of the MMP family, the role of which in SCCs is not known. Previously it was reported to be downregulated in endometrial carcinomas (Isaka et al., 2003). Our current results show that, since MMP-26 is expressed by basal keratinocytes of hyperproliferative epithelium in VC, but also at the invasive front of well-differentiated oral SCCs, it does not seem to be a specific marker of invasion for oral SCCs (V, Fig.3).

5.6. Loss of p16 from KAs or chronic wounds could be a marker for carcinogenesis (III)

p16INK4a is a tumor-suppressor gene, the loss of which may facilitate activation of cyclin D1/CDK4 or 6, which is likely to affect regulation of the G0/S checkpoint. Mutational loss of p16 and p53 has been found to be a frequent early event in the development of SCC, endowing keratinocytes with extended replicative potential and increasing the probability of neoplastic progression (Rheinwald et al., 2002). As it is previously shown that positivity of p53 and p21

might be useful for the early detection of wound malignancy (Baldursson et al., 2000b), we studied only p16. In our SCC and KA samples, p16 was strongly expressed by keratinocytes in the superficial areas (III, Fig.3.). It was, however, absent from invasive cancer tissue. In accordance with the recent *in vitro* findings of Natarajan et al., (2003), p16 was detected in this study in migrating keratinocytes in normally healing wounds as well as at the wound edge keratinocytes of a subset of chronic venous ulcers *in vivo*. Therefore, the loss of p16 from KAs or chronic wounds could be a marker for carcinogenesis. Based on our previous data on wound repair, p16 seems to be coordinately expressed with laminin-5 γ2 on tissues, since the epithelial edge of both acute and chronic wounds demonstrates cytoplasmic staining for laminin-5 γ2 (Rechardt *et al*, 2000; Saarialho-Kere *et al*, 2002).

5.7. Laminin-5 is expressed by migrating keratinocytes both in wounds and cancer but can´t be considered as a good marker for invasiveness (I, V)

Ln-5 immunohistochemistry was performed to investigate the migratory phenotype of epithelial cells (Larjava et al, 1993). Intracellular staining for Ln-5 reflects active synthesis of this protein needed for deposition of normal BM and is usually encountered in migrating cells of suction blisters and acute wound edge (Rechardt et al, 2000; Kainulainen et al, 1998). In full-thickness wounds, its deposition occurs before that of type IV and VII collagens or laminin-1 (Kainulainen et al, 1998). In this study, Ln-5 expression was qualitatively similar in both acute and chronic wounds, suggesting that the migratory capability of wound edge keratinocytes is not different in normally healing and chronic wounds. Cleavage of the precursor molecule to mature Ln-5, needed for hemidesmosome assembly, may signal the epithelial cells to become quiescent and form integrated tissue (Häkkinen et al, 2000). Unfortunately, the antibody used in this study, did not distinguish between the mature and precursor protein. Ln-5 is an in vitro substrate for MMP-14 and MMP-2, but not for MMP-9 (Giannelli et al, 1997; Koshikawa et al, 2000). We did not detect correlation between MMP-14 and Ln-5 in tissues. In chronic ulcers intracellular Ln-5 staining confined to the BM zone was surrounded by positive staining for MMP-2, suggesting that MMP-2 participates in Ln-5 processing also in the wound environment. Laminin-5 γ2 expression is a predictor of invasiveness in cancer of the larynx (Nordemar et al., 2001) and it is associated with budding cancer cells at the tip of invading malignant epithelium but not in the cancer cells inside the tumor. Laminin-5 γ2 is thought to be useful as a prognostic marker for oral cancer and malignant cells (Kainulainen et al., 1997). In our samples Ln-5 was detected already in verrucous hyperplasia and carcinoma, even though they are not invasive.

This indicates that although in SCCs the number of positive cells was much higher and the staining was more intense, Ln-5 can not solely explain the different behavior of VC versus SCCs, nor can it be considered as a marker for invasiveness.

5.8. αvβ6 integrin was present in verrucous hyperplasia and in SCC (V)

αvβ6 is an epithelial integrin that binds fibronectin and tenascin (Sheppard et al., 1990). Normal basal keratinocytes do not express αvβ6, but its expression is induced during wound healing and in SCCs (Breuss et al., 1995; Haapasalmi et al., 1995). Leukoplakia specimens positive for αvβ6 integrin show a tendency for malignant transformation (Hamidi et al., 2000) and αvβ6 enhances invasive behavior in oral SCC (Ramos et al., 2002). It is not known at which stage of transformation the expression of αvβ6 is switched on in oral epithelial cells (Breuss et al., 1995). Previously, epithelial cells of hyperplasia or chronic inflammation were not found to express αvβ6 (Hamidi et al., 2000). In this study, αvβ6 was detected already in verrucous hyperplasia. In VC, the expression was limited into a narrow area in the epithelium compared to SCC, where it was expressed widely in the marginal epithelium and also inside invasive cancer cell nests.

In vitro, αvβ6 modulates keratinocyte migration on fibronectin, vitronectin and towards the latency-associated peptide of TGF-β1 and is also associated with fibronectin-dependent upregulation of MMP-9 (Thomas et al., 2002). We did not detect co-localization of αv mRNA with MMP-9 protein *in vivo*, but caution should be exercised when interpreting these results, since a discrepancy between the protein and mRNA localization has been reported for several MMPs (see Kerkelä and Saarialho-Kere, 2003). However, in high grade SCCs there was partial colocalization of αvβ6 and MMP-3 mRNAs, although there was much more epithelial signal for αvβ6. Similar expression patterns of αvβ6, MMP-9 and MMP-3 are also detected in microinvasive cutaneous SCCs (Saarialho-Kere and Impola, unpublished observations).

6. SUMMARY AND CONCLUSIONS

Cutaneous wound healing is a multistage process during which the disrupted epithelium is restored by cell migration and proliferation and the injured BM and ECM proteins are replaced by the newly deposited matrix. MMPs have various functions during wound healing and are mainly responsible for the degradation of the dermal matrix and migration of epithelial cells. MMP expression is controlled by different growth factors and cytokines, and by many receptors and matrix molecules: actually all changes in cell-cell or cell-matrix interactions may induce MMP expression. Several MMPs have been shown to participate in wound healing and their cellular expression is generally qualititatively similar in acute and chronic wounds. However, differencies in the balance between MMPs and their inhibitors have been demonstrated. The tissue changes in cancer and chronic wounds resemble each other; they are both characterized by cell migration, neoangiogenesis, remodelling of the ECM and inflammatory reaction. Patients suffering from chronic wounds are at increased risk of developing SCCs. Wound carcinogenesis is unpredictable and often undiagnosed for long periods of time. Several MMPs, such as MMPs-1, -2, -7, -9, -13, and -14, have been implicated to be present in various cancers. The presence of specific MMPs in cancer tissue may be used as a prognostic marker to predict tumor invasiveness.

This study shows that MMP-9 expression localized in migrating epithelial cells during normal human wound healing, but is detected in stromal inflammatory cells in chronic wounds. This suggests that the absence of intact ECM molecules due to degradation by inflammatory cell-derived MMP-9 in chronic non-healing wounds may deprive wound edge keratinocytes of proper cell-matrix interactions needed for induction of migration. Equal gelatinase activity levels found in acute and chronic wounds contradict former hypotheses that chronic ulcers are caused by excessive gelatinase expression.

Furthermore, in an ex vivo fibrin-deficient skin wound healing model a broad spectrum MMP-inhibitor abrogated epithelialization, whereas serine proteinase inhibitor, aporotinin, did not. Active MMP-9 may have an important role in BM remodeling rather than in keratinocyte migration in wound repair which is accompanied by active MMP-1 and MMP-2. Like in chronic wounds, MMP-2 and MMP-14 were detected in stromal cells only and their expression partially colocalized in fibroblasts, suggesting that MMP-2 can be activated by MMP-14 *in vivo*.

My study was the first to show MMP-19 expression in keratinocytes. In all wounds, MMP-19 expression was detected in hyperproliferating and acanthotic areas of the epithelium, but not by

the migrating cells. Unlike most other MMPs, this protein was absent from the invasive skin cancer cell nests *in vivo*. Furthermore, MMP-19 was expressed in oral verrucous hyperplasias and VCs, but was absent from the invasive cancer cell nests of oral SCC. Basal expression levels of MMP-19 were low in primary keratinocytes and MMP-19 was not expressed by transformed epithelial cells (HaCaT, A5) in culture, suggesting that it is upregulated in the epithelium already early during oncogenesis in a host-response type manner to reconstitute normal cell adhesion. TNF-α induced MMP-19 mRNA expression in primary keratinocytes.

This study indicates that the epithelial expression of MMPs-7, -12, and –13, but not that of MMP-1, -3, -8, -9, -10, in a chronic wound sample provides a possible diagnostic clue for distinguishing SCCs from non-malignant chronic wounds. We also studied keratoacanthomas, which are benign rapidly growing tumors with keratinocyte proliferation and inflammation, and histologically often indistinguishable from SCCs. Epithelial expression of MMPs-7, -8, -9 and -12 was generally absent from wounds and KAs, while MMP-3, -10, -13 and -19 were present. The loss of immunohistochemical staining for MMP-19 as well as the cell cycle inhibitor p16 could aid in making the differential diagnosis between well-differentiated SCC and non-malignant chronic wound or KA.

My results showed that the invasiveness of oral cancers may also be dependent on their MMP expression profile. Typically VCs are devoid of epithelial MMP-7, -9 and -12 expression compared to oral SCCs. This phenomenon may thus serve as a prognostic marker in oral SCC. In vitro, αvβ6 is associated with fibronectin-dependent upregulation of MMP-9. We did not detect co-localization of αv mRNA with MMP-9 protein in vivo. However, in high grade SCCs there was partial colocalization of αvβ6 and MMP-3 mRNAs. MMP-13 was expressed in VCs, although the number of MMP-13 positive cells was much higher in SCCs. MMP-13 expression in VCs is most likely associated with inflammation, but as inflammation and MMP overexpression may cause malignant transformation, and because MMP-13 upregulation is detected in many types of tumors, it might have a role in oral tumorigenesis, expecially when the duration of its expression is prolonged. This study was the first to show MMP-26 expression in oral keratinocytes *in vivo*. MMP-26 is expressed by basal keratinocytes of hyperproliferative epithelium in VC, but also at the invasive front of well-differentiated oral SCCs. Therefore it does not seem to be a specific marker for invasion in oral SCCs. Eventhough laminin-5 γ2 is thought to be useful as a prognostic marker for oral cancer and malignant cells, in our samples it was detected already in VH and VC, indicating that Ln-5

cannot solely explain the different behavior of VC versus SCCs, nor can it be considered as a marker for invasiveness. In conclusion, we suggest that MMP-7, -9, -12, and -13 as well as αvβ6 integrin may be good targets for intervention therapy at the early stages of oral cancer.

I conclude that several MMPs are needed in wound re-epithelialization, and that aberrations in their expression pattern may contribute to chronic wound phenotype as well as malignant transformation. In order to produce drugs and MMP inhibitors for chronic wounds, we need more *in vivo* studies and migration assays where different MMPs, cytokines and other factors involved in the process, are tested together. In skin and oral cancers, MMPs might be useful as prognostic markes in diagnosing pre-malignant tumours from more aggressive ones. However, as tumors at their onset have been more sensitive to MMP inhibition than the advanced ones, early diagnosis is essential and therefore results that aid in diagnosis as well as prognosis are important. Several MMPs are produced by the inflammatory cells and have a dual role in cancer; in addition to tissue infiltration and destruction, they stimulate protective and adaptive immune functions and inhibition of some MMPs may end up causing harmful effects on the patient. Both *in vivo* and *in vitro* studies are needed in an attempt to understand the mechanisms of tumor cell invasion through the ECM in order to produce therapeutic invasion blocking anticancer treatments.

7. ACKNOWLEDGEMENTS

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