ROLE OF BASEMENT MEMBRANES AND THEIR BREAK-DOWN IN HUMAN CARCINOMAS

A study by *in situ* hybridization and immunohistochemistry of the expression of laminin chains, matrix metalloproteinases (MMPs) and their tissue inhibitors of metalloproteinases (TIMPs)

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Abstract

In malignancies many alterations involving matrix macromolecule synthesis, secretion and assembly into basement membranes (BMs) as well as their degradation are present. The most important groups associated with matrix turnover are matrix metalloproteinases (MMPs) and their inhibitors (TIMPs). In this study altogether 285 tissue samples were investigated comprising various malignant epithelial tumors and normal tissue structures, in which the distribution of different laminin chains was studied immunohistochemically. Laminin $\alpha5$, $\beta1$ and $\gamma1$ were detected almost in all the BMs studied including normal tissues and malignancies, whereas $\alpha1$ chain of laminin was present only in certain BMs. Laminin $\gamma2$ chain was solely expressed by epithelial BMs and was present in intracellular space especially in individual carcinoma cells infiltrating in the tumor stroma and in tumor cells in close contact with BM zone. Generally epithelial tumors contained quite well-formed BMs around their tumor clusters, except for infiltrative breast carcinoma and diffuse type gastric carcinoma. *In situ* hybridization revealed that only epithelial cells contained mRNAs for laminin $\alpha1$ and $\gamma2$ chains, whereas laminin $\beta1$ chain and $\alpha1$(IV) collagen were synthesized mainly by stromal cells.

mRNA for MMP-2 was produced mainly by stromal cells in hepatocellular carcinoma of liver (HCC) and pancreatic adenocarcinoma, whereas MMP-9 and MT1-MMP were equally synthesized by carcinoma cells and cells of tumor stroma. However, in HCCs of grade III carcinoma cells predominated in their MT1-MMP expression. All three MMPs were immunolocalized to malignant epithelial cells and showed variably stromal cell positivity. Statistically mRNA synthesis for MT1-MMP was significantly associated with the shortened survival of patients with HCC ($P \le 0.01$).

TIMP-1-3 mRNA, and especially TIMP-3, expressions in normal endometrium were significantly increased in endometrial stromal cells towards the secretory phase. In various endometrial hyperplasias TIMPs and MT1-MMP expressions were quite comparable to those seen in proliferating endometrium. In endometrial adenocarcinomas their expressions were significantly increased and the most intensified mRNA expressions were seen in grade III adenocarcinomas. Especially TIMP-3 and MT1-MMP mRNAs were synthesized by carcinoma cells.

The results indicate that epithelial malignancies are capable of active synthesis and assembly of BM macromolecules. Simultaneous matrix synthesis and degradation seen in malignancies suggest that the mechanisms involved in matrix turnover are not lost during malignant transformation. mRNA synthesis for MMPs and TIMPs is generally increased in epithelial malignancies. The results therefore strongly support the concept that MMPs have an active role in carcinoma cell invasion.

Keywords: immunolocalization, matrix degradation, mRNA synthesis, tumor invasion.

To My Family

Acknowledgements

Abbreviations

ADAM	A disintegrin and metalloprotease
APMA	p-aminophenylmercuric acetate
BAC	bronchiolo-alveolar carcinoma
BM	basement membrane
BSA	bovine serum albumin
cDNA	complementary DNA
COOH-	carboxy-
DAB	diaminobenzidine tetrahydrocloride
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	dinatrium-diamin-tetra-acetic acid
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Schwarm
FITC	fluorescein isothiocyanate
HD	hemidesmosome
HLA	human lymphocyte antigen
IL	interleukin
kDa	kilodalton
LDL	low density lipoprotein
Mab	monoclonal antibody
MMP	matrix metalloproteinase
mRNA	messenger RNA
MT-MMP	membrane-type matrix metalloproteinase
mw	molecular weight
NH_2-	amino-
Pab	polyclonal antibody
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RT	room temperature
SPARC	secreted protein acidic and rich in cysteine

SDS	sodium dodecyl sulphate
SQCC	squamous cell carcinoma
SSC	standard saline citrate
TNF-α	tumor necrosis factor-α
TIMP	tissue inhibitor of metalloproteinase

List of original articles

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

I   Soini Y, Määttä M, Salo S, Tryggvason K, & Autio-Harmainen H (1996) Expression of the laminin γ2 chain in pancreatic adenocarcinoma. J Pathol 180:290-294.

II Määttä M, Soini Y, Pääkkö P, Salo S, Tryggvason K & Autio-Harmainen H (1999) Expression of the laminin γ2 chain in different histological types of lung carcinoma. A study by immunohistochemistry and in situ hybridization. J Pathol 188: 361-368.

III Määttä M, Virtanen I, Burgeson RE & Autio-Harmainen H (2000) Comparative analysis of the distribution of laminin chains in the basement membranes of some malignant epithelial tumors. The α1 chain of laminin shows a selected expression pattern in human carcinomas. Revised version submitted.

IV Määttä M, Soini Y, Liakka A & Autio-Harmainen H (2000) Differential expression of matrix metalloproteinase (MMP)-2, MMP-9, and membrane type 1-MMP in hepatocellular and pancreatic adenocarcinoma: implications for tumor progression and clinical prognosis. Clin Cancer Res 6: 2726-2734.

V   Määttä M, Soini Y, Liakka A & Autio-Harmainen H (2000) Localization of MT1-MMP, TIMP-1, TIMP-2, and TIMP-3 messenger RNA in normal, hyperplastic and neoplastic endometrium. Enhanced expression by endometrial adenocarcinomas is associated with low differentiation. Am J Clin Pathol, 114: 401-411.

Contents

1 Introduction

The balance between the synthesis and degradation of extracellular matrix (ECM) macromolecules is critical for tissue homeostasis. Impaired tissue turnover may lead to increased tissue fibrosis and malformations. Basement membranes (BMs) are a specialized form of ECM which play an important role in separating tissue compartments from each other and mediate many essential functional properties by taking part in ultrafiltration, cell adhesion and migration and by mediating survival signals for adjacent cells. The two main networks of macromolecules in BMs are formed by laminins and type IV collagen which form a specialized integrated entity to which other BM molecules are joined. In malignancies BMs show many morphological alterations including variable amounts of attenuations, irregular accumulation of BM macromolecules to the ECM space and a considerable decrease or even total lack of BM material in some types of carcinomas.

BMs are traditionally thought to prevent tumor cell invasion by acting as structural barrier, but there is increasing evidence which underlines their active functional role in malignancies, where both synthesis and effective ECM degradation is needed during tumor cell invasion and growth. The most important group of enzymes involved in the degradative process are matrix metalloproteinases (MMPs), whose function is controlled by specific tissue inhibitors of metalloproteinases (TIMPs). MMPs efficiently degrade almost all ECM macromolecules and in malignant tumors their expression is usually highly upregulated. Also the activation of MMPs is increased in malignancies, supporting for their important role in tumor progression.

The mRNA and antigen expressions of laminin chains, type IV collagen, MMP-2, MMP-9, MT1-MMP and TIMPs-1-3 mRNA in malignant tumors was investigated here by using *in situ* hybridization and immunohistochemistry. Gelatin zymography was used to study MMP-2 and MMP-9 activation in tumors, and statistical analysis was performed to clarify the clinical significance of laminin γ2 chain and MMPs for the patients´ outcome.

2 Review of the literature

2.1   Extracellular matrix (ECM)

Extracellular matrix (ECM) underlies or surrounds tissue structures and individual cells in tissues and has a central role in maintaining the structural integrity. It is composed of collagens, noncollagenous glycoproteins and proteoglycans and its composition varies in different tissues. ECM is a highly specialized, controlled complex and dynamic meshwork, and in addition to its supportive property it directs many functional processes e.g. cell signaling, tissue differentiation, adhesion, regeneration and migration (Adams & Watt. 1993).

2.2 Basement membranes (BMs)

Basement membranes are a highly specialized form of ECM. They underlie or surround epithelial and endothelial structures, peripheral nerves and separate them from the connective tissue. They also surround some individual cell types such as muscle, decidual, fat and Schwann cells. They provide mechanical support for tissues and take part in cell migration, attachment and growth, and they also direct cellular differentiation. BMs also regulate molecular ultrafiltration by forming semipermeable barriers between different compartments. In this respect glomerular and alveolar BMs are among the most specialized structures for the ultrafiltration of molecules. The width of BMs varies between 40 and 350 nm, usually being 60-100 nm. By electron microscopy BMs can be separated into two distinct layers; *lamina rara* just beneath the cell membrane and *lamina densa* to the stromal side of it. BM proteins are produced and deposited together by adjacent epithelial, endothelial and stromal cells. (Timpl 1996, Yurchenco & Schittny 1990).

The major components of BMs are type IV collagen, laminins and other ECM macromolecules which form defined supramolecular assemblies by interacting with each other. Among them type IV collagen and laminin form independent networks to produce

a stabile structural frame to which other BM proteins are bound (for review, see Yurchenco & Schittny 1990, Colognato *et al.* 1999).

The composition of BMs is heterogenous in different tissues and during maturation they undergo morphological and qualitative changes. The reason for this heterogeneity is unknown, but it is proposed to be connected to different functions of BMs, e.g. adhesive property or cell signaling in different conditions.

2.3 Type IV collagen

Type IV collagen is the major component of BMs. To date six α chains of type IV collagen have been identified and classified (Soininen *et al.* 1987, Hostikka & Tryggvason 1988, Morrison *et al.* 1991, Sugimoto *et al.* 1993, Pihlajaniemi *et al.* 1990, Zhou *et al.* 1994). Evolutionary development of the human α chain genes has included a series of duplications and generated the genes known to date. The original genes have diverged from two quite distinct genes and other chains are classified according to their similarities to $\alpha 1$ and $\alpha 2$ chains. $\alpha 1$ and $\alpha 2$ chains can be found in almost all BMs. $\alpha 3$ and $\alpha 4$ chains are found at least in BMs of ependymal choroid plexus of brain, cornea, lens capsule and retina of eye, glomerulus, alveoli and synapses of nerves. $\alpha 5$ is present in glomerular BMs and can be found in many tissues, but shows a somewhat more restricted expression than $\alpha 1$ and $\alpha 2$ chains. $\alpha 6$ chain has been detected in BMs of esophagus, heart, placenta and skeletal muscle, but not in glomelural BMs (for review, see Sariola *et al.*).

Table 1. Collagen IV, molecular weights (mw; kDa), genes and tissue expression pattern.

Chain	Mw	Gene / gene family	Distribution
$\alpha 1$	158	COL4A1 / $\alpha 1$-like	Abundant
$\alpha 2$	164	COL4A2 / $\alpha 2$-like	Abundant
$\alpha 3$	162	COL4A3 / $\alpha 1$-like	Restricted
$\alpha 4$	164	COL4A4 / $\alpha 2$-like	Restricted
$\alpha 5$	153	COL4A5 / $\alpha 1$-like	Many BMs
$\alpha 6$	161	COL4A6 / $\alpha 2$-like	Restricted
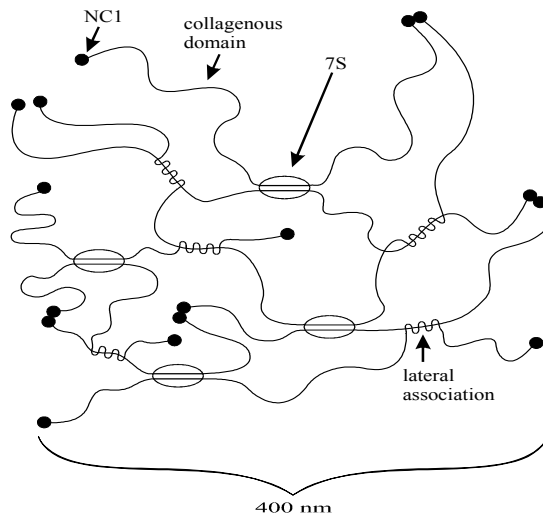
Fig. 1. Assembly of type IV collagen. Monomeric collagen IV molecules dimerize by pairing the NC1 termini. Four monomers associate by enclosing NH$_2$-terminal ends to achieve the 7S sturucture. Also lateral associations can be distinguished. Modified from Weber *et al.* (1992).

Type IV collagen is composed of three α chains, and the most wide-spread form of type IV collagen is a heterotrimer of α1(IV)$_2$α2(IV) which can be found in all BMs (Tryggvason *et al.* 1980), and α3(IV)$_2$α4(IV) is also characterized (Johansson *et al.* 1992), but other combinations may also exist.

Each chain contains three distinct globular structures; long triplehelical collagenous domain with Gly-X-Y repeats, a non-collagenous NH$_2$-terminal domain and a C-terminal globular domain (NC1). Type IV collagen molecules are assembled by joining two molecules to each other by pairing the NC1 domains to form linear dimers. Enclosing of four NH$_2$-terminal ends forms a structure called the 7S domain (Risteli *et al.* 1980), to achieve a four-armed structure. The type IV collagen network is accomplished by lateral associations to achieve a stable molecular structure (Yurchenco & Schittny 1990, Yurchenco & O´Rear 1994). The resultant type IV collagen network displays high flexibility and is highly insoluble, which is important for the mechanical strength (Timpl *et al.* 1981, Soininen *et al.* 1989).

2.4 Laminins

Laminins are a group of large heterotrimeric glycoproteins which by providing binding sites for different matrix molecules serve as integral constituent to form the second important assembly to BMs apart from type IV collagen network. Currently there is knowledge of 11 different laminin chains and 14 identified laminin molecules, but others presumably exist (Engvall *et al.* 1990, Burgeson *et al.* 1994, Miner *et al.* 1997, Koch *et*

al. 1999, Libby *et al*. 2000). The laminin molecule is composed of three chains; α, β and γ chains which are linked together by disulfide bonds to form either a cruciform or T or Y (laminins-5-9) shaped structure as visualized by electron microscopy after rotary shadowing. Complete primary structures has been presented for 10 chains; α1 (Nissinen *et al*. 1991), α2 (Vuolteenaho *et al.* 1994), α3 (Ryan *et al.* 1994), α4 (Iivanainen *et al.* 1995), β1 (Vuolteenaho *et al.* 1990), β2 (Wewer *et al.* 1994a), β3 (Gerecke *et al.* 1994), γ1 (Kallunki *et al.* 1991), γ2 (Kallunki *et al.* 1992) and γ3 chains (Koch *et al.* 1999). cDNA sequence is introduced for α5 (Durkin *et al.* 1998). Each laminin chain is the product of a single gene.

Table 2. Laminin chains, their molecular weights (mw; kDa) and genes.* ; native and proteolytically prosessed form, respectively.

Chain	mw (kDa)	Gene
α1	400	LAMA1
α2	343	LAMA2
α3	190/160/145*	LAMA3
α4	199	LAMA4
α5	380	LAMA5
β1	198	LAMB1
β2	190	LAMB2
β3	127	LAMB3
γ1	178	LAMC1
γ2	155/105*	LAMC2
γ3	170	LAMC3

Laminins form an important group of BM-associated glycoproteins and in addition to their intergral role as structural component of BMs, they also exhibit many essential functional properties, e.g. promotion of cell migration, adhesion and growth. Laminin isoforms are capable of forming three-dimensional networks through interactions with their three NH_2-terminals of short arms, but laminins 5-7 are not capable of such interaction (Cheng *et al.* 1997). Laminin-1 (α1β1γ1), also called classical laminin, was originally found in 1979 from a murine BM-forming Engelbreth-Holm-Swarm (EHS) tumor having a molecular weight of about 850 kDa (Timpl *et al.* 1979). Originally laminin chains were named A1, B1 and B2 chains, but due to the expanding number of new laminin chains and heterotrimers, it became important to create a novel nomenclature for different laminin chains and heterotrimer forms (Burgeson *et al.* 1994).

Table 3. Laminin heterotrimers, chain composition and previous names according to Engvall et al. (1990), Burgeson et al. (1994), Miner et al. (1997), Koch et al. (1999) and Libby et al. (2000).

Heterotrimer	Chain composition	Previous name
laminin-1	α1β1γ1	EHS laminin
laminin-2	α2β1γ1	merosin
laminin-3	α1β2γ1	s-laminin
laminin-4	α2β2γ1	s-merosin
laminin-5	α3β3γ2	kalinin/epiligrin/nicein
laminin-6	α3β1γ1	k-laminin
laminin-7	α3β2γ1	ks-laminin
laminin-8	α4β1γ1	-
laminin-9	α4β2γ1	-
laminin-10	α5β1γ1	-
laminin-11	α5β2γ1	-
laminin-12	α2β1γ3	-
laminin-14	α4β2γ3	-
laminin-15	α5β2γ3	-
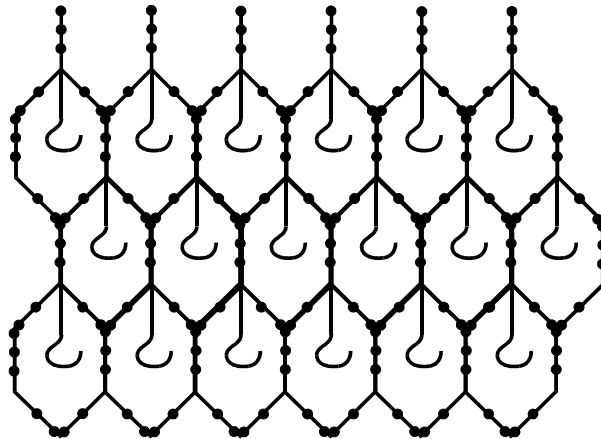
Fig. 2. Laminin polymerization. Trimeric laminin molecules pair by three NH$_2$-termini to achieve a stabile network. COOH-terminal ends remains free for receptor interactions. Modified from Yurchenco & O´Rear (1994).

2.4.1 Domain structure of laminins

Laminins are made up of several independent domains, sharing well conserved sequences among different laminin chains and also among different species. The triplehelical long arm of laminin molecule is formed by α, β and γ chains, whereas the short arms are formed solely by individual chains.
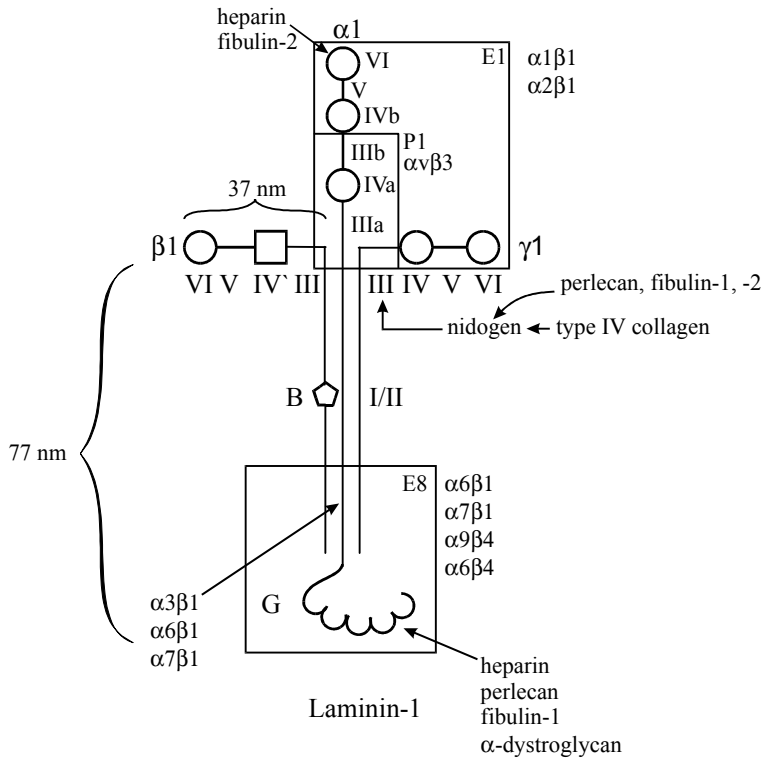
Fig. 3. Schematic pattern of the laminin molecule, domain presentation (Roman numerals), some proteolytic fragments (boxes) and some binding sites for integrins and other molecules (arrows) Modified from Engvall & Wewer (1996).

The G (globular) domain at the end of the long arm is composed only of α chain. It is a large (100 kDa) domain at the C-terminus and is composed of five repeats of about 180-200 amino acids, and it contains several conserved cysteine and glycine residues. The G domain is involved in the intermolecular assembly of laminin, since many ECM molecules are shown to bind to or near it, e.g. heparin, heparan sulfate proteoglycans, fibulins, nidogen-2 and α-dystroglycan (Talts *et al.* 1999). G domain-like repeats can be found in many other ECM macromolecules, such as perlecan and agrin. The G domain also bears the RGD sequence (Arg-Gly-Asp), which is an important receptor binding site. Domains I and II can be distinguished in the long arm of laminin in area where α, β and γ chains are joined. This region contains a rod-like structure which contains a number of

heptad repeats. Domain I is supposed to contain the data to determine the subunit composition of the laminin (Utani *et al.* 1994). The B domain (also called α domain) is only a part of the β chain and exhibits a loop-like structure. No specific function is known for this domain so far.

The short arms of laminin are each composed of a single subunit. Domain VI is found at end of each chain. Characteristic of this domain is the presense of several conserved cysteine residues and the sequence WWQS. Domain VI has been shown to be essential for polymerization of the laminins (Yurchenco & Cheng 1993). Domain IV is situated proximally in the short arms. The sequence of this domain lacks cysteines and is well conserved among laminin subunits. Two forms of the domain exist; α and γ chains contain similar domains, but domain IV in β chains (designated as IV´) differs by having a number of cysteine residues. The function of this domain is not known.

Domains VI and IV are separated by domain V and additionally, domain III can be distinguished at the beginning of the individual arm. Opposite to globular domains VI and IV, these domains exhibit several cysteine-rich repeats to form rod-like segments and are composed of many repeating EGF-like (epidermal growth factor) domains, which have also been observed in many other BM and non-BM macromolecules (for review, see Engvall & Wewer 1996, Timpl 1996 and Tryggvason 1993).

The α4 chain lacks IIIb, IVb, V and VI domains and has a molecular weight of about 199 kDa (Iivanainen *et al.* 1995). The γ2 chain lacks domains V and VI and has a molecular weight of 155 kDa (Kallunki et al. 1992).

Among laminins α3 and γ2 chains exhibit truncated forms as a result of alternative splicing and/or alternative promoter usage, so they also have lower molecular weights. α3 has been detected to exist as two forms; α3A and α3B, of which α3A has a molecular weight of 160 (Rousselle *et al.* 1991) and α3B of about 200 kDa (Miner *et al.* 1997).

The γ2 chain can be detected as two distinct alternative forms. A shorter 3´end has more restricted expression pattern, but it is not known whether shorter form is encoded for the native protein (Kallunki *et al.* 1992, Airenne *et al.* 1996).

Proteolytically digested pepsin and elastase fragments of laminin have offered a useful tool for studing receptor binding and several active sites has been introduced. The main cell surface binding sites are located on the long arm of laminin (fragment E8). Also a number of ECM molecules are bound to the G domain of laminin (see figure 3). A pentapeptide, IKVAV (Ile-Lys-Val-Ala-Val) just above the carboxyl globule on the long arm has been identified as the active site for cell adhesion and neurite outgrowth (Tashiro *et al.* 1989). RGD segment which mediates cell adhesion in mouse is found between the domains IIIa and IIIb of the laminin α1 chain (Aumailley *et al.* 1990). In human RDG segment can be found in the C-terminus (Olsen *et al.* 1989). A pentapeptide, YIGSR (Tyr-Ile-Gly-Ser-Arg) from the cysteine-rich region of the IV´ domain in β1 chain has been found to be active for cell attachment, receptor binding and migration (Graf *et al.* 1987). An additional low-affinity cell attachment site has been desribed on the short arm of α1 chain in fragment P1. It has been speculated that it is not entirely active, but can be activated during rapid tissue turnover and tumor cell invasion (Aumailley *et al.* 1987).

2.4.2 Tissue expressions of the individual laminin chains

2.4.2.1 Laminin α1, α2, α4 and α5 chains

The α1 chain is present in laminin-1 and –3. Based on RNA studies its expression is highly limited and its mRNA expression has been reported to occur mainly in placenta, kidney, testis, brain and neuroretina (Nissinen *et al.* 1991, Vuolteenaho *et al.* 1994). There has been controversy in recent years as to the α1 chain antigen expression, since immunohistochemical studies, in opposition to the results of RNA studies, have indicated that it is the most abundantly distributed laminin chain (Engvall *et al.* 1986, Virtanen *et al.* 1995a), while other studies suggested a different and more limited antigen expression pattern (Klein *et al.* 1988, Ekblom *et al.* 1990). Studies reporting a widespread and abundant expression pattern for laminin α1 chain were performed by using the 4C7 antibody described by Engvall *et al.* (1986). The antibody was believed to detect the α1 chain, but it has recently been shown to actually recognize the α5 chain instead (Tiger *et al.* 1997, Church & Aplin. 1998, Kikkawa *et al.* 1998). Currently, by using antibodies other than 4C7 the α1 chain antigen expression has not been well documented, but it has been shown to be present in glomerular and tubular BMs of kidney, many glandular BMs, follicular BM of thyroid gland, but lacking in muscular and endothelial BMs (Tiger *et al.* 1997, Falk *et al.* 1999, Virtanen *et al.* 2000).

The α2 chain can be found in laminin-2, –4 and -12. In fetal tissues its mRNA expression is detected in many tissues, including cardiac muscle, pancreas, lung, kidney, skin, but is more restricted in liver, thymus and bone. *In situ* hybridization reveals mostly mesenchymal expression pattern in fetal tissues. (Vuolteenaho *et al.* 1994). Immunohistochemically α2 chain is expressed in trophoblastic BMs of placental villi, striated muscle and peripheral nerves (Leivo *et al.* 1988), in mesangial matrix of glomeruli (Virtanen *et al.* 1995a) and in BMs of glands of gastric mucosa (Virtanen *et al.* 1995b).

The α4 chain is present in laminin-8, -9 and -14. The strongest mRNA expression can be detected in heart, lung, ovary, intestine and placenta (Iivanainen *et al.* 1995). Antigen expression has been shown to occur mainly in mesenchymally derived tissues such as endothelial BMs and alveolar BMs of lung (Miner *et al.* 1997).

Laminin-10, –11 and -15 contain the α5 chain. When it became clear that the 4C7 antibody recognizes the α5 chain, all the previous studies using this antibody indicated that the α5 chain is perhaps the most widely distributed among the laminin chains. By Northern blot analyses it has been detected in almost all tissue types, and the most intense production has shown to be present in heart, kidney, lung, skin and placenta (Miner *et al.* 1997). Parallel to mRNA studies, by immunohistochemistry it has been detected in BMs of gastric mucosa, tubular structures and glomeruli of kidney, striated and smooth muscle, trophoblastic epithelium of placenta, alveolar epithelium of lung and vascular endothelium (Engvall *et al.* 1986, Virtanen *et al.* 1995a, Virtanen *et al.* 1995b, , Miner *et al.* 1997).

2.4.2.2 Laminin β1 and β2 chains

The β1 chain is in heterotrimers of laminin-1, -2, -6, -8 and –10. It can be detected in most BMs, such as tubules and Bowman´s capsule of kidney, glandular and vascular structures of gastric mucosa, and in glomerular mesangium (Virtanen *et al.* 1995a, Virtanen *et al.* 1995b). The β2 chain is present in laminin-3, -4, -7, -9, –11, -14 and -15. It is mostly found in BMs of blood vessels, muscular BMs and muscular synapses in many locations, but rarely in epithelial BMs (Virtanen *et al.* 1995a, Wewer *et al.* 1994a).

2.4.2.3 Laminin γl and γ3 chains

The γl chain can be found in all currently known laminin heterotrimers, except for laminin-5 where it is replaced by the γ2 chain (Kallunki *et al.* 1992). There is also a newly discovered laminin containing the γ3 chain (Koch *et al.* 1999). Laminin γl chain mRNA expression occurs both in epithelial and stromal cells, and the strongest expression pattern has been detected in lung, muscle, kidney, pancreas and skin (Kallunki *et al.* 1991). As the most common component of laminin heterotrimers, it is correspondingly widely detected in BMs of different tissues including epithelial, mesenchymal and neural BMs (Virtanen *et al.* 1995a, Virtanen *et al.* 1995b).

The tissue expression of the recently discovered γ3 chain differs from chains discovered earlier and shows a highly restricted expression pattern. γ3 chain prefers apical distribution of ciliated epithelial cells and consequently forms a prominent element of the apical surface of ciliate cells of lung, oviduct, epididymis and seminiferous tubules. Authors suggested that apical presence of laminin is important in the morphogenesis and maintenance of structural stability (Koch *et al.* 1999).

2.4.2.4 Laminin-5 (α3β3γ2)

α3, β3 and γ2 chains form the laminin-5 heterotrimer. α3 is additionally present in laminins-6 and –7, but, there is no knowledge so far that β3 and γ2 chains forms laminin heterotrimers other than laminin-5. Laminin-5 was first found from anchoring filaments of skin and was originally named kalinin (Rousselle *et al.* 1991). An unique property of the α3 and γ2 chains is that they are processed in ECM space after secretion. This has not been detected for any other laminin chains. The laminin α3 chain, initially 190 kDa in size, is cleaved by matrix degrading entzyme, plasmin, into a 160 kDa sized molecule and can be additionally cleaved further into a 145 kDa sized laminin chain (Goldfinger *et al.* 1998). The γ2 chain is synthesized and secreted as a high molecular weight precursor of 155 kDa, which is processed extracellularly to 105 kDa weight (Marinkovich *et al.* 1992). Recently matrix metalloproteinase-2 (MMP-2) and membrane type-1 matrix metalloproteinase (MT1-MMP) have been shown to specifically cleave laminin γ2 chain to generate its truncated form (Giannelli *et al.* 1997, Koshikawa *et al.* 2000).

Laminin-5 was shown to be intimately involved in the attachment of keratinocytes to the BM of skin by anchoring filaments. Due to its truncated short arms it is not capable of

interations with other laminin molecules or nidogen/type IV collagen, but binds to type VII collagen of anchoring fibrils of the subjacent stroma instead. It is suggested that a single laminin-5 molecule acts as a bridge between hemidesmosomal α6β4 integrin and NC-1 domain of type VII collagen (Rousselle *et al.* 1997). Laminin-5 can interact with laminin-6 or –7 by forming disulfide-bonded complexes and thereby can be bound to BMs (Champliaud *et al.* 1996). It seems likely that laminin-5 plays a more important role in maintaining the mechanical resistance in the tissue than in forming a ubiquitous structural constituent of genuine BMs. High epithelial specificity of laminin-5 is well established and its expression occurs in BMs of both squamous and glandular epithelia in many locations, e.g. skin, lung, breast, intestine and prostate (Pyke *et al.* 1994, Virtanen *et al.* 1995a, Hao *et al.* 1996). It has a strong adhesive property and it is highly expressed especially by migrating keratinocytes in wounded skin in establishing new BM-zone (Pyke *et al.* 1994,Rousselle *et al.* 1994).
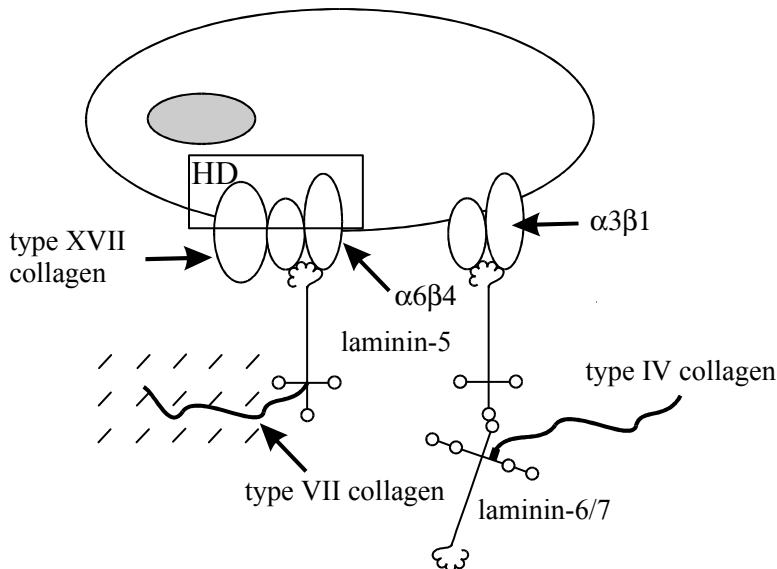
Fig. 4. Laminin-5 in BMs of squamous epithelium. Hemidesmosomes (HD) take importantly part in the attachment of polarized epithelium to the underlying stroma. Laminin-5 interacts via α6β4 integrin with the type XVII collagen (previously bullous pemphigus antigen 180; BPA-180). All these molecules are a part of hemidesmosomal system, which is bound to the type VII collagen and thereby to the underlying stroma. Alternatively, laminin-5 bound via α3β1 integrin can associate with laminins-6 and -7 and thus to be connected to the collagen IV network. Modified from Rousselle *et al.* (1997).

2.4.3 Biological implications of the laminins

Since BMs are important structural and functional structures in multicellular organisms, their significance is well known. They possess many essential properties, such as cell adhesion, migration, development and molecular ultrafiltration (Adams & Watt 1993).

Biological functions of laminins are mediated by specific or non-specific receptors present on cell membranes. Integrins are an important group of heterodimeric glycoproteins composed of α and β chains which are linked together and associated with the cell membranes. To date, at least nine different integrins have been suggested to be receptors for laminins; α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, α9β1, αvβ3 and αvβ8, of which laminin-1 can bind all (For review, see Mercurio 1995, Colognato *et al.* 1999, Ivaska & Heino 2000). They mainly bind to the G domain area of the α chain (E8 fragment) (Aumailley *et al.* 1987).

Dystroglycan, a 156 kDa sized receptor mediates laminin binding in muscle and neural tissues. It efficiently binds the G-domain of laminin α chains and is important in specifying BM formation and facilitates interactions among other BM macromolecules (Henry & Campbell 1998).

Also a 67-kDa high-affinity laminin binding protein, phospolipids and heparan sulfate can bind and mediate laminin functions (Mecham 1991, Weber *et al.* 1992), but their biological significance is not fully understood.

Previously it was thought that laminins and type IV collagen are bound to their receptors, mediating their biological functions directly. Recently it was shown that laminin polymerization would play an essential role in signaling and has an effect on the reorganization of the adjacent cytoskeleton network. Laminin polymerization occurs through NH_2-terminal ends, whereas most of the receptor interactions are mediated by COOH-terminal domains. It was shown that laminin polymerization initiates a cascade providing a targeting mechanism for the assembly of BMs. Laminin network architecture leads to changes in organization of matrix, receptors and cortical cytoskeletal components, including actin reorganization and tyrosine phosporylation (Colognato *et al.* 1999). Furthermore, it has been demonstrated, contrary to the previous concept that type IV collagen selfassembly occurs independently from other BM macromolecules, and by providing an initial signal the laminin network is essential for type IV collagen polymerization (Smyth *et al.* 1999).
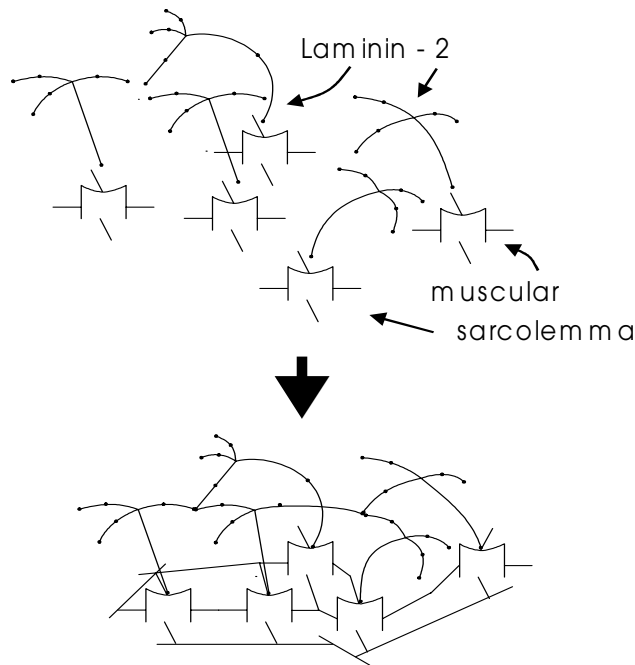
Fig. 5. Laminin-2 COOH-terminal arms bind to the cell surface receptors whereas NH$_2$-termini act to form polymer interactions. Laminin-2 polymerization at muscular sarcolemma induces receptor-cytoskeleton network. Modified from Colognato *et al.* (1999).

The function of laminins is less understood, although many studies have been performed by using proteolytic fragments of laminin or antibody perturbing technique. Laminins have a central role in cell adhesion, migration, differentiation, tissue development and mitogenic modulation (for review, see Timpl 1996, Tryggvason 1993, Engvall & Wewer 1996). Cell adhesion is essential for the cellular differentiation and also for the prevention of apoptosis, controlled cell death by which diversed cells are actively removed. Laminins and type IV collagen are known to mediate survival signals for epithelial cells (Mooney *et al.* 1999). Laminin-5 is known to inhibit T cell proliferation via $\alpha_6\beta_4$ integrin (Vivinus-Nebot *et al.* 1999). In the neurite-promoting site blocking of the $\alpha5$ chain reduces the amount of neurite-bearing cells and the length of individual neurites (Engvall *et al.* 1986). The $\beta2$ laminin chain in BMs has shown to be an effective adherence factor for neurites (Hunter *et al.* 1989). The $\gamma1$ chain of laminin is known to interact with nidogen and by that also to connect with type IV collagen. Neutralizing antibodies against the $\gamma1$ chain has shown to perturb epithelial morphogenesis by reducing branching rudiments in developing lung and by perturbing and reducing tubular formation in developing kidney (Ekblom *et al.* 1994).

Laminin chains have been shown to have some differences in their composition in BMs during development as compared to mature tissues. The best known tissue in this respect is kidney, where changes occur in laminin chain composition during embryogenesis. It is well known that blastemal mesenchyme of developing kidney converts into epithelium. This mesenchymal tissue expresses exclusively $\alpha4$, $\beta1$ and $\gamma1$

chains, whereas the mesenchyme which is not going to form epithelial structures shows α2 chain expression. As soon as pre-epithelium is formed, the α4 chain is downregulated and α1 chain replaces it. During further tubular maturation also α1 chain expression will be changed and replaced by α5 chain. Similarly, BMs in developing loops of early glomeruli display laminin α1 and α4 chains, but during maturation they will be replaced by the α5 chain. Also β1 chain in glomerular BM will be replaced during development by β2 chain which is the most prevalent laminin chain in glomerular BMs in adult kidney (Noakes *et al.* 1995). The significance of these phenomena is not known, but it is related to tissue development and maintenance of mature phenotype (for review, see Miner *et al.* 1997, Virtanen *et al.* 1995a). In fact, the laminin α5 chain has been suggested to have a more general role in epithelial and endothelial maturation and a role in the maintenance of differentiated phenotype of tissues is also implicated (Sorokin *et al.* 1997).

2.4.4 *Role of laminins in distinct diseases and knock-out mice*

Certain mutations among laminin chains are known to cause or be associated with some diseases. Point mutations in genes of laminin-5 (LAMA3, LAMB3, LAMC2) lead to an inherited blistering skin disease, junctional epidermolysis bullosa, of which many different types have been characterized in families affected by the disease (McGrath *et al.* 1995, Vailley *et al.* 1995, Uitto *et al.* 1994). LAMC2 mutation was the first mutation ever discovered among laminins leading to a specific disease. Mutations direct to perturbed adhesion of keratinocytes to the underlying BM-zone due to altered hemidesmosome structure. A minor trauma to the skin of patients can lead to severe blister formation.

Defects in α2 chain gene (LAMA2) cause congenital muscular dystrophy resulting in poor organization of muscular fibers (Helbling-Leclerc *et al.* 1995). This leads clinically to hypotonia and muscular weakness, which indicates that the α2 chain has an important role in the muscular integrity and that other α chains cannot replace it to form normally functioning muscle.

Laminin α5 gene, LAMA5, is suggested to be a candidate gene for the ragged (Ra) mutation of mouse, a phenotype which shows thin ragged fur due to the incomplete development of hair follicles (Durkin *et al.* 1998).

Investigations performed on knock-out mice which lack an individual laminin chain would be important for gaining information of the function of laminins and their different chains. Because laminin α5 is abundantly expressed in various BMs, it is not surprising that knock-out mice lacking the α5 chain died during embryogenesis. Furthermore, they exhibited multiple defects in organogenesis, such as failures to close the anterior neural tube, digit septation (syntactyly) and impaired placental villi formation. Other laminin α chains were shown to accumulate in these BMs, but this compensation was apparently functionally inadequate (Miner *et al.* 1998).

Homozygous lack of laminin γ1 chain has also been shown to lead to death on day 5 during embryogenesis. These embryos totally lacked BMs which were replaced by disorganized extracellular deposits of type IV collagen and perlecan (Smyth *et al.* 1999).

Laminin α3 deficient mice developed inherided skin and mucous blistering soon arter birth leading to neonatal death. Microskopic examination revealed the lack of the laminin

α3 chain in epithelial BMs and an abnormal hemidesmosome formation. Interestingly, isolated keratinocytes from null-mice failed to survive in culture, but were rescued by exogenous laminin-5 (Ryan *et al.* 1999).

Elimination of the laminin β2 chain has been shown to lead to impaired synapse formation and glomerular dysfunction in mice. Mice lacking the β2 chain showed abundance of β1 chain in glomerular BMs with microscopically normal structures, but they displayed severe proteinuria (Noakes *et al.* 1995)

2.4.5 *Laminins in cancer*

BMs have an essential role in cancer by modulating cell adhesion, migration, proliferation, maintaining tissue compartmentalization and controlling apoptosis (Adams & Watt 1993, Mooney *et al.* 1999). BMs serve as adhesion matrix for tumor cells to adhere to or to use them for spreading (Tani *et al.* 1997, Kitayama *et al.* 1999). Also separate laminin domains have been isolated and used for functional studies to find out which molecular regions are responsible for distinct functions (Koliakos *et al.* 1997).

Contrary to the relatively detailed desriptions of the mRNA and antigen expressions for different laminin chains in normal tissues, knowledge of their distribution in cancers is more restricted. Previously many studies were carried out by using polyclonal antibodies (Pab) against laminin and type IV collagen without knowledge of their chain specificity, and the results generally indicated that BMs structures are highly fragmented, or even largely absent in many invasive tumor types, such as breast, pancreas, prostate, colon, skin and lung carcinomas when compared to their benign counterparts or underlying normal tissues (Barsky *et al.* 1983, Kallioinen *et al.* 1984, Cam *et al.* 1984, Pääkkö *et al.* 1990). Now when monoclonal antibodies (Mab) against many BM macromolecules are available the role of BMs in cancer needs to be re-evaluated (Tani *et al.* 1996, Hewitt *et al.* 1997, Kosmehl *et al.* 1999)

It has been shown that malignancies are also able to actively produce many ECM macromolecules. Ovarian adenocarcinomas and different histological types of lung carcinomas synthesize high amounts of laminin β1 chain by tumor stromal cells (Autio-Harmainen *et al.* 1993, Soini *et al.* 1993). Human lung, colon, breast and squamous carcinoma cell lines have been shown to synthesize high levels of mRNAs for β1, β2 and γ1 chains, whereas α1 and α2 chain were produced in negligible amounts (Wewer *et al.* 1994b). The α5 chain is abundantly synthesized by various cancer cell lines, indicating that it is a widely spread laminin chain in malignancies (Tani *et al.* 1999).

2.5 Other BM proteins

Nidogen-1 (also known as entactin) is a BM-associated glycoprotein, which can be found abundantly in all BMs (Timpl *et al.* 1983). It forms equimolar complexes with laminins and it also binds to type IV collagen, and it is thus important in directing architectural interactions between these two molecules. It binds to laminin III domain of the γ chain and in type IV collagen molecule to the NH_2-region (Mayer *et al.* 1993). The molecular

weight of nidogen is about 150 kDa and it also has the ability to bind to fibulins-1 and -2 and perlecan. Recently nidogen-2 was discovered. It is a 200 kDa protein and shares many essential properties compared with nidogen-1, but it has a slightly lower affinity for laminin γ1 chain and fails to bind fibulins. Results suggested further that an alternative binding site exists for nidogen-2 (Kohfeldt *et al.* 1998).

Numerous heparan sulfate proteoglycans are also present in the BM network, perlecan (470 kDa) being the best known among them. It can be found abundantly in BMs, being an additional stabilizing component in them, and it also provides core ligands to cell receptors (Kjellen & Lindahl 1991). Recently a new BM proteoglycan, leprecan (leucine proline-enriched proteoglycan), was introduced. It has a molecular weight of 100 kDa and shares all the essential properties of BM proteins, and it preferentially localizes to BMs of glomerulus and capillaries of kidney and muscle (Wassenhove-McCarthy & McCarthy 1999).

BM-40 (also known as SPARC; secreted protein acidic and rich in cysteine) (Dziadek *et al.* 1986), is not a molecule specific for BMs, but can also be found in bone matrix (therein called osteonectin). It associates also with BMs, binds type IV collagen and interacts with cellular receptors.

Fibulins are a more novel group of BM-associated proteins. Fibulin-1 (100 kDa) (also known as BM-90) binds to the COOH-terminus of laminin α chain of laminin-1 and –4, but not of laminin-2 (Adam *et al.* 1997). It is also able to bind to type IV collagen. Fibulin-2 (195 kDa) binds to the N-terminus of α1 chain and domain IV of laminin γ2 chain of laminin-5 (Utani *et al.* 1997). (For review, see Timpl 1996, Yurchenco & O´Rear 1994).

2.6 Proteinases involved in ECM degradation

ECM homeostasis is maintained by balancing matrix synthesis and its degradation. This phenomenon is tightly regulated and involved in many physiological conditions such as embryogenesis, growth and tissue repair. Synthetic and degradative activity can be imbalanced in some cases, such as in scar formation, fibrogenesis, inflammation and in some destructive diseases such as rheumatoid arthritis. Degradation requires specific enzymes to break down the structures of ECM and this destruction is controlled by specific or more general inhibitors. The current opinion is that in cancer there is increased tissue degradation of the structural barriers formed by BMs and interstitial connective tissue. The proteinases involved in ECM degradation belong to groups of MMPs, serine proteinases and cysteine proteinases. In this thesis only proteinases belonging to a group of MMPs are investigated and this review will focused on them. (For review, see Birkedal-Hansen *et al.* 1993 and Kähäri & Saarialho-Kere 1999).

2.6.1 Domain structure of MMPs

All MMPs contain characteristic functional domains. A signal sequence, prodomain and catalytic domains are conserved between various members of the family and also the C-

terminal hemopexin-like domain and hinge region, but the last two domains are missing from MMP-7 and MMP-26, which are consequently the smallest members among MMPs. Additional fibronectin-like domains are present in MMP-2 and MMP-9 and transmembrane domains in MT-MMPs (Murphy *et al.* 1994, Sato *et al.* 1994).
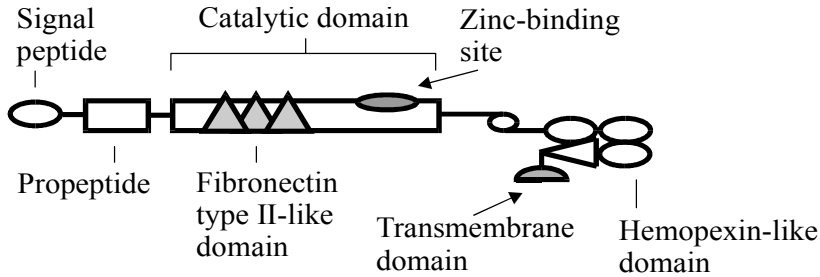
Fig. 6. Domain structure of MMPs. The fibronectin-like domain is present only in gelatinases and the transmembrane domain only in MT-MMPs. Modified from Birkedal-Hansen et al. (1993).

A hydrophobic signal sequence of 17-29 residues is present in every MMP, and it is removed when enzyme is secreted to endoplasmic reticulum. The following sequence of a 77-87 residue propeptide is cleaved during activation of the enzyme in pericellular space. The biological function of this domain is to maintain latency of MMPs until activation is needed. The propeptide has a highly conserved PRCGVPD sequence and contains a cysteine residue, which interacts with the zinc atom of the active site to maintain the latency of the enzyme (Springman *et al.* 1990, Woessner 1991).

The catalytic domain contains the conserved sequence HEXXHXXGXXH and is the putative zinc-binding site which is important for catalytic activity. The three histidine residues also act as ligands for the catalytic activity (Sanchez-Lopez *et al.* 1988, Crabbe *et al.* 1994b).

The C-terminal domain has sequence homology to hemopexin, heme-binding protein and vitronectin and it is believed to have a role in determining substrate specifity. It has been shown to have a role in binding gelatinase precursors to tissue inhibitors of metalloproteinases (TIMPs) (Jenne & Stanley 1987, Murphy *et al.* 1992, Sanchez-Lopez *et al.* 1993)

MMP-2 and MMP-9 also have additional domains; a fibronectin-like domain which is homologous to repeats found in fibronectin. MMP-9 also has a proline-rich domain found between the catalytic and the hemopexin-like domains, which has structural similarity to α2(V) collagen (Collier *et al.* 1988, Wilhelm *et al.* 1989).

MT-MMPs differ from other MMPs in that they contain a transmembrane sequence in the last hemopexin-like repeat of the C-terminal domain which essentially acts in a cell membrane binding capacity (Sato *et al.* 1994).

2.6.2 Subgroups of MMPs

To date about 20 different MMPs have been cloned. They are presented in table 4.

Table 4. The family of MMPs. Modified from Chandler et al. (1997) and Chambers & Matrisian (1997).

MMP	mw (kDa) pro/active	Some substrates	References
Collagenases			
MMP-1 (collagenase-1)	55, 52 / 42	collagens I-III, gelatin, proteoglycans	1, 2
MMP-8 (collagenase-2)	91, 75 / 58	collagens I-III, V, VII, IX and X, gelatin	3-8
MMP-13 (collagenase-3)	65 / 55	collagens I-IV, IX, X, XIV, fibronectin, gelatin	7, 9-11
Stromelysins			
MMP-3 (stromelysin-1)	60, 57 / 45	collagens III-V, IX and X, fibronectin, laminin, nidogen, proteoglycans	6-8, 12, 13
MMP-7 (matrilysin)	21, 28 / 19	collagen IV, elastin, fibronectin, gelatin, laminin	14-16
MMP-10 (stromelysin-2)	55 / 44	collagens III-V, elastin, fibronectin, gelatin	13, 17, 18
MMP-11 (stromelysin-3)	58, 55 / 27	serine protease inhibitors	6, 8, 19
MMP-12 (macrophage metalloelastase)	54 / 21	fibronectin, laminin	20, 21
Gelatinases			
MMP-2 (gelatinase A)	72 / 62	collagens I, II, IV, V, VII, X, XI, XIV, elastin, fibronectin, gelatin, laminin γ2 chain	13, 18, 22, 24-26
MMP-9 (gelatinase B)	92 / 84	collagens I, III-V, VII, X, XIV, elastin, fibronectin, gelatin, nidogen	13, 18, 22, 24, 25, 27
Membrane-type MMPs			
MMP-14 (MT1-MMP)	63	collagens I-III, fibronectin, gelatin, laminin	6, 7, 28-30
MMP-15 (MT2-MMP)	72	aggregan, collagens I-III, fibronectin, laminin, nidogen	30, 31
MMP-16 (MT3-MMP)	64	collagens I, III, fibronectin	32, 33
MMP-17 (MT4-MMP)	71 / 67	not known	34
MMP-24 (MT5-MMP)	64	not known	35
MMP-25 (MT6-MMP)	34, 28 / 19	Gelatin	36, 37
Other MMPs			
MMP-19	57	not known	38
MMP-20 (enamelysin)	54	amelogenin	39
MMP-23	44	gelatin	40, 41
MMP-26	30	casein, gelatin	42

MMP = matrix metalloproteinase, mw = molecular weight. Substrate specifity data according to 1) Wilhelm *et al*. 1986, 2) Fields *et al*. 1987, 3) Macartney & Tschesche 1983, 4) Hasty *et al*. 1987, 5) Fosang *et al*. 1994, 6) Chandler *et al*. 1997, 7) Murphy & Knäuper 1997, 8) Nagase 1997, 9) Freije *et al*. 1994, 10) Mitchell *et al*. 1996, 11) Stolow *et al*. 1996, 12) Chin *et al*. 1985, 13) Murphy *et al*. 1991a, 14) Quantin *et al*. 1989, 15) Woessner & Taplin 1988, 16) Miyazaki *et al*. 1990, 17) Nicholson *et al*. 1989, 18) Nguyen *et al*. 1993, 19) Murphy G *et al*. 1993, 20) Shapiro *et al*. 1993, 21) Chandler 1996, 22) Collier *et al*. 1988, 23) Woesser 1991, 24) Liotta *et al*. 1979, 25) Wilhelm *et al*. 1989, 26) Giannelli *et al*. 1997, 27) Murphy *et al*. 1989, 28) Sato *et al*. 1994, 29) Ohuchi *et al*. 1997, 30) D´ortho *et al*. 1997, 31) Will & Hinzmann 1995, 32) Takino *et al*. 1995, 33) Shofuda *et al*. 1997, 34) Puente *et al*. 1996, 35) Pei.1999a, 36) Pei 1999c, 37) Velasco *et al*. 2000, 38) Pendás 1997, 39) Llano 1997, 40) Velasco *et al*. 1999, 41) Pei 1999b, 42) de Coignac *et al*. 2000.

2.6.3 Some special aspects of MMPs

All collagenases cleave α-chains of interstitial collagens into ¾ and ¼ fragments. Only a limited number of cell types are capable of synthesis of MMP-8, but when it is synthesized by maturing neutrophils in bone marrow the enzyme is stored in specific granules of the circulating cells (Hasty *et al*. 1986). A splice variant has also been detected for MMP-8 which is the only member among MMPs having a splice variant so far. The splice variant is not a secreted protein and is not enzymatically active, however, and its role in cellular processes is unknown (Hu *et al*. 2000).

Stromelysins, except for MMP-11, have broad substrate specificity especially to proteoglycans and glycoproteins and therefore they have been assumed to have an important role in ECM turnover. MMP-11 is only a weak metalloproteinase, but degrades serine proteinase inhibitors (Murphy *et al*. 1993). MMP-3 also has the ability to activate many other MMPs (Chin *et al*. 1985, Murphy *et al*. 1981).

Both gelatinases, MMP-2 and MMP-9, are widely expressed by normal, as well as malignant cell types. They have similar types of substrate specifity. Important sources of MMP-9 are polymorphonuclear leucocytes in which it is stored in specific granules released upon neutrophil stimulation. It can also be detected in monocytes, macrophages and T and B lymphocytes (Murphy *et al*. 1989, Goetzl *et al*. 1996, Murphy & Crabbe 1995, Trocmé 1998). A unique property for MMP-2 and MMP-9 is the complex formation with TIMPs (Tissue Inhibitor of MetalloProteinase). TIMP-2 has higher affinity to bind proMMP-2, while TIMP-1 has higher affinity to proMMP-9 (Goldberg *et al*. 1989, Goldberg *et al*. 1992). MMP-2 activation differs greatly from other MMPs, since MT-MMPs play a central role in that process (Sato *et al*. 1994). Gelatinases have been implicated as having an important role in cell invasion due to their ability to degrade laminin and type IV collagen of BM (Liotta *et al*. 1980).

MT-MMPs share a common feature with the unique membrane-spanning sequence in the head of C-terminus (Sato *et al*. 1994). This domain is believed to direct the integration of MT-MMPs into cell membranes (Cao *et al*. 1995). MT4-MMP differes from the others, having only 65% sequence identity to other members and MT4-MMP

lacks a cytoplasmic tail, which is present in all other MT-MMPs. Recently evidence was presented that MT4-MMP is connected to cell membrane through a glycosylphosphatidylinositol anchor system, as are e.g. cytokine receptors and integrins (Itoh *et al.* 1999). This aspect can indicate a specific role for MT4-MMP in proteolytic events. All known MT-MMPs, except for MT4-MMP, are able to take part to the activation of MMP-2 (Sato *et al.* 1994, Will & Hinzmann 1995, Takino *et al.* 1995, English *et al.* 2000, Pei 1999a, Pei 1999c, Velasco *et al.* 2000).

There are also some new members of MMPs which cannot be classified into previous MMPs subgroups. MMP-19 was found from human liver. It has some differences in its domain structure, and some reports have speculated that it might belong to a totally new group of MMPs (Pendás *et al.* 1997). MMP-20 is present in human odontoblasts. It has been shown to participate in the development of teeth (Llano *et al.* 1997). The MMP isoform discovered simultaneously by different laboratories is designated to MMP-21/22, MMP-23 or its mouse counterpart CA-MMP, and it was demonstrated to be expressed mainly by reproductive organs, heart, spleen and lung tissues. It contains a conserved MMP catalytic domain, but lacks a classic cysteine switch structure and is also devoid of other structural features distinctive of the diverse MMP subclasses and may represent a novel MMP group (Gururajan *et al.* 1998, Velasco *et al.* 1999, Pei 1999b). The most recently discovered MMP, named MMP-26, was found to lack the hemopexin domain and showed close similarity to MMP-12. Its mRNA was shown to be exclusively expressed by human placenta, but not by many other organs (de Coignac *et al.* 2000).

2.6.4 Regulation of MMP synthesis and activation

There are numerous individual factors which can influence MMP synthesis either by increasing or reducing it. These include e.g. cytokines, hormones, growth factors and proto-oncogene products (for review, see Birkedal-Hansen *et al.* 1993). The ECM signaling role for MMP synthesis and activation has been emphasized in recent years. Laminin-1 and synthetic laminin α1 globular domain peptide stimulate neural cells *in vitro* to secrete increasing amounts of MMP-2 (Weeks *et al.* 1998). When grown in three-dimensional collagen skin fibroblasts upregulate MMP-13 synthesis and activation (Ravanti *et al.* 1999). It is also suggested that remodeling of actin cytoskeleton may be directly linked to regulation and activation of MMPs in general (Thomasek *et al.* 1997). Moreover, integrin α3β1-tetraspaning protein complex, consisting of two groups of cell surface transmembrane proteins have been shown to control the expression of microvilli-like protrusions involved in cell migration in breast cancer cells grown in reconstructed ECM. Neutralizing antibodies against that complex stimulated the expression and activation of MMP-2 by tumor cells and induced formation of long invasive protrusions within ECM, resulting in potentiated invasion (Sugiura & Berditchevski 1999). Integrings α1 and α2 have been shown to regulate both transcriptional and protein expression of MMP-3 in tumor cells *in vitro*, but antibodies against α6 and β1 integrins did not alter the expression (Lochter *et al.* 1999). Integrin α2β1 is a positive regulator for MMP-1 expression in three-dimensional collagen gel (Riikonen *et al.* 1995). The results together

suggest that mechanisms involved in the control of matrix proteolysis are complex and largely unknown so far.

MMPs are secreted as latent proenzymes and require removal of the propeptide in order to function. The latency of MMPs is maintained by a co-ordinated bond between the cysteine and the zinc atom, which links the cysteine residue of the propeptide to the zinc atom at the active site. During activation, dissociation between the cysteine and the zinc atom reveals the zinc binding site either as a result of conformational change or by proteolysis allowing it to interact with H_2O. After the cysteine-zinc site is disrupted, the proenzyme undergoes several autolytic cleavages to generate the fully activated form (Springman *et al.* 1990, Van Wart & Birkedal-Hansen 1990, Grant *et al.* 1987, Nagase *et al.* 1990).

MMP-2 differs from other MMPs in that it is not activated by serine proteases (Okada *et al.* 1990). In recent years it has been shown that MMP-2 has a special activation cascade in which MT1-MMP and TIMP-2 play an essential role. The first observation was introduced by Sato *et al.* (1994) who presented a wholly new member of MMPs, MT1-MMP, and showed its ability to activate proMMP-2. MT1-MMP is known to interact with TIMP-2 to form a ternary complex in cell membrane. The initial process is TIMP-2 binding via C-terminus to C-terminus of proMMP-2 to form a stabile complex. This binary complex has an affinity to bind activated MT1-MMP in cell membranes to form a ternary complex, resulting in proMMP-2 activation (Strongin *et al.* 1995). Another study has shown that TIMP-2 can directly bind active MT1-MMP to form a complex (Imai *et al.* 1996) and it can therefore act as a receptor for proMMP-2 in cell membranes. However, higher amounts of TIMP-2 are inhitory for MMP-2 activation (Kinoh *et al.* 1996). Studies with recombinant MT1-MMP with truncated C-domain have shown that this domain is essential for the activation of proMMP-2 (Cao *et al.* 1995). TIMP-2 concentration in cell membranes has an essential role for MT1-MMP activation; low amounts of TIMP-2 decrease its activation, leading to generation of a membrane-bound inactive 44 kDa species, but higher amounts are suitable, leading to fully active 57 kDa MT1-MMP (Hernandez-Barrantes *et al.* 2000). MT1-MMP initiates proMMP-2 autoactivation by its soluble catalytic domain cleaving a bond from proMMP-2 (Lichte *et al.* 1996, Sato *et al.* 1996) and recently, a crystal structure of the full-length proform of human MMP-2 was introduced (Morgunova et al. 1999). The authors showed that MT1-MMP cleaves proMMP-2 within the loop that connects helices H1 and H2 containing a disulfide bridge unique only for proMMP-2. The tightly controlled activation mechanism of proMMP-2 by TIMP-2 and MT-MMPs differs form the activation of other MMPs and may reflect the important role of MMP-2 in tissue remodeling.

MT2-MMP, MT3-MMP, MT5-MMP and MT6-MMP have also been shown to act as activators of proMMP-2 (Sato *et al.* 1994, Will & Hinzmann 1995, Takino *et al.* 1995, Pei 1999a, Pei 1999c, Velasco *et al.* 2000), but it is not known whether the interaction site is the same as with MT1-MMP. Also many other agents have been shown to activate proMMP-2. In co-operation with MT-MMPs, collagen I has been shown to induce proMMP-2 activation by stromal fibroblasts situating in close contact to epithelial tumor cells of ovarian cancer (Boyd & Balkwill 1999). Intracellular activation of proMMP-2 was suggested to occur by an activator which was found in a Golgi-enriched fraction (Lee *et al.* 1997), but its significance *in vivo* is unknown. Tumor-associated trypsin-2 has been

shown to activate both proMMP-2 and proMMP-9 (Sorsa *et al.* 1997). Thrombin can also convert proMMP-2 to its active form by vascular endothelial cells (Nguyen *et al.* 1999).
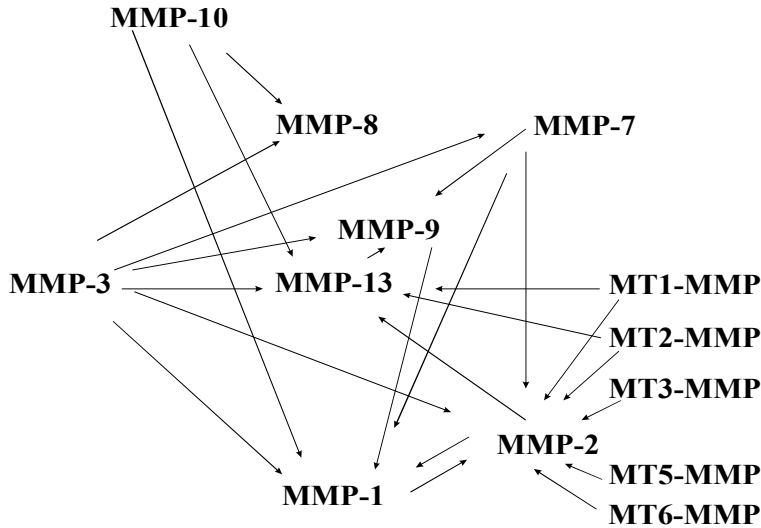
Fig. 7. Ability of MMPs to activate each other. Arrows indicate the activation cascade. Data according to Crabbe *et al.* 1994a, Crabbe *et al.* 1994b, Chandler *et al.* 1997, Vater *et al.* 1983, Knäuper *et al.* 1993, Knäuper *et al.* 1996a, Quantin *et al.* 1989, Windsor *et al.* 1993, Knäuper *et al.* 1996b, Fridman *et al.* 1995, Murphy & Knäuper 1997, Sato *et al.* 1994, Will & Hinzmann 1995, Takino *et al.* 1995, Pei 1999a, Pei 1999c, Velasco *et al.* 2000.

2.6.5 MMP and TIMP knock-out mice

Knock-out mice offer valuable data on the roles of individual MMPs and TIMPs in different biological events. To date at least seven different individual MMP and one TIMP deficient knock-out mice have been generated. Results from all these experiment showed more or less disturbed tissue turnover or suppressed tumorigenesis.

When malignant tumor cells were injected intradermally to MMP-2 deficient mice, tumor growth was significantly decreased compared to control mice. Also angiogenesis was significantly suppressed in tumor areas (Itoh *et al.* 1998).

Decreased angiogenesis was also observed in an MMP-9 experiment. The knock-out mice exhibited an abnormal pattern of skeletal growth plate vascularization and ossification, resulting in proggressive lengthening of the growth plate. Endothelial cells in such mice showed impaired proliferation and migration over newly organized cartilage. Abnormally low apoptotic activity in hypertrophic chondrocytes was also observed in null mice (Vu *et al.* 1998). Interestingly, MMP-9 deficient mice were resistent to bullous pemphigoid, showing that MMP-9 secreted from neutrophils is involved in blister formation in that skin disease (Liu *et al.* 1998).

Perhaps the most severe inherited changes were observed in MT1-MMP deficient mice. They exhibited severe ECM turnover problems resulting in dwarfism, osteopenia and arthritis. Angiogenesis of epiphyseal plates was also decreased. The authors also showed increased fibrosis of soft tissues due to impaired collagenolytic activity (Holmbeck *et al.* 1999).

MMP-3 knock-out mice were used to study experimental arthritis. However, when comparing MMP-3 deficient mice and null mice there were no significant changes in cartilage destruction (Mudgett *et al.* 1998). When mice were treated with dinitrofluorobenzene they exhibited an impaired response to contact hypersensitivity of skin compared to wild type mice, but responded normally to acute skin damaging chemical (Wang *et al.* 1999). This shows that MMP-3 plays a role in initiation of hypersensitivity response in skin. The same study also showed that MMP-9 is required to resolve hypersensitivity response, since MMP-9 deficient mice initiated the response normally, but had a prolonged, persisting reaction.

MMP-7 has been previously shown to be expressed in a high percentage of early-stage human colorectal tumors. The authors generated MMP-7 knock-out mice and demonstrated a reduction in the average tumor diameter when colon carcinoma cells were grown in them (Wilson *et al.* 1997).

MMP-11 gene inactivation in mice was tested with experimental tumorigenesis. Knock-out and null mice were stimulated with carcinogenic chemicals to produce maligant tumors. MMP-11 deficient mice exhibited a significant reduction in tumor production. When tumor cells were injected subcutaneously tumor volume grew at a lower rate in MMP-11 deficient mice (Masson *et al.* 1998).

MMP-12 deficient mice had a markedly diminished capacity to degrade ECM components, especially casein. In addition, macrophages in these mice were essentially unable to penetrate reconstituted BMs *in vitro* and *in vivo*. This result shows the role of MMP-12 in inflammatory processes (Shipley *et al.* 1996).

Up to date only one TIMP knock-out mice is indroduced. TIMP-1 deficient mice was used to study the role of this MMP inhibitor in left ventricular hypertrophy. Result showed that in TIMP-1 deficiency group heart mass increased while myocardial fibrillar collagen content was reduced. Mice had increased end-diastolic volume, but ejection faction and systolic pressure remained unchanged compared to wild-type mice (Roten *et al.* 2000).

2.6.6 Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are natural inhibitors of MMPs. They are low-molecular-weight proteins with the ability to form noncovalent irreversible complexes with active MMPs to block the active site of the enzyme. So far four distinct TIMP molecules have been isolated and characterized. They all have two main domains of a large N-terminal 3-loop and a small C-terminal 3-loop (Murphy *et al.* 1991b). The N-terminus contains the active site taking part in interaction with zinc atom of MMPs and is responsible for the inhibitory activity (O´Shea *et al.* 1992). The C-terminal domain is thought to determine protein localization

and TIMP-1 and TIMP-2 stoichiometric complex formation with the progelatinases (Murphy *et al.* 1991b).

TIMP-1 is a 28.5 kDa soluble glycoprotein (Docherty *et al.* 1985). It can form 1:1 complexes with the latent MMP-9 (Goldberg *et al.* 1989). It has also erythroid potentiating activity and its strong expression prevents angiogenesis (Gasson *et al.* 1985, Moses *et al.* 1990). TIMP-1 can bind all active MMPs, but prefers MMP-1.

TIMP-2 is a 21 kDa non-glycosylated soluble protein and forms complexes with proMMP-2 (Goldberg *et al.* 1989, Stetler-Stevenson *et al.* 1989). TIMP-2 prevents proMMP-2 activation in the complex (Howard *et al.* 1991), but if it is activated, TIMP-2 does not disturb its active function, whereas additional TIMP-2 is inhibitory. TIMP-2 inhibits most effectively MMP-1, MMP-2, MMP-8 and MMP-9 (Howard 1991, Kolkenbrock *et al.* 1991, Stetler-Stevenson *et al.* 1993). Similarly to TIMP-1 also TIMP-2 has erythroid-potentiating activity and growth factor activity (Stetler-Stevenson *et al.* 1992, Nemeth & Goolsby 1993, Hayakawa *et al.* 1994). It has an inhibitory effect against microvascular endothelial cell proliferation during angiogenesis (Murphy AN *et al.* 1993).

TIMP-3 is a 21 kDa protein, 27 kDa when glycosylated, it is insoluble and binds to components of the ECM (Apte *et al.* 1994, Yang & Hawkes 1992). It has the ability to specifically interact with the C-domain of both proMMP-2 and proMMP-9 to form complexes (Butler *et al.* 1999). The inhibitory function is most effective against MMP-1, MMP-2, MMP-3, MMP-9 and MT1-MMP (Apte *et al.* 1995, Will *et al.* 1996). TIMP-3 is also able to inhibit tumor necrosis factor α (TNF-α) converting enzyme (TACE; ADAM-17; A disintegrin and metalloproteinase) and ADAM-10 (Amour *et al.* 1998, Amour *et al.* 2000). This suggests an important role for TIMP-3 in the modulation of inflammatory processes in which TNF-α is required.

The latest member of TIMP family is TIMP-4. It has a molecular weight of 22 kDa (Greene *et al.* 1996). It can form complex with proMMP-2 in a similar manner as TIMP-2, but at a slightly lower rate (Bigg *et al.* 1997). Its mRNA tissue expression seems to be very limited, and it is present in heart, placenta, kidney, colon and testis. It shows efficiency in preventing tumor invasion and metastases in breast cancer lines, especially by limiting angiogenesis (Greene *et al.* 1996, Wang *et al.* 1997).

In addition to TIMPs, serum α2-macroglobulin is a potent inhibitor of MMPs, but prefers MMP-1 (Sottrup-Jensen 1989). Chelating agents, such as dinatrium-diamin-tetra-acetic acid (EDTA) *in vitro*, act as inhibitors of MMPs by interacting with the zinc-binding site of MMPs (Birkedal-Hansen *et al.* 1993).

Recently a specific inhibitor for gelatinases was introduced (Koivunen *et al.* 1999). It is a synthetic cyclic peptide containing the sequence HWGF, which is a potent inhibitor of MMP-2 and MMP-9, but not of several other MMPs.

2.7 MMPs and TIMPs in biological processes

2.7.1 Tissue development

MMPs and TIMPs are important modulators of matrix metabolism and they have been shown to have role in processes with increased ECM turnover, such as wound healing, bone remodeling, acute and chronic inflammation, placental development, embryogenesis, mammary gland and lung development.

In fresh wound MMP-1 and MMP-9 are strongly expressed in migrating keratinocytes, but MMP-2 shows a more stable expression pattern (Saarialho-Kere *et al.* 1994, Salo *et al.* 1994). MMP-13 is expressed by dermal fibroblasts in chronic ulcers of the skin (Vaalamo *et al.* 1997).

In bone remodeling osteoclasts participate in resorption of the surrounding matrix, and MMP-9 is expressed very strongly by these cells (Reponen *et al.* 1994). In developing bone, besides MMP-9 also MMP-1 is present in osteoclasts and in bone matrix synthesizing osteoblasts, but MMP-2 expression has been shown to remain weak (Bord *et al.* 1997). MMP-13 is exclusively synthesized by chondrocytes of hypertrophic cartilage and osteoblasts of newly forming bone, but not by osteoclasts (Johansson *et al.* 1997).

In developing placenta MMP-2, MMP-9, MT1-MMP and TIMPs-1-3 have been all shown to be expressed by cells of cytotrophoblastic columns. One part of these cells forms new villous structures and another part invades the uterine wall. Decidual stromal cells also express MMPs and TIMPs, most notably TIMP-3 (Autio-Harmainen *et al* 1992, Hurskainen *et al.* 1996). Intermediate trophoblasts residing in columns are capable of controlled cell invasion during early pregnancy. They express an abundance of MT1-MMP, the enzyme which is proposed to have a significant role in placentation and even more generally in cellular invasion (Hurskainen *et al.* 1998).

Fibrillins are one of the most important structural components of elastic fibers abundantly found in ECM and their progressive loss is proposed to be associated with some diseases, such as skin aging, pulmonary emphysema and atherosclerosis. Serine proteases were previously believed to be the main enzymes involved in fibrillins turnover, but recently the degradative role of MMPs among fibrillins was shown. MMP-2, MMP-9, MMP-12, MMP-13 and MT1-MMP were shown to degrade fibrillins efficiently, emphasizing their important role in that specific ECM turnover (Hindson *et al.* 1999).

2.7.2 Neovascularization

Neovascularization is important in many normal processes, such as organ development, tissue repair, wound healing and inflammation. It is especially essential for malignant growth, since ineffective blood supply acts as a highly growth-limiting factor (Weidner 1998).

Previous decades have underlined the role of serine proteases in endothelial invasion and degradation of debris from neovessels (Koolwijk *et al.* 1996). More recent studies

have shown that also MMPs and TIMPs have important roles in neovascularization. Recently MT1-MMP was demonstrated to have an independent role in acting as pericellular fibrinolysin and improving endothelial invasion through fibrin barries. Also MMP-3, MMP-7, MMP-9, MMP-11 and MMP-12 were highly expressed in tissues actively engaged in the angiogenic response, while the expression of MMP-2 was quite stable (Hiraoka *et al.* 1998). Also various inflammatory cytokines, such as TNF-α, interleukin-1α (IL-1α) and IL-1β and oxidized low density lipoproteins (Ox-LDL), but not native LDL can induce MT1-MMP mRNA expression by endothelial cells (Rajavashisth *et al.* 1999). This may indicate an important role for MT1-MMP also in atherosclerosis by generating focal intimal lesions.

Endothelial cell culture studies have shown that MMP activity is essential for endothelial proliferation, and that inhibition of MMP activity by TIMPs or MMP blocking chemicals leads to reduction in endothelial cell invasion and proliferation, inducing their maturation as characterized by an increased deposition of BM structures around them (Kräling *et al.* 1999). Thrombin, which is present in vessels and has an important role in blood coagulation, has also been shown to activate MMP-2 by endothelial cells rapidly and efficiently (Nguyen *et al.* 1999). Also activated protein A, an anticoagulating agent occurring in blood, has been shown to activate proMMP-2 by endothelial cells independently without the function of MT1-MMP (Nguyen *et al.* 2000).

Angiostatin is an endogenous biologically active cleavage product of plasminogen, which has been shown to inhibit endothelial cell proliferation (O´Reilly *et al.* 1994). In experiments exogenous angiostatin has been shown to inhibit tumor vasculature and metastatic tumor cell growth. Interestingly, many MMPs, such as MMP-3, MMP-7, MMP-9 and MMP-12, have been shown to generate angiostatin from plasminogen (Cornellus *et al.* 1998, Lijnen *et al.* 1998, Patterson & Sang 1997). This suggest that although MMPs act as tumor promoting enzymes they also have the ability to generate agents against endothelial proliferation. Also BM macromolecules themselves can contain domains which selectively inhibit angiogenesis in malignant tumors (Maeshima *et al.* 2000).

2.7.3 Apoptosis and cell cycle regulation

Apoptosis is a term for programmed cell death, which differs clearly from necrosis by being an active process. It is characterized by tightly controlled steps, leading to dissociation and destruction of nuclei and cell bodies (for review, see Soini *et al.* 1998).

Recently p53, a tumor suppressor protein involved in cell cycle control and DNA repair was demontrated to down-regulate the human MMP-1 gene. However, p53 mutations, which are commonly present in various human tumors as well as in some somatic diseases have been shown to repress this effect and lead to increased MMP-1 activity in these conditions (Sun *et al.* 1999). MMP-9 knock-out mice showed in addition to impaired growth also an abnormally low amount of apoptosis in hypertrophic condrocytes. This effect may be due to the direct functional role of MMP-9 on the cells, or it may be coupled with delayed ossification of hypertrophic cartilage (Vu *et al.* 1998).

New links have also been shown to exist between TIMPs and cell growth and survival. In a study by Baker et al. (1998) overexpression of all TIMPs inhibited cell invasion similarly through reconstituted BMs. However, TIMP-3, but not synthetic MMP inhibitors, had unique capacity for inducing DNA synthesis, but also apoptotic cell death in smooth muscle cells. The role of TIMP-3 in the induction of apoptosis was observed also in invasive melanoma cells (Ahonen *et al.* 1998), HT1080 fibrosarcoma cells and HeLa cervical carcinoma cells (Baker *et al.* 1999). Recently, Baker et al. (1999) also demonstrated a significant elevation in the number of cells in S phase in response to the presence of TIMP-3 and showed further that the cell death was initiated extracellularly. Apoptosis initiated in the ECM space is therefore conveyed by TIMP-3, which is known to be bound to components of ECM (Yang & Hawkes 1992). In another study, opposite to the inducing effect of TIMP-3 on apoptosis, murine melanoma cells transfected by TIMP-2 cDNA and grown subcutaneously were found to be more resistant to apoptosis than parenteral cells, though necrosis was increased. They also found a correlation between TIMP-2 expression in tumor tissue and the amount of necrosis as seen in light microscope (Valente *et al.* 1998). Similarly, also TIMP-1 has been shown to protect human breast carcinoma cells against apoptosis, and bcl-2 overexpression also induces TIMP-1 expression in carcinoma cells (Li *et al.* 1999). These studies show that TIMPs may also have some other function besides inhibition of MMPs, but can also affect cell cycle regulation.

2.8 MMPs and TIMPs in tumor progression

2.8.1 Binding of MMPs and TIMPs to tumor cell membranes

Binding of MMPs to plasma membranes of malignant cells is thought to have a significant role in degradation of ECM and cancer cell invasion due to the fact that proteolytically active molecule operates directly on the invasive site.

MMP-2 binding to cell membranes via MT-MMPs and TIMP-2 has received a lot of attention. Additionally, it has also been shown that the active MMP-2 can be bound through C-terminus to cell membrane of malignant melanoma cells by interaction with integrin $\alpha v \beta 3$ (Brooks *et al.* 1996). This provides another important binding system for MMP-2 in addition to MT-MMPs for being bound to cell membranes. A specific binding system to cell membranes has also been demonstrated for MMP-9. This binding is mediated through CD44, which is a receptor for hyaluronate in ECM space (Yu & Stamenkovic 1999). Moreover, MMPs have also been shown to cleave this receptor type from cancer cell membranes and this cleavage is inhibited by TIMP-1, but not by serine protease inhibitors. Inhibition of CD44 receptor by TIMP-1 was demonstrated to suppress lung carcinoma cell migration on a hyaluronate substrate (Okamoto *et al.* 1999). In another study MMP-9 was present in cell membrane vesicles of breast cancer cells in association with β_1 integrins and HLA I antigens. The authors also showed that the active MMP-9 as well as proMMP-9 specifically accumulate to the membrane vesicles, and the

presence of HLA I molecules suggests a mechanism for tumor cells to escape immune surveillance (Dolo *et al.* 1998).

It has also been demonstrated that TIMP-1 can be bound to cell membranes in breast carcinoma cells, but not in normal epithelial cells of breast. After incubations, however, TIMP-1 was shown to be localized to the cell nucleus. This indicates so far unknown function for TIMP-1 in cell nucleus of malignant cells (Ritter *et al.* 1999).

2.8.2 *Experimental evidence of the role of MMPs in tumor cell invasion*

An original observation by Liotta *et al.* (1980) demonstrated the importance of MMP-2 for malignant cell invasion. They showed that the invasion of melanoma cells through reconstituted ECM is dependent on the level of MMP-2. Gelatinases have been under intensive investigation since then. Tissue activation of MMPs has proved to be most important, because only the active form of MMP is functioning. In bladder carcinoma it has been shown by quantitative zymography that MMP-2 and MMP-9 activation is significantly increased in tumor samples when compared to normal bladder mucosa, and moreover, this activation is significantly higher in tumors invading the muscular layer than in more superficial ones (Davies *et al.* 1993).

Gene transfection techniques have been a useful tool for studing the role of MMPs and TIMPs in cell invasion and tumor formation in animals. Transfection of malignant cells with MT1-MMP cDNA showed an increased MMP-2 activation by tumor cells and also correlated with increased tumor cell invasion which was inhibited by exogenous TIMP-2 (Deryugina *et al.* 1997). Recently it was shown that normal breast epithelial cells transfected with MMP-3 cDNA formed invasive mesenchymal-like tumors, which, once initiated, became independent of continued MMP-3 expression. Tumorigenicity was blocked by TIMP-1 (Sternlicht *et al.* 1999). Also transfection of mice normal breast epithelium with MMP-7 to generate high and stable concentrations of matrilysin was shown to enhance tumor formation (Rudolph-Owen *et al.* 1998). Overexpression of TIMP-2 by transfection of tumorigenic rat embryo cell line also exhibited reduced MMP activity detected by zymography, suppressed tumor growth *in vivo* and a lower lung metastasis rate (DeClerk *et al.* 1992).

The importance of the stromal cells for the production of MMPs is also well known, and synthesis and activation of many MMPs is increased in normal and neoplastic cells when grown in collagen or laminin containing matrix (Ravanti *et al.* 1999, Weeks *et al.* 1998, Basset *et al.* 1997, Riikonen *et al.* 1995). In an experiment where tumor cells that were unable to produce MMPs were transfected with TIMP-2 cDNA and grown in nude mice were able to abolish the tumor-promoting effect of fibroblasts, whereas untransfected tumor cells produced solid tumors (Noel *et al.* 1998). It has become clear that surrounding tumor stroma tissue is a dynamic meshwork which takes actively part in tumor growth.

To date there are numerous synthetic MMP inhibitors and their potential inhibitory role in tumor growth and angiogenesis have been studied. Batimastat® is one of the best known inhibitors. Its suppressive effect was demonstated to prevent tumor growth in rat peritoneum and also to prolong survival of the rats in that study (Aparicio *et al.* 1999).

However, it was not suitable for human cancer treatment in preliminary trials. At the moment the most interesting inbitor is Marimastat®, which is in clinical trials and is well tolerated (Primrose *et al.* 1999). There are also many other synthetic inhibitors which have given the same kind of result by reducing tumor growth and intratumoral angiogenesis (Price *et al.* 1999). (For review, see Kähäri & Saarialho-Kere 1999).

Interestingly, the commonly used non-steroidal anti-inflammatory drug aspirin was shown to inhibit tumor cell invasion in an *in vitro* and *in vivo* mice tumor model. The activity of aspirin against tumor invasion was related to the inhibition of MMP-9 expression, but levels of MMP-2 remained unaffected. Could aspirin, a cheap drug commonly used for preventing vascular thrombosis in the elderly also prevent cancer?

2.8.3 Expressions of MMPs and TIMPs in tumors on light microscopic level

In addition to the immunohistochemical technique, the *in situ* hybridization technique, has essentially expanded our knowledge of the cell types responsible for the synthesis of matrix proteins and their degrading enzymes. In many studies stromal cells have been seen to be the most important producers of mRNAs for most MMPs. However, since corresponding antigens have been immunolocalized also to neoplastic epithelial cells, it suggests that a tumor-host response and receptor-like mediated mechanisms are involved in MMP expression.

Expression patterns of MMP-2 and MMP-9 in malignant tumors are perhaps the best documented among all MMPs. MMP-2 mRNA expression has been reported to occur mainly by stromal fibroblasts and endothelial cells e.g. in tumors of ovary, lung, skin, breast and colon (Autio-Harmainen *et al.* 1993, Soini *et al.* 1993, Poulsom *et al.* 1993, Pyke *et al.* 1993). *In vitro* co-culture studies have shown that the presence of fibroblasts increases MMP-2 content and tumor cell invasion through the reconstituted ECM, indicating their important contribution to MMP-2 production (Boyd *et al.* 1999, Westerlund *et al.* 1997). However, besides fibroblasts immunolocalization of MMP-2 antigen has been detected abundantly also in epithelial tumor cells (Autio-Harmainen *et al.* 1993, Soini *et al.* 1993, Nakada *et al.* 1999, Murray *et al.* 1998). Discrepancy between the results of MMP-2 in *in situ* hybridization and in immunohistochemistry has been confusing until MT1-MMP was found (Sato *et al.* 1994) and its role in binding MMP-2 to cell membranes through a complex formed by MT1-MMP and TIMP-2 molecules was characterized (Strongin *et al.* 1995), although alternative binding systems do exist (Brooks *et al.* 1996). MMP-9 mRNA expression has been reported both in epithelial and stromal components. In pancreatic adenocarcinoma it is expressed by neoplastic epithelial cells and stromal fibroblasts and also by inflammatory cells such as neutrophils and macrophages (Gress *et al.* 1995). In colon carcinoma MMP-9 mRNA is expressed mainly by stromal fibroblasts and endothelial cells (Pyke *et al.* 1993). Contrary to findings of MMP-2, in epithelial malignancies mRNA and protein expression of MMP-9 is localized to the same cells, both stromal and epithelial tumor cells (Murray *et al.* 1998, Lampert *et al.* 1998).

The expression patterns of MT1-MMP has been under a lot of interest due to its activation role of MMP-2 on the cell membranes (Sato *et al.* 1994). Depending on tumor type, MT1-MMP is mainly expressed by neoplastic epithelial or stromal cells. Tumors of ovary (Afzal *et al.* 1998), colon, breast, head and neck (Okada A *et al.* 1995) express MT1-MMP mRNA mainly by stromal fibroblasts and endothelial cells in *in situ* hybridization. On the other hand, similarly to immunohistochemical staining for MMP-2 in epithelial malignancies the MT1-MMP antigen has also been localized predominantly to the malignant cell population and to a lesser extent to the stromal cells in immunohistochemical studies (Ueno *et al.* 1997, Nomura *et al.* 1995, Nakada *et al.* 1999).

TIMP-1 mRNA expression has been reported in the tumor stroma in adenocarcinoma of colon and breast (Zeng *et al.* 1995, Heppner *et al.* 1996), while in pancreatic adenocarcinoma and ovarian cancers mRNA synthesis has been reported to be present both in the stromal and neoplastic epithelial cells (Gress *et al.* 1995, Naylor *et al.* 1994). Corresponding TIMP-1 mRNA and antigen expressions have been reported both in epithelial tumor cells and stromal cells in adenocarcinomas of pancreas and stomach (Gress *et al.* 1995, Nomura 1995). TIMP-2 mRNA expression has been mainly found in desmoplastic stromal fibroblasts in carcinomas of the breast and ovary (Poulsom *et al.* 1993, Naylor *et al.* 1994). Immunohistochemical stainings have localized TIMP-2 both to neoplastic epithelial cells and stromal fibroblasts in adenocarcinomas of stomach and colon (Murray *et al.* 1998, Ring *et al.* 1997). TIMP-3 mRNA expression in malignant tumors is not well documented, but it has been shown to be expressed by the stromal cells of colorectal adenocarcinomas, breast carcinomas and squamous cell carcinomas (SQCCs) of skin (Powe *et al.* 1997, Byrne *et al.* 1995, Airola *et al.* 1998), but by carcinoma cells of basal cell carcinoma of skin (Airola *et al.* 1998).

2.9 Incidence in Finland of the main tumor types of the thesis

About 350 females and 300 males per year get malignant pancreatic tumor in Finland (Pukkila *et al.* 1997). Most of the pancreatic carcinomas are adenocarcinomas which are usually located in the head of the organ. Main clinical symptom is caused from obstruction of the main hepatic duct. In some cases diagnostic problems can arise in differentiating chronic pancreatitis from well differentiated adenocarcinomas, which both are histologically characterized by a marked desmoplastic stromal reaction (Gibson 1978). 5 year survival is very poor being less than 5%.

In Finland the annual incidence of malignant lung tumor is 1500 among males and 400 among females and it is the second commonest tumor type among males after prostatic adenocarcinoma (Pukkila *et al.* 1997). It is well documented, that smoking is directly associated to this tumor type. On an average one half of the tumors are SQCCs. Usually they are located hilarly and arise from bronchial epithelium and are associated to squamous metaplasia and different grades of epithelial dysplasia. Adenocarcinoma of lung is usually peripherally localized and the tumor arises either from glandular structures of bronchial epithelium or of alveolar epithelial structures, in which cases the tumor may show multifocal and diffuse growth pattern and are called for bronchioloalveolar lung

carcinomas. Small cell carcinoma, also known as oat cell carcinoma, is a tumor type which usually arises from hilar bronchus. The histology is typical; highly cellular tumour composed of small cells with hyperchromatic nuclei. It has many distinct features including ability to secrete neuroendocrinologically active peptides. Large cell undifferentiated carcinoma is composed of cells of varying carcinoma types. The cells are close together and a diameter of cell varies. (Travis *et al.* 1999). Among lung carcinomas common 5 year prognosis is 30-40%, though the prognosis of small cell carcinoma is somewhat less favourable.

Incidence of liver cancer is about 200 cases per year, equally among females and males (Pukkila *et al.* 1997). Hepatocellular carcinoma (HCC) of liver is quite uncommon in Western countries, but is much more common in parts of Far-East and Africa due to high prevalence of viral hepatitis, cirrhosis and exposure to aflatoxins. Tumors usually arise in a liver that is the seat of advanced cirrhosis where tumors are often seen to grow as cancer nodules. (Ishak *et al.* 1994). 5 year prognosis is about 25%.

Annual indicence for malignant tumors of uterus is about 600 cases (Pukkila *et al.* 1997). Most of the cases are endometrial adenocarcinomas. The main clinical symptom for malignant tumor of uterus is postmenopausal bleeding. Histological differentiation between grade I adenocarcinomas and endometrial hyperplasias can sometimes cause diagnostic problems. (Scully *et al.* 1994). 5 year survival is about 50%.

3 Aims of the present study

Synthesis and degradation of ECM macromolecules is critical to the maintenance of tissue architecture. BMs form a highly specialized structural and functional barrier which separates different tissue structures from each other. So far their role in malignancies has been controversial, and they are believed to prevent tumor cell invasion, but on the other hand to serve as an important adhesion matrix for malignant cells. In tumor cell invasion MMPs have been shown to play an important role in controlling the ECM degradation.

To study the synthesis and degradation of BM macromolecules the expression of different laminin chains, type IV collagen, MMP-2, MMP-9, MT1-MMP and TIMP-1-3 were investigated in a series of normal tissues and malignancies. The detailed aims of the present study were:

1. to show the mRNA synthesis and antigen expression for laminin-5 constituent laminin γ2 chain in pancreatic adenocarcinoma and lung carcinomas and their regional lymph node metastases by *in situ* hybridization and immunohistochemistry to clarify the role of γ2 chain in tumor progression. Statistical analysis was also performed to investigate the possible significance of the γ2 chain for prognosis in these conditions,
2. to systematically investigate the antigen expressions of individual laminin chains and corresponding mRNA synthesis for laminin α1, β1 and γ2 chains and α1(IV) collagen in a series of normal tissues and epithelial malignancies to analyze their synthesis levels and cell types which are responsible of their production,
3. to investigate mRNA synthesis and corresponding antigen expressions for MMP-2, MMP-9, MT1-MMP and TIMPs-1-3 in HCCs and pancreatic and endometrial adenocarcinomas to study their role in these tumors,
4. to analyze the gelatinolytic activity of MMP-2 and MMP-9 in HCCs and endometrial adenocarcinomas and to compare that in the surrounding normal tissues.

4 Materials and methods

4.1 Materials

The tissue material was either retrospectively or prospectively collected from the files of the Department of Pathology, Oulu University Central Hospital. Retrospective material had been fixed in 10 % buffered formalin and embedded in paraffin. Samples of prospectively collected material were also treated in liquid nitrogen for cryosections. The clinical data were collected from patients´ files and included the patients´ sex, age and tumor grade as well as the size and clinical stage or TNM classification.

42 surgically resected pancreatic adenocarcinomas (I) including 15 grade I, 16 grade II and 11 grade III tumors were collected for *in situ* hybridization and immunohistochemical stainings to analyze the laminin γ2 chain expression. The diagnoses and gradings of all cases were based on the WHO classification of pancreatic tumors (Gibson 1978).

Altogether 64 primary lung tumors and 12 of their corresponding regional lymph node metastases (II) were collected for the laminin γ2 chain *in situ* hybridization analysis and immunohistochemical stainings. The material comprised 21 SQCCs (5 grade I, 7 grade II and 9 grade III), 19 adenocarcinomas (5 grade I, 4 grade II and 7 grade III) and 3 bronchiolo-alveolar carcinomas (BACs), 5 large cell carcinomas, 19 small cell carcinomas, and 12 regional lymph node metastases, including 6 SQCCs and 6 adenocarcinomas. There was also additional frozen material used for immunohistochemical stainings, comprising 6 SQCCs and 5 adenocarcinomas, which had been immediately frozen in -70°C liquid nitrogen. The diagnoses and gradings were based on the WHO classification of lung tumors (Travis *et al.* 1999).

47 malignant tumors were collected prospectively to analyze laminin α1, α3, α5, β1, β2, β3, γ1, γ2 and type IV collagen distribution by immunohistochemistry and laminin α1, β1, γ2 and α1(IV) collagen mRNA levels by *in situ* hybridization (III). The material included; 5 lung carcinomas (3 SQCC and 2 adenocarcinomas), 3 SQCC of tongue, 7 adenocarcinomas of stomach (3 intestinal type and 4 diffuse type), 6 colon adenocarcinomas, 6 ovarian adenocarcinomas (2 serous papillary cystadenocarcinomas, 2 mucinous cystadenocarcinomas and 2 endometrioid adenocarcinomas), 13 breast

carcinomas (10 ductal carcinomas comprising invasive and intraductal carcinomas and 3 lobular carcinomas) and 7 carcinomas of the thyroid gland (4 follicular and 3 papillary carcinomas). As a control for laminin α1 chain *in situ* hybridization experiments samples of five early human placentas were obtained from the files of the Department of Pathology.

36 HCCs (13 grade I, 13 grade II and 10 grade III) and 35 pancreatic adenocarcinomas (13 grade I, 15 grade II and 7 grade III) were collected for MMP-2, MMP-9 and MT1-MMP *in situ* hybridization and immunohistochemical stainings (IV). HCC and pancreatic adenocarcinoma diagnoses and gradings were based on the WHO classification (Ishak *et al.* 1994, Gibson 1978).

Altogether 38 endometrial samples were collected representing different conditions; 14 endometrial samples of normal cyclic phases and atrophy, 10 hyperplasias comprising simplex, complex and atypical complex hyperplasias and 14 adenocarcinomas (4 representing grade I, 5 grade II and 5 grade III) for MT1-MMP and TIMPs-1-3 *in situ* hybridization analysis (V). Hyperplasias and tumors were graded according to the WHO classification (Scully *et al.* 1994). All the adenocarcinomas studied were of the endometrioid type.

4.2 mRNA probes for *in situ* hybridization (I-V)

Table 5. Probes used in in situ hybridization.

Probe	Size (bp)	Source	Restriction sites
Ln α1	348	Nissinen *et al.* 1991	SalI-NotI 3100-3448
Ln β1	906	Pikkarainen *et al.* 1987	PstI 920-1826
Ln γ2	845	Kallunki *et al.* 1992	PstI-EcoRI 2995-3840
α1(IV)collagen	916	Pihlajaniemi *et al.* 1985	BamHI-HindIII 528-1444
MMP-2	635	Huhtala *et al.* 1990	ScaII-SacI 578-1213
MMP-9	574	Huhtala *et al.* 1991	HindIII-EcoRI 1751-2325
MT1-MMP	420	Sato *et al.* 1994	* 218-638
TIMP-1	626	Carmichael *et al.* 1986	* 62-688
TIMP-2	388	Stetler-Stevenson *et al.* 1992	EcoRI-KpnI 710-1098
TIMP-3	500	Apte *et al.* 1994	EcoRI-PstI 31-531

bp = base pair, Ln = laminin, MMP = matrix metalloproteinase, TIMP = tissue inhibitors of metalloproteinases, * = amplified by PCR.

The pGEM-4Z (Promega, Madison, WI, USA) and BluescriptSK+ (Stratagene, La Jolla, Ca, USA) vectors were linearized with suitable restriction enzymes. For RNA probes labeling a riboprobe transcription kit and ^{35}S-UTP (800 Ci / mmol, Pharmacia-Biotech, Buckinghamshire, England) were employed (Promega, Madison, WI, USA). All the probes were sequenced and subjected to Northern hybridization.

4.3 *In situ* hybridization method (I-V)

For *in situ* hybridization, paraffin was removed from the sections with xylene and the slides were dehydrated. Then the sections were treated with 0.2 M HCl for 20 min at room temperature (RT) and washed in diethylpyrocarbonate-treated water (depc-H_2O) for 5 min. For proteolysis the sections were incubated with proteinase K (1 mg/ml) (Roche Diagnostics, Indianapolis, USA) for 30 min at 37°C and the reaction was stopped with 0.2% glycine in phosphate buffered saline (PBS). After that the sections were washed twice in PBS for 30 sec. The sections were fixed with 4% paraformaldehyde in PBS for 20 min and washed in PBS. Acetylation was done in 0.25% to 0.5% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were washed once in PBS, dehydrated and air-dried for 1 to 2 hours at RT. The sections were then treated for 2 h with a prehybridization mixture containing 10 mM dithiothreitol (DTT) (Sigma, St. Louis, Mo, USA), 10 mM Tris-HCl, 10 mM $NaPO_4$, 5 mM EDTA, 0.3 M NaCl, 1 mg/ml yeast tRNA, deionized formamide 50% and dextran sulphate 10% (w/v); 0.02% (w/v) Ficoll (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.02% (w/v) polyvinylpyrrolidone and 0.02 mg/ml bovine serum albumin (BSA) and washed once in PBS and dehydrated. In the hybridization step probes were first denatured by boiling for 1 min and placed on ice. 3 x 10^6 cpm of the ^{35}S-labeled antisense or sense probe in 40 µl prehybridization buffer was applied on each section and the hybridization was carried out at +50°C overnight. The posthybridization washes were performed as follows: twice at +50°C for 1 h in prehybridization mixture except for dextran sulphate and tRNA, 15 min in 0.5 M NaCl in 10 mM Tris-HCl, 1 mM EDTA (TE) at +37°C, 30 min incubation in 0.5 M NaCl in TE containing 40 µg/ml RNAase A (Sigma) at +37°C, washed in 15 min 0.5 M NaCl in TE at +37°C, twice for 15 min in 2 x standard saline citrate (SSC) and twice for 15 min in 1 x SSC, both in 50°C. The sections were dehydrated in ethanol containing 300 mM ammonium acetate and air-dried at RT for 1 h. In autoradiography the slides were dipped into NTB-2 film emulsion (Kodak, New York, USA) and were then placed in light-tight boxes for 10-14 days. The slides were developed in GBX developer (Kodak) fixed in Agefix (Kodak) and counterstained in hematoxylin and eosin. All the solutions were treated with 0.1 % depc solution (Fluka, Buch, Switzerland), and corresponding sense probes were always used as negative controls.

4.4 Antibodies for immunohistochemical stainings (I-IV)

Table 6. Antibodies for immunohistochemical stainings.

Antibody	Clone	Source
Ln α1	161 EB7 / M	Virtanen *et al.* 2000
Ln α3	BM-2 / M	Rousselle *et al.* 1991
Ln α5	4C7 / M	Engvall *et al.* 1986
Ln β1	114 DG10 / M	Virtanen *et al.* 1997
Ln β2	C4 / M	Hunter *et al.* 1989
Ln β3	6F12 / M	Marinkovich *et al.* 1992
Ln γ1	113 BC7 / M	present study (III)
Ln γ2	P	Pyke *et al.* 1995
IV collagen	P	Risteli *et al.* 1980
MMP-2	CA-4001 / M	Höyhtyä *et al.* 1994
MMP-9	GE-213 / M	Nikkari *et al.* 1996
MT1-MMP	M	Sato *et al.* 1994

Ln = laminin, M = monoclonal antibody, MMP = matrix metalloproteinase, P = polyclonal antibody.

4.5 Production and characterization of Mab BC7 against laminin γ1 (III)

A new Mab against laminin γ1 chain was raised by Dr. Ismo Virtanen, University of Helsinki, Helsinki, Finland, and is characterized here. The new Mab (113BC7) was raised using a pepsin digest of human placental laminin (Wewer *et al.* 1983). BalB/c mice were immunized with ca. 50 µg of the antigen in Freund´s complete adjuvant, followed by two immunizations with Freund´s incomplete adjuvant. The spleens of the immunized mice were then collected and suspended cells were fused with X63-Ag8-653 myeloma cells by standard techniques (Köhler & Milstein 1976).

In order to show the specificity of the new Mab, hybridomas were screened by immunoprecipitation, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoreactivity on selected human tissues, and positive hybridomas were cloned manually under the microscope. JAR human choriocarcinoma cells (American Type Culture Collection, Manassas, VA, USA) were then radioactively labeled with S^{35}-methionine (Amersham Pharmacia Biotech, Uppsala, Sweden) and the labeled culture medium was subjected to reduction and alkylation to dissociate the laminin-1 and laminin-10 trimers secreted by this cell line (Tani *et al.* 1999). The supernatant was then subjected to immunoprecipitation using the hybridoma supernatant. For this purpose GammaBind®-Sepharose® (Amersham Pharmacia Biotech) was pretreated with the

hybridoma supernatant by standard techniques. The bands were then mixed with the culture medium and after the washes the binding was detected by SDS-PAGE (Laemmli 1970) using 5% gels under non-reducing conditions. After electrophoresis the gels were subjected to fluorography. As seen in figure 8, MAb 113BC7 when reduced and alkylated precipitated only with the M_r 200 000 laminin γ1 chain, see figure 8. For further domain specificity, see Geberhiwot et $al.$ (2000).
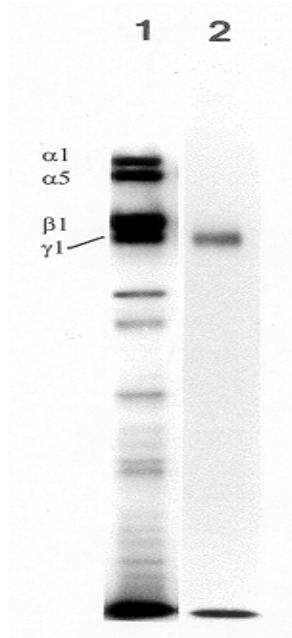
Fig. 8. Monoclonal antibody (113BC7) for laminin γ1 chain. The MAb 113BC7 precipitated two high M_r α chains (α1 and α5, lane 1) as well as the M_r 220 000 and M_r 200 000 β1 and γ1 chains, respectively. On the other hand, when the reduced, alkylated culture supernatant was precipitated with Mab 113BC7 under non-reducing conditions only the M_r 200 000 laminin γ1 chain was seen (lane 2).

4.6 Immunohistochemistry (I-IV)

For immunohistochemical staining 4 μm thick paraffin sections were deparaffinized and treated with 0.4% pepsin and incubated in purified water and hydrogen peroxide solution (9:1) for 15 min and rinsed with H_2O and PBS. 5 μm frozen sections were first air-dried for 15 min, followed by fixation in cold acetone for 10 min. After this the paraffin and frozen sections were incubated with fetal calf serum (Finnzymes, Espoo, Finland) (1:5 in PBS) for 20 min to block nonspecific binding of IgGs. The sections were then incubated with the primary antibody in +4 °C for 2-10 hours, followed by biotinylated anti-mouse or anti-rabbit IgG secondary antibody (Dako, A/S, Glostrup, Denmark) for 30-60 min,

and avidin-peroxidase complex (Dako, Denmark) for 30-60 min. The color was developed by diaminobenzidine tetrahydrocloride-H_2O_2 (DAB) (Sigma, Steinheim, Germany) in Tris buffer, pH 7.4. Finally, the sections were lightly counterstained by hematoxylin and eosin.

As negative controls the sections were incubated with PBS instead of the primary antibody. There was also a series of tissue material containing colon carcinomas and samples from early human placenta which were used for simultaneous immunohistochemistry using all the antibodies used in this study and mouse or rabbit non-immune serum (Zymed, San Fancisco, Ca, USA) as negative control. Sections incubated with non-immune serum did not show any specific reaction.
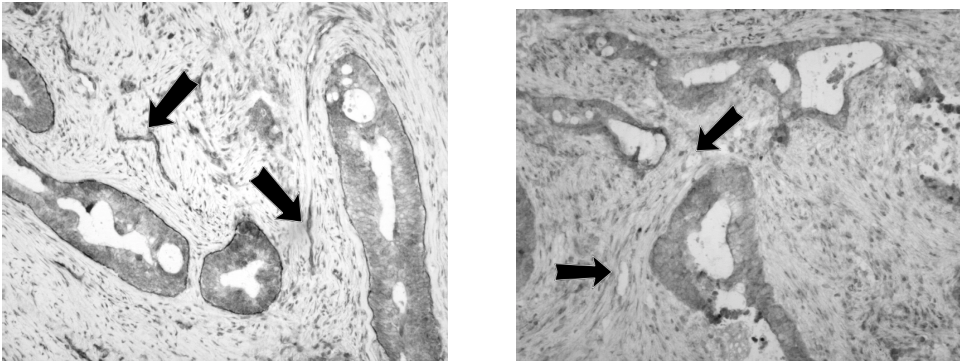
Fig. 9. Colon carcinoma stained with laminin α5 chain antibody (left figure) and the same sample incubated with mouse non-immune serum instead of primary antibody (right figure). Laminin α5 stains BMs around tumor clusters and endothelium (arrows), but the reaction is lacking in section incubated with non-immune serum.

4.7 Indirect immunofluorescence (III)

Indirect immunofluorescence stainings were performed by using Mab for laminin β2 chain. Unfixed frozen tumor samples of 4μm were first washed twice in PBS for 15 min and then incubated with fetal calf serum (Finnzymes) for 20 min. Primary antibody was applied for overnight, followed by incubation with FITC secondary conjugate (Molecular Probes, Oreganon, USA) for 30 min, and finally the sections were washed in PBS for 30 min. In the control stainings, PBS was used instead of the primary antibody. An Olympus microscope, model BHT, together with a fluorescence epi-illuminator, was used for analysis and photography of the stainings. The microscope was equipped with a mercury lamp (HBO 100W), a dichroic mirror B (DM-500) with a built-in barrier filter (O-515), and an excitation filter (EY 455) for fluorescein isothiocyanate fluorescence.

4.8 Gelatin zymography (IV, V)

In order to assess the MMP-2 and MMP-9 activity in hepatocellular carcinoma and endomerial adenocarcinoma tissues and adjacent normal liver and endometrium, gelatin zymography modified from Heussen & Dowdle (1980) was performed. Tissue samples of tumor or non-tumor tissues were obtained and homogenized. 30 µl aliquots of both tumor and non-tumor samples were dissolved in the SDS sample buffer in the absence of reducing agents without boiling. The samples were then run in SDS-PAGE in gels which also contained copolymerized gelatin (1.5 mg/ml). After electrophoresis, the gels were washed twice for 15 minutes in TC-buffer containing 5 mM calcium chloride, 1 µM zinc cloride, 50 mM-Tris-HCl, pH 7.6, supplemented with 2.5 % Triton X-100. The gels were then incubated in a buffer containing 1 mM p-aminophenylmercuric acetate (APMA) or treated directly with Triton X-100 for 3 hours at +37°C in order to activate the latent enzyme. Finally, the gel was incubated for 16 hours in the TC-buffer containing 0.2 M NaCl, 0.02 % sodium trinitrate and 10 mM calcium dichloride at 37°C for 16 hours. The enzyme activity was visualized by staining the gels with a solution containing 50 % methanol, 10 % acetic acid and 0.1 % Coomassie Brilliant Blue and destaining in 10 % methanol and 10 % acetic acid.

4.9 Western blot analysis (II)

Frozen tumor material was homogenized in the sample buffer and run on 7.5 % SDS-polyacryamide gel, and transferred onto nitrocellulose filter (Schleicher & Schuell, Dassel, Germany). The filter was incubated with Tris buffered saline supplemented with 10% non-fat dry milk for 60 min. After washing, the filter was incubated with primary antibody of laminin γ2 chain (1:80) overnight at +4 °C. Then the filter was incubated with biotinylated secondary antibody (Dako) for 1 hour at RT and the avidin-biotinylated peroxidase complex (Dako) for 30 min. The reaction was visualized by DAB solution for 10 min.

4.10 Evaluation of the immunohistochemical and *in situ* hybridization results

The staining reactions in immunohistochemical and immunofluorescence stainings and in *in situ* hybridization were scored as follows; negative (-) when the reaction or labeling was indistinguishable from the background, weak (+) when the reaction or labeling was slightly increased or only a few cells were observed to express, moderate (++) when a clear reaction or labeling was observed in a moderate number of cells and strong (+++) when the reaction or labeling was abundant and observed in the majority of the cells. In one study (II) very strong (++++) was used when the reaction was very abundant.

4.11 Statistical analysis (I, II, IV)

Statistical analysis was performed by the Kaplan-Meyer method with log rank analysis. Clinical data were collected from the patients´clinical records and survival times were correlated with the expression of laminin γ2 chain (I, II) or MMP-2, MMP-9 and MT1-MMP mRNA (IV). The results from survival analysis were expressed by reporting *P*-values, and values ≤ 0.05 were referred to as significant. The significance of the other associations was determined using Fisher´s exact test (I, II).

5 Results

5.1 Laminin γ2 chain antigen and mRNA expressions in pancreatic adenocarcinomas and lung carcinomas

5.1.1 Pancreatic adenocarcinomas (I)

Altogether 42 pancreatic adenocarcinomas were analyzed. Common to all cases was that laminin γ2 chain synthesis and distribution were highly increased in the tumor area compared to adjacent normal pancreas tissue or chronic pancreatitis area.

In pancreatic adenocarcinomas 41/42 (98%) cases investigated expressed the laminin γ2 chain antigen. The intracellular staining pattern was mainly seen in tumor cells located at the periphery of the tumor islands at the epithelial-stromal interphase. Strong laminin γ2 chain positivity was seen especially in tumors forming more or less well-defined small glandular structures or in scattered individual infiltrating cells. On the other hand, tumors forming solid epithelial islands or large epithelial ducts showed less expression.

Diffuse stromal γ2 chain positivity was detected in 22/42 (52%) tumors. Laminin γ2 chain deposition in BMs of the neoplastic glands was seen in 7/42 (17%) tumors. In a few tumor samples, weak intracytoplasmic γ2 chain positivity could be seen in non-neoplastic epithelial cells in association with inflammation and glandular destruction.

Positive signals for the laminin γ2 chain mRNA were observed in 35/42 (85%) tumors. Transcripts for γ2 chain mRNA were detected only in tumor cells; stromal cells showed no detectable labeling. Particularly strong expression was seen in individual carcinoma cells near the stroma and small invasive tumor glands. In non-neoplastic epithelial cells weak signals could occasionally be seen in areas with inflammation and epithelial destruction.

5.1.2 Lung carcinomas (II)

Altogether 64 lung tumors were analyzed. Laminin γ2 chain synthesis was seen solely in neoplastic epithelium. The most intensive mRNA synthesis and antigen distribution was seen in SQCCs and adenocarcinomas, followed by large cell carcinomas. Small cell carcinomas showed only a low expression level. Immunohistochemical and *in situ* hybridization results are summarized in table I of original publication II.

In SQCCs of lung using paraffin embedded sections intracytoplasmic immunohistochemical laminin γ2 chain positivity was seen in all 21 cases and in 4/6 lymph node metastases. The positivity was mainly seen in cells at the epithelial-stromal interphase of the tumor islands. Particularly strong positivity was seen in individual scattered carcinoma cells infiltrating desmoplastic fibrous stroma. Stromal positivity was present in 4/21 tumors (19%) and positively stained BM structures were seen in 6/21 (29%) tumors. In 6 frozen tumor samples the intracellular immunoreaction was weak, but clearly defined BM structures were seen to surround tumor clusters in each case.

In the *in situ* hybridization 20/21 (95%) SQCCs and 4/6 metastases showed signals for laminin γ2 chain mRNA. The signals were usually observed in the tumor cells at the epithelial-stromal interphase of tumor islands. Stronger laminin γ2 chain mRNA expression was often seen in smaller tumor islands and in individual tumor cells infiltrating the stroma.

All 19 adenocarcinomas and 4/6 metastases showed intracytoplasmic positivity for the laminin γ2 chain. Positivity was mainly seen in tumor cells at the epithelial-stromal interphase, but it was striking that individual tumor cells infiltrating the fibrous stroma expressed laminin γ2 chain. A clear diffuse stromal immunoreactivity was seen in 8/19 (42%) primary tumors, of which two were BACs, and in one metastasis. Positively staining BM structures around neoplastic glands were seen in 9/19 (47%) tumors and in 2/6 metastases. In frozen sections weak intracytoplasmic positivity was seen in all 5 cases. Extracellular thick and constant BM structures were seen around tumor clusters in every case, while clear stromal positivity was detected only in one tumor.

In the *in situ* bybridization 13/19 (68%) primary adenocarcinomas and 4/6 metastases were positive for laminin γ2 chain mRNA. Signals were observed in tumor cells at the epithelial-stromal interphase. The strongest expression was seen in well defined, small infiltrative tumor glands, as well as in individual tumor cells in the tumour stroma. In BACs the tumor cells generally expressed weakly mRNA for laminin γ2 chain.

All 5 large cell carcinomas were laminin γ2 chain positive by immunohistochemistry. The staining reaction located intracytoplasmically and positive cells were diffusely scattered in tumor tissue without any notable tendency to occur at the epithelial-stromal interphase. Diffuse stromal reaction and short strips of BM structures were seen only in one tumor.

In the *in situ* hybridization 4/5 large cell carcinomas were positive for laminin γ2 chain mRNA. The signals were seen in diffusely scattered cells throughout the tumor tissue.

In small cell carcinomas weak intracytoplasmic staining for laminin γ2 chain was present in scattered individual tumor cells of 10/19 (53%) tumors. Positive cells tended to be localized at the epithelial-stromal interphase. Most clearly defined positivity appeared in tumor cells infiltrating rich fibrous stroma. 3/19 (16%) of tumors showed some ECM

positivity and BM staining was seen in 2/19 (11%) cases, though similar linear BM structures around tumor islands as were seen in SQCCs and adenocarcinomas were not present.

Only 7/19 (37%) small cell carcinomas were weakly positive for laminin γ2 chain mRNA. Signals were mainly seen in cells at the epithelial-stromal interphase and in corresponding immunohistochemistry, staining was usually stronger in cells infiltrating tight fibrous stroma.

5.1.3 Statistical analyses

Pancreatic adenocarcinomas of stages other than T1AN0M0 or T1BN0M0 (tumors infiltrating peripancreatic tissues or showing regional or distant metastases) displayed less than 20% of laminin γ2 chain immunohistochemically positive cells or less than 20% of cells with positive γ2 chain mRNA signals significantly more often than other tumors (P= 0.029 and 0.025, respectively, Fisher´s exact test). There were significantly more cases with less than 20% of cells immunohistochemically positive for the laminin γ2 chain in tumors with regional or peripheral metastases than in tumors with no detectable metastases at the time of operation (P= 0.043, Fisher´s exact test). A similar statistically significant association could not be found in the *in situ* hybridization experiments (P= 0.23, Fisher´s exact test). Tumors infiltrating peripancreatic tissues (T2) contained less than 20% of laminin γ2 chain positive tumor cells or less than 20% of tumor cells showing mRNA signals for the γ2 chain significantly more often than T1 tumors (P= 0.046 and 0.022, respectively, Fisher´s exact test). There was no significant association between tumor grade and immunohistochemical laminin γ2 chain expression (grades I-II/III, P= 0.27; grades I/II-III, P= 0.62, Fisher´s exact test). There was no statistically significant difference in survival between patients showing less than or more than 20% of laminin γ2 chain positive tumor cells (P= 0.77, log rank) or between patients with a tumor expressing positive labeling for the laminin γ2 chain mRNA in less than or more than 20% of the tumor cells (P= 0.63, log rank). The presence of laminin γ2 chain in stroma or in BMs did not affect survival (P= 0.70 and 0.71, respectively, log rank).

In lung tumors grade I-II SQCCs and adenocarcinomas showed more often moderate or strong (≥++) laminin γ2 chain immunoreactivity and mRNA expressions than grade III tumors (P= 0.046 and P= 0.05, respectively, Fisher´s exact test). There was no significant difference in the quantitative mRNA expression for laminin γ2 chain between primary (SQCCs and adenocarcinomas together) and metastatic tumors. Small cell carcinomas expressed significantly less laminin γ2 chain protein and mRNA (≤+) than non-small cell carcinomas (P= < 0.00001 and P= 0.00062, respectively, Fisher´s exact test). There was no significant association between the TNM stages of tumors and laminin γ2 chain expression, and neither was there any difference in survival between patients with tumors showing strong or weak laminin γ2 chain mRNA expression in the paraffin tumor material.

5.2 Immunohistochemical distribution of different laminin chains and type IV collagen in normal tissues and malignancies (III)

5.2.1 Normal tissues

Distribution of different laminin chains in normal tissues is summarized in table 3 and in malignancies in table 2 in the original publication III.

BMs of normal tissue structures were always surrounded by continuous BM layer of laminin chains and type IV collagen. Generally laminin chains showed a different expression pattern in different BMs. Among laminin chains α5, β1 and γ1 chains were the most widely spead, whereas α1 and β2 chains were the most restricted in epithelial BMs, but β2 was abundant in endothelial BMs, however. Laminin α1, α3, β3 and γ2 chains were generally the laminin chains of epithelial BMs, whereas laminin α5, β1, β2 and γl chains were also present in other BMs, such as vascular endothelium, neural structures and stromal myofibroblasts.

BMs of normal peribronchial glands, glands of tongue and breast expressed all laminin chains investigated and type IV collagen. This staining pattern was otherwise similar in glands of gastric mucosa, except for a negative result for the laminin α1 chain. An undulating staining pattern beneath myoepithelial cells of peripheral mammary ducts was sometimes seen. Normal follicular BMs of the thyroid gland expressed laminin chains α1, α5, β1, γ1 and type IV collagen, but lacked the chains of laminin-5 (α3β3γ2).

In vascular BMs laminin chains α5, β1, β2 and γ1 and type IV collagen were all abundantly present. Occasional expression was seen for the laminin α3 chain, but the staining reaction was either negative or unconvincing for α1, β3 and γ2 chains. BMs of smooth muscle cells in larger vessels, as well as striated muscle cells expressed abundantly laminin α5, β1, β2 and γ1 chains and type IV collagen. Fibroblasts in areas rich in stromal tissue contained in some areas intracytoplasmic β1 and γ1 chains and type IV collagen. Stromal myofibroblasts showed BM structures around them containing α5, β1 and γ1, and in some cases also β2 chains and type IV collagen.

In neural structures, perineural BMs and BM around Schwann cells expressed α5, β1 and γ1 chains of laminin and type IV collagen, but they lacked other chains of laminin.

5.2.2 Lung carcinomas

In SQCCs of lung all laminin chains α1, α3, α5, β1, β2, β3, γ1 and γ2 were moderately or strongly expressed in BMs around tumor islands, except for the β2 chain which was only weakly present. Laminin chains were present in almost all tumor clusters, except for the laminin α1 chain which was detected only in about 30% of the islands even after repeated stainings. The expression pattern for various laminins was in most cases linear, but there were discontinuities seen in some invasive tumor islands. There were no significant differences in the BM staining reaction between large tumor clusters and smaller invasive islands. The laminin β2 chain was generally weakly or moderatively

expressed in tumor epithelial BMs, showing only short strips of positivity. In some areas there was a diffuse staining reaction for laminin chains α3 and β3 in the stroma around tumor islands.

In adenocarcinoma of lung all laminin chains, except for α1, were present in tumor BMs. Mostly the staining reaction was fragmented and contained clear disruptions. The laminin γ2 chain was detected also intracellularly in tumor cells in close contact to BM and tumor stroma.

Type IV collagen was strongly present in all lung carcinomas and generally showed a linear staining pattern, but there were discontinuities in some areas. Smaller and larger tumor islands expressed type IV collagen similarly in their BMs. In some areas tumor BMs stained for type IV collagen were seen to be thicker than BMs in normal bronchi or in peribronchial glands.

5.2.3 Tongue carcinomas

In SQCCs of tongue all laminin chains, except for α1, were present in tumor BMs. In areas of obvious active invasion, the staining reactions were clearly weaker or totally absent, showing a disrupted pattern. However, larger tumor islands showed linear staining of their BMs. In one case in which the growth pattern was more diffuse compared with the other tongue carcinomas of the study, the BM staining reaction was clearly reduced, and it was generally lacking in the invasive areas. These areas were also characterized by intracytoplasmic staining of carcinoma cells for laminin chains α5, α3, β1 and γ2. In paraffin embedded sections intracytoplasmic staining reaction was slightly more pronounced, and in invasive areas diffusely scattered tumor cells exclusively expressed strong intracytoplasmic laminin γ2 chain.

Type IV collagen stainings showed a comparable result with that seen in laminin stainings; smaller invasive tumor islands expressed poorly or totally lacked type IV collagen, though larger clusters were surrounded by quite linear and well formed BMs.

5.2.4 Gastric carcinomas

Intestinal type carcinomas totally lacked the laminin α1 chain, while other laminin chains were seen to be present in tumor BMs. The most intense expression was observed for α5 and β1 chains, which showed a rather continuous staining pattern. Other chains were more limited in their expression, but when present showed quite linear staining of tumor BMs. The laminin α3 chain differed from the other chains by showing a more fragmented staining pattern.

Type IV collagen was present in about 80% of tumor glands. Especially in areas showing active invasion through muscularis mucosa tumor clusters were characterized by highly discontinuous or totally lacking immunoreactivity for type IV collagen.

Among diffuse type gastric carcinomas there was one case in which there was no extracellular BM material for either laminins or type IV collagen. In this case there was, however, intracellular positivity for laminin α1, α3, β2 and γ2 chains in some diffusely

scattered individual carcinoma cells. Other diffuse carcinoma samples had very weak and fragmented, but still visible BM outlines containing all laminin chains, except for laminin α1, in tumor areas forming trabecules and small clusters within the mucosal part of the tumor. However, more diffuse infiltrative parts did not contain BM material.

5.2.5 Colon carcinomas

Laminin α1 chain was generally not detected in tumor BMs, but was present in one tumor sample. Also laminin β2 was present only occasionally in the tumors examined. Laminin chains other than α1 and β2 were moderately or strongly present in BMs of colon carcinomas. Of these laminin chains α5, β1 and γl were seen to be linearly expressed around tumor clusters, whereas   laminin chains α3, β3 and γ2 had a more disrupted staining pattern showing fragmentations especially in invasive areas. Laminin γ2 chain showed intracytoplasmic positivity in tumor cells close to tumor stroma in each sample. Type IV collagen was weakly or moderately expressed by tumor BMs which variably showed disruptions and fragmentations.

5.2.6 Ovarian carcinomas

All laminin chains were present in ovarian carcinomas of this study. In the serous papillary cystadenocarcinomas laminin chains α1, α3, β2 and γ2 were present in about 50% of neoplastic structures, while other chains were seen in BMs of over 80% of them. Only laminin α5 chain antibody stained the tumor BMs quite linearly, whereas other laminin chains had focal disruptions in their staining pattern. Laminin γ2 chain was also present intracytoplasmically in tumor cells opposing the tumor stroma.

In mucinous cystadenocarcinomas all laminins were also present in tumor BMs. In this tumor type laminin α1 was seen only in about 30% of neoplastic glands and the other laminin chains in 50-70% of glands. The most continuous staining pattern was seen for laminin α5 chain, though all chains, to a variable degree, showed disruptions in their BM stainings, and there were areas of active invasion in which only small fragments of BM positivity could be seen.

In endometrioid adenocarcinomas of ovary the results were comparable to those seen in mucinous cystadenocarcinomas, but one of the tumors lacked laminin α1 chain staining in the tumor BMs.

In ovarian tumors the presence of type IV collagen was seen in about 50% of neoplastic glands. BM structures were more or less attenuated thin membranes in all three carcinoma types.

5.2.7 Breast carcinomas

Carcinoma *in situ* of breast strongly and linearly expressed all laminin chains and type IV collagen. Invasive ductal and lobular carcinomas generally lacked immunoreactivity for all laminin chains. However, type IV collagen was detected occasionally as short strips around neoplastic growth. In some cases laminin α3, α5 and γ2 chains were present intracellularly in invasive tumor cells.

5.2.8 Carcinomas of thyroid gland

Laminin chains α1, α5, β1, β2 and γ1 were strongly present in BMs of neoplastic follicular and papillary structures of thyroid gland. Large tumor follicles showed quite linear BMs, but BMs of smaller ones in the peripheral areas had a clearly reduced staining pattern. Laminin chains α3, β3 and γ2 forming laminin-5 heterotrimer were seen either as occasional faint strips around neoplastic growths, or the result was negative.

Type IV collagen was strongly present in BMs around neoplastic follicles, though a reduced staining pattern was seen in the follicles in peripheral parts of tumors.

5.3 Distribution of mRNAs for laminin chains α1, β1 and γ2 and α1(IV) collagen in malignancies (III)

Generally, the level of mRNA synthesis for laminin α1 chain was low or at most moderate in carcinomas. For that reason samples of early human 8-10 gw (gestational week) placenta and decidua from legal abortions were simultaneously hybridized as a positive control. Human placentas gave clear signals and positive grains were localized to double-layered trophoblastic epithelium. Corresponding antigen localized to BMs of trophoblastic epithelium.

Of carcinomas the strongest expression for laminin α1 chain mRNAs was seen in SQCCs of lung, where neoplastic epithelial cells contained positive grains. Invasive cells of infiltrative breast carcinomas showed only occasional labeling, but cells adjacent to BMs of *in situ* carcinoma of breast were clearly seen to synthesize the mRNA. Occasional labeled carcinoma cells were seen in ovarian and thyroid carcinomas, but SQCCs of tongue and adenocarcinomas of stomach and colon were negative or unconvincing in their laminin α1 chain mRNA expressions.

Laminin γ2 chain mRNA was solely produced by carcinoma cells. The most intense expression was observed in SQCCs of lung and tongue, followed by adenocarcinomas of colon and intestinal type of stomach. mRNA production was seen especially in cancer cells of tumor-stromal interphase, but scattered invasive carcinoma cells in the tumor stroma and individual smaller tumor islands showed focal, but strong expressions. Central parts of larger tumor clusters contained only low amounts of transcripts or were negative.

In SQCCs intense synthesis was also seen some cases in cells adjacent to necrotic areas. Relatively lower synthesis was observed in breast and ovarian carcinomas and diffuse type of gastric adenocarcinoma, where only occasional labeling was observed in carcinoma cells. Thyroid carcinomas remained negative for laminin γ2 chain mRNA in *in situ* hybridization.

mRNAs for laminin β1 chain and α1(IV) chain were mainly synthesized by stromal cells in all tumors. Tumors expressed low levels of laminin β1 chain mRNA by carcinoma cells, while only occasional mRNA transcripts for α1(IV) chain were present in them. The most intense expression for both transcripts was observed in endothelial cells and in fibroblasts around tumor clusters, α1(IV) chain mRNA being more abundant in endothelial cells. In tumors accompanied by excessive fibrous desmoplasia, such as colo-rectal adenocarcinomas, there was an especially strong mRNA expression for laminin β1 chain and α1(IV) collagen in stromal fibroblasts.

5.4 mRNA synthesis of MMP-2, MMP-9 and MT1-MMP and TIMPs-1-3 in malignancies (IV, V)

5.4.1 *Hepatocellular and pancreatic adenocarcinoma (IV)*

Detailed results are presented in tables 1 and 2, respectively, in the original article (IV).

5.4.1.1 *MMP-2 expression*

MMP-2 mRNAs were detected in 14/36 (38 %) of HCCs. The expression occurred in stromal cells of 13/36 (36%) tumors. The mRNA level varied from weak to moderate. In 8 (22%) tumors, mRNAs were also detected in carcinoma cells. Of stromal cells both fibroblasts and, to a lesser extent, endothelial cells showed positive signals, usually in areas of abundant fibrosis either around tumor islands or more diffusely in the tumor stroma.

In immunohistochemical stainings MMP-2 antigen localized abundantly to carcinoma cells. Staining postitivity was also detected in stromal fibroblasts and vascular endothelium.

MMP-2 mRNAs were detected in 23/27 (85 %) pancreatic adenocarcinomas. Expression in stroma clearly predominated over that in neoplastic epithelium. Positive transcripts were localized to fibroblasts and endothelial cells, especially in areas of abundant fibrous desmoplasia. mRNA expression for MMP-2 by neoplastic epithelium was seen in 12/27 (44%) tumors, and the labeling was weak in 11 and moderate in one of them. Epithelial expression never occurred without simultaneous stromal synthesis.

In immunohistochemical stainings for MMP-2 pancreatic adenocarcinomas showed a clear positive staining reaction in carcinoma cells of neoplastic glands, but also in stromal

fibroblasts and endothelial cells. Occasional staining could also be seen in inflamed ductal and acinar epithelium of non-neoplastic pancreas.

5.4.1.2 MMP-9 expression

MMP-9 mRNAs were observed in 12/36 (33 %) HCCs. They all showed expression in carcinoma cells and four also in the stromal cells. The intensity of the mRNA signal varied from weak to moderate and was generally not associated with the amount of fibrous stroma. Within tumor tissue, the expression was more pronounced in the more dysplastic nodules. In stroma, both fibroblasts and endothelial cells contained the mRNA. Occasionally, mRNAs were also seen in non-neoplastic hepatocytes of cirrhotic areas and in cells of proliferating bile ducts.

In immunohistochemical staining MMP-9 was localized to the individual scattered neoplastic epithelial cells. Occacionally also stromal fibroblasts and vascular endothelial cells showed positive staining.

23/26 (91 %) of pancreatic adenocarcinomas expressed the MMP-9 mRNA. Generally, carcinoma cells and stromal cells were equally labeled for the mRNA. mRNAs in the tumor stroma were mainly detected in widely spread fibroblasts and to a lesser extent also in vascular endothelium.

In immunohistochemical stainings the MMP-9 antibody showed abundant positive staining for almost all neoplastic epithelium. Occasionally stromal fibroblasts and vascular endothelial cells expressed the antigen.

5.4.1.3 MT1-MMP expression

mRNA for MT1-MMP was detected in 12/36 (33 %) HCCs. Of these, the expression was seen exclusively in carcinoma cells in 7 tumors. In 5 cases, there was also expression in fibroblasts and, more weakly, in endothelial cells. The stromal signal was generally weak and most distinct in areas of abundant fibrosis, and it was often localized to cells adjacent to islands of tumor cells. In some areas, positive labeling was also seen in epithelial cells of proliferating, but not of normal bile ducts. In carcinoma cells of HCCs, the MT1-MMP mRNA expression was independent from that in stroma and no clear co-expression with fibroblasts was observed. The expression level in carcinoma cells varied from negative to strong positivity and had a tendency to be associated with a low degree of differentation; 5/10 (50%) of grade III tumors showed positive labeling.

MT1-MMP stainings were not so successful in HCCs, but there was, however, some intracytoplasmic or cell membrane staining of the carcinoma cells for MT1-MMP in grade III tumors.

In pancreatic adenocarcinomas, MT1-MMP mRNA expression was seen in 34/35 (97 %) cases. The strongest stromal MT1-MMP mRNA signal was seen in the fibroblasts of newly formed fibrous tissue in the vicinity of invasive epithelial ducts or islands. Normal acinar or ductal structures were negative.

In immunohistochemical stainings intracellular positivity was seen in both carcinoma and also to a lesser extent in stromal cells. In some areas, a positive staining reaction could also be seen in cell membranes of the neoplastic epithelial cells.

5.4.1.4 Statistical analysis

In order to analyze whether the expression of MMPs was associated with the outcome of the patients, the semiquantitative results of *in situ* hybridization were compared with the survival data of the patients. A significant association was seen between the expression of MT1-MMP mRNA by carcinoma cells of HCC and shortened survival of patients ($P= < 0.01$, log rank). No such association was seen in cases where the mRNA expression was present only in stromal cells ($P= 0.81$, log rank). Generally, there was a tendency toward increased MT1-MMP mRNA expression in grade III tumors when compared to grade I tumors, but the difference was not statistically significant ($P= 0.07$).

In HCCs there was no statistically significant difference in survival between patients showing negative or positive results for the presence of MMP-2 or MMP-9 mRNA in carcinoma cells ($P= 0.24$, log rank and $P= 0.97$ log rank, respectively) or in stromal cells ($P= 0.94$, log rank and $P= 0.70$, log rank, respectively). There was, however, a tendency for positive expression to be associated with a less favorable prognosis of patients.

In pancreatic adenocarcinomas MT1-MMP mRNA expression by neoplastic cells did not associate with survival when compared to prognosis ($P= 0.32$, log rank), but the Breslow test gave a significant value ($P= 0.04$). No significant association was seen when correlated to the mRNA expression by the stroma ($P= 0.12$, Log rank). Neither was there any difference in the mRNA expression between various differentiation grades (I-III) of pancreatic adenocarcinomas.

Since none of the pancreatic adenocarcinomas expressed moderately or strongly mRNA for MMP-2 by carcinoma cells statistical analysis was not performed. The association of stromal expression with the patients´ outcome did not show statistical significance ($P= 0.27$, log rank). Nor was there any significant association between the patients´ prognosis and the expression of mRNA for MMP-9 by cancer cells ($P= 0.65$, log rank) or by stromal cells ($P= 0.35$, log rank). However, according to life tables there was a tendency towards a less favorable prognosis in patients with increased MMP-9 mRNA expressions by tumor cells.
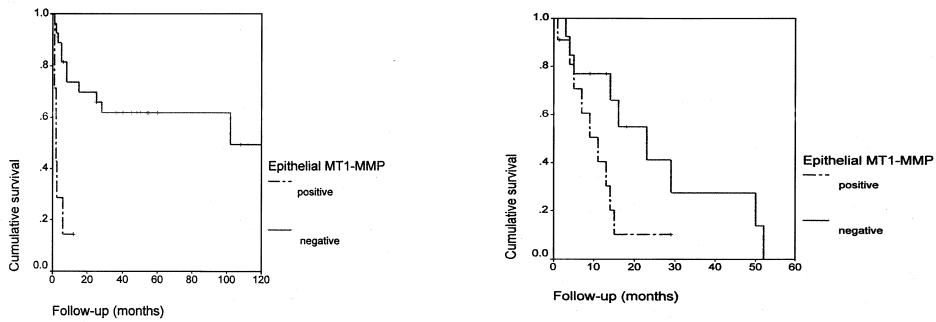
Fig. 10. Statistical life tables for hepatocellular carcinoma (left panel) and pancreatic adenocarcinoma (right panel). MT1-MMP mRNA expression in HCC was associated to shortened survival of patients (P= < 0.01, Log rank), but in pancreatic adenocarcinomas the value did not reach statistical significance (P= 0.32, Log rank).

5.4.2 Normal endometrium (V)

Detailed results are presented in table 1 in the original article (V).

5.4.2.1 Cyclic menstrual phases

In situ hybridization for MT1-MMP mRNA expression did not reveal any convincing labeling in proliferative or early secretory endometrium. In the late secretory phase weak MT1-MMP expression was seen in the stromal cells, being accentuated around glands and in endothelial cells and smooth muscle cells of spiral arteries. In normal endometrium there was no convincing evidence of the MT1-MMP mRNA expression in glandular epithelium. Atrophic endometrium did not contain specific labeling.

TIMP-1 and TIMP-2 mRNA were not detectable in proliferative and early secretory phases. In late secretory endometrium, stromal cells were seen to express both TIMP-1 and –2 mRNAs weakly. Positivity was localized to the stromal cells around the secretory glands, especially to the endothelial cells of small capillaries. In some cases there was weak labeling for TIMP-1 and –2 in smooth muscle cells of spiral arteries. TIMP-2 expression in the stroma was especially localized to the superficial parts of endometrium where the early progesterone effect first became visible. TIMP-1 was localized only to stromal components, but in one case of late secretory menstrual phase, TIMP-2 was expressed also by glandular epithelial cells.

TIMP-3 mRNA expression was present throughout the menstrual cycle. In the proliferative phase positivity was seen in endothelial cells of small endometrial capillaries and in endothelial, and to a lesser extent smooth muscle cells of spiral arteries. Proliferative and early secretory endometrium also showed mRNA synthesis in glandular epithelium in some cases. Late secretory endometrium showed abundant TIMP-3 mRNA

synthesis in endometrial stromal cells, in areas showing morphological features of predecidualization. Hence, the most intensive expression was seen in cells of periglandular stroma, around spiral arteries and under the surface epithelium. In this endometrial phase, epithelial expression for TIMP-3 mRNA was not seen.

5.4.2.2 Endometrial hyperplasias

In *in situ* hybridization for MT1-MMP mRNA expression in hyperplasias there were only occasional positive grains present in periglandular and periarterial stromal cells. Generally, MT1-MMP mRNA expression was very weak. In only one case, which represented atypical complex hyperplasia, was there evidence of epithelial mRNA expression. Contrary to the relatively negligible findings in intact hyperplastic areas MT1-MMP mRNA expression was seen particularly in desquamative stromal cells around collapsed dispersing glands. These areas expressed the mRNA very intensively, but focally.

In *in situ* hybridization the expressions for TIMP-1, -2 and –3 mRNAs remained negative in most cases, regardless of the histologic type of hyperplasia. TIMP-1 and –3 mRNAs were expressed mainly by endothelial cells and to a lesser extent by smooth muscle cells of spiral arteries and by stromal cells around glandular structures. In some cases mRNA expression for TIMP-3 could also be localized to hyperplastic glandular epithelial cells in complex and atypical complex hyperplasias. TIMP-2 was negative or unconvincing in its expression in all cases of hyperplasias.

5.4.2.3 Endometrial adenocarcinomas

Common to all mRNAs investigated was their increased expression, with relation to poorer differentation grade in adenocarcinomas of endometrium.

Both neoplastic epithelial and stromal cells showed weak to moderate mRNA expression for MT1-MMP mRNA in grade I-II adenocarcinomas. The most intense mRNA expression was seen in grade III tumors, where carcinoma cells were the main producers of this mRNA. Increased expression was usually present in the peripheral parts of tumors, especially at the invasive front, where individual tumor cells showing high-grade anaplasia were often strongly labeled for the MT1-MMP mRNA. In one case endothelial cells of myometrial vessels expressed MT1-MMP mRNA extremely abundantly.

Weak to moderate TIMP-1 mRNA expression was seen in endothelial and smooth muscle cells of spiral arteries and occasionally in endometrial stromal cells in grade I and II adenocarcinomas. Grade III tumors showed mostly strong expression in desmoplastic stromal fibroblasts and also in vascular structures around neoplastic glands and cell clusters. There was no TIMP-1 mRNA expression in neoplastic epithelial cells.

Similarly to the findings in different types of hyperplasias TIMP-2 mRNA synthesis was not clearly seen in any of grade I adenocarcinomas. Grade II tumors showed weak mRNA expression in neoplastic epithelial cells and in vascular stuctures. In grade III

adenocarcinomas there was stronger epithelial expression especially in the pheripheral invasive parts of the tumor. In some cases this expression was localized to cells of invasive front. Stromal desmoplastic fibroblasts and vascular structures also showed increased mRNA expression for TIMP-2 in grade III adenocarcinomas.

Weak TIMP-3 mRNA synthesis in grade I tumors was only seen in some stromal and endothelial cells, while the neoplastic epithelium remaining negative. In grade II and III adenocarcinomas there was weak to moderate expression level in neoplastic epithelial cells, especially at the tumor-stromal interphase of grade III adenocarcinomas. More anaplastic carcinoma cells at the invasive front were usually seen to express TIMP-3 mRNA strongly. Stromal fibroblasts and vascular structures as well as endothelial cells of myometrial vessels also expressed the TIMP-3 mRNA, but to a lesser extent.

5.5 Gelatinolytic activity in malignancies

Gelatin zymography was performed to show gelatinolytic activity in samples from HCC and endometrial adenocarcinoma and comparing them to their normal surrounding tissues. Results showed that latent 72 kDa of MMP-2 and 92 kDa of MMP-9 were both present in normal liver and endometrium representing the secretory phase, as well as in HCCs and endometrial adenocarcinomas. Relatively higher amounts of proMMP-9 were seen especially in HCCs. Only tumor samples clearly contained the activated forms 62 kDa of MMP-2 and 82 kDa of MMP-9. Also larger bands of about 115 kDa were seen in normal as well as endometrial adenocarcinomas, probably representing formation of the complex with TIMPs. One tumor sample of endometrium also showed a faster migrating band of about 50 kDa.

6 Discussion

The present thesis demonstrates the expression of the laminin α1, α3, α5, β1, β2, β3, γ1, γ2 chains, type IV collagen, MMP-2, MMP-9, MT1-MMP and TIMPs-1-3 in epithelial malignancies and normal tissues by immunohistochemical and *in situ* hybridization methods. Gelatin zymography was used to determine the gelatinolytic activity in carcinomas, and the results were compared to those in their normal surrounding tissue. Additionally, statistical analysis was used to clarify the clinical role of the laminin γ2 chain and MMPs in malignancies.

6.1 Expression of laminins in BMs of normal tissues and malignancies

6.1.1 General aspects

Laminins and type IV collagen form the structural and functional integrity for BMs, separating different tissue structures from each other, e.g. epithelium from the underlying connective tissue. BMs serve as an important adhesion matrix for cells, take part in cell signaling and migration and protect cells from apoptosis (Yurchenco & O´Rear 1994, Timpl 1996, Colognanto *et al.* 1999, Mooney *et al.* 1999). Their mandatory role in tissue organogenesis has been shown in several experiments (Adams & Watt 1993, Ekblom *et al.*1994, Miner *et al.*1998).

In this thesis individual laminin chains showed quite a unique expression pattern. Some of them had a more generalized distribution, such as laminin chains α5, β1 and γ1, components of laminin-10, which were expressed in all the locations investigated, including nearly all the tumor BMs of the thesis, BMs of normal squamous epithelia, glandular structures, vascular epithelia, muscle and nerves. This indicates the ubiquitous presence of laminin-10 in carcinomas as well as in their normal surrounding tissues. Interestingly, it has been previously shown that the expression of the laminin α5 chain is related to differentiated phenotype (Sorokin *et al.* 1997). However, in carcinomas which comprise various levels of differentiation its expression was quite comparable to that seen

in normal tissue structures. Some laminin chains were present mainly in epithelial BMs, such as α1, α3, β3 and γ2. In the studies of this thesis the most restricted expression in epithelial BMs was seen for laminin chains α1 and β2.

It is noteworthy that BMs of carcinomas and their normal surrounding tissues expressed many laminin chains in their BMs, which suggests the presence of more than one laminin heterotrimer in them. Why do individual BMs bear more than one laminin heterotrimer? Why do two different BMs contain a different laminin heterotrimer composition? Individual laminins mediate different functions such as adhesion and migration, and the expression of a certain laminin may reflect more its functional properties than structural characteristics. Or vice versa, a structural feature in the laminin molecule, such as truncated short arms, may result in an inability of the molecule to form laminin networks, but an ability to take part in the anchoring system instead, as in the case of laminin-5 (Rousselle *et al.* 1997). The results of the studies of this thesis showed that generally the same heterotrimers were expressed in carcinomas and in their normal surrounding tissue. This indicates that the synthesis of laminins as to quality and composition of the synthesized laminin chains is generally not lost during malignant transformation. However, the neoexpression of laminin-5 has been demonstrated to occur in carcinomas of the thyroid gland (Lohi *et al.* 1998), which shows that tumors are also capable of active modulation of their surrounding environment.

6.1.2 γ2 chain of laminin-5 is upregulated in carcinomas

Laminin-5 (α3β3γ2) is believed to have a specific role in normal as well as in malignant tissues. It belongs to the so-called truncated laminins and, as was mentioned earlier, it is not capable of forming three-dimensional networks, but mediates interactions via hemidesmosomes between cells and extracellular environment (Cheng *et al.* 1997, Rousselle *et al.* 1997). Originally laminin-5 was found in keratinocyte cultures of skin (Rousselle *et al.* 1991) and was shown to either inhibit or promote cell migration (Rousselle & Aumailley 1994, Goldfinger *et al.* 1998). It is proposed that integrin α6β4 mediates the anchoring phenotype of laminin-5 whereas integrin α3β1 functions in cell spreading and migration (Xia *et al.* 1996, DiPersio *et al.* 1997). The γ2 chain is characteristic of the molecule and is expressed only by epithelial BMs. Its mRNAs are synthesized by epithelial cells, whereas many other laminin chains are produced together by stromal and epithelial cells (Autio-Harmainen *et al.* 1993, Pyke *et al.* 1994, Hao *et al.* 1996, Perreault *et al.* 1998). Laminin-5 has been shown to be overexpressed in carcinomas and physiological conditions with strong epithelial turnover, such as inflammation and cell migration (Pyke *et al.* 1994, Ono *et al.* 1999, Sordat *et al.* 1998, Kainulainen *et al.* 1997). Results from this study demonstrated a strong mRNA and antigen expression for the laminin γ2 chain in pancreatic adenocarcinomas and in different histological types of lung carcinoma, in which an especially strong expression was seen in those carcinoma cells which were located peripherally in the tumor clusters and apposing the tumor stroma and in the small invasive tumor islands and individual tumor cells infiltrating the tumor stroma. This fits well the function of laminin-5 in the interaction of forming contacts with cells and BMs. Recently γ2 chain processed either by

MMP-2 or MT1-MMP caused a strong migration effect on tumor cells grown on laminin-5 surfaces (Giannelli *et al.* 1997, Koshikawa *et al.* 2000). It was proposed that laminin-5 produced by the tumor cells is digested by MMPs to achieve the invasive phenotype. This proposal may well be valid also *in vivo* since the expressions of the MMP-2, MT1-MMP and laminin-5 are commonly present in malignant tumors (Bramhall *et al.* 1997, Imamura *et al.* 1998, Pyke *et al.* 1994).

The laminin γ2 chain is synthesized as a 155 kDa molecule and is then further processed into the ECM space as a 105 kDa form (Marinkovich *et al.* 1992, Goldfinger *et al.* 1998). Interestingly, the Pab against the γ2 chain used in this study detected both the intracellular unprocessed form and extracellular proteolytically processed form, as demonstrated by Western blot analysis and by the strong intracytoplasmic staining reaction in tumor cells in the peripheral aspects of tumor clusters as well as by extracellular staining of BMs around the clusters. The strong intracytoplasmic expression indicates either a highly upregulated production of the laminin γ2 chain or incapable secretion, processing or assembly to laminin-5 molecule by carcinoma cells. A recent study of gastric carcinomas demonstrated that the laminin γ2 chain can also be secreted to the ECM space alone without the assembly with α3 and β3 chains (Koshikawa *et al.* 1999). This observation is also supported by the results of this thesis and suggests an important role for the γ2 chain of laminin-5 in malignant growth. However, even though the increased expression of laminin γ2 chain was clearly associated with carcinoma cell invasion it did not seem to have influence on the general prognosis of patients with pancreatic and lung adenocarcinomas, though there were more often cases with low laminin γ2 chain mRNA and antigen expression in tumors infiltrating peripancreatic tissues and tumors with regional or distant metastases. There is one previous study, however, in which the overexpression of the γ2 chain was shown to be associated with a poor outcome of patients with tongue carcinomas (Ono *et al.* 1999).

6.1.3 *Laminin-10, but not laminin-1, is commonly present in carcinomas*

A confusing topic during recent years has been the expression of the laminin α1 chain, present in laminin-1 (α1β1γ1) and laminin-3 (α1β2γ1) in normal tissues and carcinomas. Antisera against laminin isolated from EHS tumor has been widely used to detect the immunohistochemical distribution of laminin in human tissues. This antibody has been usually referred to as representing the laminin-1 molecule (Aumailley & Smyth 1998). Later, the Mab 4C7, which was raised before there was any knowledge of the existence of laminin heterotrimers other than laminin-1, was also thought to detect the Ln α1 chain (Engvall *et al.* 1986). Antisera against EHS laminin and Mab 4C7 have been used for many morphological studies (Engvall *et al.* 1986*,* Virtanen *et al.* 1995a, Hewitt *et al.* 1997). Gene expression studies aimed at specific detection of mRNAs for the human laminin α1 chain have shown a rather restricted distribution, however (Nissinen *et al.* 1991, Vuolteenaho *et al.* 1994). The discrepancy was resolved when the 4C7 antibody was demonstrated actually to detect the laminin α5 chain (Tiger *et al.* 1997). Results from this study showed that the laminin α1 chain by immunohistochemistry and

supported by *in situ* hybridization can be detected in BMs of certain carcinomas, including carcinomas of the thyroid gland and carcinoma *in situ* of breast, but in other carcinomas investigated laminin α1 chain expression was very weak or negative. Secondly, when present in BMs of normal tissue structures the laminin α1 chain was also expressed in carcinomas derived from that tissue. Thirdly, laminin α5 chain, present in laminins-10 (α5β1γ1) and –11 (α5β2γ1), in coexpression with β1 in epithelial BMs, but very rarely with β2, showed a widespread expression pattern, being present in all the BMs studied, which shows the abundant presence of laminin-10, but not laminin-11, in them. Vascular BMs seem to contain laminin-11 commonly, however.

The spatial differences in the laminin chain distribution may be related to their differences in mediating cell adhesion via specific receptors. It is also possible that in BMs some kind of general laminin skeleton is required to mediate ordinary cell functions, such as adhesion and general survival signal for cells, and other heterotrimers in association with it could modulate more specific functional aspects. Laminin-10 may therefore represent such a general laminin, though more studies are needed to clarify its role among laminins. Knock-out mice model for laminin α5 chain has showed its lethality during early embryogenesis, confirming the fundamental role of laminin-10 in BMs. Such knock-out models would tell us more about the functional requirement of laminin α1 chain for development, as well.

6.1.4 Evidence that BMs take part in malignant growth

In carcinomas BMs have been traditionally thought to prevent tumor cell invasion. In addition, decreased synthesis and assembly and increased degradation of BM macromolecules has been a generally accepted fact in tumor biology (Liotta *et al.* 1980, Barsky *et al.* 1983, Bosman *et al.* 1985). However, there has been an increasing number of reports recently which have indicated a more specialized role of BMs for malignant growth than just being structural barriers. BMs serve as an important adhesion matrix for tumor cells, and malignant cells actively express several receptors to recognize BM macromolecules (Ivaska & Heino 2000, Mercurio 1995). They also constitutively migrate on laminin substratum and exhibit a different cell morphology *in vitro* when grown on them (Klein *et al.* 1988, Gonzales *et al.* 1999). Finally, malignant tumors also actively synthesize BM macromolecules and deposit them into BMs (Pyke *et al.* 1995, Soini *et al.* 1996, Tani *et al.* 1997, Tani *et al.* 1999). There is also evidence that BM macromolecule composition is modified during tumor progression (Catusse *et al.* 2000). In this study there were carcinomas which contained well-formed and linear BMs around tumor clusters, and on the other hand there were tumors which showed hardly any immunoreactivity against laminin chains and type IV collagen. Based on the results of the studies of this thesis, and also supported by previous reports, carcinomas could be classified into two main groups, of which one group comprises tumors with well-organized BMs around carcinoma clusters, e.g. carcinomas of lung, colon and thyroid and intestinal-type of gastric carcinoma, and another group in which the tumors are characterized by the loss of BM macromolecules around tumor infiltrates, e.g. invasive breast carcinoma and diffuse type gastric carcinomas (Tani *et al.* 1996, Tani *et al.* 1997,

Sordat *et al.* 1998, Henning *et al.* 1999). However, malignant tumors generally contained less BM material in parts of the obvious active invasion, which supports the concept that under active invasion BMs are either degraded by proteolytic enzymes, or the synthesis or assembly of BM macromolecules is disturbed.

There are reports which have shown both decreased and increased mRNA synthesis for components of BM in malignant tumors (Martin *et al.* 1998, Pfohler *et al.* 1998). In this study the synthesis of the mRNAs for laminin β1 and γ2 chains and collagen α1(IV) chain was clearly increased in tumors compared to adjacent non-tumor area. It can be concluded that malignant tumors in many cases, except for infiltrative breast and diffuse-type gastric carcinomas, are characterized by increased synthesis of BM macromolecules of laminin β1 and γ2 chains, as well as α1(IV) collagen, reflecting an upregulated ECM turnover in them. It may also be concluded that malignant epithelial tumors use BM macromolecules as their growth substratum more notably than what has been previously known. Interestingly, invasive breast carcinomas seems to totally lack BM macromolecules around tumor infiltrates. This suggest that in these carcinomas ECM is efficiently degraded or they grow is independent of BM macromolecules. It is also interesting that the stromal or carcinoma cells which expressed the mRNAs for Ln β1 chain and α1(IV) collagen also simultaneously synthesized matrix degrading enzymes, MMP-2, MMP-9 and MT1-MMP, as well as their inhibitors TIMPs. This indicates that the elements for a balanced matrix synthesis and degradation are also present in malignant tumors. Recently, a new term for biologically active cryptic sites found in all matrix molecules, matricryptic sites, was introduced. These are revealed after proteolytic processing or conformational changes and mediate many biological processes, such as inflammation, cell migration and proliferation, and are called matricryptins. MMPs are shown to act as enzymes which cleave matricryptic sites, e.g. MMP-2 and MT1-MMP for γ2 chain of laminin-5 and MMP-1 and MMP-8 for triple helical collagens. (For review, see Davis *et al.* 2000). On the other hand, in recent years distinct domains from BM macromolecules has been found which selectively inhibit angiogenesis and tumor growth *in vivo* (Maeshima *et al.* 2000). When we understand better the regulatory mechanisms involved in the ECM turnover of malignancies as well as those involved in carcinoma cell adhesion and migration over BMs we will have more opportunities to develop techniques to control such events, e.g. to modulate cell adhesion by neutralizing antibodies and migration by antimigrative agents.

6.2 Expression of MMP-2, MMP-9, MT1-MMP and TIMPs in normal endometrium and malignancies

6.2.1 General aspects

ECM turnover and cell invasion are dynamic processes and require active degradation of ECM components. Along with serine proteases much attention has been paid to MMPs which form an expansive group of proteinases with similar structural features and require

activation in order to function (for review, see Birkedal-Hansen *et al.* 1993, Kähäri & Saarialho-Kere 1999). To date about 20 different MMPs have been discovered. They are differentially expressed in tissues and they are believed to have a pivotal role in controlling matrix remodeling.

6.2.2 TIMPs, but not MT1-MMP, are induced during secretory phase in normal endometrium

The cyclic changes in the ECM turnover in normal endometrium are an important topic for study, because endometrium of early pregnancy provides the only tissue in which "physiologic invasion" can be studied, since the intermediate trophoblasts of early placenta infiltrate the endometrium and endometrial vessels in anchoring the placenta to the uterine wall (Fisher *et al.* 1985). Fertile endometrium is a very dynamic tissue which undergoes cyclic changes which can be classified into three phases: proliferative phase under estrogen effect when mitotic activity and synthesis of ECM material is high, secretory phase under progesterone effect which is characterized by the functional changes in endometrial glands and predecidualization of stroma, and finally desquamative phase when the functional layer of endometrium is removed in menstruation. MMPs and TIMPs have been suggested to have an important role in maintaining the tissue architecture in endometrium. This study confirms the previous reports that TIMPs, especially TIMP-3, are greatly induced during secretory phase (Higuchi *et al.* 1995, Salamonsen *et al.* 1997). Most intense mRNA synthesis for TIMP-3 was seen in endometrial stromal cells showing morphological changes of predecidualization and situating in the superficial parts of endometrium, while glandular epithelium did not seem to produce significant amounts of any TIMPs. This supports the concept that expression of TIMPs is under the control of progesterone (Rodgers *et al.* 1994). The role of TIMPs in endometrium would be to maintain tissue architecture and to prevent uncontrolled tissue degradation. High mRNA levels of TIMPs and especially of TIMP-3 in implantation are maintained also during pregnancy (Reponen *et al.* 1995, Hurskainen *et al.* 1996). There has been discussion as to whether there might be an imbalance in the amounts of MMPs and TIMPs in abnormal intrauterine bleeding, spontaneous abortions and conditions where the blastocyst is not capable of implantation. Imbalance in synthesis and degradation would lead to small breaks of BMs of small capillaries, leading to spontaneous bleeding (for review, see Salamonsen & Woolley 1994). There is also a possibility that an imbalance in the amount of TIMPs could affect inflammatory processes unfavourably since TIMP-3 has been shown to inhibit TACE (ADAM-17) (Amour *et al.* 1998).

   MMPs have been shown to be induced most notably just before menstruation, and their mRNA levels are low during proliferation and secretion (Hampton & Salamonsen 1994, Salamonsen *et al.* 1997). In this study MT1-MMP mRNA was expressed in low amounts during these phases, which speaks for the fact that its regulatory mechanisms differ from TIMPs and it is not significantly under the control of progesterone or estrogen. Its expression pattern by endometrial stromal cells and vascular pericytes and smooth muscle cells fits well the concept of other known MMPs, except that MMP-7 and

-9 are expressed also by glandular epithelium (Rodgers *et al.* 1994, Soini *et al.* 1997). However, MT1-MMP is highly expressed by invasive cytotrophoblasts in early pregnancy and it is proposed to be one of the key enzymes among MMPs during implantation (Hurskainen *et al.* 1998).

In future investigations of the functional role of MMPs and TIMPs in endometrium may lead us to better understanding of those regulatory mechanisms which are involved in normal implantation and early pregnancy and give us opportunities to develop therapeutical implications to treat e.g. infertility and repeated spontaneous abortions.

6.2.3 MMP-2, MMP-9, MT1-MMP and TIMPs are commonly present in malignancies

The observation by Liotta *et al.* (1980) showed that MMP-2 has a specific role in tumor invasion. Since then the group of structurally and functionally similar proteinases has expanded to form the MMP family, and there is increasing evidence of their importance for malignant growth. Early experimental and morphological studies by using *in vitro* studies and immunohistochemistry showed that carcinoma cells expressed the immunoreactivity for MMP-2 and it became generally accepted that tumor cells themselves synthesize and express the MMPs (Liotta *et al.* 1983, Tryggvason *et al.* 1993). However, when *in situ* hybridization became a relevant research method it showed that *in vivo* the tumor stromal cells actually synthesized the mRNA for MMP-2, even though the antigen was predominantly immunolocalized to the carcinoma cells (Autio-Harmainen *et al.* 1993, Soini *et al.* 1993, Poulsom *et al.* 1993). The discrepancy was explained by a possible existence of specific receptors in tumor cells to which MMP-2 could be bound (Autio-Harmainen *et al.* 1993). Finally MT-MMPs, a wholly new subgroup of MMPs which was capable of both binding MMP-2 into the tumor cell membranes and regulating its activation via complex formation with TIMP-2, were introduced (Sato *et al.* 1994, Strongin *et al.* 1995). Now it has been become clear that tumor stroma and neoplastic cell population have many connections, including carcinoma cell proliferation and induction of tumor stroma (for review, see Basset *et al.* 1997).

Also in this study the importance of tumor stromal cells for mRNA synthesis of MMP-2, MMP-9, MT1-MMP and TIMPs-1-3 was noticed. However, MMP-9 was either mainly synthesized by tumor epithelial cells or equally with stromal cells. This type of expression pattern of MMPs may represent a tumor-host response. Many cytokines and growth factors, such as IL-1β, TGF-β and TNF-α, induce stromal cells to express these proteinases (Sehgal *et al.* 1999, Watari *et al.* 1999, Johansson *et al.* 1997). Also stromal matrix proteins, such as laminin, have been shown to increase tumor cell secretion of MMPs (Turpeenniemi-Hujanen *et al.* 1986). It was also observed that the surrounding non-tumor tissue did not show any significant mRNA expression for the MMPs investigated. Also in endometrial hyperplasias MT1-MMP and TIMPs-1-3 as well as MMP-2 and MMP-9 (Soini *et al.* 1997) mRNA expressions were very low, pointing out that in conditions which are characterized by high estrogen influence, the MMP machinery is not induced and the type of synthesis pattern may be related to that in normal proliferating endometrium. However, in areas of endometrial desquamation MT1-

MMP mRNA synthesis was focally increased by endometrial stromal cells, but not by epithelium. This may represent a new active role for MT1-MMP in endometrial desquamation. Endometrial adenocarcinomas and HCCs showed enhanced mRNA expressions most notably in poorly differentiated tumors, though such a connection could not be observed as clearly in adenocarcinomas of pancreas. The expression pattern seems, therefore, to be variable and may be associated with individual tumor type, and the amount and type of tumor stroma may modulate the mRNA expressions of MMPs. Interestingly TIMP-3 was abundantly synthesized by endometrial adenocarcinoma cells and more notably by low differentiated tumors. There are some previous reports which have revealed that TIMP-3 gene is inactivated in many malignant tumors, suggesting a tumor suppressor role for TIMP-3 in general (Bachman *et al.* 1999, Ueki *et al.* 2000). However, endometrial adenocarcinomas retained their ability to synthesize high levels of TIMP-3 mRNA. Malignant tumors are also commonly accompanied by variable amounts of inflammarory cells, which can also affect the levels of MMPs and TIMPs expressions. It is also noteworthy that generally in malignancies several MMPs can be simultaneously detected in tumor tissues and by their differing substrate specificities, together with other known proteolytic enzymes they can provide an effective degradative machinery in malignant tumors.

6.3 Evidence that MMPs and TIMPs are actively involved in neovascularization

An effective blood supply is mandatory for malignant tumor growth. Blood vessels also allow hemotogenous metastases to spread to distant organs. In recent years much attention has been paid to a concept that the amount of tumor neovascularization could be an independent prognostic factor for the disease (for review, see Weidner 1998). There is also increasing interest in concentrating specific therapies against the tumor neovasculature (Chaplin & Dougherty 1999). In neovascularization endothelial cells must proliferate and penetrate the tumor matrix. In such growth the role of serine proteases was previously underlined (Koolwijk *et al.* 1996), but recent evidence supports the active role of MMPs and TIMPs in that process as well (Hiraoka *et al.* 1998). In the studies of this thesis tumor endothelial cells were seen to actively synthesize mRNAs for all the mRNA investigated. MMP-2, MMP-9 and MT1-MMP antigens were also localized to the endothelial cells. The amount of mRNA synthesis by endothelial cells was variable in different pathological conditions, reflecting the state of ECM turnover in them. Tumor cells are also known to secrete many growth factors which stimulate endothelial cell migration, increase proliferation and upregulate the synthesis and activation of proteolytic enzymes (Kräling *et al.* 1999, Mach *et al.* 1999). Interestingly, in animal experiments inhibition of MMPs by synthetic inhibitors or transfection of tumor cells with TIMP cDNA has caused a clear reduction in tumor neovascularization (Wang *et al.* 1997, Price *et al.* 1999). On the other hand, several MMPs also have the ability to converge angiostatin from plasminogen (Cornellus *et al.* 1998, Lijnen *et al.* 1998, Patterson & Sang 1997). This further supports the concept that MMPs are involved both in tumor and

endothelial cell proliferation and invasion, but also have the ability to reduce neovascularization.

6.4 Active species of MMPs are present in malignant tumors

Only activated MMPs have proteolytic capacity. Therefore it is important to determine whether expressed MMPs are actually capable of matrix proteolytic degradation. In this study MMP-2 and MMP-9 activation in HCC and endometrial adenocarcinoma was determined by using gelatin zymography and the result was compared to non-tumor samples. In both tumor types the activated forms of MMP-2 and MMP-9 were present only in tumor samples, but not in nearby non-tumor normal tissue. Tumor samples also contained increased amounts of latent forms of MMP-2 and MMP-9. The results support previous studies which have reported increased MMP activation in malignant tumors in many locations (Davies *et al.* 1993, Koshiba *et al.* 1997, Garbett *et al.* 1999). On the other hand, in benign tumors the expression pattern of MMP is usually lower as compared to their malignant counterparts, and MMPs are either in latent forms or their activation rate is low (Forsyth *et al.* 1999). There are also studies which have shown that increased amounts of both latent and activated MMPs can be detected in the urine and increased amounts of TIMP-1 in the serum of patients with cancer (Moses *et al.* 1998, Holten-Andersen *et al.* 1999).

To date there is knowledge of substantial amounts of agents which have been identified as activators of an individual MMP. MT-MMPs have received a lot of interest because they bind MMP-2 into cell membranes. By complex formation with TIMP-2 MT1-MMP can simultaneously activate and regulate first of all the activity state of MMP-2, but also of MMP-13 (Knäuper *et al.* 1996b). The expression level of MT-MMPs has also been shown to correlate with MMP-2 activation on tumor tissues (Ueno *et al.* 1997, Nakada *et al.* 1999). Many other MMPs also have the ability to activate each other and, indeed, in some cases MMP activation has been suggested to occur in a cascade manner (Ramos-DeSimone *et al.* 1999). *In situ* zymography has also demonstrated the actual occurrence of gelatinolytic activity *in situ* around an individual malignant cell (Nakada *et al.* 1999). Cell culture experiments have also shown that tumor cell lines can in some cases be divided into highly and poorly invasive lines based on their expression pattern of MMPs (Kurschat *et al.* 1999). Taken together, malignant tumors generally express higher amounts of MMPs, increased amounts of both latent and activated species of MMPs are present in tumor tissues, and in some cases increased activation has direct correlation with tumor invasion.

6.5 Increasing evidence that MMPs have clinical significance

Numerous individual factors are involved in malignant transformation, and matrix proteolysis is essential for invasive growth (Murphy & Gavrilovic 1999). There is a considerable number of studies in which immunohistochemistry has been used to detect antigen expression in tumor tissues, Northern hybridization and *in situ* hybridization to

show mRNA synthesis, zymography to demonstrate MMP activation in tumor samples, and synthetic MMP inhibitors to study their reducing effect on MMPs. In the studies of this thesis *in situ* hybridization was used to detect MMP-2, MMP-9 and MT1-MMP mRNA expression in HCCs and adenocarcinomas of pancreas, and the results were correlated with the survival of patients. These comparisons showed that the mRNA expression of MT1-MMP in HCCs was associated with shortened survival of patients. A similar tendency was also present in pancreatic adenocarcinomas, though the association did not reach statistical significance. The mRNA levels of MMP-2 and MMP-9 did not reach statistical significance, although they did have a tendency to be associated with a less favorable prognosis in both tumor types. This shows that MMP expression is significantly associated with malignant growth and possesses important clinical significance. It is noteworthy that in addition to the three MMPs studied in this thesis the remaining members of the known MMPs might all have their own contribution as well.

However, the findings of the studies of this thesis and those of other researchers underline most notably the role of MT1-MMP as prognostic marker in certain malignancies. MT1-MMP operates not only by regulating the activity of MMP-2, but also by its own independent proteolytic activity. Previously, MT1-MMP antigen or mRNA expressions have been shown to have an association with more aggressive clinical behavior or distant organ or lymphnode metastases, these malignancies including astrocytic brain tumors, pediatric neuroblastoma, gastric and breast carcinomas (Nakada *et al.* 1999, Sakakibara *et al.* 1999, Caenaezzo *et al.* 1998, Ueno *et al.* 1997). There are also some studies which have shown that immunohistochemically detected expression of MMP and TIMP antigens in tumor tissues have an association with the poor prognosis of patients, these including MMP-2 in skin melanoma and breast carcinoma, MMP-2 and MMP-9 in pancreatic adenocarcinoma, MMP-1 in breast carcinoma and TIMP-2 in colon carcinoma (Väisänen *et al.* 1998, Talvensaari-Mattila *et al.* 1998, Kuniyasu *et al.* 1999, Nakopoulou *et al.* 1999, Ring *et al.* 1997). Increased MMP-2 and -9 activation have been shown to correlate with the more aggressive growth and invasion in pancreatic adenocarcinoma, bladder carcinoma, breast carcinoma and various brain tumors (Koshiba *et al.* 1998, Davies *et al.* 1993, Davies *et al.* 1992, Lampert *et al.* 1998).

Animal experiments have provided an interesting tool to study the role of MMPs *in vivo*. In these experiments tumor cell lines are transfected with cDNAs for TIMPs or MMPs, followed by inoculation into an experimental animal. Tumors which have over-expressed TIMPs or which have been treated with MMP inhibitors have usually shown reduced growth potential, a lower metastasis rate and histologically less blood vessel formation (DeClerck *et al.* 1992, Wang *et al.* 1997, Valente *et al.* 1998). This kind of results provide an interesting challenge for the use and development of synthetic modulators of MMP production for cancer treatment in the future. More information and experimental data are needed, however, to evaluate the usability, safety and efficacy of such treatment in man.

7 Conclusions

BMs are a specialized form of ECM which separate different tissue conpartments from each other. Laminins, together with type IV collagen and other BM macromolecules, form the structural and fuctional integrity for BMs. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) together form a group of molecules which control tissue degradation. In this thesis immunohistochemistry and *in situ* hybridization were used to analyze the expression of individual laminin chains α1, α3, α5, β1, β2, β3, γ1 and γ2, type IV collagen, MMP-2, MMP-9, MT1-MMP and TIMPs-1-3 in normal tissues and epithelial malignancies. Also MMP-2 and MMP-9 activity was determined by gelatin zymography in malignancies.

1. Normal tissue structures were always surrounded by linear BMs. With the exception of infiltrative breast and diffuse type gastric carcinomas BMs were quite well-formed around tumor clusters in carcinomas. In some areas BMs showed more or less fragmented phenotype, however. This shows that the ability to synthesize and deposit laminins and type IV collagen is not totally lost during malignant transformation, and that malignant epithelial tumors generally may use BM macromolecules as their growth substratum more notably than previously has been emphasized.

2. Among laminins the most abundant expression in normal and malignant tissues was seen for α5, β1, γ1 and γ2 chains, suggesting the ubiquitous presence of laminin-5 and -10 in their BMs. Laminin α1 and β2 were the most restricted chains in epithelial BMs, confirming that laminin-1 and –11 are rarely present in them. Laminin-1 was present only in certain BMs, such as many glandular structures and BMs of the thyroid gland, but was not seen in squamous epithelia. In line with immunohistochemistry the *in situ* hybridization showed that α1 and γ2 chains of laminin were solely synthesized by epithelial cells, whereas the the β1 chain of laminin and α1(IV) collagen were synthesized mainly by stromal cells.

3. Laminin γ2 chain mRNAs were especially expressed by infiltrative tumor cells at the leading edge of carcinoma clusters. The same invasive tumor cells also contained, together with BM and stromal immunoreactivity, intracytoplasmic positivity, as well. Generally the expression of laminin γ2 chain of laminin-5 was increased in epithelial malignancies, and this may reflect the need to mediate contacts between ECM and malignant cells and to serve as growth substratum for the infiltrative cells. Since

laminin γ2 chain degraded by MMP-2 or MT1-MMP causes a strong migratory effect on epithelial cells the presence of all these mediators in malignancies, as seen in the studies of these thesis, may provide a mechanism to potentiate the invasion of tumor cells in general.

4. Increased expression of mRNAs for MMP-2, MMP-9 and MT1-MMP was observed in HCC and pancreatic and endometrial adenocarcinomas, and their expression was low in surrounding normal tissues and hyperplastic endometrium. Elevated MT1-MMP mRNA expression was observed in HCC with low differentiation and statistically it was associated with less favorable outcome of patients. A similar tendency was observed in pancreatic adenocarcinomas, although the correlation did not reach statistical significance. In pancreatic adenocarcinomas MMP-2, MMP-9 and MT1-MMP were quite equally expressed by tumors with high and low differentiation, but in endometrial adenocarcinomas increased expression was connected with low differentiation. Endometrial tumors also expressed increased amounts of TIMPs-1-3 mRNAs. The *in situ* hybridization results showed that MMP and TIMPs mRNA synthesis is upregulated in malignancies which strongly supports their important role in matrix turnover and tumor cell invasion.

5. Increased gelatinolytic activity was present in HCC and endometrial adenocarcinoma as detected by gelatin zymography. Only latent forms of MMP-2 and MMP-9 were present in samples of normal tissues. MMP-2 and MMP-9 activation was detected only in tumor samples. Gelatin zymography showed that synthesized and secreted latent MMP-2 and MMP-9 are also activated in malignant tumors, which further demonstrates their involvement in the degradation of matrix macromolecules in malignant tumors.

MMPs and TIMPs form potential group of molecules to facilitate tumor cell invasion. In this process MT1-MMP, which by complex formation with TIMP-2 regulates MMP-2, is suggested to play pivotal role. As the γ2 chain of laminin-5 is degraded by MT1-MMP and MMP-2 and the processed γ2 chain induces increased cell migration, the selective modulation of the synthesis and activity of MMPs in malignant tumors may provide a new tool for cancer treatment in the future. The studies of this thesis showed that epithelial tumors may use BMs, most notably laminin-5 and -10, as their growth substratum and that proteolysis by MMPs may serve as an effective modulator for the growth and invasion.

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