COLLAGEN XVII AND TIMP-1 IN EPITHELIAL CELL MIGRATION

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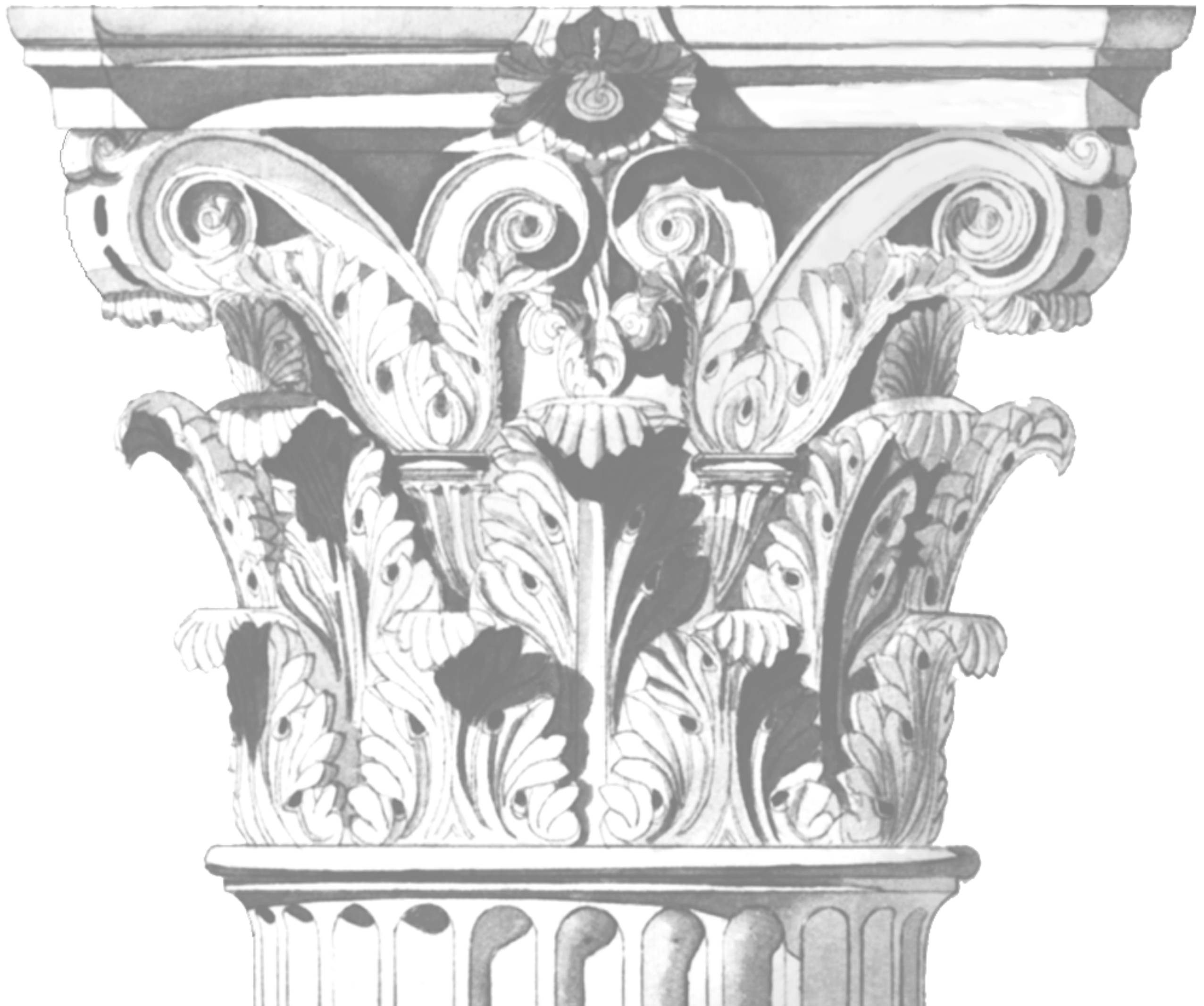

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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Auditorium 1 of the Institute of Dentistry, on November 28th, 2003, at 12 noon.

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Abstract

Collagen XVII (BP180) is a transmembrane component of hemidesmosomes, which connect basal keratinocytes to the basement membrane. The extracellular domain of collagen XVII is proteolytically shed from the cell surface and released to the extracellular matrix. Apart from its function in epithelial cell adhesion, collagen XVII has been suggested to participate in keratinocyte motility. The collagen XVII expression pattern was studied in wounds of oral mucosa and in epithelial tumors. During re-epithelialization, collagen XVII was expressed in the keratinocytes distal to the wound edge, but not in the leading cells of the epithelial tip. Collagen XVII upregulation was observed in moderate/severe dysplasias of oral mucosa. In follicular ameloblastomas and basal cell carcinomas, collagen XVII expression was reduced in peripheral cells, whereas cytoplasmic staining was detected in central tumor cells. Tongue squamous cell carcinomas showed increased collagen XVII expression in grade II/III tumors, particularly in areas of invasive growth. The results suggest a correlation between overexpression of collagen XVII and the invasive potential of the tumor.

For the first time, the role of collagen XVII in the regulation of malignant migration was explored. The presence of COL15, the cell adhesion domain of collagen XVII, induced migration of tongue squamous cell carcinoma cells in transmigration assays. Experiments with specific function-blocking integrin antibodies revealed that the promigratory function of COL15 is mediated by αv and α5 integrins.

The role of the matrix metalloproteinase (MMP) family of proteolytic enzymes in wound re-epithelialization was studied in a transgenic mouse model. In these mice, a specific inhibitor of MMPs, TIMP-1, was overexpressed in cells that normally produce MMP-9. The healing of cutaneous wounds was found to be significantly delayed, but not prevented, due to the impaired ability of keratinocytes to migrate to the wound area.

These results suggest that collagen XVII may participate in epithelial tumor progression and invasion by promoting migration of tumor cells. Based on the present study, epithelial cell-derived MMPs play a significant role in the migration of wound keratinocytes during re-epithelialization.

Keywords: carcinoma, collagen XVII, epithelial migration, matrix metalloproteinases, neoplasm invasiveness, odontogenic tumors, re-epithelialization, tissue inhibitor of metalloproteinase-1, wound healing

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Oulu, November 2003 Mataleena Parikka

Abbreviations

ADAM	a disintegrin and metalloproteinase
BCC	basal cell carcinoma
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BMZ	basement membrane zone
bp	base pair
BP	bullous pemphigoid
BSA	bovine serum albumin
C-	carboxy-
cDNA	complementary DNA
CLAC	collagen-like Alzheimer amyloid plaque component
COL	collagenous domain
cRNA	complementary RNA
DEAE	diethylaminoethyl cellulose
DMEM	Dulbecco's Modified Eagle's Medium
DTT	dithiotreitol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
ECM	extracellular matrix
EFG-R	epidermal growth factor -receptor
FCS	fetal calf serum
FITC	fluorescent isothiocyanate
hTIMP-1	human TIMP-1
Hh	hedgehog
IL	interleukin
ISZ	*in situ* zymography
JEB	junctional epidermolysis bullosa
kDa	kilodalton
mAb	monoclonal antibody
mRNA	messenger RNA
MARCO	macrophage receptor with collagenous structure
MMP	matrix metalloproteinase

N-	amino-
NBT	nitroblue tetrazolium
NC	noncollagenous domain
OSCC	oral squamous cell carcinoma
PBS	phosphate-buffered saline
PMA	phorbol-12-myristate-13-acetate
PTCH1	Patched1
RPA	ribonuclease protection assay
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TACE	TNF-α converting enzyme
TBS	tris-buffered saline
TCA	trichloroacetic acid
TGF	transforming growth factor
TNF	tumor necrosis factor
TIMP	tissue inhibitor of matrix metalloproteinases
tRNA	transfer RNA

Original articles

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

I Parikka M, Kainulainen T, Tasanen K, Bruckner-Tuderman L & Salo T (2001) Altered expression of collagen XVII in ameloblastomas and basal cell carcinomas. J Oral Pathol Med 30: 589-595.

II Parikka M, Kainulainen T, Tasanen K, Väänänen A, Bruckner-Tuderman L & Salo T (2003) Alterations of collagen XVII expression during transformation of oral epithelium to dysplasia and carcinoma. J Histochem Cytochem 51: 921-929.

III Parikka M, Nissinen L, Kainulainen T, Bruckner-Tuderman L, Salo T, Heino J & Tasanen K. The cell adhesion domain of collagen XVII promotes tongue carcinoma cell transmigration. Manuscript.

IV Salonurmi T, Parikka M, Kontusaari S, Pirilä E, Munaut C, Salo T & Tryggvason K (2003) Overexpression of TIMP-1 under the MMP-9 promoter interferes with wound healing in transgenic mice. Cell Tissue Res, in press.

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1 Introduction

Epithelial cell migration occurs during various physiological events, such as fetal development, regeneration and wound healing. It is dynamic process, involving coordinated function of various extracellular matrix (ECM) components and proteolytic enzymes. Controlled proteolytic processing of ECM molecules is essential for the detachment and migration of epithelial cells. Many pathological conditions, including impaired wound healing and epithelial tumor invasion, are associated with alterations in the regulation of epithelial cell migration.

Collagen XVII (BP180) is a transmembrane component of hemidesmosomes, which participate in keratinocyte adhesion to the basement membrane. The ectodomain of collagen XVII is constitutively shed from the keratinocyte surface, forming a soluble basement membrane collagen (Hirako *et al.* 1998, Schäcke *et al.* 1998, Franzke *et al.* 2002). The shedding of collagen XVII has been proposed to affect cellular processes, such as detachment and motility.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes generally considered important in degrading the ECM in tissue resorption and remodelling. The proteolytic activity of MMPs is strictly regulated during their activation and inhibition. (Nagase & Woessner, Jr. 1999). Tissue inhibitors of metalloproteinases (TIMPs) are capable of inhibiting all MMPs by forming 1:1 complexes (Bode *et al.* 1999). In physiological situations, the destructive activity of MMPs is mainly under the control of TIMPs. It is known that MMPs are needed for normal wound healing, and that imbalance in the MMP-TIMP ratio may lead to impaired wound healing (Bullen *et al.* 1995, Vaalamo *et al.* 1999).

In this work, the expression pattern of collagen XVII was studied in basal cell carcinomas (BCC), ameloblastomas, oral dysplasias, and oral squamous cell carcinomas (OSCC) by immunohistochemistry and *in situ* hybridization. A recombinant fragment of collagen XVII was used to study the effect of collagen XVII on carcinoma cell migration in cell transmigration experiments. The role of keratinocyte MMPs in wound re-epithelialization was investigated in a transgenic mouse model, with TIMP-1 overexpression in MMP-9-producing cells.

2 Review of the literature

2.1 Mucocutaneous basement membrane zone

The mucocutaneous basement membrane zone (BMZ) is a thin, continuous, sheet-like extracellular matrix structure composed of hemidesmosomes, basement membrane and anchoring fibrils. Basement membranes are specialized structures located at the junction between epithelial cells and the underlying connective tissue stroma (Fig. 1). As defined by electron microscopy, basement membranes consist of two layers, *lamina lucida* and *lamina densa*. *Lamina lucida* is a layer with low electron density located adjacent to the cell membrane. *Lamina densa* is observable as an electron-dense layer next to the connective tissue stroma. However, it is possible that these two layers do not exist *in vivo*: *lamina lucida* is proposed to be an artefact resulting from rapid dehydration during tissue processing (Chan *et al.* 1993). The molecular structure of basement membranes is mainly composed of type IV collagen and laminin networks, connected with nidogen, and the proteoglycans perlecan and agrin. In addition, there are several minor connecting or adhesive constituents. Basement membranes have many important functions. They both separate and connect two distinct tissue compartments. Basement membranes allow migrating cells to pass under physiological conditions, but act as barriers against tumor cell invasion and regulate the passage of molecules based on their electronic charge and molecular size. Many BMZ ligands interact with cell surface receptors, influencing epithelial cell behavior during morphogenesis, fetal development, and wound healing by regulating cell shape, proliferation, differentiation, and motility as well as gene expression and apoptosis. (Timpl 1996, Burgeson & Christiano 1997, Ghohestani *et al.* 2001)

2.1.1 Hemidesmosomal adhesion complex

Hemidesmosomes are multi-protein complexes that connect the basal keratinocytes to the basement membrane (Fig. 1). Together with the anchoring filaments and anchoring fibrils, they form a functional unit of the hemidesmosomal adhesion complex, which integrates the cytoskeleton of the epithelial cell to the ECM. Ultrastructurally, hemidesmosomes appear as small electron-dense complexes at the ventral cell membrane of basal keratinocytes, composed of an internal plaque, an external plaque, and a sub-basal dense plate. In epidermal keratinocytes, actin, intermediate, and microtubule filament systems form a cytoskeletal network, which is connected to adjacent keratinocytes via desmosomes and to the underlying basement membrane via hemidesmosomes. The internal plaque of the hemidesmosome consists of two plakin family proteins, plectin and BP230, and a novel accessory protein erbin (Favre *et al.* 2001). Plectin functions as a cross-linker of the three cytoskeletal filament networks, binding them to cell membrane structures, including the hemidesmosomes. BP230 attaches the intermediate filaments to the hemidesmosomal adhesion complex. Plectin and BP230 bind to the transmembrane proteins of the external plaque, i.e. $\alpha6\beta4$ integrin and collagen XVII (BP180). The association of BP230 and $\alpha6\beta4$ integrin may be mediated by erbin. The cytoplasmic as well as the extracellular part of $\alpha6\beta4$ integrin binds to collagen XVII. A third transmembrane component of the external plaque is tetraspanin CD151, which is thought to participate in the clustering of integrin receptors to facilitate cell binding. (Jones *et al.* 1998, Hirako & Owaribe 1998, Nievers *et al.* 1999, Borradori & Sonnenberg 1999, McMillan *et al.* 2003)

Outside the basal keratinocyte, thread-like anchoring filaments extend from the hemidesmosomes to the basement membrane (Fig. 1). Laminin-5 is the major constituent of the anchoring filaments, which connect hemidesmosomes to the basement membrane. Laminin-5 is a heterotrimer composed of $\alpha3$, $\beta3$, and $\gamma2$ chains. Association of laminin-5 with hemidesmosomal $\alpha6\beta4$ integrin is likely to occur via the carboxy(C)-terminal globular G-domain of the $\alpha3$ chain. Laminin-5/$\alpha6\beta4$ integrin interaction is essential for hemidesmosome formation *in vivo* and the maintenance of epithelial adhesion, as emphasized by the fact that mutations in the genes encoding laminin-5 chains result in a hereditary blistering skin disease, junctional epidermolysis bullosa. In addition to $\alpha6\beta4$ integrin, laminin-5 also binds the extracellular domain of collagen XVII. For a review, see (Franzke *et al.* 2003). Unlike other laminins, laminin-5 is unable to bind nidogen and therefore incapable of being directly attached to the collagen IV/perlecan network of the basement membrane. Instead, it is likely that laminin-5 is incorporated in the basement membrane via laminins 6 and 7, which are able to connect to nidogen. Laminin-5 binds to the amino(N)-terminal domain of collagen VII, providing a bridge between hemidesmosomal $\alpha6\beta4$ integrin and collagen VII of anchoring fibrils. (Jones *et al.* 1998, Nievers *et al.* 1999)

Anchoring fibrils extend from the basement membrane to the structures called anchoring plaques in the connective tissue matrix or loop back to the basement membrane, connecting it to the underlying connective tissue (Fig. 1). The anchoring fibrils entrap collagen fibrils in the connective tissue, which is a way of ensuring epithelial anchorage. Anchoring fibrils are mainly composed of collagen VII polymers,

which are connected to the hemidesmosomal anchoring complex via attachment to laminin-5 within the basement membrane. The role of anchoring fibrils is vital for the function of the anchoring complex: mutations in the collagen VII gene result in a heritable blistering skin disease, dystrophic epidermolysis bullosa, in which the epithelium is detached below the basement membrane. (Bruckner-Tuderman 1999)

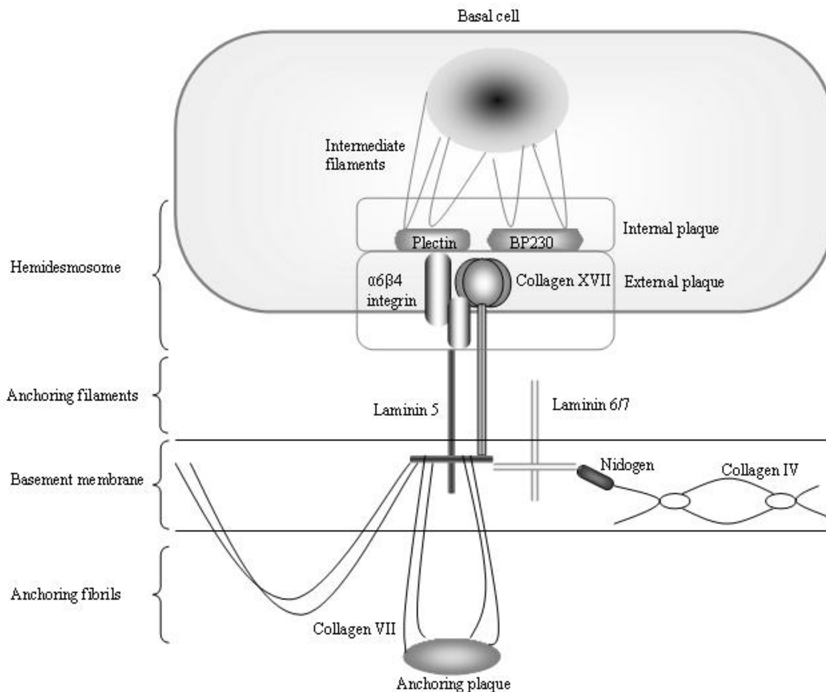

Fig. 1. Hemidesmosomal adhesion complex and basement membrane zone. (Modified from Jonkman 1999, Ghohestani *et al.* 2001)

2.2 Collagen XVII

Collagen XVII (Fig. 2) is a member of the transmembrane collagen family, which also includes three cell adhesion molecules (collagens XIII and XXIII and ectodysplasin A); a brain-specific component of Alzheimer amyloid plaques (collagen XXV/ CLAC (collagen-like Alzheimer amyloid plaque component) precursor); and three receptors involved in host defence (type I and II macrophage scavenger receptors and MARCO (macrophage receptor with collagenous structure) receptor). Transmembrane collagens share similarities in their structure: all the seven molecules are synthesized as integral membrane proteins in type II orientation, i.e. with extracellular C-terminus and cytoplasmic N-terminus. They are homotrimers of α-chains containing a relatively short

intracellular domain, a hydrophobic transmembrane stretch, and a large extracellular domain with at least one collagenous subdomain. The transmembrane collagens can act both as cell surface receptors and as matrix molecules: the ectodomains of several, if not all, transmembrane collagens are proteolytically shed from the cell surface, yielding soluble extracellular matrix collagens. (Franzke *et al.* 2003)

The cDNA sequence of collagen XVII encodes a protein of 1497 amino acids in length (Fig. 2). Collagen XVII consists of three 180 kDa α1(XVII) chains, composed of an intracellular domain of 466 amino acids, a short 23 amino acid transmembrane domain and an extracellular domain of 1008 amino acids. The C-terminal ectodomain consists of 15 collagenous subdomains (COL 1-15) and 16 short stretches of non-collagenous sequences (NC 1-16). (Giudice *et al.* 1992) The ectodomain of collagen XVII is triple-helical and folds in the N- to C-terminal direction (Hirako *et al.* 1996, Areida *et al.* 2001). Rotary shadowing electron microscopy studies of purified bovine collagen XVII have shown that the molecule is composed of a globular cytoplasmic head, a central rod corresponding to the largest collagenous domain COL15, and a flexible extracellular tail. (Hirako *et al.* 1996)

The important role of collagen XVII in keratinocyte-matrix adhesion is clearly demonstrated by the fact that a lack of collagen XVII function leads to hereditary and autoimmune blistering skin diseases. Collagen XVII was first discovered as a target molecule for autoantibodies in bullous pemphigoid, an aquired subepidermal blistering skin disease. In addition, herpes gestationis, cicatricial pemphigoid and linear IgA disease are characterized by an autoimmune response directed against collagen XVII. The autoantigenic sites of the molecule are located highly clustered within the NC16A domain (Fig. 2)(Zillikens *et al.* 1997). Collagen XVII is also involved in human hereditary blistering skin disease: mutations in COL17A1 are associated with a rare type of junctional epidermolysis bullosa (JEB), with generalized blistering, loss of hair in infancy, dental anomalies and nail dystrophy (McGrath *et al.* 1995). (Zillikens & Giudice 1999, Zillikens 1999, Uitto & Pulkkinen 2001)

2.2.1 Proteolytic shedding

The collagen XVII ectodomain is constitutively shed from the cell surface, forming a shorter, soluble basement membrane collagen (Fig. 2)(Hirako *et al.* 1998, Schäcke *et al.* 1998). The cleavage product is a trimer of 120 kDa polypeptides, which includes almost the entire extracellular domain. Studies with domain-specific antibodies have indicated that the cleavage occurs close to the cell membrane, within the NC16A domain, but the exact site remains unknown. Furin was first presumed to be responsible for the shedding, based on experiments using specific inhibitors (Schäcke *et al.* 1998). Recently, it was shown that collagen XVII is actually shed by three members of the ADAM (a disintegrin and metalloproteinase) family of proteases, which are activated by furin (Franzke *et al.* 2002). ADAM-9, -10 and TACE (tumor necrosis factor (TNF)-α converting enzyme, ADAM-17) were confirmed to cleave collagen XVII, TACE being the major sheddase (Franzke *et al.* 2002). The biological role of collagen XVII ectodomain shedding remains

elusive. It is likely to affect keratinocyte detachment, motility, and differentiation. The shed ectodomain of collagen XVII can be extracted from the epidermis and amniotic fluid *in vivo*, implicating a role as a ligand-binding matrix molecule (Schumann *et al.* 2000).

In an experimental bullous pemphigoid mouse model, cleavage of the collagen XVII ectodomain by neutrophil elastase and MMP-9 has been suggested to mediate blister formation (Ståhle-Bäckdahl *et al.* 1994, Liu *et al.* 1998). However, although recombinant MMP-9 and neutrophil elastase are both capable of degrading recombinant collagen XVII *in vitro*, a study with MMP-9-deficient and neutrophil elastase-deficient mice showed that MMP-9 does not cleave mouse collagen XVII *in vivo*. The main role of MMP-9 in the experimental bullous pemphigoid model is the proteolytic inactivation of α1-proteinase inhibitor, allowing neutrophil elastase to degrade collagen XVII. (Liu *et al.* 2000)

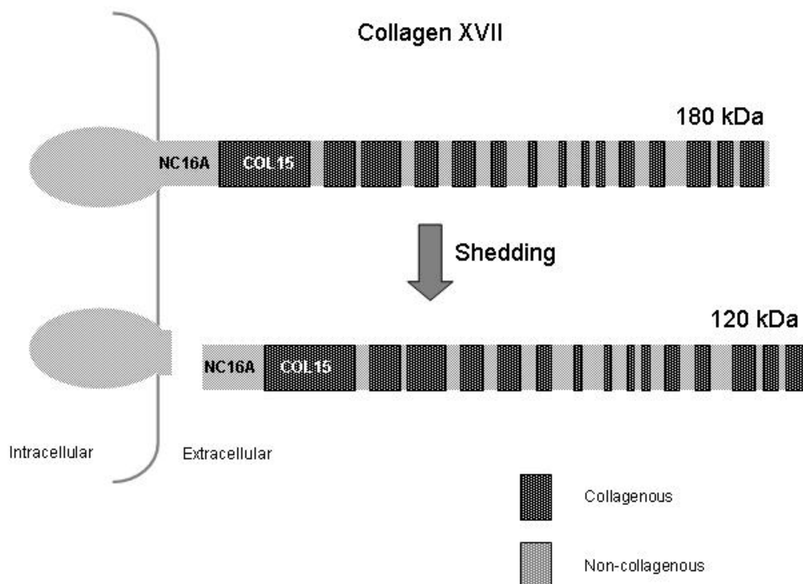

Fig. 2. Proteolytical shedding of collagen XVII. (Modified from Franzke *et al.* 2003)

2.2.2 Ligands

Collagen XVII is a structural component of hemidesmosomes, participating in keratinocyte-matrix adhesion via various protein-protein interactions, and it has multiple

ligands in the hemidesmosome (Fig. 1). The cytoplasmic N-terminus of collagen XVII is located within the external plaque of the hemidesmosome, and the intracellular ligands of collagen XVII include hemidesmosome components β4 integrin subunit, plectin, and BP230 (Koster *et al.* 2003). The extracellular C-terminus of collagen XVII is situated in the region of anchoring filaments, where the juxtamembranous NC16A (Fig. 2) domain binds to the α6 integrin subunit (Hopkinson *et al.* 1995, Aho & Uitto 1998). Another extracellular binding partner for collagen XVII is laminin-5 (Tasanen *et al.*, manuscript). Furthermore, the results of a recent study suggest that collagen XVII may also interact with actinin family members, in regions of cell-cell contacts (Gonzalez *et al.* 2001). COL15, the largest collagenous subdomain of collagen XVII (Fig. 2), is reported to promote adhesion and spreading of epithelial cells via β1 integrins (Tasanen *et al.* 2000, Nykvist *et al.* 2001). COL15 interacts with the fibronectin receptor integrins α5β1 and αvβ1, instead of the typical collagen-binding integrins (Table 1.) (Nykvist *et al.* 2001).

2.3 Mechanisms of epithelial cell migration

Epithelial cell migration, including normal keratinocyte migration in wound healing and malignant invasion of tumor cells, requires alterations in actin cytoskeleton dynamics and cell adhesions. First, cells extend actin-rich protrusions, lamellipodia and filopodia, at the leading edge of the cell. Then, stable adhesions are formed close to the leading edge of the protrusions, after which the cell is translocated forward. Adhesions at the leading edge provide the traction force necessary for movement. Cells move forward by contracting the actin cytoskeleton and simultaneously releasing the attachments at the rear end. Cell adhesions are structures undergoing constant remodelling. The coordinated processes of adhesion assembly, disassembly and turnover are still mainly unclear. Subclasses of cell adhesions have been described, including focal complexes, fibrillar adhesions, podosomes, and focal adhesions, which differ in molecular composition. Altogether, more than 50 proteins have been detected in cell adhesions. (Horwitz & Parsons 1999, Webb *et al.* 2002).

Physiologic and pathologic epithelial cell migration is modulated by the microenvironment consisting of the ECM and stromal cells. Integrins are the main cellular receptors involved in epithelial migration (Table 1.), and they act as sensors and integrators between the ECM and the cytoskeleton. Specifically binding to ECM ligands, integrins mediate the signals that activate the intracellular signalling pathways. Signal transduction via integrins regulates many essential cellular processes, such as proliferation, differentiation, survival, and motility. Integrins are components of cell adhesions and play a central role in stabilizing adhesions during cell migration. Alterations in integrin-ligand binding strength are needed in the cyclic process of attachment and detachment during migration. Integrin function can be modulated by the regulation of either receptor affinity or receptor density. Integrin clustering on the cell membrane can be induced by outside-in signalling, leading to enhanced ligand capture. Binding of ECM by integrins enhances integrin clustering and assembly of cytoskeletal proteins into aggregates. This process induces integrin clustering and ECM organization,

forming a positive feedback system. As a migrating cell moves forward over stable adhesions, integrins as well as other components of adhesions accumulate at the rear end. There is evidence that integrin-containing vesicles move from the rear to the front of the cell, indicating that intracellular transportation may exist, moving integrins back to the front. (Lauffenburger & Horwitz 1996, Giancotti & Ruoslahti 1999, Friedl & Brocker 2000, Martin *et al.* 2002, Webb *et al.* 2002)

Table 1. Keratinocyte surface integrin receptors and their ligands. (Modified from Steffensen et al. 2001).

Integrin	Quiescent keratinocytes	Wound keratinocytes	Ligands
α1β1	+	+ (*in vivo*)	Collagen I, IV, V, VI
α2β1	+	+	Collagen I, III, IV, V, VI, denatured collagen I
α3β1	+	+	Lam-5
α5β1	+	+	Fibronectin, collagen XVII*
α9β1	+	+	Tenascin-C
α6β4	+	+	Lam-5, collagen XVII**, plectin***
αvβ1	+(*in vitro*)	+(*in vitro*)	Fibronectin, vitronectin, collagen XVII*
αvβ5	+/-	+	Vitronectin
αvβ6	-(*in vivo*)	+	Fibronectin, tenascin-C, vitronectin

* (Nykvist *et al.* 2001) ** (Hopkinson *et al.* 1995, Borradori *et al.* 1997) *** (Koster *et al.* 2003)

In wounds of various organs, such as oral and intestinal mucosa, skin, and cornea, mechanisms of keratinocyte migration onto the wound bed are influenced by the differences in the underlying ECM. In wounds of oral mucosa, healing proceeds faster and with less scarring compared to epidermis (Häkkinen *et al.* 2000). In skin wounds, epidermal keratinocytes migrate under the fibrin clot on the dermal matrix containing type I collagen, whereas in gingival wounds, keratinocytes migrate through the clot, i.e. on the fibrin-fibronectin matrix. Migration on these ECMs is facilitated by different integrins, and the requirements for proteolytic processing of the ECM are different, leading to variations in the expression of integrins and proteolytic enzymes. (Steffensen *et al.* 2001)

The behavior of malignant epithelial cells is regulated by the presence of various factors in the ECM on the one hand and by the altered response of the cells to these factors on the other. To a certain degree, the genotype of the neoplastic cells determines their response to the motility factors. However, through ECM-derived signalling, it is even possible to restore a differentiated phenotype in cells with a neoplastic genotype (Weaver et al. 1997), emphasizing the importance of cell-matrix interactions in the regulation of tumor invasion.

Cell motility can be defined as random (-kinesis) or directed (-taxis), and it can be stimulated by soluble (chemo-) or solid (hapto-) substrates. Soluble factors contributing

to epithelial migration include various growth factors and cytokines, and they have been widely studied. Cytokines have been suggested to participate in invasion and metastasis by directly promoting carcinoma cell motility (Murphy 2001). Solid motility factors are still poorly known, although BMZ molecules are obvious candidates. By proteolytic processing, the function of certain BMZ molecules can be altered from adhesive to motility-promoting. For example, pro-laminin-5, a migratory molecule, is converted into a statically adhesive hemidesmosome-associated protein by serine protease cleavage of the α3 chain (Goldfinger et al. 1998). Furthermore, the laminin-5 γ2 chain can be proteolytically processed into fragments that act as ECM motility factors (Giannelli et al. 1997, Koshikawa et al. 2000, Pirila et al. 2003). Similarly, fragments of vitronectin, tenascin, and collagen IV are reported to promote epithelial motility (Stefansson & Lawrence 1996, Xu et al. 2001, Swindle et al. 2001). (Giancotti & Ruoslahti 1999, Friedl & Brocker 2000, Schock & Perrimon 2002, Quaranta 2002).

To some extent, mesenchymal transition occurs in migrating epithelial cells, including loss of polarization and cell-cell attachments, which are typical features of quiescent keratinocytes. This process is characteristic of normal keratinocytes during wound healing and fetal morphogenesis as well as of invasive carcinoma cells. In wound healing, mesenchymal transition only occurs in the leading cells, and it is partial with only minor loss of cell-cell contacts, whereas malignant cells exhibit more complete transition to the mesenchymal phenotype. Carcinoma invasion may take place by at least two cellular patterns, migration of cell clusters and migration of single cells. Single invading cells migrate either by integrin-dependent, fibroblast-like motion or by faster ameboid, integrin-independent migration. Cell cluster invasion follows paths of low resistance in tissues, and invading cell clusters have been detected in lymphatic vessels and peripheral blood. The leading cells of the cluster are highly motile, while the following cells are more passive. (Friedl & Brocker 2000, Quaranta 2002)

2.4 BMZ components in re-epithelialization

The process of re-epithelialization during wound healing involves epithelial cell migration and proliferation and leads to closure of the epithelial injury (Fig. 3). Keratinocyte migration begins within 3-6 hours of wounding, presumably initiated by exposure to various growth factors and cytokines released by the damage. Keratinocytes at the wound edge are converted into a migratory phenotype, a process associated with alterations in cell-matrix adhesion systems, ligand-binding affinity of cellular receptors as well as the expression of BMZ ligands and proteolytic enzymes. Some hours after the onset of migration, keratinocyte proliferation is increased distal from the migrating edge. The migrating keratinocytes deposit provisional basement membrane, and the mature basement membrane is re-established later on, from the margins of the wound inwards, after the completion of re-epithelialization. (Martin 1997, Jacinto *et al.* 2001)

Initiation of the migratory process requires alteration of hemidesmosome-based keratinocyte anchorage to dynamic adhesion mediated by different cellular receptors. Basal cells at the wound edge detach from the basement membrane and come into contact

with type I collagen in the wound bed. The leading cells attach transiently to the exposed type I collagen via α2β1 integrin and deposit laminin-5 precursor in the provisional basement membrane of the wound. In the leading keratinocytes at the wound edge, laminin-5 expression is increased, and laminin-5 precursor is also present in the cytoplasm of the leading cells. The following cells attach to the deposited precursor laminin-5 through α3β1 integrin. An endogenous protease plasmin cleaves the α3 chain of the laminin-5 precursor, releasing mature laminin-5. This cleavage inhibits epithelial cell migration and promotes hemidesmosome formation. The quiescent cells adhere to the mature laminin-5 in the basement membrane through hemidesmosomal α6β4 integrin. (Nguyen *et al.* 2000) In addition to the α3 chain, the γ2 chain of laminin 5 may also be proteolytically processed into a shorter form. This cleavage is achieved by MMPs, resulting in the formation of a 80 kDa fragment, which is capable of inducing cell motility. The cleavage of the γ2 chain occurs in tumors and in tissue remodelling, i.e. wound healing, but not in quiescent tissues. (Giannelli *et al.* 1997, Pirilä *et al.* 2003) Immunohistochemical and *in situ* hybridization studies have revealed induction in laminin-5 expression in the leading keratinocytes of the wound edge, regardless of the wound model (Larjava *et al.* 1993, Ryan *et al.* 1994, Kainulainen *et al.* 1998, Salo *et al.* 1999a).

In addition to laminin-5, α6β4 integrin is another BMZ component synthesized by the migrating keratinocytes (Larjava *et al.* 1993). After hemidesmosome disassembly, the localization of α6β4 integrin is switched to actin protrusions instead of hemidesmosomes. It is suggested that α6β4 integrin has migration-enhancing functions apart from its role as a hemidesmosomal adhesion protein. (Gipson *et al.* 1993, Rabinovitz *et al.* 1999, Lotz *et al.* 2000)

Other BMZ components are synthesized and deposited at a later stage, during basement membrane maturation after re-epithelialization: type IV collagen, laminin-1 and type VII collagen are only seen in quiescent keratinocytes and not in the epithelial outgrowth (Larjava *et al.* 1993). The last components of the hemidesmosome adhesion complex to reach maturity are the anchoring fibrils (Compton *et al.* 1989).

Hemidesmosomes are reassembled to accomplish anchorage of keratinocytes to the newly synthesized basement membrane. The process of hemidesmosome assembly is complex and not well known. Initiation of hemidesmosome assembly may occur as α6β4 integrin binds to epidermal growth factor (EGF) receptor, which induces phosphorylation of the β4 integrin subunit by activating tyrosine kinase Fyn (Mariotti *et al.* 2001). Hemidesmosome assembly is nucleated by interaction of α6β4 integrin with plectin, which changes the configuration of β4, enabling it to directly interact with collagen XVII. Collagen XVII is incorporated into the complex by binding to both β4 integrin subunit and plectin (Koster *et al.* 2003). This is followed by association of collagen XVII with BP230. (Gagnoux-Palacios *et al.* 1997, Hopkinson *et al.* 1998, Schaapveld *et al.* 1998) The results of several studies indicate a major role for α6β4 integrin and laminin-5 in hemidesmosome formation. Mice lacking α6 or β4 are not able to form hemidesmosomes (van der *et al.* 1996, Dowling *et al.* 1996, van der Neut *et al.* 1996, Georges-Labouesse *et al.* 1996). Furthermore, JEB patients with mutations in the genes encoding laminin-5 fail to form normal hemidesmosomes. (Jones *et al.* 1998, Nievers *et al.* 1999, Borradori & Sonnenberg 1999)

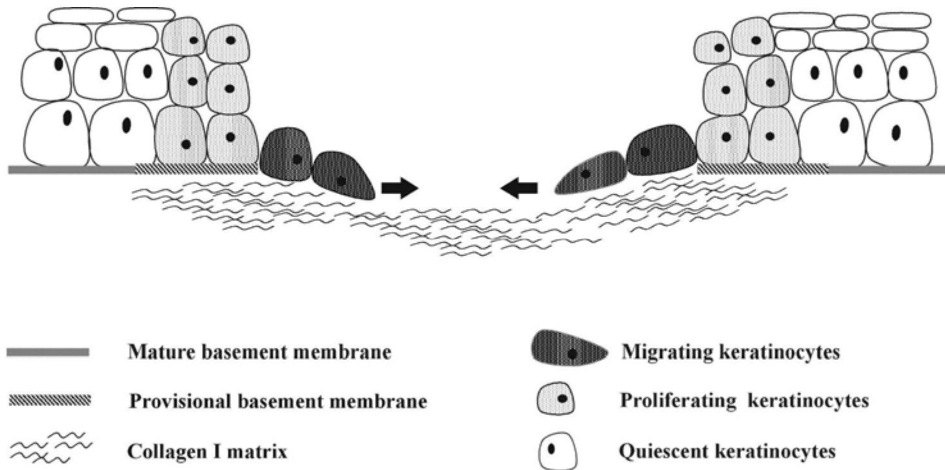

Fig. 3. Re-epithelization. (Modified from Parks 1999)

2.5 MMPs in re-epithelialization

Strictly regulated degradation and reorganization of the extracellular matrix, including the BMZ, is required for the normal process of wound healing. Various proteolytic enzymes are needed for modulation of the wound matrix to facilitate the migration of epithelial cells and the subsequent wound closure (Table 2). ECM-degrading enzymes are divided into four main groups: serine proteases, cysteine proteases, aspartic proteases, and metalloproteinases, based on their catalytic sites. The migrating cells must be detached from the basement membrane, and a path must be generated for their migration through the fibrin clot. Moreover, enzymatic activity is necessary to regulate the function of various molecules by proteolytic processing. (Murphy & Gavrilovic 1999, Steffensen *et al.* 2001, Overall & Lopez-Otin 2002)

MMPs are a multigene family that belongs to the superfamily of metalloproteinases. Altogether 23 human MMPs are known at present, classified as collagenases, gelatinases, stromelysins, membrane-type MMPs, and others. All members of the MMP family are structurally related. Three domains are common to all MMPs: the N-terminal hydrophobic (pre)domain, the propeptide domain, and the catalytic zinc-binding domain. The predomain directs the synthesis of MMPs to the endoplastic reticulum, after which it is removed. The catalytic domain of MMPs has a cleft containing the catalytic Zn^{2+}, in which the substrate is bound and then cleaved. MMPs are synthesized as inactive zymogens, with the prodomain masking the catalytic site. A conserved cysteine residue in the prodomain forms a “cysteine switch”, which needs to be disrupted before removal of the propeptide domain and subsequent exposure of catalytic Zn^{2+} are possible. The

disengagement of the propeptide may take place through proteolytic processing or as a result of the latent enzyme binding to a ligand or a substrate (Bannikov *et al.* 2002).

Once active, MMPs can be inhibited by either general endogenous inhibitors, such as α2-macroglobulin in plasma and tissue fluids, or by specific inhibitors, such as TIMPs, which are anchored or secreted to the ECM. TIMPs inhibit active MMPs as well as proMMP activation. Four homologous TIMPs are known to date: TIMP-1 to TIMP-4. TIMPs are relatively small proteins with two structurally and functionally distinct domains. They are able to bind MMPs non-covalently, forming enzyme-inhibitor complexes at molar equivalence. All TIMPs bind to most MMPs, but some differences exist between the inhibitory functions of TIMPs. TIMPs posess various functions, including inhibition of cell invasion, tumor progression, and metastasis and induction of apoptosis. TIMPs also exhibit some additional biological activities, such as growth-factor-like and anti-angiogenic activity, which are independent of their MMP-inhibitory functions. (Bode *et al.* 1999, Nagase & Woessner, Jr. 1999, Nagase *et al.* 1999, Van den Steen *et al.* 2001, Overall & Lopez-Otin 2002)

With few exceptions, MMPs that are expressed in wounded skin, are not synthesized in normal skin. Most known MMPs are expressed during wound healing, but their specific biological functions are mainly unknown. Although specific MMPs act on some substrates better than others, many MMPs that are expressed in wounds have overlapping substrate specificities. Substrate selectivity may be directed by the differences in enzyme affinities, and by compartmentalization: activity of a specific MMP is strictly regulated both temporally and spatially during wound repair process. (Parks 1999)

2.5.1 Keratinocyte-derived MMPs

Together with MMP-8 (collagenase 2) and MMP-13 (collagenase 3), MMP-1 (collagenase-1) participates in the degradation of fibrillar collagens, especially type I collagen, the most abundant protein in the dermis. The importance of MMP-1 in the context of human skin wound healing is well characterized. MMP-1 is expressed by the wound edge keratinocytes (Table 2), in both acute and chronic wounds, and its expression is rapidly shut off after the re-epithelialization is complete (Saarialho-Kere *et al.* 1992, Saarialho-Kere *et al.* 1993). In blisters, however, keratinocytes migrating on the basement membrane do not express MMP-1(Saarialho-Kere *et al.* 1995b). As the wound edge keratinocytes move off the basement membrane, they come to contact with type I collagen in the dermal matrix. The interaction of α2β1 integrin with type I collagen leads to induction of MMP-1 expression in the wound edge keratinocytes (Pilcher *et al.* 1997). Furthermore, MMP-1 activity is essential for the onset of keratinocyte migration at the initiation of re-epithelialization (Pilcher *et al.* 1997).

Although it is known that BMZ type IV and type VII collagens can be cleaved by MMP-2 (gelatinase A) and MMP-9 (gelatinase B), the specific biological functions of these enzymes during re-epithelialization remain elusive. In addition to the processing of BMZ molecules, the gelatinases may play an important role in the remodelling of wound ECM, because of their ability of degrading fibrillar collagens after the initial cleavage by

collagenases. MMP-2 is constitutively expressed by basal keratinocytes both in uninjured skin and during wound repair (Table 2). It is capable of inducing epithelial cell migration by cleavage of laminin-5, which takes place in tissue remodelling and tumor invasion (Giannelli *et al.* 1997). The relevance of this processing in normal wound re-epithelialization is unclear. MMP-9 has been suggested to participate in the detachment of epithelial cells from the basement membrane, in the degradation of the fibrin clot, in the migration of keratinocytes on the provisional matrix and in processing components of the maturing basement membrane. Temporal and spatial expression pattern of MMP-9 expression during wound repair has been widely studied in different experimental models. MMP-9 is not expressed in quiescent human skin keratinocytes, but transient upregulation is observed in the migrating keratinocytes of acute wounds (Table 2), decreasing as re-epithelialization is complete (Tarlton *et al.* 1997, Madlener 1998). In contrast to epidermis, MMP-9 is expressed in the quiescent keratinocytes of oral mucosa (Salo *et al.* 1994). MMP-9 expression is also detected in the migrating epithelial outgrowth of mucosal wounds and experimental skin suction blister wounds (Oikarinen *et al.* 1993, Salo *et al.* 1994). In chronic wounds, however, MMP-9 expression is ectopic and persistent (Yager *et al.* 1996, Wysocki *et al.* 1999, Mirastschijski *et al.* 2002a). In MMP-9-deficient mice, re-epithelialization of corneal wounds is accelerated as a result of their increased keratinocyte proliferation rate, suggesting that MMP-9 affects epithelial wound healing (Mohan *et al.* 2002). The accelerated wound closure is correlated with an accumulation of cytokine IL-1α and a delayed inflammatory response (Mohan *et al.* 2002). During re-epithelialization, cytokine activity is essential for triggering keratinocyte migration. MMP-9 is known to be able to process various cytokines, converting pro-forms into active molecules, and on the other hand, proinflammatory cytokines and growth factors regulate pro-MMP-9 expression and activation. In rat mucosal keratinocytes, pro-MMP-9 expression is upregulated by interleukin-1β (IL-1β), transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) (Lyons *et al.* 1993). In organ-cultured human skin, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) synergistically induce pro-MMP-9 expression (Han *et al.* 2002). Furthermore, conversion of pro-MMP-9 into active MMP-9 is promoted by TNF-α (Han *et al.* 2002). (Nagase & Woessner, Jr. 1999, Steffensen *et al.* 2001, Van den Steen *et al.* 2001)

MMP-3 (stromelysin-1) and MMP-10 (stromelysin-2) are able to degrade a wide variety of substrates relevant to wound healing, including type IV collagen, fibronectin, gelatine, and laminin. MMP-3 is expressed by keratinocytes distal to the wound edge, in the proliferating cell subpopulation in normal and chronic wounds (Table 2) (Saarialho-Kere *et al.* 1994). Mice lacking MMP-3 show delayed wound healing, resulting from impaired early wound contraction (Bullard *et al.* 1999). MMP-10 is expressed by keratinocytes at the migrating front in normal and chronic wounds (Table 2) (Vaalamo *et al.* 1996, Saarialho-Kere 1998), upregulation taking place later than that of MMP-1 (Rechardt *et al.* 2000). In contrast to MMP-1, expression of MMP-10 is upregulated by cytokines rather than cell-matrix interactions (Rechardt *et al.* 2000). In retarded wound healing, MMP-10 is not overexpressed (Rechardt *et al.* 2000)

MMP-28 (epilysin), a novel member of MMP-family, is a caseinolytic enzyme which is expressed by basal keratinocytes distally from the wound edge, in the proliferative cell population (Table 2) (Lohi *et al.* 2001, Saarialho-Kere *et al.* 2002). In quiescent

epidermis, MMP-28 is expressed by basal and suprabasal keratinocytes (Lohi *et al.* 2001). The role of MMP-28 during re-epithelialization is not known.

MMP-7 (matrilysin) is expressed in ductal and secretory cells of eccrine and apocrine glands (Saarialho-Kere *et al.* 1995a). Activity of MMP-7 is required for healing of airway wounds, but it is not expressed in cutaneous wounds, and apparently not necessary for the healing of epidermal injury (Parks 1999, Parks *et al.* 2001).

2.5.2 TIMP-1

It is essential that the proteolytic activity of the MMPs participating in ECM degradation is under exact control. TIMPs have an important role in regulating MMP activity, and an imbalance in the levels of MMPs and TIMPs is detected in many pathological processes, including chronic degenerative diseases, tumor invasion, and chronic ulcers. There are very few reports on the effects of MMP inhibitors on normal or chronic wound healing. Interestingly, TIMP-1 is expressed by migrating keratinocytes in an acute wound (Table 2), while it is completely missing from chronic wounds (Vaalamo *et al.* 1996, Vaalamo *et al.* 1999). TIMP-1 inhibits all known MMPs, including the keratinocyte-derived MMPs essential for epithelial migration in wound healing (Table 2). A recent study identified a tissue-bound chymotrypsin-like proteinase in human skin, which mediates the proteolytic activation of MMP-9, and which can be blocked by TIMP-1. Furthermore, inhibition of MMP-9 by TIMP-1 is down-regulated by tumor necrosis factor (TNF)-α. (Han *et al.* 2002) Studies with truncated recombinant TIMP-1 or TIMP-1 with site-directed mutations indicate that MMP-inhibitory activity is located in the N-terminal domain. TIMP-1 is capable of binding proMMP-9, associating via the C-terminal domain, which leaves the N-terminal domain free to interact with other MMPs. (Murphy *et al.* 1991, O'Shea *et al.* 1992, Nagase *et al.* 1999). (Gomez *et al.* 1997, Bode *et al.* 1999, Nagase & Woessner, Jr. 1999, Brew *et al.* 2000, Van den Steen *et al.* 2001)

Table 2A. MMP expression in keratinocytes during human mucocutaneous wound healing. (Salo *et al.* 1994, Vaalamo *et al.* 1996, Vaalamo *et al.* 1999, Rechardt *et al.* 2000, Saarialho-Kere *et al.* 2002)

MMP	Expression in wound healing	Main substrates
MMP-1	Keratinocytes at the wound edge	Collagen I
MMP-2	Basal keratinocytes	Gelatin, type I collagen, laminin-5*
MMP-3	Keratinocytes distal to the wound edge	Collagen IV, gelatin, nidogen, perlecan, fibronectin, elastin
MMP-9	Keratinocytes at the wound edge	Gelatin, collagen IV, VII, nidogen, fibronectin, elastin
MMP-10	Keratinocytes at the wound edge	Collagen IV, gelatin, nidogen, fibronectin, elastin, laminin
MMP-28	Keratinocytes distal to the wound edge	Physiological substrates not determined

*(Giannelli *et al.* 1997)

Table 2B. TIMP expression in keratinocytes during human mucocutaneous wound healing. (Vaalamo et al. 1996, Vaalamo et al. 1999)

TIMP	Expression in wound healing
TIMP-1	Keratinocytes distal to the wound edge
TIMP-2	Keratinocytes at the wound edge
TIMP-3	Keratinocytes distal to the wound edge
TIMP-4	Not detected in keratinocytes

2.6 BMZ components in epithelial tumors

Transformation of epithelium into a malignant tumor involves disturbances in the normal features of keratinocytes, such as attachment to the basement membrane, polarized morphology, and specialized cell-cell adhesions. This process is characterized by changes in the expression and function of various cellular receptors and BMZ proteins. Abnormal expression of BMZ components, which is observed in epithelial tumors of various types, is believed to play a role in tumor invasion and metastasis. BMZ proteins, acting as ligands for cell surface receptors, play a major role in normal tissue maintenance, wound healing, and carcinogenesis. (Ziober *et al.* 2001) Recent studies have suggested a major role for α6β4 integrin in tumor progression as well as in physiological migration during wound healing. α6β4 is able to stimulate carcinoma cell migration by interacting with the actin cytoskeleton (Rabinovitz & Mercurio 1997). In addition, it has been reported that α6β4 integrin is capable of triggering apoptosis in carcinoma cells expressing wild-type but not mutant p53, which means that the invasion-promoting activity of α6β4 is enhanced in tumor cells expressing mutant p53 (Bachelder *et al.* 1999).

Laminin-5 γ2 chain has been extensively studied in epithelial tumors, and it is considered a marker of invasiveness in these lesions. Strong cytoplasmic γ2 chain expression has been reported in carcinomas of different origin and in malignant melanomas, but not in sarcomas. (Pyke *et al.* 1995, Skyldberg *et al.* 1999, Lenander *et al.* 2001) The γ2 chain overexpression, compared to lower levels of α3 and β3 chains, implicates that γ2 chain occurs as a monomer in invading cancer cells (Koshikawa *et al.* 1999). The exact biological function of laminin-5 in tumor invasion is not known.

Altered expression of BMZ molecules appears to contribute to the transformation of pre-malignant epithelium into a malignant tumor. Downregulation of collagen XVII and other hemidesmosome components is detected in various epithelial cancers not originating from stratified epithelia, i.e. prostate cancer, undifferentiated carcinoma of the parotid gland, and ductal mammary carcinoma (Bergstraesser *et al.* 1995, Nagle *et al.* 1995, Kim *et al.* 2000). On the other hand, in thyroid carcinoma, hemidesmosomal adhesion complex components are neoexpressed, even though they are abcent from normal thyroid epithelium (Lohi *et al.* 1998).

2.6.1 Basal cell carcinoma

Basal cell carcinoma (BCC) is the most common skin cancer, localizing only in hair-bearing skin. It is a rarely metastatic, but locally invasive and recurrent neoplasm. The histological features of BCC include well-demarcated tumor islands surrounded by fibrous stroma and peritumoral cystic spaces. The tumor cells resemble normal basal keratinocytes. Many BCC subtypes of different histological growth patterns exist, including the solid, keratotic, and superficial types (Elder 1997). Recently, it was discovered that mutations in the Patched1 (PTCH1) gene, a member of the hedgehog (Hh)-signalling pathway, leads to the development of multiple BCCs in Gorlin's syndrome. These mutations cause overexpression of the target gene product, a cell surface receptor for the Hh protein family. Afterwards, mutations of the PTCH1 and related genes have also been reported in sporadic BCCs. However, it is unclear how this cellular defect results in tumorigenesis. In addition to PTCH1, other genetic alterations, such as P53 mutation, have been reported in BCC tumors. (Dicker *et al.* 2002, Saldanha *et al.* 2003). As a tumor with limited invasive potential, BCC is an interesting model for studies on the functions of the BMZ. In spite of the non-metastasizing growth pattern, basement membrane breaks occur in BCCs, particularly in the areas of invasive tumor growth (Hewitt *et al.* 1996). Furthermore, ultrastructural studies have revealed an absence or decrease of hemidesmosomes in the BMZ of BCC (Jones *et al.* 1989), and immunohistochemical studies on hemidesmosomal adhesion complex molecules suggest that these components are not synthesized or assembled properly. Diminished levels of BP230, α6β4 integrin, collagen XVII, laminin-5, and type VII collagen have been reported in the BMZ of BCC tumors. The alterations in the hemidesmosomal adhesion complex have been suggested to affect the cell adhesion to the basement membrane, and to facilitate BCC tumor progression. (Savoia *et al.* 1993, Korman & Hrabovsky 1993, Fairley *et al.* 1995, Bahadoran *et al.* 1997, Chopra *et al.* 1998)

2.6.2 Ameloblastoma

Ameloblastoma, a usually benign but locally invasive tumor, is the most common odontogenic epithelial neoplasm. It has a strong tendency to recur, but malignant ameloblastoma is rare. The tumors typically occur as slowly growing intrabony swellings and rarely as soft-tissue overgrowths classifiable as peripheral ameloblastomas. The name 'ameloblastoma' is derived from the tumor's histological resemblance to developing tooth germs: ameloblastoma is believed to originate from the odontogenic epithelium. Neoplastic islands are composed of peripheral ameloblast-like columnar epithelial cells and central stellate reticulum-like tissue. Ameloblastomas are classified according to their distinct histopathologic growth patterns as follicular, plexiform, granular cell, acanthomatous, desmoplastic and basal cell types. (Neville BW 2002) The basement membrane proteoglycan perlecan is synthesized by ameloblastoma cells and accumulates into the intercellular space of the stellate reticulum-like tissue, participating in the development of the characteristic myxoid histology of the tumors (Ida-Yonemochi *et al.*

2002). The basement membrane surrounding the tumor islands of ameloblastoma is mostly continuous and visualized as linear tenascin, type IV collagen, and laminin immunostaining (Thesleff & Ekblom 1984, Sauk 1985, Heikinheimo *et al.* 1991, Nakano *et al.* 2002). However, the α chain distribution of type IV collagen is altered compared to the normal basement membrane of oral mucosa, suggesting a role in tumor progression (Nakano *et al.* 2002). The BMZ of the rare malignant ameloblastoma is clearly abnormal, characterized by focal, discontinuous type IV collagen staining (Sauk 1985). The focal loss of immunoreactivity to collagen VII seen at the BMZ of some ameloblastomas may be associated with their aggressive behavior (Heikinheimo *et al.* 1991). Invading small ameloblastoma islands and peripheral neoplastic cells exhibit intracellular staining for laminin-5 γ2 chain, reflecting the invasive and migratory phenotype of these cells (Salo *et al.* 1999b).

2.6.3 Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is a common, highly invasive and metastasizing carcinoma that occurs in the oral mucosa. Histologically, the tumors consist of keratinized carcinoma cell islands, which invade into the surrounding tissue. Large numbers of inflammatory cells are seen in the tumor stroma. According to a histopathological evaluation of the differentiation level, OSCC tumors are divided into grades I-IV. (Neville BW 2002) Although well differentiated SCCs express all main structural BMZ components (Stanley *et al.* 1982), at the ultrastructural level the basement membrane-like structure surrounding the tumor islands of OSCCs appears to be discontinuous or absent (Stanley *et al.* 1982, Kannan *et al.* 1994, Cheng & Hudson 2002). Abnormal expression patterns have been described for various BMZ components in OSCC. Laminin-5 γ2 chain is overexpressed in the invasive areas of OSCC tumors and observed as intense, cytoplasmic staining of carcinoma cells (Kainulainen *et al.* 1997a, Kosmehl *et al.* 1999). However, the level of heterotrimeric laminin-5 molecules is decreased in the basement membrane-like structure of the neoplastic OSCC islands, inversely correlating with the degree of tumor differentiation (Haas *et al.* 2001). Type VII collagen, which is a component of the anchoring fibrils, is synthesized at a high level by OSCC cells, but with abnormal cytoplasmic distribution (Kainulainen *et al.* 1997b). Variable changes in integrin expression have been documented in OSCC, including decreased expression of β1 integrins. The reduced expression of α6β4 integrin correlates with poor tumor differentiation and with a loss of BMZ proteins. Furthermore, alterations in αv integrins have been described in OSCC, with a decrease in αvβ5 expression and neo-expression of αvβ6. (Thomas *et al.* 1997, Thomas & Speight 2001) Induced expression of αvβ6 has been shown to promote OSCC invasion *in vitro*, suggesting that it may contribute to tumor progression *in vivo* (Ramos *et al.* 2002).

2.7 *In vivo* and *in vitro* models of epithelial migration

To study epithelial migration *in vivo*, different experimental wound models, both in humans and in animals, have been developed. Full-thickness wounds can be generated on the epidermis or mucosa by punch biopsy, after which the area is allowed to heal for the desired time, and removed for analysis. In full-thickness wounds, the BM is removed along with the epithelium. In the suction blister method, separation of the epidermis from the dermis takes place at the level of *lamina lucida* as a result of negative pressure applied using a specific device, which leaves the basement membrane intact (Kiistala 1968). Cell motility and behavior *in vivo* can be directly monitored with intravital microscopy in various experimental animal models. For monitoring cell migration, fluorescently labelled cells are injected locally into tissues. In transparent organs, native cells can also be monitored. (von Andrian & M'Rini 1998, Friedl & Brocker 2000)

By *ex vivo* epithelialization assays, it is possible to study, for example, the protein function in epithelial cell migration in gene-manipulated mouse models. Mouse skin biopsies are spread flat on tissue culture plates. Explants are grown in cell culture medium, and keratinocyte outgrowth is determined at frequent intervals from phase-contrast micrographs. The explants can be fixed and immunostained to differentiate keratinocytes from fibroblasts. Similarly, skin explants can be seeded and cultured on the membrane of Transwell tissue culture inserts, to assess cell transmigration. (Mazzalupo *et al.* 2002)

In vivo cell migration is a complex and poorly understood process. Two- or three-dimensional *in vitro* models of migration are able to provide information of the basic principles involved. Haptokinetic epithelial cell migration has been widely studied on two-dimensional migration models. In scratch assays, cells are grown to confluency, an approximately 2 mm wide area is scraped clear of cells using a pipette tip, and cells are allowed to migrate to the area. Another way of studying lateral migration is to plate the confluent cells inside a steel cylinder on a culture dish and and to remove the cylinder after the cells have adhered. The cells at the edge are allowed to migrate. Lateral migration assays can be monitored by photographing at regular intervals. Different aspects of migration can be studied by coating the culture plate with ECM proteins, on which the cells migrate. The directionality and speed of cell migration can be assessed *in vitro* using time-lapse video microscopy. Cells are plated on culture dishes and allowed to migrate, and the coordinates of cells are frequently recorded by image processing software. In cell-mixing experiments, different cell types can be labelled with fluorescent markers. (Friedl & Brocker 2000, Maheshwari *et al.* 2001)

In three-dimensional models mimicking the basement membrane structure, cells migrate from an upper chamber to a lower chamber through a porous filter. In Transwell chambers (Costar), the filter can be coated with basement membrane proteins. In Matrigel™ chambers, the filter is covered by a layer of gel prepared from murine EHS tumor, which mainly contains basement membrane components. Matrigel assay can be considered a model for quantitating the invasive potential of cells, as the cells must invade the gel as well as transmigrate to the other side of the filter (Albini 1998). In both types of transmigration assays, chemotactic agents may be added to the lower chamber to attract the cells. The migration level is quantified by counting the fixed and stained cells

on the lower side of the filter by light microscopy. Three-dimensional collagen matrix models provide a defined model of studying cell migration, resembling connective tissue *in vivo*. Collagen solutions are polymerized, whereupon they form interconnected fibers and bundles. The cell suspensions can be added into the collagen solution before plating or on top of the polymerized collagen gel. Migration can be quantified by counting the fixed and stained cells that have invaded after a given period, or it can be monitored by computer-assisted cell tracking. (Friedl & Brocker 2000)

3 Aims of the present study

Epithelial migration is a complex, dynamic process, which plays a key role in development, regeneration and repair of various tissues and organs. In pathologic processes, such as developmental defects, chronic ulcers and epithelial tumors, the underlying molecular mechanisms of epithelial migration are similar to those in physiologic processes. Collagen XVII is a hemidesmosomal protein, which adheres keratinocytes to the basement membrane. It has been proposed to participate in the regulation of epithelial motility. MMPs secreted by keratinocytes have been suggested to facilitate re-epithelialization during wound healing. The aim of the present work was to explore the role of collagen XVII and the inhibition of keratinocyte-derived MMPs in epithelial migration. Epithelial tumors, and mucocutaneous wounds were chosen as models of physiologic and pathologic epithelial migration.

The specific aims of the study were:

1. to determine collagen XVII expression pattern in epithelial tumors with different invasive potential: in locally spreading ameloblastomas and BCCs, and in highly invasive tongue SCCs
2. to study the role of the cell adhesion domain of collagen XVII, COL15, in regulation of epithelial migration
3. to investigate the effect of targeted inhibition of keratinocyte-derived MMPs by TIMP-1 on wound healing of transgenic mice

4 Materials and methods

4.1 Antibodies and RNA probes (I, II, III, IV)

For immunostainings and immunoblotting, the following polyclonal human collagen XVII antibodies (Fig. 4) were used: NC16A, raised against the juxtamembranous NC16A domain (Schumann *et al.* 2000), Ecto-3, raised against the amino acids 1365-1413 (Schumann *et al.* 2000), and Ecto-5, recognizing the most distal 51 amino acids of the C-terminus (Franzke *et al.* 2002). The polyclonal antibody, recognizing the domain III of the γ2 chain of human and murine laminin-5 was a generous gift from Prof. Karl Tryggvason (Pyke *et al.* 1995). The polyclonal rabbit anti-mouse pan-cytokeratin antibody was from Zymed (Zymed Laboratories, Inc., San Francisco, CA, USA). The antibodies against human α5 (Akiyama *et al.* 1989), αv (L-230; American Type Culture Collection, Manassas, VA, USA) and β1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) integrins were used in flow cytometry experiments. In cell transmigration experiments, integrin function was blocked with anti-human α5 (Akiyama *et al.* 1989), αv (L-230; American Type Culture Collection, Manassas, VA, USA) and β1 (R.322, rabbit polyclonal)(Heino *et al.* 1989) antibodies. The mouse keratinocyte proliferation marker Ki-67 was from DAKO (DAKO, A/S, Glostrup, Denmark).

To prepare a non-radioactive *in situ* hybridization probe for human collagen XVII (Fig. 4) with the DIG RNA Labeling kit (Boehringer Mannheim, Mannheim, Germany), a cDNA fragment, Ecto-4, covering the amino acids 1365-1497 of the collagen XVII ectodomain, was amplified from keratinocyte mRNA with reverse transcriptase polymerase chain reaction (RT-PCR) (Titan reverse transcriptase PCR, Roche Molecular Biochemicals, Mannheim, Germany) with the sense primer 5'-CGCGGATCCGCTGACT TTGCTGGAGATCT-3' and the antisense primer 5'-CGCGGAATTCCGGCTTG ACAGCAATACT-3' and, after digestions with BamHI and EcoRI (restriction sites underlined), cloned into the pGEM.4Z vector (Promega, Madison, WI, USA). The cDNA fragment for the human TIMP-1 probe (626 bp) was generated by digestion with BamHI and HindIII restriction enzymes (Hurskainen et al., 1996), and the probe for murine MMP-9 (323 bp), was prepared from a SmaI-EcoRI cDNA fragment (Reponen *et al.* 1994).

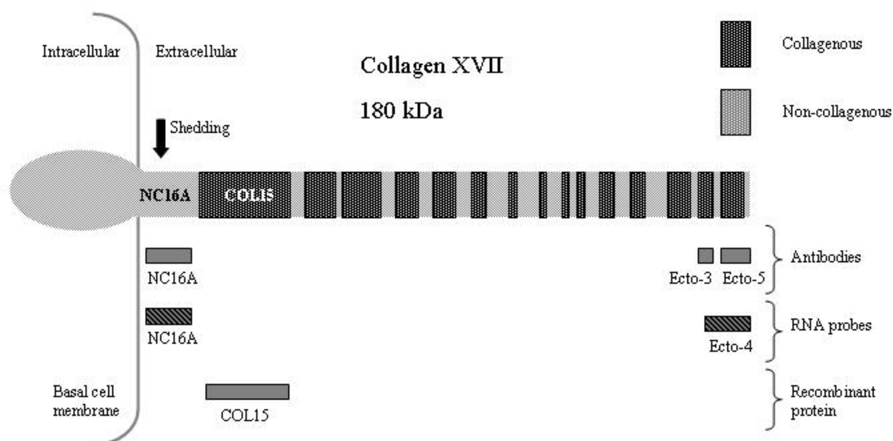

Fig. 4. Schematic illustration of collagen XVII, the specificities of the antibodies and RNA-probes, and the recombinant fragment used. (Modified from Franzke *et al.* 2003)

4.2 Immunohistochemistry and *in situ* hybridization (I, II, III, IV)

Immunoperoxidase staining was performed on 5-10 μm thick paraffin sections. Deparaffinized sections were pretreated to retrieve antigens, according to the specific requirements of each antibody, with glycine buffer (0.2 M, pH 3.6, 10 min, +100˚C) (NC16A, laminin γ2 chain), citrate buffer (10 mM, pH 6.0, 4 min, +100˚C)(laminin γ2 chain), or pepsin (0.4 % in 0.01 M HCl, 10 min, RT)(pan-cytokeratin). Endogenous peroxidase activity was prevented by incubation in H_2O_2 (3%, 3.5 h). Nonspecific binding of the antibodies was blocked by normal goat serum treatment (1:20 in PBS, 0.1% BSA), after which the optimally diluted primary antibodies were applied on the sections (in PBS with 0.1% BSA, overnight, 4˚C). On negative control sections, non-immune or pre-immune rabbit serum was applied instead of the primary antibodies. The secondary antibody, biotinylated anti-rabbit IgG, was then applied (Vector Elite kit, Abbott, Chicago, IL, USA, 30 min, RT), followed by the avidin-biotin-peroxidase complex (Vector Elite kit, Abbott, Chicago, IL, USA, 30 min, RT) and the peroxidase substrate (AEC, 3-amino-9-ethylcarbazole, Vector laboratories, Burlingame, CA, USA, 8

min, RT). Double staining was performed by repeating the following steps: blocking of non-specific antibody binding with normal serum, incubation with a second primary antibody, secondary antibody, and the avidin-biotin-peroxidase complex. The second antibody was detected with another peroxidase substrate, SG (Vector SG substrate kit for peroxidase, Vector Laboratories, Burlingame, CA, USA). The sections were counterstained (Mayer's haematoxylin, Histolab Products AB, Göteborg, Sweden) and mounted (GVA-mount, Zymed, or Aquamount improved, BDH Laboratory Supplies, Poole, England).

For *in situ* hybridization, 6 µm thick paraffin tissue sections were deparaffinized, pretreated with Triton X-100 (0.3% in PBS, 15 min, 37˚C), and washed in PBS. For proteinase K treatment (37˚C), the concentration of the enzyme and the incubation time were optimized for each probe (10-50 µg/ml, 5-20 min). The enzyme reaction was finished by incubation with glycine (0.2% in 0.1 M triethanolamine, 10 min), and the sections were acetylated in acetic anhydride (0.5% in 0.1 M triethanolamine, 10 min). After pretreatment in 4 x SSC, the samples were prehybridized (2 h, 58˚C) in hybridization buffer (4 x SSC, 250 µg/ml yeast t-RNA, 10 mM DTT, 10% dextran sulphate, 2 x Denhardts solution, 50% formamide). Digoxigenin-labelled antisense or sense RNA probe (500 ng/ml in hybridization buffer) was denatured (5 min, 80˚C) and applied on the sections (overnight, 58˚C). Post-hybridization washes were done by incubation in SSC (2 x and 0.1 x, 60˚C). The sections were equilibrated in buffer I (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), and non-specific binding of anti-digoxigenin was prevented using normal goat serum (2% in buffer I, 0.1% Triton X-100, 30 min). The hybridized probe was traced by alkaline-phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim, 1:200 in buffer I, 0.1% Triton X-100, 1% normal goat serum, 90 min). Following washes in buffer I and detection buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the antibody was detected using the Fast Red substrate (20-90 min)(Boehringer Mannheim). The sections were counterstained with Mayer's haematoxylin (Histolab Products AB, Göteborg, Sweden) and mounted (Aquamount improved, BDH Laboratory Supplies, Poole, England).

4.3 Production and purification of recombinant COL-15 (III)

Recombinant COL15, representing the largest collagenous domain of collagen XVII, was produced for cell transmigration assays. When produced in the presence of ascorbic acid, the recombinant COL15 protein is triple-helical (Tasanen *et al.* 2000). Production and purification of COL15 was carried out as previously described in detail (Tasanen *et al.* 2000). Human kidney 293-EBNA cells (Invitrogen, Groningen, Netherlands) were transfected with an expression vector, pCEP-Col15, encoding the amino acids 567-807 of human collagen XVII, with the calcium phosphate method. The transfected cells were selected with puromycin (Sigma) and grown to confluency in the presence of 50 µg/ml ascorbic acid. The medium was collected every 48 h and centrifuged to remove cellular debris, after which proteinase inhibitors Pefablock (1mM, Merck) and N-ethylmaleimide (NEM) (1mM, Sigma) were immediately added. The COL15 recombinant fragment was

dialyzed against 50 mM Tris, pH 8.6, and purified of contaminating proteins with a DEAE-cellulose column (Whatman). COL15 was concentrated by 60% ammonium sulphate precipitation and finally dialyzed against PBS at +4˚C for transmigration experiments.

4.4 Cell culture and cell migration assays (II, III)

IHGK cells (HPV-16-transformed oral keratinocyte cells, a kind gift from prof. Dolphin Oda, Seattle, USA) (Oda et al., 1996) and HaCaT cells (skin keratinocytes, a generous gift from Dr. N. Fusening, German Cancer Research Center, Heidelberg, Germany) were cultured in serum-free keratinocyte medium supplemented with bovine pituitary extract, recombinant epidermal growth factor (Gibco BRL, Grand Island, NY), 1% penicillin, and 0.5% nystatin. The human SCC cell lines SCC-25 (tongue, ATCC, CRL-1628, Rockville, MD, USA) and HSC-3 (tongue, Japan Health Science Research Resources Bank, JRCB 0623) were cultured in 1:1 Dulbecco's Modified Eagle's Medium (DMEM) and Ham´s Nutrient Mixture F-12, containing 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 IU/ml nystatin, 25 µg/ml amphotericin-B, and 0.4 µg/ml hydrocortisone. EA.hy926 cells (immortalized human endothelial cells, a generous gift from prof. Cora-Jean S. Edgell, North Carolina, USA) were cultured in DMEM containing 10% FBS and HAT supplement (Gibco BRL, Grand Island, NY). The human myeloma cell line RPMI 8226 (ATCC Number: CCL-155; Rockwille, MD, USA) was cultured in RPMI 1640 medium (Gibco BRL, Basel, Switzerland) supplemented with 10% NCS, 1% lactate glutamate and 1% penicillin-streptomycin. Cells were grown in a humidified atmosphere at 37°C with 5% CO_2, culture media were replaced once every 2-3 days, and subcultures were obtained by trypsin/EDTA treatment. 48 h prior to and during the experiments, cells were cultured in the presence of 50 µg/ml ascorbic acid, and the supplements were excluded from the keratinocyte medium.

In transmigration assays, the undersides of Transwell (8.0 µm pore size, Costar, Cambridge, MA) filters were coated with fibronectin (10 µg/ml in PBS, 1 h, RT). Membranes were washed with PBS and blocked with 1% BSA (30 min, RT). HSC-3 cells were serum-starved overnight prior to the experiments. Cells were plated in the upper chambers at a density of 1.5 x 10^5 cells/well in serum-free culture medium. Denatured or native COL-15, a recombinant fragment of collagen XVII, was added to the lower chambers (20-60 µg/ml in serum free culture medium). In the control wells, proteinase-digested type I collagen or vehicle (PBS) was added into the lower chambers. Antibodies were added into both chambers. After 6 h incubation (37˚C), the cells on the filter were fixed with 10% TCA (15 min), and the upper, uncoated side of each filter was wiped to remove the cells that had not migrated. The cells on the lower surface were stained with crystal violet and counted in multiple fields. Each experiment was repeated 3-6 times.

4.5 Protein extractions and immunoprecipitation (II)

The cultured cells and the conditioned media were processed separately for protein extractions. The full-length collagen XVII was extracted from the cell layer as described earlier (Schäcke *et al.* 1998). In brief, the cell layer was incubated with extraction buffer containing detergent (1% Nonidet P-40) and proteinase inhibitors (14 µg/ml chymostatin, 7 µg/ml antipain, 7 µg/ml leupeptin, 14 µg/ml pepstatin, 1 mM Pefablock, and 10 µl/ml NEM). The lysate was scraped from the dish and centrifuged to remove cellular debris. The frozen SCC tissue (approximately 50 mm^3) was homogenized in 2 ml of extraction buffer with a glass homogenizer and incubated for 3 h at 4 °C in a mixer. Tissue debris was removed by centrifugation at 14,000 X g for 30 min at 4 °C. The extracts and the medium concentrate were analyzed by immunoblotting.

For immunoprecipitation of the shed ectodomain of collagen XVII, the culture medium was collected, cooled, centrifuged to remove cellular debris, and supplemented with 1 mM Pefablock (Merck, Darmstadt, Germany) and 1 mM NEM (Sigma-Aldrich Deisenhofen, Germany). Prior to immunoprecipitation, 0.2 g of Protein A Sepharose (Amersham Pharmacia Biotech Uppsala, Sweden) was prepared as indicated by the supplier, washed once with phosphate-buffered saline, once with phosphate-buffered saline-0.5 % Tween, and once again with phosphate-buffered saline. Then, 50 µl of the NC16A antibody (Fig. 4) was added to Protein A Sepharose in 0.5 ml of phosphate-buffered saline and rotated for 3 h at 4 ºC. The Protein A Sepharose/antibody complex was washed three times as above, and 30 ml of medium was added to the antibody complexes and rotated at 4 ºC overnight. After three washes as above, the pellet was packed into a small column using 50 mM Tris-HCl, ph 7.0, at room temperature, as column buffer. After extensive washing with the same buffer, the ectodomain of collagen XVII was eluted using 6 ml of 0.1 M glycine buffer, pH 3.0, at room temperature, and the pH of the fractions was neutralized with 1 M Tris-HCl, pH 9.0, RT, immediately after elution.

4.6 Immunoblotting (II)

Protein samples, including immunoprecipitated media and cell extracts from oral keratinocytes (IHGK) and SCC cells (SCC-25, HSC-3), as well as carcinoma tissue extracts were separated on 12% SDS-PAGE gel and transferred on to nitrocellulose. Nonspecific antibody binding was blocked with 2% milk in tris-buffered saline (TBS) (30 min). The polyclonal antibodies NC16A (1:1000), Ecto-5 (1:500), and Ecto-3 (1:500) (Fig. 4) were diluted in 2% milk-TBS and incubated overnight with the membrane. After washes and incubation with the secondary antibody, the collagen XVII signal was detected by the NBT-BCIP substrate.

Semiquantitative analysis of collagen XVII in extracts of IHGK, SCC-25, and HSC-3 cells was performed by enhanced chemiluminescence (ECL) Western blot. The samples were run on 12% SDS-PAGE gel and transferred on to Immobilon™ -P nitrocellulose membrane (Millipore, Bedford, MA, USA). Nonspecific binding of the antibody was

blocked by incubation with 10% milk in TBS for 60 min. The membrane was washed with TBS and incubated overnight with polyclonal collagen XVII antibody NC16A, diluted 1:500. After washes in TBS, the filter was incubated with peroxidase-conjugated swine anti-rabbit immunoglobulins (1:500, DAKO, A/S, Glostrup, Denmark) for 60 min, washed again, and incubated with avidin-biotinylated horseradish peroxidase (ABComplex/HRP, DAKO, A/S, Glostrup, Denmark) for 60 min. The filter was treated with ECL detection solution for 1 min, and collagen XVII was visualized by X-ray film exposure (Hyperfilm-ECL, Amersham Life Science, England). The relative amount of collagen XVII in the samples was calculated by scanning the bands on the X-ray film and dividing the scanning unit values by the respective values of the total loaded protein visualized by Coomassie staining (ScionImage PC, Scion Corporation, MD, USA).

4.7 Flow cytometry analysis (III)

Cultured HSC-3 carcinoma cells were washed with PBS after detachment with trypsin. The cell suspensions were incubated in PBS containing 1% FCS for 20 min at +4˚C, with 1:20 dilution of the anti-α5 and -β1 integrin antibodies and with 1:50 dilution of the anti-αv integrin antibody, in PBS with 1% FCS for 30 min at +4˚C. The cells were then washed with PBS and incubated with FITC-conjugated anti-mouse or anti-rat secondary antibody for 30 min at +4˚C. After washing with PBS, the cells were resuspended in PBS and analyzed by FACSscan flow cytometry.

4.8 RT-PCR and ribonuclease protection assay (II)

4 ng of human total mRNA extracted from immortalized keratinocytes of oral cavity (IHGK) and skin (HaCaT), SCC cells (SCC-25, HSC-3), endothelial cells (EA.hy926), and myeloma cells (RPMI 8226) was reverse-transcribed using Superscript II RnaseH^{-} Reverse Transcriptase (Gibco BRL, Roskilde, Denmark) and random hexamer primers. A human cDNA fragment of 230 bp corresponding to the NC16A domain of collagen XVII (amino acids 490-567)(Fig. 3) was amplified using the sense primer 5’-GAGGAGGTGAGGAAGCTGAA-3’ and the antisense primer 5’-TCGGAGATT TCCATTTTCC-3’. The PCR reaction was performed in a thermal reactor with thermostable Dynazyme™ DNA polymerase (Finnzymes Oy, Espoo, Finland), using 35 cycles. The PCR products were analyzed by electrophoresis on 1 % agarose gel containing 1.0 μg/ml ethidium bromide.

Ribonuclease protection assay (RPA) was carried out using the RPA III™ Ribonuclease Protection Assay Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. [α-32P]UTP-labeled 399 bp Ecto-4 cRNA probe (nucleotides 4198-4596) (Fig. 4) was hybridized with 10 μg of total RNA. The protected double-stranded RNA fragment was run on a 5% denaturing polyacrylamide gel and visualized on an X-ray film (Kodak Company, Rochester, NY). A 115 bp 18S cRNA

probe (Ambion, Austin, TX, USA) was included in the hybridization reaction as an internal control for semiquantitative analysis of the protected collagen XVII fragment. Statistical analysis was performed by Student's t-test, and statistical significance was set at $p<0.05$.

4.9 *In situ* zymography (IV)

In situ zymography was performed according to the protocol as described elsewhere (Pirila *et al.* 2001). Briefly, 10 µm serial frozen tissue sections were preincubated for 30 min at 37˚C with 500 µM CTT peptide (Koivunen *et al.* 1999) or control peptide (negative control peptide for CTT peptide) diluted in *in situ* zymography (ISZ) buffer (50 mM Tris-HCl, pH 7.4; 1 mM $CaCl_2$). The samples were then covered with a solution containing 1:1 1 mg/ml Oregon Green 488-conjugated gelatin (Molecular Probes, Inc., Eugene, OR) and 1% low melting temperature agarose (Sigma, St. Louis, MO) with or without 500 µM CTT peptide or control peptide. The sections were covered with coverslips, allowed to gel for 1 h at RT, and incubated for 7 h in a dark, humidified chamber. Gelatinolytic activity was detected with a fluorescence microscope as dark areas in the otherwise uniform green fluorescent substrate layer.

4.10 Generation of hTIMP-1 transgenic mice (IV)

Transgenic mice containing the murine MMP-9 promoter cloned in front of human TIMP-1 cDNA were generated, expressing human TIMP-1 in cells normally expressing MMP-9. A plasmid construct was created containing the human TIMP-1 gene under the control of the MMP-9 promoter. The linearized DNA constructs were injected into fertilized mice oocytes, which were then transferred into pseudopregnant mice. DNA was isolated from the tails of three-week-old mice for the identification of transgenic animals using PCR analysis. The primers were specific to human TIMP-1 (5'-CACAACCGCAGCGAGGAGTTT-3' and 5'-CACTGTGCAGGCTTCAGCTTC-3'). Integration of the transgene was verified by PCR, and its expression confirmed using RT-PCR and *in situ* hybridization.

4.11 Tissue samples (I, II, III, IV)

4.11.1 Experimental wounds of skin and oral mucosa

In healthy volunteers, 3 mm full-thickness punch wounds were generated on the oral mucosa. The wounds were allowed to heal for 1-14 days, after which the wound area was biopsied using a 6 mm punch. The tissue samples were fixed with 4% formalin and embedded in paraffin. Using a similar method, wounds were made on the dorsal skin of hTIMP-1 transgenic mice and wild-type control mice.

4.11.2 Oral dysplasias and epithelial tumors

Thirty-five cases of human tongue squamous cell carcinomas, 25 cases of epithelial dysplasias of oral mucosa, four cases of fibrotic hyperplasia, 13 cases of ameloblastomas and 10 cases of BCC were included in the study. The samples were obtained from the archives of the Department of Pathology, University of Oulu. The tumors and mucosa samples were diagnosed and graded by a pathologist/oral pathologist.

All parts of the study were approved by the Ethical Committee of the University of Oulu.

5 Results

5.1 Expression of collagen XVII in basal cell carcinomas and ameloblastomas (I)

Basal cell carcinomas and ameloblastomas show similar histomorphological features as well as growth patterns with local invasion. Collagen XVII expression was studied in these tumors by immunohistochemical and *in situ* hybridization methods (Fig. 5). In solid and keratotic basal cell carcinomas, variable immunostaining for collagen XVII was observed in different parts of the tumors. In the epidermis overlying the tumor area, the cell membranes of the basal keratinocytes were stained with collagen XVII antibody, similarly to normal epidermis. In the neoplastic islands of the tumor, diffuse cytoplasmic immunoreaction was seen in some central and peripheral carcinoma cells. In superficial BCC, the invading epithelial buds were not stained with the collagen XVII antibody, whereas the basal cells in the other parts of the epithelium showed a positive immunoreaction. In double immunostainings, collagen XVII and laminin-5 γ2 chain mostly colocalized. In solid and keratotic BCCs, collagen XVII was mainly observed as a diffuse cytoplasmic signal in the carcinoma cells, while the γ2 immunoreaction was restricted to the BM-like structure surrounding the tumor nests. In contrast to collagen XVII, γ2 chain was also detected in the BMZ of the invading epithelial buds in superficial BCCs.

Collagen XVII mRNA expression varied in tumor islands. A positive *in situ* hybridization signal was detected in some peripheral and central tumor cells of the neoplastic islands. The basal keratinocytes of the epidermis close to the tumor or within the tumor area showed a clear signal for collagen XVII mRNA, whereas the basal cells in healthy epidermis were negative.

In ameloblastomas, collagen XVII immunostaining was variable in different parts of the tumors (Fig. 5). In plexiform ameloblastomas, including both unicystic and solid types, the cell membranes of the peripheral cells in the tumor nests displayed a strong immunoreaction. In follicular ameloblastomas, a diffuse cytoplasmic signal was seen in the central and peripheral cells of the neoplastic islands. Compared to plexiform

ameloblastomas, however, the staining was mostly very weak, being partly undetectable in the peripheral cells. In double immunostainining of collagen XVII and laminin-5 γ2 chain, the immunoreactions usually overlapped. The staining pattern of γ2 chain was variable and fragmented in the basement membrane-like structures under the peripheral tumor cells.

An *in situ* hybridization signal for collagen XVII mRNA was observed in the peripheral cells of plexiform ameloblastomas as well as in both central and peripheral tumor cells of follicular ameloblastomas. In stromal cells, no signal was visible. The results of *in situ* hybridization analysis agreed well with the results of immunostainings.

5.2 Expression of collagen XVII in oral dysplasias and SCCs (II)

Mild to severe dysplasias of oral mucosa and grade I-III tongue squamous cell carcinomas were studied with immunohistochemical and *in situ* hybridization methods, to examine deregulation of collagen XVII expression at various stages of carcinogenesis.

By *in situ* hybridization, normal oral mucosa did not show any detectable mRNA signal, and in mild dysplasias, too, the signal was mostly negative, with the exception of the local areas with a weak signal in the basal keratinocytes. In contrast, all the layers of epithelium in moderate and severe dysplasias contained significant levels of collagen XVII mRNA in localized zones of the epithelium. In tongue SCCs, collagen XVII mRNA was expressed in both the central and the peripheral cells of carcinoma islands. A particularly strong signal was observed in the areas of invasive growth.

By immunostaining, the presence of collagen XVII in normal oral mucosa was verified to be similar to that of skin epidermis, localizing to the BM area and to the cell membranes of basal keratinocytes (Fig. 5). In non-dysplastic fibrotic hyperplasia of oral mucosa, the staining pattern was identical to that seen in normal mucosa. In all cases of mild dysplasias, however, the collagen XVII immunoreaction was mainly negative in the basal keratinocytes and BMZ, with only a faint signal detectable in some areas. All moderate and severe dysplasias showed local areas of epithelium with collagen XVII expression throughout all epithelial layers. In grade I SCCs, an intense collagen XVII immunoreaction was observed in the peripheral cells of the tumor islands. Grade II/III carcinomas showed a different pattern of expression, with positive tumor cells in the central regions of the tumor nests as well. In the invasive parts of the tumors, the carcinoma cells displayed intense collagen XVII staining.

To evaluate the immunostainings quantitatively, immunostaining scores were calculated for each tissue section by grading the number of positive cells and their staining intensity. Statistical analyses showed a significantly higher score for collagen XVII expression level in suprabasal keratinocytes of moderate and severe dysplasias compared to mild dysplasias or normal mucosa. The collagen XVII level was significantly lower in the basal keratinocytes of mild dysplasias compared to healthy mucosa. Grade II/III SCCs were found to express significantly higher levels of collagen XVII in the central carcinoma cells of the tumor islands compared to grade I tumors. The differences were considered statistically significant if $p<0.05$.

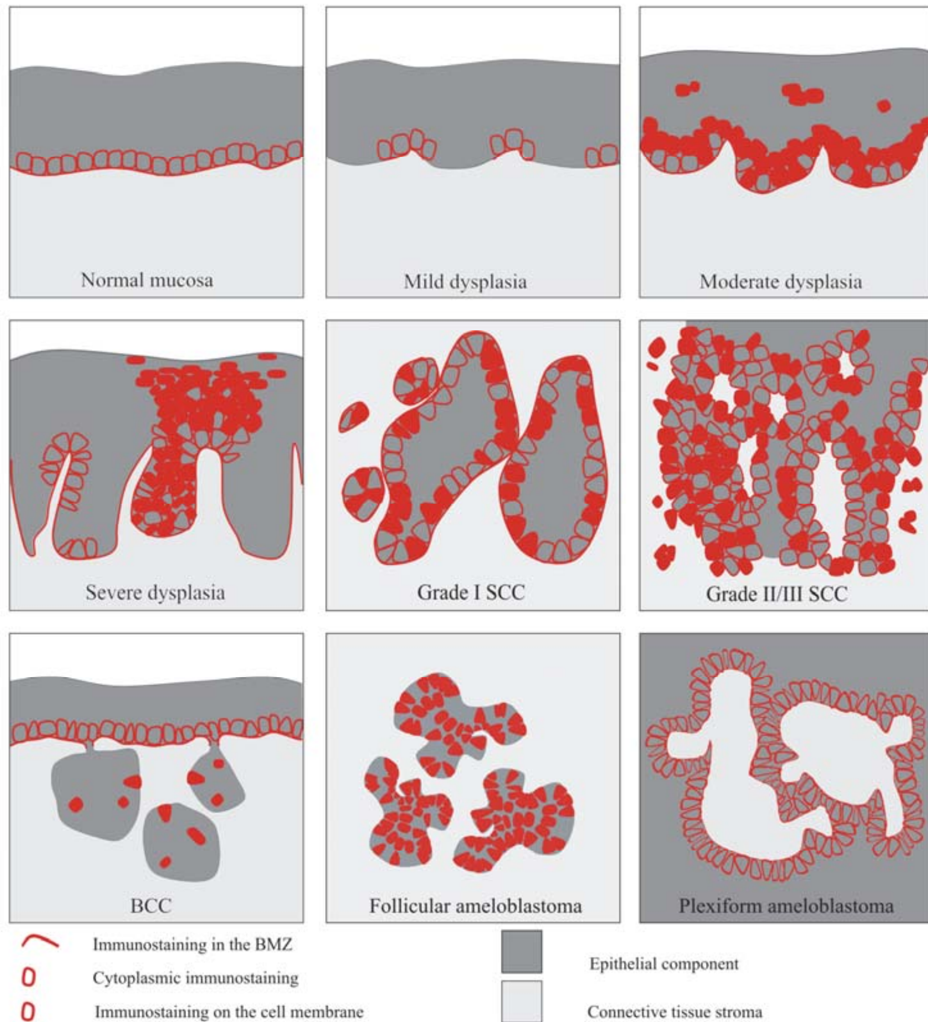

Fig. 5. Collagen XVII immunostaining in epithelial tumors.

5.3 The two forms of collagen XVII in cultured oral keratinocytes, SCC cells and carcinoma tissue (II)

Two cell lines of tongue SCC (SCC-25, HSC-3) and oral keratinocytes (IHGK) were analyzed for collagen XVII mRNA synthesis using RT-PCR, the amplified sequence covering the juxtamembranous NC16A domain (Fig. 4). Collagen XVII mRNA could be demonstrated in all the three cell lines. Skin keratinocytes were included as a positive

control, and endothelial cells (EA.hy926) as well as plasmacytoma cells (RPMI 8226) as negative controls.

The level of collagen XVII expression in the two SCC cell lines, compared to oral keratinocytes, was studied by Western blot analysis with NC16A antibody (Fig. 4). The collagen XVII expression level in HSC-3 cells was approximately 1.3-fold and that in SCC-25 cells 1.6-fold compared to IHGK keratinocytes, indicating clear upregulation in invasive carcinoma cells.

Collagen XVII could be extracted from oral keratinocytes (IHGK), two tongue SCC cell lines (SCC-25, HSC-3), and tongue SCC tissue. The soluble ectodomain was immunoprecipitated with the NC16A antibody from the conditioned media. The cell extracts and the precipitates were analyzed by immunoblotting with three specific collagen XVII antibodies (Fig. 4): NC16A, Ecto-3 and Ecto-5. The 180 kDa full-length protein could be detected in the cell extracts, and the shed 120 kDa ectodomain in the media precipitates by all of the three antibodies. The NC16A antibody has strong affinity for the shed ectodomain, and is able to detect low levels of it (Schumann *et al.* 2000). The 120 kDa protein was also observed in the SCC tissue extract by the NC16A antibody, and the 180 kDa form was detected by the Ecto-5 antibody.

5.4 Regulation of collagen XVII gene expression by cytokines (II)

IHGK keratinocytes were treated with different concentrations of cytokines tumor necrosis factor (TNF)-α, epidermal growth factor (EGF), transforming growth factor (TGF)-β1, interleukin (IL)-1β, IL-6, and tumor promoter PMA, to study the effect on collagen XVII gene expression. The level of collagen XVII mRNA was analyzed semiquantitatively by RPA. Treatment with TNF-α (20 ng/ml) or TGF-β1 (1ng/ml) resulted in 1.4-fold collagen XVII expression, compared to the control level. Incubation with PMA (100 ng/ml) led to 1.5-fold expression, which is significantly higher than the steady-state level ($p<0.05$). Other cytokines did not cause significant changes in the collagen XVII mRNA level.

5.5 Expression of collagen XVII during re-epithelialization (III)

Experimental punch wounds of oral mucosa and oral squamous cell carcinomas were analyzed for collagen XVII expression by immunohistochemical and *in situ* hybridization methods. Laminin-5 γ2 chain immunostaining was performed on parallel sections. Collagen XVII was detected on the cell membranes of the following kertinocytes in the epithelial outgrowth and in the BMZ underlying these cells. The leading keratinocytes did not show any staining, with the exception of a faint immunoreaction in the BMZ. On the other hand, collagen XVII upregulation was observed in carcinoma cells in the invasive parts of oral SCCs (II), indicating a role in malignant migration. In accordance with previous wound healing studies (Larjava *et al.* 1993, Pyke *et al.* 1994), intense

cytoplasmic γ2 chain staining was seen in the leading keratinocytes of the epithelial outgrowth and a continuous band-like signal in the BMZ underneath the keratinocytes distal to the wound edge. A clear γ2 chain reaction was observed in the carcinoma cells of the invasive tumor areas, protruding into the surrounding tissue. These findings indicate that collagen XVII expression is differentially regulated in epithelial cells during physiological and malignant epithelial migration.

5.6 Effect of COL15, the cell adhesion domain of collagen XVII, on carcinoma cell migration (III)

Haptotactic migration of HSC-3 SCC cell line and HaCat keratinocytes was analyzed by Transwell chamber transmigration assays with fibronectin-coated membranes. Addition of denatured COL15, the largest collagenous domain of collagen XVII (Fig. 4), into the lower Transwell chamber caused an up to 4.3-fold concentration-dependent increase in the transmigration of HSC-3 cells. Native COL15 resulted in a smaller (2.8-fold), concentration-dependent increase in the transmigration of HSC-3 cells. COL15 had, however, no effects on the migration of HaCaT kertinocytes. As a positive control, transmigration of HaCaT cells was induced by EGF in the lower chamber (data not shown), demonstrating the ability of the cell line to migrate. Addition of vehicle or trypsin-digested type I collagen did not affect the transmigration level of HSC-3 or HaCat cells. When COL15 was present in both Transwell chambers (i.e. without gradient) or only in the upper chamber, the basal transmigration level of HSC-3 cells was not significantly altered. Furthermore, HSC-3 cells migrated poorly through COL-15-coated membranes as compared to fibronectin-coated ones. These results indicate that COL15 induces the chemotactic transmigration of HSC-3 carcinoma cells onto fibronectin.

Flow cytometry analysis was carried out on HSC-3, using antibodies to α5, αv, and β1 integrin subunits, the previously reported ligands of COL15 (Nykvist *et al.* 2001). These integrin receptors were found to be expressed by HSC-3 cells. We next investigated whether the promigratory effect of COL15 can be inhibited by specific function-blocking antibodies to α5, αv, and β1 integrins. The anti-β1 integrin antibody completely blocked the transmigration of HSC-3 cells through fibronectin-coated membranes, independently of the presence of COL15. Anti-αv or anti-α5 had no significant inhibitory effect on the basal transmigration level of HSC-3. Nevertheless, the combination of antibodies against αv and α5 integrins caused a 53 % decrease in transmigration. When COL15 was present in the lower Transwell chamber, addition of anti-αv or anti-α5 inhibited transmigration (by 57 % and 53 %, respectively). As a combination, anti-αv and anti-α5 inhibited COL15-induced transmigration by 63 %. These experiments demonstrate that the motility-promoting function of COL15 is partially mediated by αv and α5 integrins. However, the results also implicate that additional COL15-binding receptor(s) are likely to exist.

5.7 Cutaneous wound healing of transgenic mice carrying human TIMP-1 under MMP-9 promoter (IV)

To study the overexpression of TIMP-1 in MMP-9-expressing cells, mice carrying the human TIMP-1 transgene under the MMP-9 promoter were generated. The integration of the transgene was confirmed by PCR, and its expression was detected by RT-PCR. Expression of the transgene could also be shown in adult and newborn mouse bone samples by RT-PCR, using human TIMP-1 primers. More prominent expression was observed in newborn and young adult mice, and the expression decreased significantly by the age of two years. The transgenic mice appeared normal at birth, exhibiting normal postnatal growth. No defects were observed in their long bones or skin. However, the second-generation mice started to present skin manifestations as they aged: by the age of 6-7 months, the mice started to develop crust on the skin of the back, neck and ears. This was typical in animals sharing their cage with others. Histological examination revealed distinct differences in the skin of transgenic and wild-type animals. The skin of hTIMP-1 mice showed local epithelial hyperplasia, cyst-like structures and erosive areas. *In situ* hybridization analysis with a human (h) TIMP-1-specific probe (the sequence homology of human and mouse TIMP-1 is 78.4%) showed a strong signal in epithelial cells in erosive skin areas. No signal was detected in intact epidermis.

5.7.1 Delayed wound healing in hTIMP-1 transgenic mice (IV)

To study the effects of hTIMP-1 overexpression on wound healing, experimental punch wounds 3 mm in diameter were made into the dorsal skin of control and transgenic mice. The wounds were allowed to heal for 1-14 days, after which they were removed for analysis. Significant delay of wound healing was observed in the hTIMP-1 transgenic mice. The difference in the wound healing capacities of hTIMP-1 mice and wild-type mice was obvious after 7 days of healing: re-epithelialization was completed in the wounds of the control mice, whereas the wounds of the hTIMP-1 mice were nearly of the same diameter as at the time of wounding. Staining with the Ki-67 proliferation marker demonstrated that the keratinocyte proliferation rate was not altered in the hTIMP-1 mouse wounds compared to the control wounds. Histological analysis revealed significant retardation in the wound healing process in the transgenic mice, when the wound areas were measured by scanning. *In situ* hybridization analysis with a probe specific to hTIMP-1 mRNA showed expression of the transgene in keratinocytes of the wound edge. Human TIMP-1 mRNA expression was detectable 24 h after wounding, and a weaker signal was still observed after two weeks of repair. To confirm the cell type expressing hTIMP-1, anti-cytokeratin staining was performed on serial sections. The laminin-5 γ2 chain antibody was used as a marker for the regenerating basement membrane, on sections obtained at various time points of wound repair from hTIMP-1 and wild-type mice. In the transgenic mice, the staining of the BMZ was discontinuous and weaker than in the control mice.

5.7.2 Detection of gelatinolytic activity in the migrating epithelial cells of the wound edge (IV)

Gelatinolytic activity in tissues can be localized using *in situ* gelatin zymography (Galis *et al.* 1995). With a gelatinase (MMP-2 and -9)-specific inhibitor, a synthetic CTTHWGFTLC (CTT) peptide (Koivunen *et al.* 1999), it is possible to discriminate between the active gelatinolytic enzymes localizing to the tissue (Pirila *et al.* 2001). In the wounds of the wild-type mice, intense gelatinolytic activity was seen at the edge of the wound, whereas no gelatinolytic activity could be observed in the wounds of the hTIMP-1 mice. This indicates that MMP-9 is the major gelatinase active during wound healing. When the wounds of the wild-type mice were incubated with CTT peptide, most gelatinolytic activity was abolished. However, slight activity was detected around the hair follicles, indicating local activity of some gelatinolytic enzymes other than MMP-2 and -9. Incubation of the wild-type mouse wounds with the control peptide did not affect gelatinolytic activity.

6 Discussion

Epithelial cell migration is essential for various biological processes. Physiological migration of epithelial cells occurs during fetal morphogenesis and reparative processes, such as wound healing. Malignant migration of tumor cells involves similar molecular mechanisms as physiological migration. The different cellular behavior may result from differences in the ECM signalling molecules and from the different ability of tumor cells to respond to these signals. Wound healing and tumor cell invasion are the most commonly used models for studying the mechanisms of epithelial migration. The molecular mechanisms of epithelial migration must be exquisitely controlled, involving processing of receptor-ligand and protein-protein interactions by proteolytic enzymes. Progress has been made recently in characterizing the molecular basis of cell migration and the biological functions of the various components involved. However, knowledge of how these molecules work together as a dynamic, integrated system is still limited. Understanding of the molecular process of cell migration is of great interest, as it could lead to new diagnostic and therapeutic applications in pathological conditions, such as cancer, hypertrophic scarring, chronic ulcers, and developmental defects.

6.1 Role of collagen XVII in the physiological migration of epithelial cells (III)

In various wound healing models, collagen XVII is localized at the basement membrane underneath the following keratinocytes of the epithelial outgrowth (Dabelsteen *et al.* 1998, Leivo *et al.* 2000). Comparison of the putative ligands, collagen XVII and laminin-5, during mucosal wound healing demonstrated a clear difference in the temporal expression patterns of these molecules. Cytoplasmic staining of laminin-5 γ2 chain was seen in the leading keratinocytes, whereas the same cells showed no signs of collagen XVII. In concert with our findings, previous reports indicate that laminin-5 is upregulated and deposited by the leading wound keratinocytes, which then migrate on precursor laminin-5 utilizing α3β1 integrin (Nguyen *et al.* 2000). The leading cells exhibit transient

actin-associated cell-matrix adhesions, whereas stable hemidesmosomal adhesions occur more distally from the wound edge. Here, collagen XVII and laminin-5 colocalized at the BMZ underneath the following cells, most likely coinciding with the formation of new hemidesmosomes. During hemidesmosome assembly, collagen XVII is incorporated into a complex first formed by laminin-5, α6β4 integrin, and plectin (Koster *et al.* 2003).

The physiological shedding of collagen XVII by ADAM proteinases is an example of proteolytical processing of collagenous transmembrane proteins, often leading to alterations in the functions of the molecule. Many of these physiologically active proteins occur in two forms: they are synthesized as membrane-bound proteins, which undergo selective proteolytical shedding, releasing soluble forms to the ECM. The shedding may lead to many changes in the biological activities of the molecules, by altering their functions in lignd binding, cell attachment or signal transduction. (Franzke *et al.* 2003) It has been hypothesized that collagen XVII shedding from the keratinocyte surface is mainly an anti-adhesive event, releasing the basal cell from its attachment to the basement membrane and thereby enabling migration. However, it was recently shown by *in vitro* re-epithelialization assays that induced shedding of collagen XVII results in decreased keratinocyte motility (Franzke *et al.* 2002). Therefore, the shed ectodomain is likely to play a role in cell-matrix signalling through interaction with cell surface receptors, leading to changes in lateral migration of keratinocytes. It is possible that induced collagen XVII shedding occurs during the activation of the wound edge keratinocytes immediately after wounding, and the released ectodomain may participate in the regulation of cellular events at the initiation of re-epithelialization.

6.2 Role of collagen XVII in malignant migration of epithelial cells (I, II, III)

Several studies have shown aberrant expression of hemidesmosomal adhesion complex components in epithelial tumors (Kainulainen *et al.* 1997a, Chopra *et al.* 1998, Skyldberg *et al.* 1999, Herold-Mende *et al.* 2001). In this work, the collagen XVII expression pattern was studied in three epithelial tumor types: ameloblastoma, basal cell carcinoma (BCC), and squamous cell carcinoma (SCC). All these tumors originate from the epithelium, but differ from each other in their invasive potential. Ameloblastoma and BCC, both locally invasive but rarely metastasizing tumors, showed similarities in collagen XVII localization. In follicular ameloblastomas and BCCs, collagen XVII expression was reduced in the peripheral cells of the tumor islands, but was clearly detectable as cytoplasmic immunostaining in single central tumor cells. In plexiform ameloblastomas, the staining pattern was more similar to that of normal epithelium: collagen XVII was expressed at the cell membranes of polarized basal and suprabasal cells in close connection with the basement membrane-like structure. The decreased expression in peripheral tumor cells of BCC and follicular ameloblastoma could coincide with hemidesmosome disassembly and reflect the reduced epithelial cell adhesion to the extracellular matrix. Similarly, decreased collagen XVII expression was observed in mild dysplasias of oral mucosa, meanwhile moderate/severe dysplasias and grade II/III SCCs

displayed collagen XVII upregulation. Compared to BCC and ameloblastoma, tongue SCC is a highly invasive carcinoma with a tendency to metastasize. In this work, collagen XVII was most notably overexpressed in SCCs by the carcinoma cells in areas invading into the surrounding tissue. Laminin-5 overexpression has been reported in the invasive areas of SCC tumors, and the laminin γ2 chain is considered a marker for microinvasive SCC of the cervix (Skyldberg *et al.* 1999, Koshikawa *et al.* 1999, Katoh *et al.* 2002). In our studies, collagen XVII and laminin-5 γ2 chain mostly colocalized in epithelial tumors. The elevated level of collagen XVII in dysplasias and SCCs implicates a potential of collagen XVII as a predictive biomarker for malignant transformation and early invasiveness in lesions of oral mucosa. Upregulation of collagen XVII is not found in suprabasal proliferating keratinocytes following the leading migratory front during wound healing, suggesting that the overexpression is associated with other features of malignant conversion than cell hyperproliferation. It can be hypothesized that collagen XVII overexpression is related to the increased migratory potential of tumor cells.

In oral keratinocytes, none of the cytokines tested were able to upregulate collagen XVII, and the synthetic tumor promoter PMA caused only modest induction, suggesting that the role of soluble factors may not be important for the regulation of collagen XVII synthesis. This is supported by the fact that collagen XVII is not upregulated in the wound edge keratinocytes, which is a site rich in multiple cytokines and growth factors. However, the differential regulation of collagen XVII in the invasive areas of SCC tumors, as compared to the wound edge, indicates that the composition of the ECM, through cell-matrix interactions, may play a central role in the induction of collagen XVII expression.

Apart from immunohistochemical studies, there are no previous data concerning the role of collagen XVII in malignant epithelial migration. It is not known if constitutive shedding of collagen XVII occurs in tumors. The *in vivo* presence of the shed collagen XVII ectodomain is difficult to study, as there are no specific antibodies available. In the present work, we were able to extract the shed ectodomain from SCC tissue and to demonstrate it by immunoblotting. Purification of sufficient amounts of collagen XVII from tissues for functional studies is very difficult, and a recombinant protein COL15 was therefore used in the present *in vitro* cell migration experiments. COL15, the largest collagenous domain of collagen XVII ectodomain, has been designated a cell adhesion domain for its ability to promote epithelial cell adhesion (Tasanen *et al.* 2000). By cell transmigration experiments, it was shown in this work that COL15 is capable of strikingly inducing tongue SCC cell migration through fibronectin-coated membranes. Interestingly, the promigratory function of denatured COL15 was more evident than that of the native fragment. Moreover, denatured COL15 is more efficient as an *in vitro* cell adhesion ligand compared to its native counterpart (Tasanen *et al.* 2000). This evidence implicates that the shedding of the collagen XVII ectodomain may lead to generation of new binding sites by denaturation of the ectodomain.

The promigratory function of COL15 could be partially prevented by blocking α5 and αv integrin receptors with specific antibodies, suggesting that the process is mainly mediated by α5β1 and αvβ1 integrin receptors. Since transmigration was not completely prevented, additional receptors may play a role in the process. Transmigration was totally blocked by a β1 integrin antibody, regardless of COL15 stimulation. This is in agreement with a recent *in vivo* study showing that β1 integrins are crucial for epithelial cell

migration (Grose *et al.* 2002). Interestingly, transmigration of HaCaT keratinocytes was not influenced by the presence of COL15. Comparison of cell surface integrin expression patterns of HSC-3 carcinoma cells and HaCaT keratinocytes revealed that, whereas HaCaT cells express low levels of α5β1 integrin (Koivisto *et al.* 1999), a very high level was detected in a subpopulation of HSC-3 cells. Another reason for the different response of the two cell lines could be that αv integrins dimerize with a different β subunit in HSC-3 cells, compared to HaCaT cells, in which αvβ1 and αvβ6 are prominent (Koivisto *et al.* 1999). Thirdly, HSC-3 cells were found to express αIIb subunit (data not shown), which is a platelet fibrinogen receptor. This receptor has been detected in malignant melanoma cells, in addition to platelets, but not in keratinocytes (Chopra *et al.* 1992, Chang *et al.* 1992, Tang *et al.* 1993, Puerschel *et al.* 1996). The integrin receptor binding sites within COL15 are the numerous KGD motifs (Nykvist *et al.* 2001), which bind to αIIb with a greater affinity than to α5β1 or αv (Scarborough *et al.* 1991). This suggests that the presence of αIIb-integrin in HSC-3 cells may cause the different response of HSC-3 cells to COL15.

6.3 Effects of *in vivo* overexpression of TIMP-1 in migrating epithelial cells during wound healing (IV)

To gain new insight into the functional significance of keratinocyte-derived MMPs in cutaneous wound healing, experimental wounds were generated on transgenic mice carrying the hTIMP-1 gene under the murine MMP-9 promoter and enhancers. The mice expressing hTIMP-1 in cells normally expressing murine MMP-9 were viable and fertile, with normal growth and development. However, the skin of aging hTIMP-1 mice gradually began to show alterations, such as unhealed scratches and crust formation. The overexpression of TIMP-1 in keratinocytes caused severe delay in wound healing. Seven days post-injury, re-epithelialization was nearly complete in control wounds, whereas hTIMP-1 wounds were barely smaller than at the time of wounding.

A few hours after wounding, keratinocytes start migrating to cover the injured dermis. The onset of cell movement is followed by an increased proliferation rate in keratinocytes behind the migratory front. Consequently, the delayed wound closure in hTIMP-1 mice could result from retarded cell motility or proliferation. Nevertheless, the present results revealed no differences in the cell proliferation rates of hTIMP-1 and control mice *in vivo*. Histologically, the keratinocytes in hTIMP-1 mice formed hyperplastic wound edges as compared to the thin epithelial outgrowth in the wounds of wild-type animals. Proliferating keratinocytes piled at the wound edges, apparently unable to migrate on the dermis. Eventually, however, the hTIMP-1 keratinocytes succeeded in covering the wound, indicating that wound closure is delayed, but not prevented, in this model. *In situ* hybridization showed strong expression of human TIMP-1 mRNA in the migrating keratinocytes of the wound edge, colocalizing with murine MMP-9 expression.

The results showed that the ability of keratinocytes to migrate over the wound was affected by the hTIMP-1 overexpression. TIMP-1 is able to form complexes with virtually all known MMPs (Bode *et al.* 1999), indicating that inhibition of MMPs

secreted by murine wound edge keratinocytes, namely MMP-2, MMP-3, MMP-9, MMP-10 and MMP-13, could have contributed to the decreased re-epithelialization. The expression patterns of the keratinocyte-derived MMPs in normal and impaired wound healing have been well characterized, but the specific functions of the distinct MMPs are still mainly unknown. In human, MMP-1 activity is essential for the keratinocyte migration on dermal matrix (Pilcher *et al.* 1997). MMP-13, the murine orthologue for human MMP-1, is responsible for the remodelling of fibrillar collagen in mouse. Emphasizing the importance of collagenolytic activity in re-epithelialization, wound healing is severely delayed in collagenase-resistant mice (Beare *et al.* 2003). The expression and function of the recently identified mouse MMP-1 in wounds is still unknown (Balbin *et al.* 2001). MMP-2 has been proposed to participate in migration of keratinocytes *in vitro* (Mäkelä *et al.* 1999), but its *in vivo* function in wound healing is not known. In MMP-3 knockout mice, wound healing is retarded as a consequence of impaired wound contraction (Bullard *et al.* 1999). Results of these studies, in concert with the present study, suggest that MMPs play an important role in wound repair. In MMP-9 deficient mice, however, the re-epithelialization rate of corneal wounds is accelerated, due to increased epithelial cell proliferation (Mohan *et al.* 2002). This is the first report showing that MMPs may exert negative control on cell migration. Nevertheless, notable differences in wound healing exist between distinct organs, such as skin, mucosa and cornea, and the results may be influenced by the choice of wound model.

In line with our findings with the hTIMP-1 mouse model, there are many reports implicating that a correct balance between MMPs and their inhibitors is essential for normal wound healing. In chronic wounds, elevated levels of activated MMPs and altered expression patterns of TIMPs-1, -2, and -3 have been observed (Bullen *et al.* 1995, Saarialho-Kere 1998, Vaalamo *et al.* 1999). Studies with broad-spectrum MMP-inhibitors in human *in vivo* and *ex vivo* wounds show that inhibition of MMP-activity leads to delayed epidermal regeneration, suggesting that MMPs are required for normal wound healing (Agren *et al.* 2001, Mirastschijski *et al.* 2002b).

By *in situ* gelatin zymography, it was demonstrated that hTIMP-1 mice exhibited no gelatinolytic activity at the wound edge. Addition of MMP-9 and -2-specific inhibitor (CTT peptide) totally abolished the gelatinase activity at the margins of control wounds. These results suggest that the loss of MMP-2 and MMP-9 activity is not compensated by other gelatinolytic enzymes during re-epithelialization, and that the total lack of gelatinolytic activity does not prevent wound re-epithelialization in the hTIMP-1 mice. However, as there is variation in the ability of TIMP-1 to inhibit different MMPs, it is possible that other than gelatinolytic MMP-activity is not totally blocked at the wound edge. In this case, other MMPs present may partially compensate for the loss of degradative activity at the wound edge, leading eventually to wound closure. The functional overlap of proteolytic enzymes is observed in knockout mouse models, in which the loss of activity of one enzyme is often compensated by others, leading to only minimal changes in the development and viability. Mice deficient in the serine protease precursor plasminogen exhibit delayed wound healing, similarly to wild-type mice with pharmacologically blocked MMP-activity, whereas wound healing is totally prevented if MMP-activity is blocked in plasminogen-deficient mice (Lund *et al.* 1999).

MMPs secreted by keratinocytes participate in the processing of BMZ molecules during wound repair, required for the correct remodelling of the basement membrane. In the wounds of MMP-9 deficient mice, BMZ defects were found, including large fibrinogen deposits and an abnormal laminin-5 expression (Mohan *et al.* 2002). In the wounds of hTIMP-1 mice, the laminin-5 staining pattern was weak and discontinuous. Although the proteolytical shedding of collagen XVII ectodomain by ADAM proteinases is not influenced by TIMP-1(Franzke *et al.* 2002), preliminary studies showed alterations in collagen XVII distribution in the epidermis of hTIMP-1mice (data not shown). Immunostaining of collagen XVII was reduced in the BMZ of quiescent epithelium in hTIMP-1 mice, compared to wild-type littermates. In wound keratinocytes distal to the migrating edge, collagen XVII was expressed similarly in the hTIMP-1 and wild-type wounds. These data suggest that alterations in the activity of keratinocyte MMPs may lead to multiple changes in the organization of the BMZ. Although MMPs are not responsible for the shedding of collagen XVII (Franzke *et al.* 2002), the loss of MMP-activity may alter the distribution of collagen XVII indirectly, by disturbing the normal processing of the BMZ.

Inhibition of the keratinocyte-derived MMPs by the targeted overexpression of hTIMP-1 at the wound edge resulted in delayed re-epithelialization, due to impaired keratinocyte migration. The results confirm previous studies showing that keratinocyte MMPs have an important role in cutaneous wound healing. However, the inhibition of MMP-activity at the wound edge, including total blockage of gelatinolytic activity, could not prevent re-epithelialization.

7 Conclusions

This study demonstrates disturbed expression of collagen XVII in epithelial precancerous lesions and tumors with variable invasive potential. In moderate or severe dysplasias of oral mucosa, collagen XVII upregulation was observed as cytoplasmic immunostaining in suprabasal cells. In follicular ameloblastomas and basal cell carcinomas (BCC), collagen XVII expression was reduced in peripheral cells, whereas cytoplasmic staining was detected in central tumor cells. Tongue squamous cell carcinomas (SCC) showed increased collagen XVII expression in grade II/III tumors, particularly in areas of invasive growth. In summary, these results indicate that the level of collagen XVII expression correlates with the invasive potential of tumors originating from squamous epithelium. Behind the leading keratinocytes of the wound outgrowth, collagen XVII was absent from the proliferating suprabasal keratinocytes, implicating that collagen XVII upregulation is not merely a feature associated with cell hyperproliferation. In addition, the increased expression in SCC cells was confirmed *in vitro*. During wound healing collagen XVII was not expressed by keratinocytes at the migrating epithelial tip, suggesting that collagen XVII may be differentially regulated in the migrating cells of wounds and epithelial tumors.

The present results show that recombinant COL15, a fragment of the collagen XVII ectodomain, is capable of dramatically inducing migration of SCC cells *in vitro*. The role of collagen XVII in the regulation of malignant migration was explored for the first time in this work. The promigratory effect of COL15 was shown to be mediated by αv, α5, and β1 integrins, supporting the previous evidence of collagen XVII binding to αvβ1 and α5β1 integrins (Nykvist et al., 2001). Based on this work and previous reports, it can be postulated that collagen XVII may also have another function in cell-matrix signalling and regulation of cell motility in addition to its role as a hemidesmosomal adhesion molecule. It is likely that the function of collagen XVII, as well as certain other extracellular matrix molecules, is altered as a result of proteolytic processing.

The significance of MMPs in cutaneous wound healing was studied using a transgenic mouse model with human TIMP-1 overexpression in cells normally expressing MMP-9. The mice were otherwise normal, but exhibited cutaneous lesions over aging. Wound healing was significantly delayed though not prevented, in hTIMP-1 mice, due to impaired keratinocyte migration to cover the wound bed, meanwhile keratinocyte

proliferation rate was not affected by the transgene. Strong hTIMP-1 expression was detected in the leading keratinocytes of the migrating epithelial tip, colocalizing with MMP-9. hTIMP-1 overexpression was shown to totally block gelatinolytic activity at the wound edge. With a specific inhibitor, it was demonstrated that MMP-9 is the main gelatinase at the wound edge, and that MMP-2 does not compensate for the MMP-9 activity blocked by hTIMP-1. The present study provides a new model for studying the effects of targeted MMP-inhibition *in vivo*. It supports the previous evidence showing that MMP function is important for normal re-epithelialization of cutaneous wounds.

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