MATRIX DEGRADING PROTEASES AND COLLAGEN-DERIVED ANGIOGENESIS INHIBITORS IN THE REGULATION OF CARCINOMA CELL GROWTH

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Faculty of Medicine,
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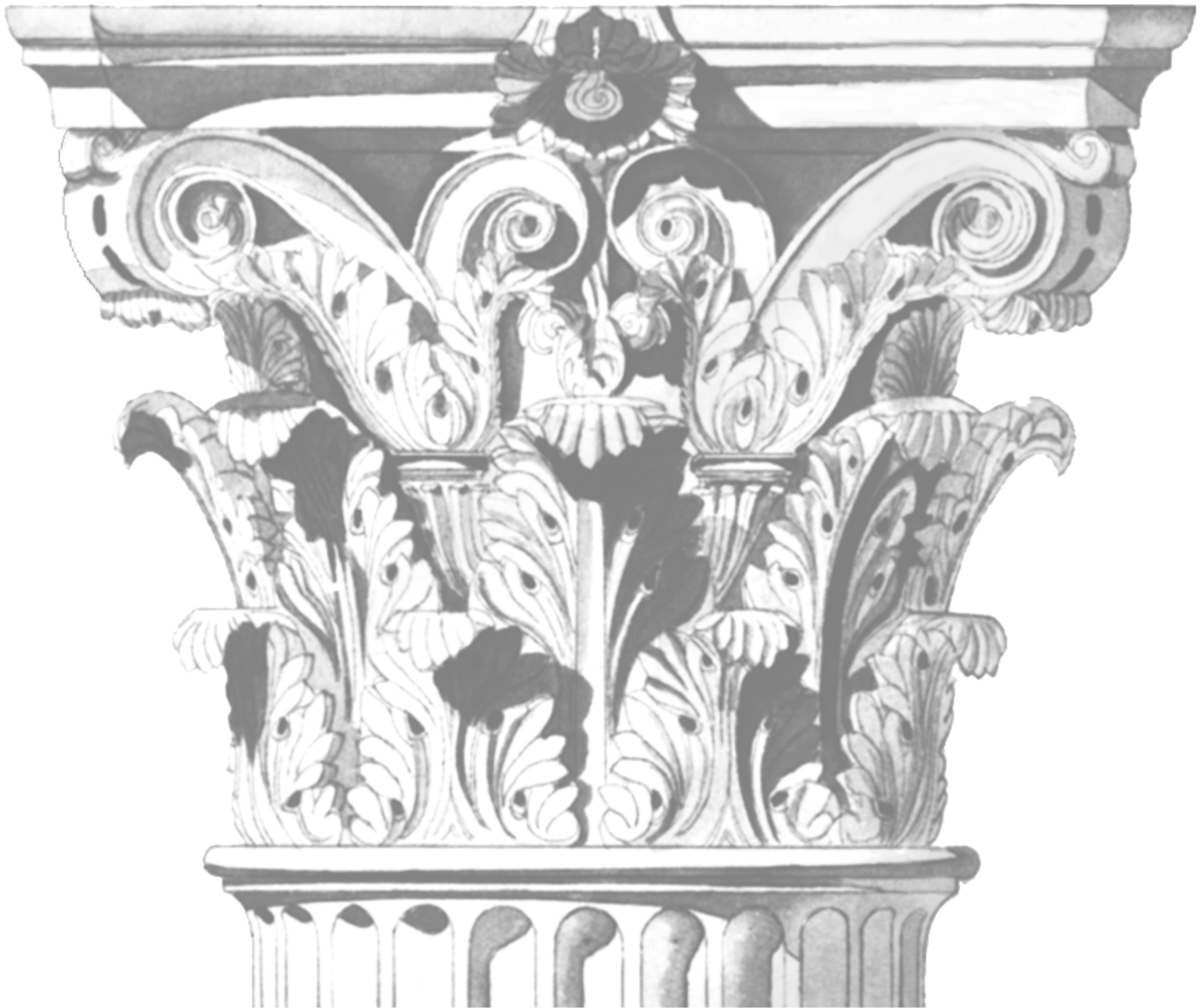

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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Auditorium 1 of the Institute of Dentistry, on April 15th, 2005, at 12 noon

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Abstract

Cancer progression is a complex multi-step process. Two critical steps in tumor growth and invasion are the proteolytic processing of the extracellular matrix environment and the angiogenic switch enabling blood supply into the tumor.

Matrix metalloproteases (MMPs) are a group of proteolytic enzymes involved in physiological and pathological extracellular matrix processing. Trypsinogen, a serine protease, is one of the first proteolytic enzymes characterized. The amount of one of its isoforms, tumor associated trypsinogen-2 (TAT-2) correlates with the malignant phenotype of several forms of cancers. Both of these protease groups are critically dependent on their activation from latent proforms to fully active enzymes. We found that the overproduction of TAT-2 in malignant oral squamous cell carcinoma cell line was associated with elevated proMMP-9 (but not proMMP-2) activation, as well as enhanced cancer cell intravasation in an *in vivo* model. This indicates that TAT-2 and MMP-9 activation play a role in the invasive growth of oral carcinomas.

Proteases are involved in angiogenesis, the formation of new blood vessels, in several ways. One mechanism is the release of cryptic anti-angiogenic molecules from larger extracellular matrix components. Endostatin is one such cryptic endogenous inhibitor of angiogenesis. Certain MMPs were able to cleave endostatin from its parent molecule, collagen XVIII. The endostatin fragments generated by MMP-3, -7, -9, -13 and -20 inhibited angiogenesis in a similar fashion as the native endostatin. The regulation between MMPs and endostatin was shown to be reciprocal, as endostatin was able to block the activation and activities of MMP-2, -9 and -13. The inhibition of these tumor-associated MMPs explains at least in part the anti-tumor activity of endostatin. Endostatin not only affects endothelial cell growth as is usually thought, but it also inhibits the migration of oral carcinoma cells. In addition, cell density and proper concentration were proven to be critical for the activity of endostatin. Arresten is another endogenous inhibitor of angiogenesis and tumor growth derived from type IV collagen. We confirmed that arresten binds to integrin $\alpha 1\beta 1$ on endothelial cell surface. We found that this binding is functionally significant for the anti-angiogenic properties of arresten, as tumors planted to integrin $\alpha 1$ knockout mice or endothelial cells derived from those mice did not respond to arresten treatment.

Keywords: angiogenesis inhibitors, arresten, carcinoma, collagen, endostatins, extracellular matrix, integrins, matrix metalloproteases, trypsinogen

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Oulu, February 2005 Pia Nyberg

Abbreviations

Akt/PKB	protein kinase B
AP-1	activator protein-1
APMA	p-aminophenyl mercuric acetate
bFGF	basic fibroblast growth factor
BM	basement membrane
BSA	bovine serum albumin
CAM	chorioallantoic membrane
cDNA	complementary deoxyribo nucleic acid
CMT	chemically modified tetracyclin
CO_2	carbon dioxide
cRGD	cyclic arginine-glysine-aspartate
DMEM	Dulbecco's Modified eagle's medium
DMSO	dimethyl sulfoxide
dNTP	deoxy nucleotide triphosphate
ECM	extracellular matrix
EDTA	ethylene diamino tetraacetic acid
EGF	epiderman growth factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal regulated protein kinase
ETS	E-twenty-six specific sequence
FBS	fetal bovine serum
GPI	glycosyl-phosphatidyl-inositol
HepG2	hepatoblastoma cell line
HSC-3	human tongue squamous cell carcinoma cell line
HSPG	heparin sulphate proteoglycan
HUVEC	human umbilical vein endothelial cell line
IFMA	immunofluorometric assay
IGF	insulin-like growth factor
IHGK	human papillomavirus 16 immortalized human gingival keratinocytes
IL	interleukin
kDa	kilodalton

KGF	keratinocyte growth factor
LEF/TCF	lymphoid enhancer binding factor/T-cell factor
MAPK	mitogen activated protein kinase
MLEC	mouse lung endothelial cell
MMP	matrix metalloprotease
mRNA	messenger ribonucleic acid
MT	membrane-type
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetradodium bromide
NC	non-collagenous
NF-κB	nuclear factor κB
NGF	nerve growth factor
NO	nitric oxide
PAI	plasminogen activator inhibitor
PAR	plasminogen activator receptor
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEA	polyomavirus enhancer A element
PEDF	pigment epithelium derived factor
PSTI	pancreatic secretory trypsin inhibitor
PVDF	polyvinylidene difluoride
RAD	arginine-alanine-aspartate
rh	recombinant human
RT-PCR	reverse transcriptase polymerase chain reaction
SCC	squamous cell carcinoma
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacryl amide gel electrophoresis
SE	standard error
SFCM	serum free conditioned media
SP-1	stimulating protein
SPARC	secreted protein acidic and rich in cysteine
TAT-2	tumor-associated trypsinogen-2
TGF-β	transforming growth factor β
TIMP	tissue inhibitor of matrix metalloprotease
TNF-α	tumor necrosis factor α
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
VEGF-R	vascular endothelial growth factor receptor

List of original articles

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

I. Nyberg P, Moilanen M, Paju A, Sarin A, Stenman U-H, Sorsa T & Salo T (2002) MMP-9 activation by tumor trypsin-2 enhances *in vivo* invasion of human tongue carcinoma cells. J. Dent. Res. 81: 831-835

II. Heljasvaara R, Nyberg P, Luostarinen J, Parikka M, Heikkilä P, Rehn M, Sorsa T, Salo T & Pihlajaniemi T (2005) Generation of biologically active endostatin-containing fragments from human collagen XVIII by distinct matrix metalloproteases. Exp. Cell Res., in press

III. Nyberg P, Heikkilä P, Sorsa T, Luostarinen J, Heljasvaara R, Stenman U-H, Pihlajaniemi T & Salo T (2003) Endostatin inhibits human tongue carcinoma cell invasion and intravasation and blocks the activation of matrix metalloproteases-2, -9 and -13. J. Biol. Chem. 278:22404-22411

IV. Nyberg P, Sugimoto H, Colorado P, Sund M, Soubasakos M, Holthaus K, Lively J, Sudhakar A, Salo T, Pozzi A & Kalluri R Arresten, a constituent of extracellular matrix degradome, is an $\alpha 1\beta 1$ integrin dependent angiogenesis and tumor growth suppressor. Manuscript, 2005

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1 Introduction

The search for the pathogenesis and treatment of cancer has been continuing for over half a century, and the complexity of the scientific literature is at times overwhelming. However, detailed understanding of the principal pathogenic mechanisms common to all cancers is emerging. Among the key mechanisms there are two pivotal critical steps in cancer growth and progression: the proteolytic processing of the extracellular matrix environment, especially the basement membranes, and the angiogenic switch, which have been characterized during this work. Matrix metalloproteases (MMPs) are a group of highly conserved endopeptidases that are collectively capable of degrading almost all components of the extracellular matrix and basement membranes. This protease group is critically dependent on the activation of latent proforms to fully active enzymes. The activity of MMPs needs to be strictly regulated. Trypsinogen was one of the first matrix serine proteases to be isolated and characterized; it was later discovered that one of its isoenzymes, tumor associated trypsinogen-2 (TAT-2), correlates with the malignant phenotype of several cancers. Angiogenesis, the formation of new blood vessels, is likely regulated by a delicate balance between endogenous pro-angiogenic and anti-angiogenic factors. It has been suggested that the progression of tumors requires the disruption of this balance. Many of the endogenous anti-angiogenic factors are cryptic fragments of larger extracellular matrix constituents that display no effects on angiogenesis as intact proteins. Endostatin, a fragment of collagen XVIII, and arresten, a fragment of collagen IV, are two examples of such cryptic endogenous inhibitors of angiogenesis.

The current results address the complex activation cascade networks of TAT-2 and MMP. In addition to the involvement of MMPs in enhancing tumor cell migration by digesting the extracellular matrix, we found that they are also capable of inhibiting cancer progression by liberating endostatin. The anti-angiogenic and anti-tumor mechanisms of endostatin have been extensively characterized; one of them seems to be the ability of endostatin to inhibit the activities of certain MMPs. Cell surface integrins have been proposed to play a major role in the action of endogenous inhibitors of angiogenesis. The anti-angiogenic activity of arresten seems to be dependent on integrin $\alpha1\beta1$.

2 Review of the literature

2.1 Tumor growth and invasion

Recent literature indicates that tumorigenesis is a complex multi-step process, and that these steps reflect alterations that drive the progressive transformation of normal cells into highly malignant ones (reviewed by Hanahan & Weinberg 2000). Pathological analyses of several organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normal cells via series of premalignant states into invasive cancer cells (Foulds 1954). Transformation of cultured cells follows a similar multi-step pattern: rodent cells require at least two introduced genetic changes, while in human cells numerous genetic changes are needed for malignancy (Hahn *et al.* 1999). There seem to be six essential alterations in cell physiology that collectively dictate malignant growth (Figure 1): (i) self-sufficiency in growth signals, (ii) insensitivity to growth inhibitory signals, (iii) evasion of programmed cell death, (iv) limitless replicative potential, (v) sustained angiogenesis and (vi) tissue invasion and metastasis (Hanahan & Weinberg 2000).

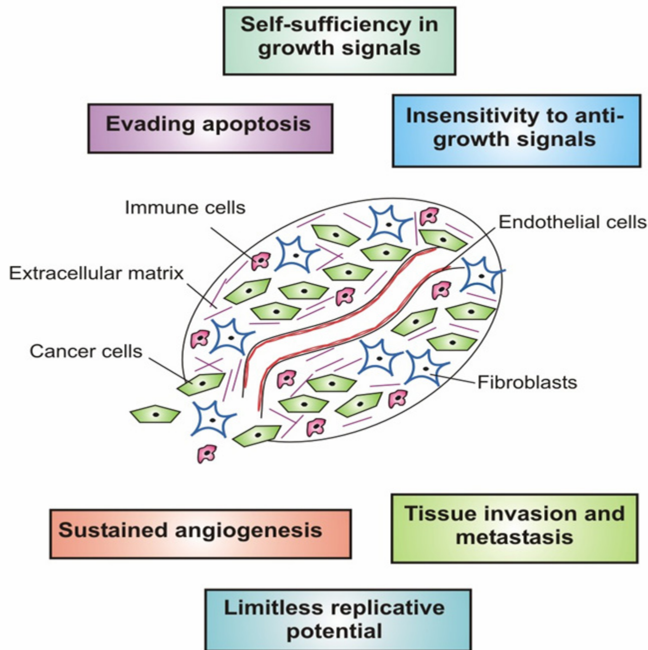

Fig. 1. Acquired capabilities of cancer. Most cancers have acquired six essential alterations that enable limitless growth. The order in which these alterations are acquired varies quite a lot within various cancer types and subtypes. In some cases, one genetic alteration can lead to more than one of these capabilities (e.g. the loss of p53 tumor suppressor can facilitate both angiogenesis and resistance to apoptosis), and in other cases the alteration happens in collaboration with two or more genetic changes. Tumors are complex tissues: in addition to cancer cells also other cell types, including surrounding stromal fibroblasts, endothelial cells and immune cells as well as the extracellular matrix play a role in cancer progression. (Modified from Hanahan & Weinberg 2000)

At some point during the development of most human cancers, pioneer cells move out from the primary tumor mass, invade the tissue and travel to distant sites where they may succeed in founding new colonies called metastases. Metastases are the cause of about 90% of human cancer deaths (Sporn 1996). During invasion and metastasis, the physical coupling of cells to each other and to the microenvironment changes, and the proteases in the extracellular milieu or on cell membranes will be both upregulated and activated (Hanahan & Weinberg 2000). Intravasation, when cells invade into the bloodstream or lymphatic vessels, and extravasation are key steps of the carcinoma process leading to metastasis. The metastatic cells break various physical barriers consisting of basement membranes, extracellular matrix and layers of tightly associated cells. The activity of proteolytic enzymes is involved (Mignatti & Rifkin 1993).

There are more than 100 distinct types of cancer, and subtypes can be found within specific organs (Hanahan & Weinberg 2000). Oral squamous cell carcinoma is a malignant tumor of the squamous epithelial cells of the oral cavity. Oral squamous cell

carcinomas (SCC) are aggressive, and they have high potential for invasiveness associated with high mortality (Silverman 1988, Muir & Weiland 1995, Macfarlane *et al.* 1996). Globally, oral SCC is the twelfth most common cancer, representing about 5% of all malignancies (Parkin *et al.* 1990). In Finland, there are about 250 new cases per year, and the number has been increasing (Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi last updated in August, 2004). In the United States, 15,600 people died of oral SCC in 2000 (American Cancer Society 2000). Nearly half of the patients with oral SCC present evidence of lymph node metastasis. The 5-year survival rate is less than 50% for patients with a single unilateral lymph node metastasis and less than 25% for patients with bilateral metastases (American Cancer Society 2000, Som 1992).

2.2 Proteolysis of the extracellular matrix

The extracellular matrix (ECM) is a three-dimensional structure of heterogeneous macromolecules. In addition to providing structural support to cells and tissues, this network supports adhesion of cells, transmits signals through adhesion receptors, and binds, stores and presents growth factors. The ECM can take many different shapes depending on the requirements of the tissue, such as bone, cartilage and skin (reviewed by Gustafsson & Fässler 2000). Different cells and even different types of tumor cells secrete a characteristic pattern of matrix proteins (Alitalo *et al.* 1981). Basement membranes are specialized sheet-like matrix structures being closely attached to cells. They are present practically everywhere in the body, separating organ cells, epithelia and endothelia from each other and from interstitial connective tissue. The main constituent of basement membranes is type IV collagen that forms a network together with other basement membrane molecules, such as laminins, nidogens, fibulins, SPARC (secreted protein acidic and rich in cysteine), fibronectin, type XV and XVIII collagens, and heparin sulphate proteoglycans, such as perlecan (Yurchenco *et al.* 2004). Basement membranes function as barriers, polarize epithelial cells, shape tissue structures, guide and support migrating cells and act as selective filters in the kidney (Gustafsson & Fässler 2000). The constituents of the basement membranes can vary depending on the location, and even within the same tissue; not all vascular basement membranes are the same, for example. The BMs can also have some structural abnormalities in pathological conditions, such as in blood vessels and endothelial sprouts in tumors (Kalluri 2003, Baluk *et al.* 2003).

The degradation of the extracellular matrix promotes cell migration (Tryggvason *et al.* 1987). As early as in 1949 investigators noted that there was an association between a variety of protease activities and the invasive behavior of tumor cells (Gersch & Catchpole 1949). Later in the 1970s these early findings were explored further (Taylor *et al.* 1970, Poole *et al.* 1978, Liotta *et al.* 1979, Liotta *et al.* 1980). There are four major groups of proteases: the aspartate and cysteine enzymes, which function at low pH and are mainly involved in the intracellular proteolysis within lysozymes, and the serine and metal-dependent enzymes that are active at neutral pH and are responsible for extracellular proteolysis (Curran & Murray 1999). Although representatives of all four classes of proteolytic enzymes have been implicated in tumor invasion and metastasis

(Table I), serine proteases, particularly plasmin, plasminogen activators and trypsinogens, and matrix metalloproteases (MMPs) have been studied most extensively.

Table 1. Protease families involved in tumor invasion and metastasis (adapted from Mignatti & Rifkin 1993, Overall & Lopez-Otin 2002 and Netzel-Arnett et al. 2003)

Serine proteases	Matrix metalloproteases	Other proteases
Cathepsins G and E	Collagenases	Aspartate proteases (Cathepsins D and E)
Chymase	Gelatinases	Cysteine proteases (Cathepsins B, H, K, L, M, N, O and S)
Chymotrypsin	Matrilysins	Threonine proteases
Membrane bound serine proteases	Membrane-associated MMPs	
Elastase	Stromelysins	
Plasmin	Other MMPs	
Plasminogen activators		
(Tumor-associated) trypsins		
Tryptase		

When proteolytic activity is required, the protease genes are upregulated, the protease inhibitor genes are usually downregulated and the inactive zymogen forms are converted into active enzymes (Werb 1997, Stetler-Stevenson 1999). Serine proteases and MMPs form complex cascades in which they can activate each other. These cascades affect tumor progression in many stages (Fig. 2).

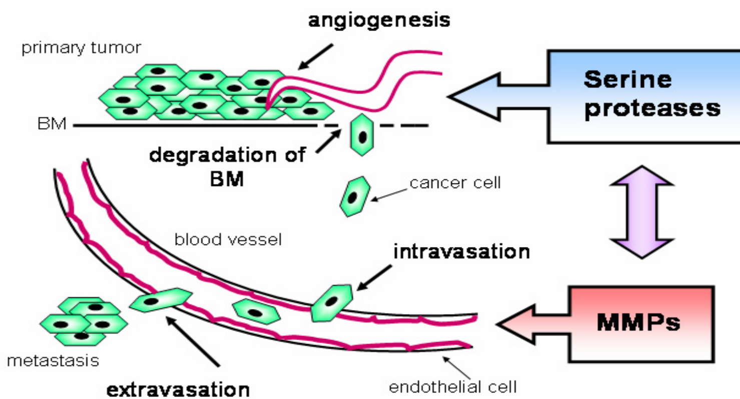

Fig. 2. Schematic presentation of the many steps of cancer growth and invasion affected by serine proteases and metalloproteases.

2.2.1 Plasminogen and plasminogen activators

The plasminogen activator/plasmin system consists of the serine proteases plasminogen and the corresponding active form plasmin, two plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor (PAI) and cell surface receptors (PAR). Plasmin is involved in the proteolysis of the fibrin clot, but it is also able to degrade various extracellular matrix components (Ellis 2003). Plasmin can also activate some MMPs, such as progelatinase A (Keski-Oja *et al.* 1992; Mazzieri *et al.* 1997), prostromelysin-1 (Nagase *et al.* 1990) and latent elastase (Chapman & Stone 1984). Plasminogen has important functions in metastasis, as the spread of tumor from the primary origin is significantly delayed in plasminogen-deficient mice (Bugge *et al.* 1998). The plasminogen factors are serine proteases that were first called cell factors and discovered from the media of transformed cell lines. PAs were later identified from stimulated macrophages and human tumor cell lines (Quigley *et al.* 1974, Rifkin *et al.* 1974, Unkeless *et al.* 1974a, Unkeless *et al.* 1974b). The two types of plasminogen activators seem to have different physiological functions, since tPA is primarily important in wound healing and intravascular fibrinolysis (Saksela & Rifkin 1988, Stubbs *et al.* 1998), while uPA is mainly involved in cell migration and invasion, thus making it important for cancer cell invasion as well (Andreasen *et al.* 1997). Previously it was thought that uPA was only synthesized by the surrounding stromal cells, not by the tumor cells themselves (Bugge *et al.* 1998). Later it turned out that invading tumor cells as well as migrating endothelial cells and leukocytes express the components of the uPA/uPAR system (Mazar *et al.* 1999). The uPA/uPAR interaction is important for tumor-associated angiogenesis, primary tumor growth and metastasis (Crowley *et al.* 1993, Min *et al.* 1996, Ossowski 1996). Plasminogen activation is controlled by plasminogen activator inhibitors, PAI-1, -2 and -3, and α_2-antiplasmin (Juhan-Vague *et al.* 1984, Ginsburg *et al.* 1986, Ellis *et al.* 1990). PAI-1 seems to play the most relevant role in cancer progression (Danø *et al.* 1985). PAI-1 is the primary physiological inhibitor of uPA and tPA. It regulates not only the proteolytic activity of uPA, but also determines the level of uPA bound to uPAR by promoting the rapid endocytosis of the trimolecular uPA–PAI-1–uPAR complex (Conese & Blasi 1995, Blasi 1997). High levels of all uPA, uPAR and PAI-1 in breast cancer tissue are associated with poor prognosis (Grøndahl-Hansen *et al.* 1993 and 1995). The use of antisense mRNA for uPA and uPAR, the use of natural or synthetic serine protease inhibitors or uPAR antagonists all reduced tumor invasion (Min *et al*. 1996, Carmeliet & Collen 1998). Consequently, uPA/uPAR/plasmin antagonists are currently being developed as therapeutic strategies to inhibit tumor angiogenesis and progression (Bajou *et al.* 2001).

It is generally believed that uPA initiates a proteinase cascade at the cell surface, which in turn leads to breakdown of the extracellular matrix and thereby promotes cellular migration. Unexpectedly high levels of PAI-1 are predictive of poor survival prognosis in patients suffering from a variety of different cancers (Pedersen *et al.* 1994a, Pedersen *et al*. 1994b). Even though uPA in general promotes, and PAI-1 suppresses tumor growth in most available experimental tumor models, PAI-1 was recently found to promote tumor growth and angiogenesis (Bajou *et al.* 1998). It has been suggested that plasmin is

involved in the formation of new vessels, but that PAI-1-mediated control of proteolytic breakdown is required, probably to allow vessel stabilization and maturation (Bajou *et al.* 2001).

2.2.2 Trypsinogens

Although plasmin and plasminogen activators are so far the most thoroughly studied tumor-associated serine proteases, trypsinogens also play a significant role in tumor progression. Trypsinogen and its active form trypsin was one of the first enzymes to be isolated and characterized; it was crystallized from human pancreas as early as in 1936 (Kunitz & Northrop 1936). Eight trypsinogen genes have been found, but only three of them, T4, T8 and T9, have been demonstrated to encode a protein (Emi *et al.* 1986, Tani *et al.* 1990, Wiegand *et al.* 1993, Rowen *et al.* 1996, Nyaruhucha *et al.* 1997). Figarella *et al.* (1969) purified two trypsinogen isoforms from pancreatic juice and named them trypsinogen-1 and -2. These isoforms were later called cationic and anionic trypsinogen, respectively, and a third isoform, mesotrypsinogen, was found (Rinderknecht *et al.* 1979, 1984). Scheele *et al.* called these same pancreatic isoforms trypsinogen-3, -1 and -2, respectively (Scheele *et al.* 1981). A fourth trypsinogen isoform was found in the brain in 1993; it has been suggested to be a splicing variant of the same gene that encodes trypsinogen-3/mesotrypsinogen (Wiegand *et al.* 1993). To clarify the nomenclature of trypsinogen isoforms, from now on the trypsinogen isoform (trypsinogen-1/cationic/trypsinogen-3) encoded by T4 gene is called trypsinogen-1, the second isoform (trypsinogen-2/anionic/trypsinogen-1) encoded by T8 gene is called trypsinogen-2 and the isoforms encoded by the T9 gene (mesotrypsinogen/trypsinogen-2 and trypsinogen-4) are called trypsinogen-3 and -4.

Trypsinogen-1, -2 and -3 consist of 247 amino acids, and trypsinogen-4 contains 259/260 amino acids. All trypsinogens contain five disulphide bridges (Guy *et al.* 1978, Wiegand *et al.* 1993). The substrate specificity of trypsinogens is determined by the hydrophobic substrate-binding pocket including three critical conserved amino acids: Asp 189, Gly 216 and Gly 226. Trypsin hydrolyzes the peptide bond on the carboxyl-terminal side of arginine or lysine residues (Craik *et al.* 1985). In addition to digesting dietary proteins, trypsinogens and the corresponding active forms trypsins from various sources are important in proteolytic cascades activating other proteases, such as pro-uPA (Koivunen *et al.* 1989), proMMP-9 (Sorsa *et al.* 1997, Duncan *et al.* 1998) and -2 (Sorsa *et al.* 1997), proMMP-14 (Will *et al.* 1996) and a chymotrypsin-like serine proteinase PSA (prostate specific antigen) (Paju *et al.* 2000).

The latent trypsinogens need to be activated into catalytically competent trypsins. In the digestive tract, secreted trypsinogens are activated by the serine protease enterokinase/enteropeptidase (Lu *et al.* 1999). Normal tissues produce small amounts of enterokinase-like protease (Miyata *et al.* 1998). Trypsin-1 and -2 are also able to autoactivate trypsinogen-1 and -2 (Colomb & Figarella 1979). Trypsinogens can be activated at least *in vitro* by cathepsin B, and *in vivo* cathepsin B participates in the premature activation of trypsinogen during pancreatitis (Figarella *et al.* 1988, Halangk *et al.* 2000). The main endogenous inhibitors of trypsins are α_2-macroglobulin, α_1-

proteinase inhibitor, or α_1-antitrypsin, Kazal-type trypsin inhibitor or pancreatic secretory trypsin inhibitor (PSTI) and tumor-associated trypsin inhibitor (TATI) (Kazal *et al.* 1948, Pubols *et al.* 1974, Huhtala *et al.* 1982). Trypsinogens are not only expressed in the pancreas, as pancreatectomized patients have detectable levels of trypsinogens in the serum (Itkonen *et al.* 1996). Trypsinogen expression has been detected in vascular endothelial cells (Koshikawa *et al.* 1997), epithelial cells of the skin, esophagus, stomach, small intestine, lung, kidney, liver, bile ducts, splenic and neuronal cells (Koshikawa *et al.* 1998) and male genital track and seminal fluid (Paju *et al.* 2000) as well as in many tumors and tumor cell lines that are described in the next chapter.

2.2.2.1 Tumor-associated trypsinogen-2 (TAT-2)

Trypsinogens were first thought to be pancreatic enzymes only involved in the digestive process, but in the early 1980s LaBombardi *et al.* identified a trypsin-like protease in the cell membrane of Walker-256 carcinoma cells (LaBombardi *et al.* 1983). First it was called protease T, but it is currently known as tumor-associated trypsinogen (TAT) (Stenman *et al.* 1988, Koivunen *et al.* 1989). There are two isoforms of tumor-associated trypsinogen, TAT-1 and TAT-2, the latter being the more predominant one (Koivunen *et al.* 1990). TAT-2 and trypsin-2 derived from pancreas have identical aminoterminal sequence, molecular weight (28 kDa) and immunoreactivity, but they differ in their activity against synthetic substrates, enzyme stability and isoelectric point. These differences are probably due to post-translational modification that is different in pancreas and carcinomas (Koivunen *et al.* 1989, 1991, Itkonen *et al.* 1996, Sorsa *et al.* 1997). The nucleotide sequence of TAT-2 and pancreatic trypsin-2 was identical except for one base substitution (G to A) at position 276 (Sorsa *et al.* 1997). TAT-2 was identified when investigators were looking for the target molecule for the tumor-associated trypsin inhibitor (TATI) that is known to be a marker molecule in ovarian carcinomas (Halila *et al.* 1988). TAT-2 could be demonstrated for the first time in ovarian neoplasms (Koivunen *et al.* 1989). Later, tumor-associated trypsinogens have also been shown to be expressed in other carcinomas, such as pancreatic cancer (Ohta *et al.* 1994), hepatocellular and cholangiocarcinomas (Terada *et al.* 1995), ovarian carcinomas (Hirahara *et al.* 1995), lung neoplasms (Kawano *et al.* 1997) and colorectal cancers (Oyama *et al.* 2000), and by various cancer cell lines, such as colon carcinoma, fibrosarcoma, erythroleukemia (Koivunen *et al.* 1991), pancreatic carcinoma (Miszczuk-Jamska *et al.* 1991) and gastric cancer (Koshikawa *et al.* 1992, Kato *et al.* 1998, Miyata *et al.* 1998).

The production of TAT-2 has been shown to correlate with the malignant phenotype of cancers. TAT-2 is the predominant isotype in malignant cancers. The level of TAT-2 correlates with the malignancy and the metastatic potential of tumors (Koivunen *et al.* 1990). Other studies have shown that the amount of TAT-1 is also upregulated in colorectal tumors with a more malignant phenotype, although TAT-2 is the dominant trypsinogen isoform in colon tissue (Williams *et al.* 2001). There is evidence of several possible mechanisms how tumor-associated trypsins increase tumor aggressiveness. Addition of trypsin increases the growth of various cells lines, such as lymphoma cells,

fibroblasts and keratinocytes, by activating their thrombin receptors and plasminogen receptor-2, and possibly also other cell surface receptors, such as growth factor receptors, integrins (Burger 1970, Miyata *et al.* 1998) and proteinase activated receptor PAR-2 (Alm *et al.* 2000). Integrins in particular may be of functional significance, as esophagus carcinoma cells transfected with TAT-1 attached more to vitronectin and fibronectin, and treatment of human melanoma cells with trypsin, chymotrypsin or plasmin stimulates the integrin-mediated cell attachment to vitronectin and fibronectin (Fujii & Imamura 1995, Miyata *et al.* 1998). Furthermore, trypsin promotes integrin α5β1-mediated adhesion to fibronectin, and less efficiently integrin αvβ3-mediated adhesion to vitronectin, and regulates the adhesion and proliferation of human gastric carcinoma cells by inducing PAR-2/G protein signaling (Miyata *et al.* 2000). One important reason for the ability of trypsin to make cells more invasive is its effect on the degradation of the extracellular matrix either on its own or by activating other proteases (Koshikawa *et al.* 1992, Miyata *et al.* 1998). TAT-2 can initiate protease cascades by activating proMMPs, and thus it may promote tumor spreading. It can activate proMMP-9 *in vitro* at an extremely low molar ratio, 1:1000. Furthermore, TAT-2 activated proMMP-9 complexed with the tissue inhibitor of matrix metalloproteases, which is thought to be the major MMP form *in vivo*. TAT-2 also activates proMMP-2 *in vitro*, but much less efficiently (Sorsa *et al.* 1997). This phenomenon has also significance *in vivo*: the higher the amount of trypsin-2 in ovarian tumor cyst fluids, the higher the level of MMP-9 activation. On the other hand, trypsin-2 had no effect on the activation of MMP-2 in these experiments (Paju *et al.* 2001a). Recently, it has been shown that trypsin-2 can also activate other proMMPs, MMP-1, -3, -8, and -13, and it can directly degrade native type I collagen on its own (Moilanen *et al.* 2003). Down-regulation of TAT-2 expression by chemically modified tetracyclines results in reduced human colon adenocarcinoma cell migration (Lukkonen *et al.* 2000). Cancer is not the only pathological condition that is associated with trypsin-2. In inflammatory lung diseases the activity of MMP-9 and -8 was increased in trypsin-2 dependent manner in bronchoalveolar lavage fluid (Prikk *et al.* 2001). The levels of trypsinogen-2 are higher in preterm infants who subsequently develop bronchopulmonary dysplasia leading to inflammation and respiratory distress (Cederqvist *et al.* 2003).

Tumor-associated trypsin is inhibited by tumor-associated trypsin inhibitor, TATI, a 6-kDa peptide originally detected in the urine of ovarian cancer patients (Stenman *et al.* 1982). It is expressed by several tumors and cancer cell lines (Stenman *et al.* 1991) as well as in normal renal tissue (Lukkonen *et al.* 1999). TATI is also a useful serum marker for ovarian cancers (Halila *et al.* 1988, Venesmaa *et al.* 1998), renal cell carcinomas (Paju *et al.* 2001) and squamous cell carcinomas of the head and neck (Goumas *et al.* 1997). The TATI gene is identical with the Kazal-type trypsin inhibitor/pancreatic secretory trypsin inhibitor (PSTI) discovered earlier (Pubols *et al.* 1974, Horii *et al.* 1987). Lys-18 and Ile-19 form the reactive site for TATI that serves as a specific target substrate for trypsin in a 1:1 molar ratio (Pubols *et al.* 1974, Bartelt *et al.* 1977). In tumors, high concentrations of TATI may protect the ECM from trypsin-mediated proteolysis, thus inhibiting invasion (Stenman *et al.* 1991). However, lower concentrations might only partially protect the ECM to an extent that is needed for cell adhesion. This might explain the fact that ovarian cancer patients with elevated amounts of TATI in serum sometimes have a worse prognosis than patients with normal TATI levels (Andreasen *et al.* 1997, Venesmaa *et al.* 1998).

2.2.3 Matrix metalloproteases (MMPs)

Members of the metzincin superfamily belonging to the metalloproteinase family have a highly conserved motif containing three histidines that bind zinc at the catalytic site and a conserved methionine turn that sits beneath the active site zinc (Stöcker *et al.* 1995). The metzincins are further subdivided into four groups: 1) serralysins, 2) astacins, 3) adamalysins, ADAMs (proteases with a disintegrin and metalloprotease domain), ADAMTs (the disintegrin domain is followed by thrombospondin type I repeats) and 4) matrix metalloproteases (Stöcker *et al.* 1995, Sternlicht & Werb 2001).

The MMPs, also called matrixins, are a family of zinc-dependent endoproteases that are able to degrade essentially all extracellular matrix and basement membrane components. The first MMP discovered was a collagenase (MMP-1) in the tail of a tadpole undergoing metamorphosis (Gross & Lapier 1962). At present, 24 different vertebrate MMPs have been identified, 23 of which have been found in humans (Visse & Nagase 2003). In addition, there are many non-vertebrate MMPs, such as the embryonic sea urchin hatching enzyme envelysin (Lepage & Cache 1990), several *Caenorhabditis elegans* MMPs (Wada *et al.* 1998), a *Drosophila* MMP (Llano *et al.* 2000), an MMP in *Hydra* regulating foot process development (Leontovich *et al.* 2000) and an MMP in the flowering mustard plant *Arabidopsis thaliana* (Maidment *et al.* 1999). The members of the MMP family have many common features. They are genetically distinct but structurally related. Their amino acid sequences are homologous to each other, they all have the zinc-binding motif HEXGHXXGXXH in the catalytic domain, and nearly all of them have the cysteine switch motif PRCGXPD in the propeptide maintaining the latent zymogen form (Visse & Nagase 2003). Individual MMPs are referred to with their common names or according to a sequential numeric nomenclature based on the order of discovery. On the basis of specificity, sequence similarity and domain organization vertebrate MMPs are divided into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs (Table 2 and Figure 3). The MMPs can also be classified according to their structural properties into eight subgroups: simple hemopexin domain-containing MMPs (MMP-1, -3, -8, 10, -12, -13, -19, -19, -20, -22 and -27), gelatin-binding MMPs (MMP-2 and -9), furin-activated secreted MMPs (MMP-11 and -28), vitronectin-like insert MMPs (MMP-21), minimal domain MMPs (MMP-7 and -26), type I transmembrane MMPs (MMP-14, -15, -16 and -24), GPI-linked MMPs (MMP-17 and -26) and type II transmembrane MMPs (MMP-23) (Egeblad & Werb 2002).

Table 2. Vertebrate MMPs and substrates (modified from Sternlicht & Werb 2001, Overall 2002, Visse & Nagase 2003, Pirilä et al. 2003)

Name	Common names	Some substrates
MMP-1	Collagenase-1	Coll III>I>II, VII, VIII, X,XI, Gel, En, Tn, Per, Lm, Ag, Cas, proTNF-α, proIL-1β, IL-1β, IGF-BP, proMMP-1 and -2, α1-PI, α1-ACT, α2-MG, MCPs
MMP-2	Gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase	Gel, Coll I, III, IV, V, VII, X, XI, El, Fn, Lm, Ag, Vn, Dc, Pl, proTGF-β1, proTNF-α, proIL-1β, IGF-BP, FGF-R1, proMMP-1, -2 and -13, α1-PI, α2-MG, MCP-3, On
MMP-3	Stromelysin-1, transin-1	Ag, Ln, Fn, Coll III, IV, V, IX, X, XI, XVIII, Gel, Dc, En, Per, Tn, Vn, Fb, El, Lm, Cas, proTNF-α, pro-HB-EGF, proIL-1β, Per, Pl, E-cadherin, IGF-BP, proMMP-1, -3, -7, -8, -9, and -13, α1-PI, α1-ACT, α2-MG, MCP-3, On
MMP-7	Matrilysin, PUMP-1	Fn, Lm, Coll I, IV, V, IX, X, XI, XVIII, Gel, Ag, En, Tn, Vn, Dc, Fb, Cell surface FasL, proTNF-α, E-cadherin, β4 integrin, Pl, proMMP-1, -2, -7, and -9, α1-PI, α2-MG, On
MMP-8	Collagenase-2, neutrophil collagenase	Coll I>II>III, VII, X, Gel, En, Ag, Tn, proTNF-α, IGF-BP, proMMP-8, α1-PI, α2-MG, MCP-1
MMP-9	Gelatinase B, 92-kDa gelatinase	Gel, Coll I, IV, V, VII, X, XI, XVIII, El, Dc, Fn, Lm, Ag, Vn, Cas, proIL-8, PF-4, proTGF-β1, proTNF-α, proIL-1β, FGF-R1, Pl, proMMP-2, -9 and -13, α1-PI, α2-MG. ICAM-1, On
MMP-10	Stromelysin-2, transin-2	Coll I, III, IV, V, Gel, El, Cas, Fn, Lm, Pgl, proMMP-1, -8 and -10
MMP-11	Stromelysin-3	Fn, Lm, Ag, IGF-BP, α1-PI, α2-MG
MMP-12	Metalloelastase, macrophage elastase	El, Coll I, IV, Fn, Lm, Pgl, Fb, Pl, proTNF-α, α1-PI
MMP-13	Collagenase-3	Coll II>III>I, VII, X, XVIII, Gel, En, Tn, Ag, Lm, proTNF-α, proMMP-9 and 13, α1-ACT, α2-MG, MCP-3, On
MMP-14	MT1-MMP	Coll I, II, III, Gel, Fn, Lm, Vn, Ag, Cell surface CD44 and tTG, proTNF-α, proMMP-2 and -13, pro αv integrin, α1-PI, α2-MG, MCP-3
MMP-15	MT2-MMP	Pgl, proMMP-2, Cell surface tTG, proTNF-α
MMP-16	MT3-MMP	Coll III, Fn, proMMP-2, Cell surface tTG, proTNF-α
MMP-17	MT4-MMP	Gel, Fb, proMMP-2, proTNF-α
MMP-18 (frog)	Collagenase-4	Coll I, II, III, Gel
MMP-19	RASI, stromelysin-4	Coll I, IV, Gel, Lm, Fn, Tn, En, Ag, Fb, Cas
MMP-20	enamelysin	Amg, Ag, Coll XVIII, Lm

Abbreviations used: α1-ACT, antichymotrypsin; α1-MG, macroglobulin; α1-PI, α2-proteinase inhibitor; Amg, amelogenin; Ag, aggregan,, Cas, casein; Coll, collagen; Dc, decorin; El, elastin; En, entactin; Fb, fibrin/fibrinogen; FGF-R, fibroblast growth factor receptor; Fn, fibronectin; Gel, gelatin; HB-EGF, heparin binding epithelial growth factor-like growth factor; ICAM, intercellular cell adhesion molecule; IGF-BP, insulin-like growth factor binding protein; IL, interleukin; Lm, laminin; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteases; On, osteonectin; Per, perlecan; PF, platelet factor; Pgl, proteoglycans; Pl, plasminogen; TGF, transforming growth factor; Tn, tenascin; TNF, tumor necrosis factor; tTG, tissue transglutaminase; Vn, vitronectin

Table 2. (Continued)

Name	Common names	Some substrates
MMP-21	Xenopus MMP (human homolog)	Gel
MMP-22 (chicken)	Chicken homolog of MMP-27	
MMP-23	Cysteine array (CA) MMP	Gel
MMP-24	MT5-MMP	Fn, Pgl, Gel, proMMP-2
MMP-25	MT6-MMP	Coll IV, Gel, Fn, Pgl, Ln-1, Fb, proMMP-2 and -9, α1-PI
MMP-26	Matrilysin-2, endometase	Coll IV, Gel, Fn, Fb, IGF-BP, proMMP-9, α1-PI
MMP-27		
MMP-28	Epilysin	Cas

Abbreviations used: α1-ACT, antichymotrypsin; α1-MG, macroglobulin; α1-PI, α2-proteinase inhibitor; Amg, amelogenin; Ag, aggregan,, Cas, casein; Coll, collagen; Dc, decorin; El, elastin; En, entactin; Fb, fibrin/fibrinogen; FGF-R, fibroblast growth factor receptor; Fn, fibronectin; Gel, gelatin; HB-EGF, heparin binding epithelial growth factor-like growth factor; ICAM, intercellular cell adhesion molecule; IGF-BP, insulin-like growth factor binding protein; IL, interleukin; Lm, laminin; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteases; On, osteonectin; Per, perlecan; PF, platelet factor; Pgl, proteoglycans; Pl, plasminogen; TGF, transforming growth factor; Tn, tenascin; TNF, tumor necrosis factor; tTG, tissue transglutaminase; Vn, vitronectin

In addition to the zinc-binding catalytic site, all MMPs have an N-terminal signal sequence (predomain) followed by the propeptide that maintains the enzyme in latent inactive stage until it is removed or disrupted. All MMPs, except MMP-7, -23 and -26, have a hemopexin-like domain that is linked to the catalytic domain and that can influence the binding of some substrates (Sanchez-Lopez *et al.* 1993, Gururajan *et al.* 1998, Park *et al.* 2000). There is variation in the length and composition of the hinge region of different MMPs (Knäuper *et al.* 1997). The gelatinases, MMP-2 and -9, are further distinguished by the insertion of fibronectin type II-resembling repeats within their catalytic domain (Murphy *et al.* 1994). The MT-MMPs have a single-pass transmembrane domain anchoring it to the cell membrane (Sato *et al.* 1994, Sternlicht & Werb 2001). The domain structure of MMPs is shown in Figure 3.

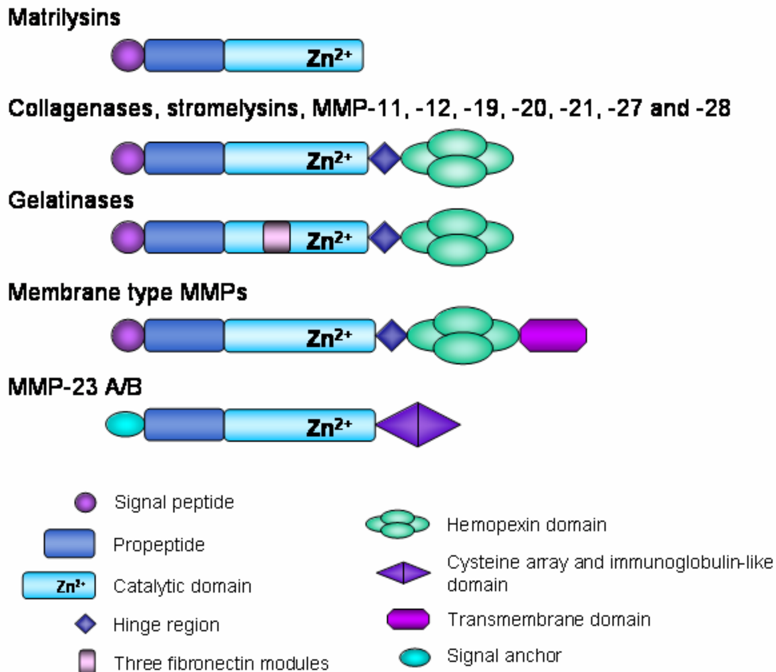

Fig. 3. The domain structure of human MMPs. (Modified from Egeblad & Werb 2002, Visse & Nagase 2003)

MMPs cleave the peptide bond before a residue with a hydrophobic side chain, such as Leu, Ile, Met, Phe or Tyr. MMP-12 also cleaves the bond before a charged residue X-Lys (Gronski *et al.* 1997). The catalytic domain dictates the cleavage site specificity through the active site cleft, through the specificity of binding sites nearby the active site cleft that bind residues next to the scissile peptide bond, and through secondary substrate binding sites outside the active site itself (Overall 2002). The hydrophobic residues fit into the active site specificity pocket, whose size and shape can differ greatly among MMPs (Bode *et al.* 1999). Even though the major function of MMPs has been thought to be the degradation of ECM in tissue resorption and remodeling, it should also be noted that the ECM serves as a reservoir for biologically active molecules that are processed and released by MMPs. Therefore the ECM processing by MMPs can alter cellular behavior and phenotypes (Visse & Nagase 2003). The MMPs are involved in various physiological and pathological conditions. They play a role in many events of reproduction, from ovulation to blastocyst implantation, embryonic development and organ morphogenesis. MMPs are also needed during wound healing, angiogenesis, bone remodeling, nerve growth, mammary gland development, inflammation and apoptosis. In addition to normal physiological events, MMPs are associated with many tissue destructive diseases, such as cancer, arthritis, skin diseases, chronic wound healing, liver fibrosis, cardiovascular diseases, kidney diseases, periodontitis and gastric ulceration (Kähäri & Saarialho-Kere

1997, Nagase & Woessner 1999). The MMPs are tightly regulated, as they need to be present in the right cell type and pericellular location at the right time and in the right amount. Regulation occurs at the transcriptional level and at the protein level via a delicate balance between the activators and inhibitors as well as cell surface localization (Sternlicht & Werb 2001).

2.2.3.1 Collagenases

The collagenase group consists of the mammalian collagenases MMP-1, -8 and -13 and frog *Xenopus* collagenase MMP-18. All collagenases are able to cleave the interstitial fibrillar collagens I, II and III at a specific site into characteristic N-terminal ¾ α^A- and C-terminal ¼ α^B-fragments (Kähäri & Saarialho-Kere 1999, Visse & Nagase 2003).

MMP-1 (collagenase-1) was the first collagenase and the first MMP to be discovered when it was identified from the tail of a tadpole going through metamorphosis (Gross & Lapier 1962). The MMP-1 gene was cloned and sequenced from human fibroblasts (Goldberg *et al.* 1986). In addition to the fibrillar collagens, MMP-1 cleaves other ECM molecules, such as type VII, VIII, X collagens, fibronectin and gelatin, and proteolytically activates proMMP-2 and -9 (Birkedahl-Hansen *et al.* 1993, Chandler *et al.* 1997). Degradation of type I collagen by MMP-1 affects keratinocyte migration and re-epithelialization (Pilcher *et al.* 1997), degradation of fibronectin affected cell migration (Watanabe *et al.* 2000). MMP-1 processes IL-1 from the precursor and thus has a proinflammatory effect (Schonbeck *et al.* 1998), but it also has anti-inflammatory effects by cleavage of monocyte chemoattractant protein-3 and by further degrading IL-1 (Ito *et al.* 1996, McQuibban *et al.* 2002). It increases the bioavailability of IGF-1 and cell proliferation (Fowlkes *et al.* 1994). The expression of MMP-1 is increased in various cancers and inflammatory diseases (Johansson *et al.* 2000). MMP-1 expression has been shown in epithelial cells within the tumor island and particularly in the fibrous connective tissue adjacent to the tumor (Gray *et al.* 1992, Thomas *et al.* 1999).

MMP-8 (neutrophil collagenase, collagenase-2) was previously thought to be synthesized only in polymorphonuclear neutrophils before the cells leave the bone marrow during neutrophil development (Mainardi *et al.* 1991). However, it was later discovered that MMP-8 is expressed by a broad range of different tissues and cell types, such as squamous cell carcinomas of the tongue (Moilanen *et al.* 2002), melanoma cells (Giambernardi *et al.* 1998), chondrocytes (Cole *et al.* 1996), odontoblasts (Palosaari *et al.* 2000) rheumatoid fibroblasts and endothelial cells (Hanemaaijer *et al.* 1997), macrophages (Kiili *et l.* 2002) and bronchial (Prikk *et al.* 2001) and gingival sulcular epithelial cells (Tervahartiala *et al.*2000). The degree of glycosylation of MMP-8 varies, and therefore the molecular mass of MMP-8 ranges between 50 and 75 kDa (Hasty *et al.* 1986, Mallya *et al.* 1990, Hanemaaijer *et al.* 1997).

MMP-13 (collagenase-3) was cloned and identified from human breast carcinomas (Freije *et al.* 1994). MMP-13 is expressed by epithelial cells at the invading tumor front and stromal fibroblasts (Airola *et al.* 1997, Johansson *et al.* 1997) as well as by many malignant tumors, cultured carcinoma cells and inflammatory diseases (Giambernardi *et al.* 1997, Kähäri & Saarialho-Kere 1999). In addition to being involved in cancer and

inflammation, such as intestinal ulcers (Kähäri & Saarialho-Kere 1999), it is involved in osteoclast activation (Holliday *et al.* 1997), enhanced collagen affinity of ECM protein BM-40/SPARC/osteonectin (Sasaki *et al.* 1997) and release of bFGF (Whitelock *et al.* 1996). It is suggested that MMP-13 might have a particular role in bone and cartilage remodeling (Vincenti & Brinkerhoff 2002).

MMP-18 was discovered from the frog *Xenopus laevis*, and the corresponding human enzyme is not known. It degrades type I collagen and gelatin (Stolow *et al.* 1996).

2.2.3.2 Gelatinases

The two members of this group, MMP-2 (gelatinase A, 72 kDa type IV collagenase) and MMP-9 (gelatinase B, 92 kDa type IV collagenase) were originally named after their ability to readily digest type IV collagen and gelatin, the denatured form of collagens (Salo *et al.* 1983, Fessler *et al.* 1984). Both of these enzymes have been shown to be important in cancer progression and invasion (Tryggvason *et al.* 1987, Stetler-Stevenson *et al.* 1993, Chambers & Matrisian 1997). The unique structural feature of gelatinases distinguishing them from other MMPs is the insertion of three repeats of type II fibronectin domain into the catalytic domain, which bind to gelatin, collagens and laminin (Allan *et al.* 1995). Although MMP-2 and MMP-9 are closely related enzymes, they differ in terms of regulation of expression, activation, and glycosylation as well as substrate selectivity (Sternlich & Werb 2001, Overall 2002).

MMP-2 was first identified and purified from metastatic murine tumors (Liotta *et al.* 1979, Salo *et al.* 1983) as well as from various normal and malignant adherent cells (Vartio & Vaheri 1981). It is secreted to the extracellular milieu as a latent 72-kDa form that is activated to 62-66-kDa forms (Murphy *et al.* 1985, Mollet *et al.* 1990). MMP-2 can activate itself by autoactivation, leading to the formation of multiple smaller activation products in addition to the 62-kDa active enzyme (Bergmann *et al.* 1995). ProMMP-2 lacking the hinge region and hemopexin-like domain has a molecular size of 48 kDa, which is converted to the 42-kDa form possessing the same kind of activity as the native 62-kDa active form (Lee *et al.* 2002). MMP-2 null mice do not have any apparent abnormalities, but mutations in MMP-2 cause bone resorption and arthritis, suggesting an important role for MMP-2 in human osteogenesis (Martignetti *et al.* 2001). MMP-2 expression has been shown to relate to lymph node metastasis (Kusukawa *et al.* 1993). However, the MMP-2 present in carcinomas *in vivo* is usually not derived from tumor cells, but from the surrounding stromal cells (Pyke *et al.* 1992). MMP-2 also affects cell migration by cleavage of fibronectin and laminin-5 (Giannelli *et al.* 1997), neurite outgrowth, inflammation and increased bioavailability of TGF-β (Imai *et al.* 1997, Visse & Nagase 2003).

MMP-9 was identified as a gelatin-binding protein synthesized by human macrophages (Vartio *et al.* 1982), and the cDNA was cloned from transformed lung fibroblasts (Wilhelm *et al.* 1989) and later from human fibrosarcoma cells (Huhtala *et al.* 1991). During activation, the latent 92-kDa enzyme is converted to the active forms of several sizes: 63- and 77-82-kDa forms (Sorsa *et al.* 1997, Duncan *et al.* 1998). Surprisingly, Bannikov *et al.* (2002) found that upon binding, the purified proMMP-9

acquired activity against gelatin and synthetic peptide substrates, although its propeptide remained intact. This suggests that sometimes MMP-9 binding to a ligand or to a substrate may lead to a disengagement of the propeptide from the active site leading to activation without proteolysis, and thus no changes in the molecular size are necessarily required for activation. In general, most cells secrete MMP-2 constitutively, whereas the expression of MMP-9 needs to be induced by adequate triggering. Monocytes, dendritic cells, lymphocytes, endothelial cells, epithelial cells and osteoblasts have been shown to produce gelatinase B (Opdenakker *et al.* 2001). During embryogenesis, MMP-9 expression is strictly regulated, and strong expression is detected especially in the osteoclasts (Reponen *et al.* 1994). Later, it was shown that MMP-9 knockout mice exhibit abnormal skeletal growth plate vascularization and ossification (Vu *et al.* 1998). MMP-9 is one of the most important MMPs in tumor progression and metastasis, in addition to being involved in reproduction, wound healing, autoimmune skin blistering disease, aortic aneurysms, angiogenesis and inflammatory diseases (Sternlich & Werb 2001). The interaction of MMP-9 and many cytokines and growth factors is one important mechanism how MMP-9 regulates cell behavior. The processing of the immunomodulating cytokine IL-1 by gelatinase B is an excellent example of the regulation of cytokine activity by proteolysis (Schönbeck *et al.* 1998). In addition, MMP-9 is involved in retrieving VEGF from the extracellular matrix (Bergers *et al.* 2000).

2.2.3.3 Stromelysins

MMP-3 (stromelysin-1) and MMP-10 (stromelysin-2) have similar substrate specificities, but MMP-3 is more efficient. (Visse & Nagase 2003). MMP-3 and -10 are expressed by fibroblasts and normal and transformed epithelial cells (Giambernardi *et al.* 1998, Kähäri & Saarialho-Kere 1999, Johansson *et al.* 2000). MMP-3 is also vastly expressed in the colon; MMP-3 deficient mice are unable to control bacterial infections in the colon, suggesting that MMP3 is important in defense against bacteria (Li *et al.* 2004). MMP-10, which was identified from mouse wound cDNA library (Madlener &Werner 1997), is much less characterized than MMP-3. However, it is unique in its exclusive expression on the tip of migrating epithelia in skin wound healing and in inflammatory bowel disease (Krampert *et al.* 2004). Although MMP-11 is also called stromelysin-3, its substrate specificity and structure are quite different from the other members of the stromelysin family (Visse & Nagase 2003). It is involved in wound healing, reproduction and cancer development (Luo *et al.* 2002).

2.2.3.4 Matrilysins

The matrilysins, MMP-7 (matrilysin-1) and MMP-26 (matrilysin-2 or endometase), are characterized by the lack of the hemopexin domain. MMP-7 was first cloned from a mixed tumor library. Elastin is the most important substrate, but it also digests various other ECM components and cell surface molecules, such as proTNF-α and E-cadherin (Wilson & Matrisian 1996). MMP-26 was isolated from human endometrial tumor

library. In addition to endometrial and other tumors, it is also expressed in normal placenta and uterus (Uria & Lopez-Otin 2000, Park *et al.* 2000).

2.2.3.5 MT-MMPs

There are six membrane-type MMPs: MMP-14, -15, -16, 17, -24 and -25, or MT1-, MT2, MT3-, MT4-, MT5- and MT6-MMP (Sato *et al.* 1994, Will & Hinzmann 1995, Takino *et al.* 1995, Puente *et al.* 1999, Llano *et al.* 1999, Pei 1999, Velasco *et al.* 2000). Four of them are type I transmembrane proteins (MMP-14, -15, -16 and -24), while MMP-17 and -25 are glycosylphosphatidyinositol (GPI)-anchored proteins (reviewed by Visse & Nagase 2003). Because the MT-MMPs are membrane-bound they provide a focalized area for ECM proteolytic degradation. Unlike other classes of MMPs, MT-MMPs are proteolytically active once inserted into the cell membrane (Spinale 2002). MT-MMPs constitute an important pathway for the activation of other MMPs within the ECM. It has been demonstrated that all MT-MMPs, except MT4-MMP, are capable of activating proMMP-2. In addition, MT1-MMP can proteolytically process the proform of MMP-8 and -13 (Woessner & Nagase 2000, Visse & Nagase 2003, Holopainen *et al.* 2003). The six different MT-MMPs appear to be expressed in both normal and diseased tissue. MT1-MMP is the best-characterized MT-MMP. It was first identified on invasive tumor cells (Sato *et al.* 1994). In addition to tumor cells, MT1-MMP is expressed on fibroblasts (Ruangpanit *et al.* 2001) and endothelial cells (Galvez *et al.* 2001), and it is thus involved in angiogenesis. MT1-MMP-deficient mice show a dramatic phenotype. The mice have severe defects in skeletal development as well as in angiogenesis, leading to early death (Holmbeck *et al.* 1999, Zhou *et al.* 2000).

2.2.3.6 Other MMPs

Seven MMPs not classified in the above-mentioned categories are MMP-12 (metalloelastase), MMP-19, MMP-20 (enamelysin), MMP-21, MMP-22, MMP-23, MMP-28 (epilysin). MMP-12 is mainly expressed in macrophages (Shapiro *et al.* 1993), and is essential for macrophage migration (Shipley *et al.* 1996). Elastin is its major substrate (Table 3). MMP-19 was identified and cloned from human liver cDNA library (Pendas *et al.* 1997), and as a T-cell-derived autoantigen from patients with rheumatoid arthritis (Kolb *et al.* 1997). MMP-20 was first found in odontoblasts, and it is primarily located within newly formed tooth enamel (Bartlett *et al.* 1998). Later it was discovered in tongue squamous cell carcinomas, tooth pulp and placenta (Väänänen *et al.* 2001). MMP-21 was characterized from human placenta cDNA. It is the human ortholog for XMMP. MMP-21 is expressed in various human fetal and adult tissues as well as in cancer cell lines and carcinomas (Ahokas *et al.* 2002). MMP-22 was cloned from chicken fibroblasts (Yang *et al.* 1998), and the human homologue is called MMP-27 (Visse & Nagase 2003). MMP-23 could be grouped with membrane-type MMPs, because it has a transmembrane domain in the N-terminal part of the propeptide. It differs from most MMPs, since it is released as an active enzyme into the extracellular space (Pei *et al.*

2000. It is mainly expressed in reproductive tissues (Velasco *et al.* 1999). The latest addition to the MMP family is MMP-28. It is mainly expressed in testis and keratinocytes (Lohi *et al.* 2001).

2.2.4 Transcriptional regulation of MMPs

As MMP substrate specificities overlap, the regulation of the expression of each individual MMPs is of major importance. This also can dictate the biological functions of MMPs (Sternlicht & Werb 2001). When alterations in extracellular matrix need to occur, ECM degradation is facilitated by induction of MMP gene expression by ECM receptors (Damsky & Werb 1992). Most cells do not constantly express MMPs, or the basal expression level is low. Instead the transcription can be induced by various extracellular stimuli that influence multiple signaling pathways (Fini *et al.* 1998). MMP-2 is an exception to that rule, as it is usually constitutively expressed and controlled mostly by a unique mechanism of cell surface-associated activation (Strongin *et al.* 1995). MMPs can be up- or downregulated by phorbol esters, integrin-derived signals, extracellular matrix proteins, cell stress, changes in cell shape and several cytokines and growth factors, including interleukins, interferons, EGF, KGF, NGF, bFGF, VEGF, PDGF, TNF-α, TGF-β and the extracellular matrix metalloproteinase inducer EMMPRIN. The efficiency of induction of different agents varies. The effect can also vary depending on cell type (Birkedal-Hansen *et al.* 1993, Borden & Heller 1997, Sternlicht & Werb 2001). The same factor can have opposite effects on distinct MMPs, for example TGF-β1 suppresses the production of MMP-1 and -3, but stimulates the production of MMP-2, -9 and -13 (Salo *et al.* 1991, Uria *et al.* 1998). In addition to inducing the expression of MMPs, transcription factors can inhibit the production of structural matrix components, thus enhancing the ECM degradative effect. That is the case for TNF-α and interleukin-1: they upregulate the production of MMP-1 and -3 by fibroblasts, and downregulate the expression of collagen I and elastin (Reunanen *et al.* 1998).

Many of these stimuli induce the expression of c-*fos* and c-*jun* proto-oncogene products, which heterodimerize and bind the activator protein-1 (AP-1) sites in various MMP gene promoter areas (Fini *et al.* 1998, Sternlicht & Werb 2001). The AP-1 binding site is about 70 nucleotides downstream of the site for beginning of transcription, and it can influence MMP gene expression on its own or together with other *cis*-regulatory elements. AP-1 binding sites enable several MMP genes to be induced by phorbol esters and to act synergistically with Ets-binding sites, such as PEA-3, in some MMP genes, such as MMP-1, but not in all of MMPs, such as MMP-13 (Benbow & Brinkerhoff 1997, Pendas *et al.* 1997). The Ets transcription factor family is important for MMP expression; the disruption of Ets-2 results in embryonic lethality and deficient MMP-3, -9 and -13 expression (Yamamoto *et al.* 1998). Several other regulatory elements within various MMP gene promoters include AP-2, SP-1, SP-3, NF-κB, CCAAT/enhancer binding protein-β, and retinoic acid response elements (Fini *et al.* 1998, Lohi *et al.* 2000, Ludwig *et al.* 2000). In addition, there are regulatory elements specific for only certain MMPs: osteoblast specific element responding core-binding factor-1in the MMP-13 promoter (Jimenez *et al.* 1999), β-catenin regulated LEF/TCF site near the MMP-7 start site

(Crawford *et al.* 1999) and functional p53 binding site in MMP-2 promoter (Bian & Sun 1997).

2.2.5 Activation of MMPs

All MMPs are produced as inactive proenzymes, and most of them are secreted to the extracellular milieu as latent proforms that need to be further processed to become active proteases. In the latent zymogen, there is an unpaired cysteine sulfhydryl group at position 73 of the propeptide domain within a conserved region of PRCG(V/N)PD. The cysteine sulfhydryl group binds to the catalytic zinc-ion. For the activation of MMPs, that bond needs to be disrupted and replaced by a water molecule (Van Wart & Birkedal-Hansen 1990). The mechanism of activation is further studied with the help of a three-dimensional structure of proMMP-3. In the proform, amino acids 83-89 corresponding to the first seven residues in the active enzyme form part of the loop that fills the active site of the enzyme. After activation the structure changes: residues 83-89 move to the outer edge of the molecule and a salt bridge is formed between Phe^{83} and Asp^{237} (Becker *et al.* 1995, Duncan *et al.* 1998).

MMPs can be activated by three mechanisms: by stepwise activation, by intracellular activation or on cell surface. Although all MMPs belong to the same protease family and their structure and function can be very similar, they can be activated with distinct mechanisms (Ramos-DeSimone *et al.* 1999). MMPs can be activated in a stepwise manner (Fig. 4) by proteinases or *in vitro* by chemical agents, such as thiol-modifying agents such as 4-aminophenylmercuric acetate and other mercurial compounds, gold (I)-compounds, oxidized glutathione, SDS, chaotropic agents, oxygen derived free radicals, low pH and heat treatment (Nagase *et al.* 1997, Visse & Nagase 2003). The chemical activation occurs in a stepwise manner. The initial cleavage occurs within the propeptide, and the propeptide is subsequently removed by intermolecular fragmentation via several intermediates (Okada *et al.* 1988, Nagase *et al.* 1990). Chemical activation of MMPs is demonstrated to occur also *in vivo*. NO activates proMMP-9 during cerebral ischemia by reacting with the thiol group of the cysteine (Gu *et al.* 2002). Especially during inflammation proMMPs can get activated by reactive oxygen species, such as hydrogen peroxide and hydroxyl radicals. The reactive oxygen species not only activate proMMPs, but they also inhibit the function of many protease inhibitors, thus further amplifying the degradation of the extracellular matrix (Sorsa *et al.* 1998). Chemical or oxidative activation is considered to be especially important *in vivo* in inflammatory diseases (Weiss 1989).

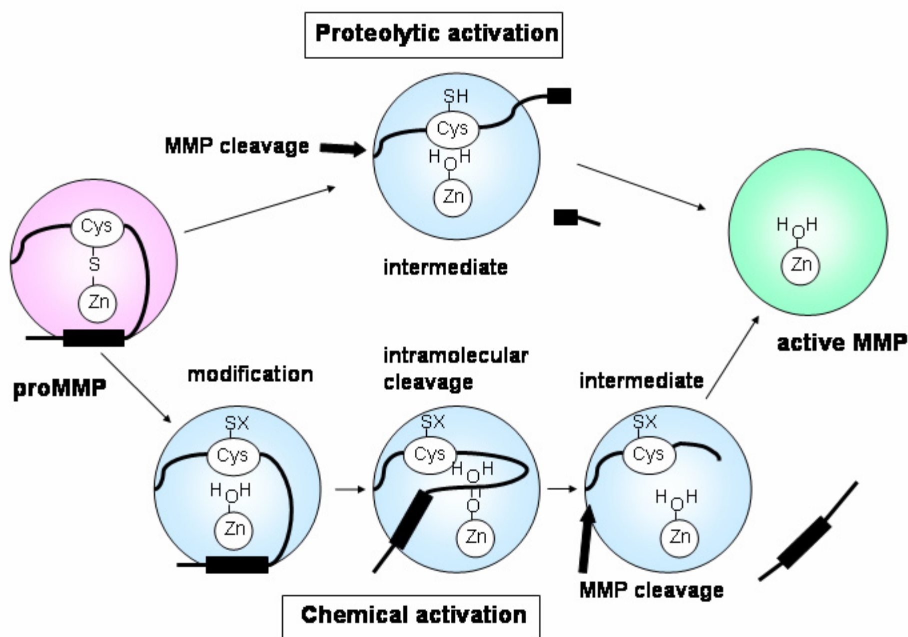

Fig. 4. The stepwise mechanism of MMP activation. The latent proMMPs can be activated either by proteolysis of by chemical activation. In the proteolytic activation, a proteinase cleaves the bait region (black rectangle) in the propeptide (thick black line) leading to an MMP intermediate, in which the catalytic site zinc is dissociated from the sulfhydryl group of the cysteine residue in the propeptide. During the second step, another MMP-molecule (thick black arrow) degrades the rest of the propeptide, resulting in an active MMP. Chemical activation relies on modification of the cysteine switch sulfhydryl (step 1), resulting in partial activation of the MMP and intramolecular cleavage of the propeptide (step 2). In the last step, similarly to the proteolytic activation, another MMP removes the rest of the propeptide. (Modified from Visse & Nagase 2003)

The proteolytic activation is believed to be more important than chemical activation *in vivo* especially in malignant diseases. It also occurs in a stepwise fashion. The initial proteolytic attack occurs at an exposed loop region at the middle of the propeptide. The cleavage specificity of this bait region is dictated by the sequence found in each MMP. Once this cleavage has occurred, it causes conformational changes that destabilize the rest of the propeptide, making it susceptible for the final activation cleavage. This final step of proMMP activation is often conducted by other MMPs (Suzuki *et al.* 1990, Nagase *et al.* 1990, Visse & Nagase 2003). MMP-3 activates proMMP-9 *in vitro* and *in vivo,* where it participates in the invasiveness of breast carcinoma cells (Ramos-DeSimone *et al.* 1999). MMP-3 also activates proMMP-7 (Wilson & Matrisian 1996), which in turn can activate proMMP-1 (Sang *et al.* 1996), -2 and -8 (Balbin *et al.* 1998). MMP-10 activates proMMP-7 (Nakamura *et al.* 1998) and -8 (Knäuper *et al.* 1996). The activation systems of MMP-9 and -13 are multifaceted and redundant, indicating their

importance. MMP-1 (Sang *et al.* 1995), -2 (Fridman *et al.* 1995), -3 (Cowell *et al.* 1998), -10 (Nakamura *et al.* 1998), -13 (Knäuper *et al.* 1997), and -26 (Uria & Lopez-Otin 2000) are all capable of activating proMMP-9. MMP-2, -3, -10, -14 and -15 can activate proMMP-13 (Knäuper *et al.* 1996, Johansson *et al.* 2000). The proMMP and the activator MMP do not have to be secreted by the same cell type. Melanoma cells produce large amounts of latent MMP-1, which are only activated in the presence of proteases secreted by fibroblasts (Benbow *et al.* 1999). The activation cascades of proMMPs are quite complex. In addition to MMPs, the activation pathways involve other proteases, such as plasmin (Mazzieri *et al.* 1997) and trypsin-2 (Sorsa *et al.* 1997, Moilanen *et al.* 2003). In general, plasmin has been regarded to be a relevant activator *in vivo*. The precursor molecule of plasmin, plasminogen, and the proteolytic activator of plasmin, uPA, are both membrane-bound, thereby creating localized MMP activation. Plasmin is known to activate proMMP-1, -2, -3, -7, -9, -10 and -13 (Lijnen 2001, Monea *et al.* 2002). Tumor associated trypsin-2 can activate proMMP-1, -3, -8, -13, -20, and less efficiently proMMP-2 (Sorsa *et al.* 1997, Väänänen *et al.* 2001, Moilanen *et al.* 2003) as well as pro-uPA (Koivunen *et al.* 1989).

Some MMPs can already be activated in the intracellular milieu. ProMMP-11 was the first MMP shown to be activated intracellularly by furin, which is a protease associated with the Golgi membrane (Pei & Weiss 1995). Also the six membrane-type MMPs, MMP-23 and -28 have a similar basic furin recognition motif, so they are most likely secreted as active enzymes, like MMP-11 (Visse & Nagase 2003). In fact, MT1-MMP is activated by furin at least *in vitro* (Sato *et al.* 1996).

ProMMP-2 is quite unique among the MMPs, since it is not readily activated by the secreted MMPs. ProMMP-2 can be activated on the cell surface by MT-MMPs. MT4-MMP is the only one that is known not to activate proMMP-2 (English *et al.* 2000); the other five MT-MMPs, MMP-14, -15, -16, -24 and -25 can activate proMMP-2 (Strongin *et al.* 1995, Butler *et al.* 1997, Sato *et al.* 1996, Llano *et al.* 1999, Velasco *et al.* 2000). The mechanism of MT1-MMP mediated activation is best known. The interesting feature about the activation is that it requires the assistance of TIMP-2 (Strongin *et al.* 1995). At least two MT1-MMP molecules are required for the activation. The N-terminal inhibitory domain of TIMP-2 binds to the catalytic domain of one MT1-MMP. This complex can act as a receptor to proMMP-2 by binding to its hemopexin domain via the C-terminal domain of TIMP-2. The formation of this trimeric complex enables the presentation of proMMP-2 to the other MT1-MMP molecule in the correct orientation, thus leading to activation of proMMP-2 (Zucker *et al.* 1998, Wang *et al.* 2000). An excessive amount of MT1-MMP is required for optimal activation of proMMP-2, as maximum activation is observed at TIMP-2/MT1-MMP ratio of 0.05 (Jo *et al.* 2000). However, TIMP-2 is not necessarily required in the activation process. ProMMP-2 activation by MT2-MMP is direct and independent of TIMP-2 (Morrison *et al.* 2001). MT1-MMP also activates proMMP-13 and -8 without the requirement of TIMP-2 (Knäuper *et al.* 1996 and 2002, Holopainen *et al.* 2003).

2.2.6 Inhibition of MMPs

For at least 30 years, MMPs have been regarded as promising targets for cancer therapy, and a lot of focus has been on the suppression of the MMPs. Studies with MMP inhibitors have also revealed information of the mechanism of MMP function both in pathological and physiological situations. The activity of MMPs can be inhibited by endogenous or synthetic inhibitors. Tissue inhibitors of MMPs (TIMPs) are endogenous specific inhibitors for MMPs. Several other proteins have also been reported to inhibit MMPs: tissue factor pathway inhibitor-2, C-terminal fragment of the procollagen C-terminal proteinase enhancer protein, membrane bound β-amyloid precursor protein, and GPI-anchored glycoprotein called RECK (reversion including cysteine rich protein with Kazai motifs). Chemically MMPs can be inhibited with chelating agents, such as EDTA, inhibiting antibodies, drugs designed as MMP inhibitors, such as Marimastat, old drugs with novel MMP inhibitory properties, like bisphosphonates, doxycycline and chemically modified tetracyclines, and small synthetic peptides (Birkedal-Hansen *et al.* 1993, Chambers & Matrisian 1997, Golub *et al.* 1998, Koivunen *et al.* 1999, Coussens *et al.* 2002, Heikkilä *et al.* 2002, Visse & Nagase 2003).

2.2.6.1 Tissue inhibitors of MMPs

An important mechanism for regulating the activity of MMPs is via binding to a family of homologous proteins called tissue inhibitors of matrix metalloproteases (TIMPs). TIMPs bind MMPs in a 1:1 stoichiometry. Four TIMPs (21-29 kDa) have been identified in vertebrates (Brew *et al.* 2000, Visse & Nagase 2003). An imbalance between MMPs and TIMPs can lead to pathological destruction or accumulation of extracellular matrix (Edwards *et al.* 1996, Kähäri & Saarialho-Kere 1997). Although different TIMPs bind to and inhibit most MMPs, there are some differences in their inhibitory properties. TIMP-2 and -3, but not TIMP-1, are effective inhibitors of MT1-MMPs, while TIMP-3, but not TIMP-1, -2 or -4, is a good inhibitor of TACE, a member of MMP-related enzymes called ADAMs (Will *et al.* 1996, Amour *et al.* 1998, Brew *et al.* 2000). In addition to being a poor inhibitor of MT-MMPs, TIMP-1 does not inhibit much of the activity of MMP-19 either. TIMP-3 inhibits the activity of MMP-1, -2, -3, -13 and very potently that of MMP-9.TIMP-4 inhibits the activity of MMP-2 and -7, and less potently also that of MMP-1, -3 and -9 (Brew *et al.* 2000, Baker *et al.* 2002). When forming a complex with the MMPs, the wedge-shaped TIMPs bind with their edge into the entire length of the active site cleft of their cognate MMPs. More specifically, the first five TIMP residues bind to the active site cleft in a substrate-like manner (Gomis-Rüth *et al.* 1997, Fernandez-Catalan *et al.* 1998).

An imbalance in the amount TIMPs affects tumor growth. Overexpression and injections of TIMPs can reduce the formation of experimental metastases (DeClerck *et al.* 1994, Schultz *et al.* 1988), and the depletion of TIMP-1 can render cells tumorigenic (Khokha *et al.* 1989). Overexpression of TIMP-3 induces apoptotic cell death in various cancer cell lines (Ahonen *et al.* 1998, Brew *et al.* 2000), but TIMP-1 and -2 suppress the apoptosis of mouse melanoma cells (Valente *et al.* 1999). TIMP-1 binds to the cell

surface of breast carcinoma cells, translocates to the nucleus and reduces tumor cell growth (Valente *et al.* 1999). TIMPs can also affect tumor growth by affecting angiogenesis. TIMP-2 inhibits bFGF induced human endothelial cell growth (Murphy *et al.* 1993). It is noteworthy that TIMPs have other biological activities in addition to their ability to inhibit MMPs, and part of the effects of TIMP on tumor growth and other biological functions may result from those activities.

2.2.6.2 Synthetic inhibitors

As early as in 1988, the broad-spectrum MMP inhibitor SC-44463 was shown to prevent experimental metastasis (Reich *et al.* 1988). These results were also confirmed with other synthetic inhibitors using various mouse models. Despite these first steps being promising and validating the idea that MMPs are potential therapeutic targets for cancer, investigators soon realized that the concept was much more complex than what was originally thought (Coussens *et al.* 2002). The effect of synthetic inhibitors can be critically dependent on the stage of tumor growth and the time of drug administration. For example, batimastat treatment decreases the number of tumors in several mouse models, but only if given early enough, before the emergence of large invasive carcinomas (Bergers *et al.* 1999). Marimastat was the first orally active MMP inhibitor. It and other inhibitors, such as prinomastat, tanomastat and BMS-275291, have been studied in phase III clinical trials, but the efficacy has been quite poor (Coussens *et al.* 2002).

One problem with MMP inhibitors has been the lack of specificity, since the first inhibitors prevented the activity of all MMPs. Koivunen *et al.* (1999) has searched phage display libraries for peptides that would selectively only inhibit gelatinases, which are regarded to be the most relevant MMPs in cancer progression. Cyclic peptides with a His-Trp-Gly-Phe (HWGF) sequence were found to be selective inhibitors. The decapeptide CTTHWGFTLC inhibits the migration of human endothelial cells and various cancer cell lines. *In vivo*, the peptide homes to tumors, suppressing their growth and inhibiting tumor angiogenesis in tumor-bearing mice (Koivunen *et al.* 1999). The peptide could be administered into the tumor cells using liposomes and combined with other anti-cancer drugs in the same liposome for extra efficacy (Medina *et al.* 2001).

The antimicrobial effect of tetracyclines was already discovered 60 years ago, but it was not until 1983 that Golub and colleagues found that tetracyclines and especially doxycycline (Suomalainen *et al.* 1992) as well as several modified non-antimicrobial analogues called chemically modified tetracyclines (CMT) inhibit MMP activity independently from their antimicrobial action (Golub *et al.* 1998). Tetracyclines and CMTs inhibit activated MMPs by interacting with the Ca^{2+} and Zn^{2+} binding sites and they can also prevent the latent proMMP activation caused by oxidation (Sorsa *et al.* 1998). They have also been shown to inhibit tumor cell proliferation and tumor growth, to reduce metastatic potential and to induce apoptosis (Hidalgo & Eckhardt 2001, Lokeshwar *et al.* 2002). In addition to MMPs, CMTs also affect other proteases, such as trypsinogen-2 (Lukkonen *et al.* 2000).

Bisphosphonates are another group of drugs that have long been in human use. It was recently found that they inhibit various MMPs. They can inhibit bone resorption and are therefore used in the treatment of Paget's disease, osteolytic tumor metastasis, osteoporosis and multiple myeloma, but the mechanisms have remained relatively unclear. Bisphosphonates can inhibit MMP-3, -12, -13, -14 and -20, but not uPA, and diminish the invasion and migration of human malignant and endothelial cell lines (Heikkilä *et al.* 2002 and 2003).

2.3 Angiogenesis in tumors

Neoangiogenesis, the formation of new capillaries is one of the key events in various pathological processes, such as tumor growth, metastasis formation and arthritis, as well as in physiological events, such as organ growth and development, wound healing and reproduction (Folkman 1995). As early as in 1971 Judah Folkman first launched the formal hypothesis that tumor growth depends on angiogenesis (Folkman 1971). The growth and expansion of tumors is critically dependent on neoangiogenesis, and the inhibition of vascular supply to tumors can suppress their growth (Hanahan & Folkman 1996). Solid tumors cannot grow beyond a few millimeters in diameter without being able to recruit their own blood supply. Angiogenesis depends plausibly on a delicate balance between endogenous stimulators and inhibitors (Fig. 5).

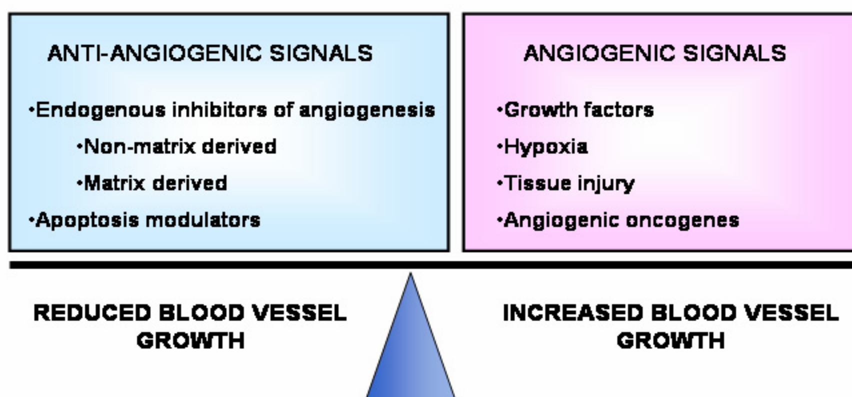

Fig. 5. The angiogenic balance (Adapted from Folkman 2003)

Stimulators of angiogenesis include hypoxic conditions that activate hypoxia inducible factor HIF-1α, which itself can upregulate angiogenic proteins, various growth factors

such as VEGF, bFGF and PDGF, as well as angiogenic oncogenes such as Ras. A lot of research has focused particularly on VEGF family members and their receptors, VEGFR-1, -2 and -3, in cancer progression. By binding to VEGF-R-1 and -2 on the endothelial cell surface, VEGF(-A) mediates vascular leakage, endothelial cell proliferation and migration (Scavelli *et al.* 2004). In addition, autocrine VEGF(-A) signaling contributes to the invasiveness of carcinomas by affecting the survival and migration of the carcinoma cells themselves (Mercurio *et al.* 2004). Very recently, an anti-VEGF monoclonal antibody (bevacizumab, Avastin) has been approved by the U.S. Food and Drug Administration as a first-line treatment for metastatic colorectal cancer in combination with chemotherapy (Ferrara 2004). VEGF-C and -D have been implicated in the development and maintenance of lymphatic vasculature that has been hypothesized to be involved in tumor metastasis (He *et al.* 2004, Saharinen *et al.* 2004). Endogenous inhibitors of angiogenesis include various anti-angiogenic peptides, hormone metabolites and apoptosis modulators, such as p53 (Folkman 1995 and 2003). p53 also affects directly the survival of tumor cells in hypoxic conditions, as cells expressing wild type p53 will apoptose during hypoxia, whereas p53 mutant or null cells do not (Graeber *et al.* 1996), demonstrating how hypoxia can select for the survival of p53 mutant cells.

Capillary endothelial cells are supported by vascular basement membranes (Paulsson *et al.* 1992, Madri *et al.* 1997). In addition to providing structural and functional support, vascular basement membrane components can modulate endothelial cell behavior (Darland & D'Amore 1999). A series of endogenous anti-angiogenic factors have been described, many of which are fragments of naturally occurring basement membrane and extracellular matrix components (Cao 2001). Angiogenesis can also be regulated by integrins, a group of transmembrane cell surface receptors (Hynes 2002a and b). They can mediate cell adhesion to the components of the extracellular matrix and to other cells, as well as make transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways. Integrins are heterodimers consisting of α and β subunits (Hynes 2002a and b).

2.4 Matrix derived endogenous inhibitors of angiogenesis

Many endogenous inhibitors of angiogenesis are cryptic fragments from larger matrix molecules, and thus these inhibitors can be divided into two major classes, matrix derived and non-matrix derived inhibitors (Table 3).

Table 3. Endogenous inhibitors of angiogenesis (adapted from Nyberg et al. 2005)

Matrix derived	Growth factors and cytokines	Others
Arresten	Interferons	Angiostatin
Canstatin	Interleukins	Antithrombin (cleaved)
Endorepellin	Pigment epithelium derived factor (PEDF)	Chondromodulin Kringle-1 –and 2 domain of tPA
Endostatin	Platelet factor (PF)	PEX
Fibronectin fragments		Prothrombin Kringle 2
Endostatin-like fragment from collagen XV		TIMPs
Thrombospondin-1 and -2		Troponin I
Tumstatin		Vasostatin

Thrombospondin-1 was the first protein to be recognized as a naturally occurring endogenous inhibitor of angiogenesis (Good *et al.* 1990). Thrombospondin was originally found as a large glycoprotein from human blood platelets (Lawler *et al.* 1978). Later it was discovered that also other cell types produced it; e.g. fibroblasts secrete and deposit it into the extracellular matrix (Jaffe *et al.* 1983). It is a large multifunctional extracellular matrix glycoprotein that regulates several biological events in addition to angiogenesis, such as cell adhesion, proliferation and survival, TGF-β activation, and protease activation (Chen *et al.* 2000). It has been shown to inhibit tumor angiogenesis, growth and metastasis (Streit *et al.* 1999a, Rodriguez-Manzaneque *et al.* 2001). Implanted melanoma and testicular teratocarcinoma tumors grow faster in thrombospondin null mice, showing that also endogenous levels are sufficient to inhibit angiogenesis (Lawler *et al.* 2001). Thrombospondin-2 has anti-angiogenic activity as well (Streit *et al.* 1999b). Endostatin is an anti-angiogenic fragment from collagen XVIII and arresten, canstatin and tumstatin are inhibitors of angiogenesis cleaved from collagen IV (Table 4) (Sund *et al.* 2004).

Table 4. Characteristics of collagen-derived inhibitors of angiogenesis (modified from Sund et al. 2004).

Inhibitor	EC proliferation	EC migration	EC apoptosis	Tumor growth	Parent collagen type	Collagen chain
Arresten	↓	↓	↑	↓	IV	α1
Canstatin	↓	↓	↑	↓	IV	α2
Endostatin	↑↓	↓	↑	↓	XVIII	α1
Endostatin from type XV coll	↓	↓	ND	↓	XV	α1
Tumstatin	↓	↔	↑	↓	IV	α3
α6 NC1 domain	↓	ND	ND	ND	IV	α6

Abbreviations and symbols used: coll, collagen; EC, endothelial cell; NC1, non-collagenous domain; ND, not determined; ↓, decrease; ↑, increase; ↔, no effect; ↑↓, decrease or no effect depending on the assay or the source of endostatin

Other matrix derived endogenous inhibitors of angiogenesis include endorepellin from perlecan (Mongiat *et al.* 2003), and the fibronectin derived fragment anastellin (Pasqualini *et al.* 1996, Yi & Ruoslahti 2001). Angiostatin is a non-matrix derived inhibitor of angiogenesis derived from plasminogen, and the first cryptic fragment of a larger parent molecule possessing novel anti-angiogenic properties that the intact molecule did not have (O'Reilly *et al.* 1994). Several members of the MMP family, including MMP-2, -3, -7, -9 and -12, hydrolyze human plasminogen to generate angiostatin fragments of 38-45 kDa (Sang 1998, O'Reilly *et al.* 1999a). Other non-matrix derived endogenous inhibitors are cleaved antithrombin III and prothrombin kringle 2 (O'Reilly *et al.* 1999b, Lee *et al.* 1998), chondromodulin I (Kusafuka *et al.* 2002), interferons (Mitsuyasu 1991), interleukins (Strieter *et al.* 1995), kringle 1-2 domains of tissue type plasminogen activator (Kim *et al.* 2003), pigment epithelium-derived factor PEDF (Bouck 2002), the non-catalytic C-terminal hemopexin-like domain of MMP-2 called PEX (Brooks *et al.* 1998), platelet factor-4 (Maione *et al.* 1990), TIMPs (Seo *et al.* 2003), troponin I (Moses *et al.* 1999) and vasostatin (Pike *et al.* 1998).

2.4.1 Endostatin

Endostatin is a collagen XVIII derived angiogenesis inhibitor identified and purified from a murine hemangioendothelioma cell line (O'Reilly *et al.* 1997), found later in the circulation of mice (Standker *et al.* 1997). It corresponds to a 20-kDa fragment derived from the C-terminal non-collagenous (NC1) domain of type XVIII collagen (Rehn *et al.* 1994, Oh *et al.* 1994, O'Reilly *et al.* 1997). The NC1 domain of type XVIII collagen consists of three functional elements: the endostatin region (183 residues), the protease sensitive hinge region (~70 residues) and the trimerization region (~70 residues) (Sasaki *et al.* 1998).

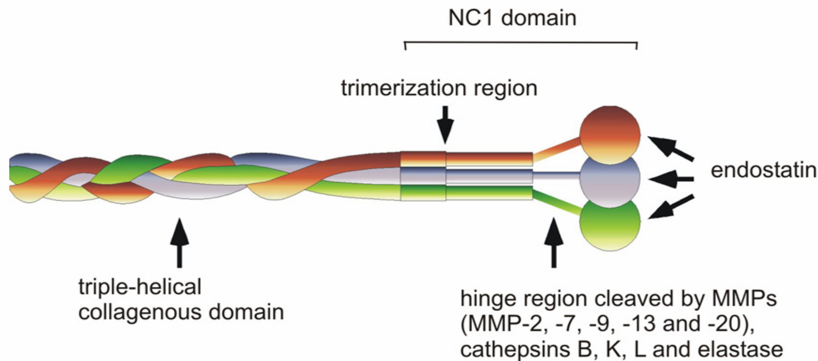

Fig. 6. Schematic illustration of the C-terminus of the type XVIII collagen molecule. Collagen XVIII is a trimer consisting of N-terminal domain, central triple-helical domain and C-terminal non-collagenous (NC1) domain. Various proteases cleave the protease-sensitive hinge region and generate 20-kDa endostatin from the parent molecule. (Adapted from Kalluri 2003)

Multiple studies have shown that recombinant endostatin efficiently inhibits angiogenesis and suppresses primary tumor growth and metastasis in animal models without any apparent side effects, toxicity or development of drug resistance (O'Reilly *et al.* 1997, Boehm *et al.* 1997, Bergers *et al.* 1999, Blezinger *et al.* 1999, Dhanabal *et al.* 1999a, Yamaguchi *et al.* 1999, Perletti *et al.* 2000, Sauter *et al.* 2000). However, collagen XVIII and thus endostatin deficient mice do not show increased carcinoma growth. Instead they have eye abnormalities (Fukai *et al.* 2002).

New insights are emerging into the molecular mechanisms behind the anti-angiogenic and anti-tumor effects of endostatin. Endostatin rapidly downregulates many genes in endothelial cells, including immediate early response genes, cell cycle related genes and genes regulating apoptosis inhibitors, mitogen activated protein kinases, focal adhesion kinases, G-protein coupled receptors mediating endothelial cell growth, mitogenic factors, adhesion molecules and cell structure component (Shichiri & Hirata 2001). Endostatin downregulates many signaling pathways associated with proangiogenic activity in human microvascular endothelial cells, while at the same time upregulating many anti-angiogenic genes. It should be noted that endostatin also affects signaling events that are not associated with angiogenesis, demonstrating the importance of inter-pathway communications in an intricate signaling network (Abdollahi *et al.* 2004). Other recent studies have reported that endostatin induces apoptosis (Dhanabal *et al.* 1999b), causes G_1 arrest of endothelial cells through inhibition of cyclin D1 (Hanai *et al.* 2002), interferes with FGF-induced signal transduction resulting in blockage of endothelial cell motility (Dixelius *et al.* 2002), blocks VEGF-mediated signaling via direct interaction with the VEGF-R2/KDR/Flk-1 receptor tyrosine kinase in human umbilical vein endothelial cells (Kim *et al.* 2002), and blocks TNF-induced activation of JNK and JNK-dependent pro-angiogenic gene expression (Yin *et al.* 2002). On the other hand, Eriksson

et al. (2003) conclude that endostatin inhibits chemotaxis, without affecting intracellular pathways known to regulate endothelial cell migration, proliferation and survival, since they did not find any effect of endostatin on phospholipase C-γ, Akt/PKB, p44/42 MAPK, p38 MAPK and p21-activated kinase activity (Eriksson *et al.* 2003). Recently, it was discovered that endostatin inhibits the VEGF induced mobilization of endothelial progenitor cells (Schuch *et al.* 2003).

Although the main focus of most studies elucidating the anti-tumor effect and mechanism of endostatin has been on the effect of endostatin on the endothelial cells, it should also be remembered that endostatin reduces significantly invasion of not only endothelial cells but also of tumor cells into the matrigel preparation of reconstituted basement membrane (Kim *et al.* 2000). Furthermore, endostatin directly affects epithelial human cells derived from tumors of patients with head and neck squamous cell carcinoma (HNSCC). Head and neck cancers are significant due to their high morbidity and associated complications. Exposure of HNSCC cells to endostatin activates the transcription-activating factors, NF-κB and AP-1 in a cell-line-dependent fashion. Endostatin also downregulates the gene expression of several pro-migratory molecules, and significantly inhibits migration and invasion that are essential for tumor progression. Endostatin co-localizes to tropomyosin-binding HNSCC microfilaments, suggesting that the suppression of HNSCC cell migration and invasion by endostatin may reflect perturbation of the microfilament function (Wilson *et al.* 2003).

Recombinant immobilized human endostatin interacts with α5- and αv-integrins on the surface of human endothelial cells (Rehn *et al.* 2001). Sudhakar *et al.* (2003) have further characterized the receptors for soluble endostatin and the downstream signaling events. Endostatin binds to the α5β1 integrin and inhibits the migration of endothelial cells by blocking signaling pathways via Ras and Raf and further downstream via ERK1 or p38 (Sudhakar *et al.* 2003). In endothelial cells, endostatin induces rapid clustering of integrin α5β1 associated with actin stress fibers and causes co-localization with the membrane anchor protein caveolin, which couples integrins to cytoplasmic signaling cascades (Wary *et al.* 1998). Later it was confirmed that endostatin binds to α5β1 integrin, which is associated with caveolin-1, and that endostatin treatment induces phosphatase dependent activation of caveolin associated Src family kinases and the loss of actin stress fibers. This disturbs the deposition of fibronectin matrix and contributes to the antimigratory effect of endostatin (Wickström *et al.* 2002). Endostatin associates with lipid rafts that are specialized cell membrane microdomains where transmembrane proteins and intracellular signaling molecules are recruited. The localization of endostatin to the lipid rafts occurs through heparin sulphate proteoglycan- and integrin-mediated interactions. It was found that endostatin induces the loss of actin stress fibers by Src-dependent activation of Rho-GTPase-activating protein (GAP) and subsequent downregulation of RhoA, and in this way possibly impairs cell migration (Wickström *et al.* 2003).

Endostatin also binds to heparin, and with low affinity to cell surface heparin sulphate proteoglycans, such as glypican-1 expressed on endothelial cells, which are known to act as co-receptors for various cytokines and to be involved in growth factor signaling (Hohenester *et al.* 1998, Sasaki *et al.* 1999, Karumanchi *et al.* 2001). The anti-angiogenic activity seems to depend on the interactions with the heparin sulphate proteoglycans, possibly by interactions between discontinuous sulphated domains in heparin sulphate proteoglycans and arginine clusters at the endostatin surface (Kreuger *et al.* 2002).

Recent work has demonstrated the identity of a specific arginine-rich sequence motif of human endostatin that interacts with endothelial cell surface β1 integrin and heparin, and that inhibits endothelial cell migration and tube formation. This RGD-independent sequence is likely to be the motif responsible for the anti-angiogenic activity of endostatin (Wickström *et al.* 2004). Interestingly, it has been demonstrated that the human collagen XVIII is the first collagen carrying heparan sulphate side-chains (van Horssen *et al.* 2002).

Recombinant human endostatin binds also to tropomyosin *in vitro* and to tropomyosin-associated microfilaments in a variety of endothelial cell types. It has been suggested that the interaction of endostatin with tropomyosin results in disruption of microfilament integrity leading to inhibition of cell motility, induction of apoptosis, and ultimately inhibition of tumor growth (MacDonald *et al.* 2001). AIDS-related Kaposi's sarcoma cells rapidly internalize endostatin, initiating activation of the transcription activating factors NF-κB and AP-1. The internalized endostatin co-localizes to tropomysin microfilaments and acts to inhibit cell migration and invasion in response to the angiogenic cytokines VEGF and bFGF (Mallery *et al.* 2003).

Endostatin binds not only to cell surface receptors but also to soluble ligands in the extracellular milieu. Endostatin binds directly to MMP-2 blocking its activity, and it also inhibits the activity of MT1-MMP, but it is not known yet whether endostatin also binds to this MMP (Kim *et al.* 2000, Lee *et al.* 2002). Interestingly, the regulation between endostatin and MMPs occurs in both directions, as certain MMPs are able to generate endostatin-containing peptides differing in molecular size (20-30 kDa) from human type XVIII collagen (Ferreras *et al.* 2000). In addition to MMPs, endostatin interferes with the actions of other proteases, such as the plasminogen activator system by downregulating the levels of secreted uPA and PAI-1 and their complexes and by removing uPAR-associated uPA from the focal adhesions on the cell surface. This is associated with the disassembly of focal adhesions and disruption of actin stress fibers (Wickström *et al.* 2001).

Endostatin can exist in two forms, as a monomer or as a trimer when present in the full-length collagen XVIII or the NC1 domain, which possess different or even opposite effects. The trimeric form is needed for endothelial cell migration, but the monomeric form inhibits the migratory activity (Ackley *et al.* 2001, Kuo *et al.* 2001). Immobilized endostatin has different functions than soluble endostatin *in vitro*, as immobilized endostatin supports endothelial cell survival and migration, whereas soluble endostatin inhibited endothelial cell functions (Rehn *et al.* 2001). It has been shown that endostatin can exist as a soluble globular form or as an insoluble form with abundant cross-β-sheets aggregating into amyloid deposits. These different conformations have distinct effects at least on plasminogen activation. Insoluble endostatin stimulates plasminogen activation, whereas soluble endostatin has no effect on the activity of plasminogen (Kranenburg *et al.* 2002). The same cross-β structure is also present in amyloidogenic polypeptides in plaques of patients with amyloidosis, such as Alzheimer disease. Many angiogenesis inhibitors stimulate tPA-mediated plasminogen activation. Because the presence of cross-β structure is a common denominator in tPA-binding ligands, it has been suggested that these endogenous antiangiogenic proteolytic fragments share features with amyloidogenic polypeptides, that the cross-β structural fold is present in these

antiangiogenic fragments, and that this structure mediates the inhibitory effects (Gebbink *et al.* 2004).

Table 5. Functions of distinct forms of endostatin (adapted from Wary et al.1998, Dhanabal et al 1999, Kim et al. 2000, Ortega & Werb 2002, Urbich et al. 2002, Kim et al. 2002, Lee et al. 2002, Kranenburg et al. 2002, Eriksson et al. 2003)

Induction	Inhibition
Reorganization of actin cytoskeleton (sE)	Proliferation and migration of endothelial cells (bFGF or VEGF induced) (sE)
Clustering of integrin α5β1 followed by co-localization with caveolin (sE)	VEGF induced mobilization of endothelial cells (sE)
Activation of Src tyrosine kinase (sE)	VEGF mediated signaling via VEGF-R1 (sE)
Down-regulation of gene transcription (sE)	Choroidal neovascularization (sE)
G1 arrest of endothelial cells (sE)	Microvessel formation in rat aortic or human vein ring assay (sE)
In vitro stabilization of microvessels (sE)	Chorioallantoic membrane angiogenesis (sE)
Apoptosis (sE)	Migration of neural and non-neural cells in *C. elegans* (sE)
Dephosphorylation of endothelial cell NO synthase (sE)	Migration and branching morphogenesis of renal epithelial cells (sE)
Cell motility of endothelial and non-endothelial cells (NC1 trimer)	Endothelial cell chemotaxis (sE)
Plasminogen activation (isE with β-sheets)	FAK phosphorylation (sE)
Endothelial cell spreading (imE monomeric)	Focal adhesion and actin stress fiber formation (sE)
FAK phosphorylation (imE monomeric)	Endothelial and cancer cell invasion through Matrigel (sE)
	Activity of certain MMPs (sE)
	Amount of uPA and PAI-1 (sE)
	Tumor growth in various mouse models (sE)
	Tumor angiogenesis (sE)
	Endothelial cell tube formation in Matrigel (NC1 trimer)

Abbreviations used in the table: bFGF, basic fibroblast growth factor; FAK, focal adhesion kinase; imE, immobilized endostatin; isE, insoluble endostatin; MMP, matrix metalloproteases; NC1, non-collagenous domain; NO, nitric oxide; PAI, plasminogen activator inhibitor; sE, soluble endostatin; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor; VEGF-R1, vascular endothelial growth factor receptor

Based on the homology search with endostatin, a 22-kDa fragment from type XV collagen was found with 70% homology and with the ability to suppress xenograft carcinoma growth (Ramchandran *et al.* 1999). There are no major defects in collagen XV deficient mice, but further analysis shows that type XV collagen appears to function as a structural component needed to stabilize skeletal muscle cells and microvessels (Eklund *et al.* 2001). While it seems that the functions of endostatin from collagen XVIII and XV

do overlap somewhat, double knockout mice show no additional defects compared to single knockout mice (Ylikärppä *et al.* 2003).

2.4.2 Anti-angiogenic fragments from collagen IV

Type IV collagen is the main component of vascular basement membranes. The mesh-like assembly of collagen IV forms the backbone of the structure together with other macromolecules such as laminin, heparin sulphate proteoglycans, fibronectin and entactin (Timpl 1996). Type IV collagen is composed of six distinct gene products, namely α1-α6 assembled into a trimer (Prockop & Kivirikko 1995). The first clue of the role of collagen IV in angiogenesis came from the observation that α1 and α2 chains isolated from Engelbreth-Holm-Swarm sarcoma tumors inhibit capillary endothelial cell proliferation (Madri 1997). To date, α1 (arresten), α2 (canstatin), α3 (tumstatin) and α6 chains of collagen IV have been shown to possess anti-angiogenic properties.

2.4.2.1 Tumstatin, canstatin and α6 chain

Cryptic activities not exhibited by the whole collagen IV molecule were first found in the α3 chain. Synthetic peptides derived from the NCI domain of the α3 chain have been shown to inhibit the proliferation of melanoma cell line *in vitro* and to prevent the activation of human polymorphonuclear leucocytes (Monboisset *et al.* 1994, Han *et al.* 1997). The 28-kDa anti-angiogenic fragment was named tumstatin (Maeshima *et al.* 2000a). MMP-9 is the most effective in cleaving tumstatin-containing fragments, but MMP-2, -3 and -13 can also release tumstatin (Hamano *et al.* 2003). It inhibits angiogenesis and tumor growth, as well as induces apoptosis of endothelial cells (Maeshima *et al.* 2000a), and interferes with αvβ3 mediated signaling pathways leading to the inhibition of cap-dependent protein synthesis specifically in endothelial cells (Maeshima *et al.* 2002). Tumstatin has two separate binding sites for integrin αvβ3: one in the N-terminal end of the molecule associated with the anti-angiogenic properties and the other in the C-terminal end associated with the anti-tumor activity (Shahan *et al.* 1999, Maeshima *et al.* 2000b). More specifically, the anti-angiogenic activity of tumstatin is localized to a 25-amino acid region within the 69-98-amino acid region, and the synthetic anti-angiogenic peptide from this area is called the T7 peptide (Maeshima *et al.* 2001). It seems that the physiological level of tumstatin is also enough to affect angiogenesis and tumor growth, as shown with collagen α3-chain/tumstatin deficient mice; the administration of exogenous tumstatin to physiological level decreases tumor growth back to wild type level (Hamano *et al.* 2003).

The 24-kDa fragment of the NC1 domain of type IV collagen α2 chain is called canstatin. It inhibits endothelial cell proliferation and migration and induces apoptosis (Kamphaus *et al.* 2000). The receptor of canstatin is not yet known, but it is likely to function via cell surface integrins. Moreover, canstatin-induced apoptosis seems to be associated with phosphatidylinositol 3-kinase and Akt signaling inhibition, and it is dependent upon signaling events transduced through membrane death receptors (Panka *et*

al. 2003). The N-terminal 1-89-amino acid fragment of canstatin inhibits neovascularization *in vivo*, potently induces apoptosis of endothelial cells *in vitro* and suppresses *in vivo* tumor growth in mice, even much more efficiently than the full-length canstatin (He *et al.* 2003). The C-terminal 157-227-amino acid fragment of canstatin also specifically inhibits the proliferation of endothelial cells, induces apoptosis and suppresses tumor growth, but the apoptosis-inducing activity, while close to that of the full-length canstatin, was much lower than that of canstatin-N. Thus, canstatin-C is primarily associated with the inhibition of proliferation, whereas canstatin-N mainly possesses potential apoptosis-inducing activity (He *et al.* 2004).

The α6 chain possesses anti-angiogenic activity and it inhibits tumor growth. Soluble NC1 domain of the α6 chain regulates endothelial cell adhesion and migration (Petitclerc *et al.* 2000).

2.4.2.2 Arresten

Arresten is one of the newly identified endogenous inhibitors of angiogenesis. It is a 26-kDa molecule derived from the NC1 domain of the type IV collagen α1 chain (Colorado *et al.* 2000). Arresten inhibits dose dependently bFGF stimulated endothelial proliferation with an ED50 value as low as 10 nM. Without arresten, aortic endothelial cells cultured on Matrigel rapidly align and form hollow tube-like structures (Grant *et al.* 1994), but arresten disrupts this tube formation. The formation of new blood vessels in Matrigel plugs in mice is reduced significantly with arresten present. It inhibits endothelial cell proliferation and migration without any significant effect on the proliferation of several cancer cell lines, even at very high doses. Arresten affects metastasis leading to a significant reduction of pulmonary nodules in arresten-treated mice and inhibition of renal cell carcinoma growth (Colorado *et al.* 2000).

The mechanism of arresten is not understood yet, but as it binds to integrin α1β1, it might function via that receptor (Colorado *et al.* 2000). Integrin α1β1 is predominantly a collagen receptor, but it also binds other basement membrane constituents, such as laminin (Plow *et al.* 2000). Although it is widely and dynamically expressed during embryogenesis, and in the adult it can be the only collagen receptor in some organs, such as liver, integrin α1β1 knockout mice are viable, fertile and have no obvious phenotype. However, knockout fibroblasts are unable to spread on or migrate into substrata of collagen IV and they are deficient in spreading on and migrating into laminin (Gardner *et al.* 1996). Later it was discovered that the integrin α1 null mice develop osteoarthritis at accelerated speed when they are aging (Zemmyo *et al.* 2003), and during bone fracture healing there is diminished cartilage synthesis in the knockout mice (Ekholm *et al.* 2002). In the vascular system, it is abundantly expressed on microvascular endothelial cells, but not on endothelial cells lining larger blood vessels (Defilippi *et al.* 1991). Lymphatic endothelial cells also express integrin α1β1 (Saharinen *et al.* 2004). The α1 integrin neutralizing antibodies can suppress angiogenesis associated with tumor growth without any effect on the pre-existing vasculature (Senger *et al.* 1997). The β1 integrins are also involved in angiogenesis (Bloch *et al.* 1997). Blocking or ablation of the interactions with α1β1 integrin inhibits angiogenesis, indicating that integrin α1β1 acts as a pro-angiogenic

factor (Senger *et al.* 1997, Pozzi *et al.* 2000). Treatment with VEGF, one of the major angiogenic growth factors, significantly induces the expression of integrin α1β1on dermal microvascular endothelial cells, suggesting particular importance for integrin α1β1 in pathological angiogenesis. (Senger *et al.* 1997). It is the only collagen receptor known to activate the Ras-Shc-mitogen activated protein kinase pathway, thus being capable of promoting cell proliferation (Pozzi *et al.* 1998). Furthermore, implanted tumors are smaller and less vascularized in the integrin α1 deficient mice compared to wild type mice (Pozzi *et al.* 2000, Pozzi *et al.* 2002).

Arresten might also function via heparan sulphate proteoglycans (HSPGs), as it has been demonstrated that both the full-length α1 NC1 domain of collagen IV and arresten itself bind to heparin sulphate proteoglycans (Keller et al. 1986, Colorado et al. 2000). While the binding of arresten to integrin α1β1 shows a high-affinity pattern, the binding to HSPGs is low-affinity (Colorado et al. 2000). Although it is not known yet whether this binding of arresten to HSPGs is significant for the anti-angiogenic function, many recent studies have shown that HSPGs are important in the regulation of angiogenesis. The two most abundant angiogenic factors, VEGF and bFGF, as well as EGF and TGF-β possess heparin-binding properties, and further, it has been shown that receptor binding of VEGF and bFGF is dependent on cell surface HSPGs (Yaon *et al.* 1991, Higashiyama *et al.* 1991, Vlodavsky *et al.* 1996, Dougher *et al.* 1997, Neufeld *et al.* 1999, Jones *et al.* 2000, Qiao *et al.* 2003). The bioavailability of these growth factors is regulated by the balance of their binding capacity onto HSPGs and the action of extracellular matrix degrading enzymes, such as MMPs, which can release heparin binding growth factors to exert their effect (Vlodavsky *et al.* 1996).

3 Outlines of the study

Proteolytic degradation of the extracellular matrix and basement membranes is essential in tumor progression. It allows cells to migrate beyond physical barriers, such as basements membranes, get to the blood stream and metastasize. On the other hand, proteolytic enzymes can also protect the individual from tumor growth. Certain proteases are able to cleave cryptic anti-angiogenic molecules from basement membrane components. The anti-angiogenic fragments can inhibit blood vessel growth to the tumor, so that the tumors cannot grow beyond a few millimeters in diameter. In order to improve our understanding of the complex protease activation cascades, the relationship between proteases and endogenous matrix derived anti-angiogenic molecules, and the mechanisms of certain anti-angiogenic molecules, the following aims were set for this work:

1. Most of the proteolytic enzymes are secreted to the extracellular milieu as inactive latent zymogens that require activation, often by other proteases. Matrix metalloproteases (MMPs), especially gelatinases (MMP-2 and -9), are important enzymes in cancer progression. The amount of tumor-associated trypsinogen-2 (TAT-2) correlates with the aggressiveness of the tumors, and it is known to activate the gelatinases *in vitro*. Therefore, we investigated whether TAT-2 could activate gelatinases and whether that would possess any significance in tumor invasion and intravasation *in vivo*.
2. Endostatin is a thoroughly studied anti-angiogenic molecule derived from collagen XVIII. Our first aim was to study which MMPs can digest endostatin and release endostatin-containing fragments, and whether these fragments have similar biological activity as the parent molecule. We also wanted to study MMPs and endostatin from a different angle: does endostatin have any effect on MMPs, and how would that affect the growth behavior of cultured oral carcinoma cells?
3. Finally, we wanted to investigate the mechanism of arresten, a recently identified anti-angiogenic molecule derived from the α1 chain of collagen IV. Arresten interacts with integrin α1β1 on endothelial cell surface, but the significance of this interaction to the anti-angiogenic activity of arresten has been unclear so far.

4 Materials and methods

4.1 Cloning of the TAT-2 construct (I)

The total RNA isolated from the COLO-205 cells with the Trizol method (according to the manufacturer's instructions, Life Technologies, Grand Island, NY, USA) was used as a template for transcribing the TAT-2 cDNA with RT-PCR. The RT-reaction was done according to the instructions of the Superscript II RNase H- Reverse Transcriptase kit (Gibco BRL Life Technologies, Paisley, Scotland). 3 µg of total RNA and 2.8 pmol of TAT-2 gene specific antisense primer was used. The cDNA was amplified with PCR. The antisense primer was 5'-ATGGGATCCTTAGCTGTTGGCAGCTATGGT-3' and the sense primer was 5'-CTGGCTAGCACCATGAATCTACTTCTGATC-3'. The TAT-2 PCR reaction was performed with 2 U DynazymeEXT DNA polymerase (Finnzymes, Espoo, Finland), 150 ng of each primer, 200 µM dNTP (Promega, Madison, WI), and 0.5 mM $MgCl_2$ in a final volume of 50 µl of 1 x EXT buffer (Finzymes). After initial denaturation (5 min at 95ºC), 25 cycles were performed (1 min at 95ºC, 1 min 15 sec at 54ºC, and 3 min at 72ºC), followed by the final extension of 10 min at 72ºC. The 760-bp TAT-2 PCR-product, purified with QIAEX II kit (Qiagen, Hilden, Germany), was cloned using bidirectional TA-cloning kit (Invitrogen) into a pCR3.1 vector, which was further purified with Quantum Prep kit (BioRad, Hercules, CA). To verify the correct orientation of the TAT-2 gene in the vector and the gene correctness, sequencing was done by ABI PRISM-310 DNA sequencer (Perkin Elmer) using dRhod Terminator Cycle Sequencing Ready reaction kit (Perkin Elmer) and T7 and pCR3.1 reverse primers (Invitrogen).

4.2 TAT-2 transfection (I)

The TAT-2 construct was stably transfected into HSC-3, SCC-25 and IHGK cell lines using Lipofectin Reagent (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. Briefly, a 1-µg quantity of control or TAT-2 plasmid 2.5 µl of Lipofectin reagents were incubated with 30% confluent cells in 24-well dishes (Nunclon,

Roskilde, Denmark) for 5 h in 250 µl of serum- and antibiotic-free medium. After 3 days in normal media, the cells were placed under G418 (Life Technologies, Paisley, Scotland) selection (300 µg/ml for HSC-3 and SCC-25 cells and 600 µg/ml for IHGK cells). After the selection, the presence of trypsinogen-2 mRNA in transfected and untransfected cells was estimated by RT-PCR. Trypsin-2 expression in HSC-3, SCC-25 or IHGK cell lines was studied with RT-PCR. PCR primers and protocols were the same as were used to create the TAT-2 construct. The amount of TAT-2 protein in different clones was measured from serum-free culture medium from TAT-2-transfected and control cells by the immunofluorometric method (Itkonen *et al.* 1990). The endogenous TATI (tumor-associated trypsin inhibitor) amounts were measured immuno-fluorometrically (Osman *et al.* 1993). Equal amount of cells were seeded per well and grown to confluency before 48 hr collection of media. Immunofluorometric assays were based on monoclonal catcher antibody (14D4, monoclonal anti-trypsin-2 IgG) and europium-labeled monoclonal tracer antibody (14F10 (Eu) monoclonal anti-trypsin-2 IgG).

4.3 Extraction of RNA and RT-PCR analysis of MMPs (II)

For RT-PCR analysis of MMPs, total RNA was extracted from HepG2 cells according to (II). Briefly, the RNA extraction and purification took place according to the instructions of the Trizol kit (Gibco). For cDNA synthesis, 4 ng of the total RNA was reverse transcribed using oligo(dT) as a primer. Gene specific primers for amplifying MMP-3, MMP-7, MMP-9, MMP-13 and MMP-20, were used as described in (II). 18S ribosomal RNA was used as a control for RNA integrity. The MMP-9 and MMP-20 PCR products were purified by the QIAEX II agarose gel extraction protocol (Qiagen) and determined by automated sequencing (ABI 310 DNA Sequencer).

4.4 Cell culturing (I, II, III, IV)

Two human tongue cell carcinoma cell lines, a more malignant HSC-3 (JCRB Cell Bank 0623, National Institute of Health Sciences, Osaka, Japan) and a less malignant SCC-25 (ATCC CRL 1628, Rockville, MD, USA), were cultured in 1:1 DMEM and Ham's Nutrient Mixture F-12 supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 units/ml nystatin, 250 ng/ml fungizone, 1 mM sodium pyruvate, 2 mM L-glutamine and 0.4 ng/ml hydrocortisone (Diosynth, Oss, The Netherlands). All cell culture reagents were from Invitrogen, Carlsbad, CA, USA unless mentioned otherwise. IHGK (human papilloma virus-16 immortalized human gingival keratinocytes, later called premalignant oral mucosal keratinocytes (Oda *et al.* 1996)) cells were grown in Keratinocyte growth medium supplemented with 12.5 µg/ml epidermal growth factor, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 units/ml nystatin. The amounts of enterokinase (Boehring Mannheim, Mannheim, Germany) and TATI (tumor-associated trypsinogen inhibitor) (Koivunen *et al.* 1991) in cell culture experiments were 50 ng/ml and 10 µg/ml, respectively. COLO-205 colon adenocarcinoma cells (CCL-222, ATCC, Rockville, MD) were cultured to obtain TAT-2

RNA. The culture media was RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Human hepatoblastoma HepG2 cells (ATCC HB8062) were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. Human umbilical vein derived endothelial cell line EA.hy926 (kindly provided by Cora-Jean Edgell, University of North Carolina, NC) was cultured in DMEM supplemented with 10% FBS, 2 mM glutamine and HAT additive containing 5 mM hypoxanthine, 20 µM aminopterin and 0.8 mM thymidine (Sigma, Saint Louis, MO). Human umbilical vein endothelial cells HUVEC (ATCC CRL-1730) were cultured in EGM-2-MV supplemented media (Clonetics, San Diego, CA, USA) (IV) or in modified Kaighn's F12K medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS, 0.1 mg/ml heparin and 0.1 mg/ml endothelial growth supplement containing bFGF (Sigma, Saint Louis, Missouri, USA) (II). CT26 colon carcinoma cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin.

4.5 Preparation and culturing of primary mouse lung endothelial cells (IV)

Primary mouse lung endothelial cell lines (MLEC) for wild type (Charles River) and integrin α1 knockout balb/c mice (Gardner *et al.* 1996) were prepared using a modified method from Dong *et al.* (1997). The mice were sacrificed with cervical dislocation. Their lungs were perfused with ice-cold PBS-heparin (1U/ml) and the lung and heart were collected into cold Ham's F-12 (Life Technologies). The lungs were minced into small pieces and digested with 0.1% collagenase for 1 hour at 37ºC, homogenized by passing through a cannula and a sieve, and plated in 0.1% gelatin coated flasks in MLEC media (40% Ham's, 40% DMEM low glucose, 20% FBS, 1% penicillin-streptomycin, 2 mM L-glutamine, 100 µg/ml heparin (Sigma) and 50 µg/ml endothelial mitogen (Biomedical Technologies)). Negative selection was done with magnetic beads (Dynabeads M-450 Sheep anti-Rat IgG; Dynal, Oslo, Norway) conjugated with Rat anti-Mouse FcγII/III (Pharmingen), and positive selection was done twice with beads conjugated with Rat anti-Mouse ICAM-2 (Pharmingen). The ratio of antibody and Dynabeads was 1:5, and that suspension was diluted to cold PBS/2% FBS in a ratio of 1:10. The antibody-bead-PBS/FBS suspension was incubated on precooled dishes for 1 h and the cells were trypsinized briefly. In the case of negative selection, cells attached to the beads were discarded, and in the case of positive selection, cell not attached to the beads were discarded.

4.6 MMP inhibitor assay (II)

HepG2 cells were grown in 6-well plates in normal media until they reached confluency. The cells were then incubated with serum-free DMEM for 3 hours. After that the incubation was started with fresh serum-free medium containing increasing

concentrations of MMP inhibitor marimastat (BB-2516) (British Biotech Pharmaceuticals Ltd, Oxford, UK) or human recombinant TIMP-1 (Oncogene Research Products, San Diego, CA). The concentrations of marimastat were 0, 1, 5, 10 and 50 μM, and the concentrations of TIMP-1 were 0, 30 and 50 nM. After 24-h incubation the inhibitors were replenished and SFCM was collected after 48 h. SFCM was concentrated by ultracentrifugation (MWCO 10 kDa), separated by 15% SDS-PAGE under reducing conditions and identified by Western blotting with the polyclonal HES.6 antibody to human endostatin. The polyclonal HES.6 antibody was raised against the C-terminal endostatin portion of the molecule as described (II, Saarela et al 1998, Rehn et al 2001).

4.7 Treatment with endostatin (III)

HSC-3 cells were plated on 24-well plates (Nunclon, Roskilde, Denmark) using cell densities of 18,000, 28,000 or 38,000 cells/well, and the cells were allowed to grow in normal media for 24 h. Then the HSC-3 cells were incubated with serum-free medium containing 0, 1, 5, 10, 12.5, 15, 17.5, 20, 30 or 40 μg/ml of recombinant endostatin for 48 h. After collection, the media were concentrated 4-fold and treated with 1 mM APMA (4-aminophenylmercuric acetate, Sigma) at 37ºC for 25 min to observe the effect of endostatin on proMMP-9 and proMMP-2. The APMA activation was stopped by adding nonreducing Laemmli buffer. MMP-9 processing was detected by gelatin zymography.

4.8 Cell proliferation assay (II, IV)

In part II of this work the proliferation assay was performed as previously described (O'Reilly et al 1997) with a few modifications. A HUVEC cell suspension was plated on 24-well plates at 11,500-12,500 cells/well and incubated (37ºC, 5% CO_2) for 24 h. The medium was replaced with 0.5 ml of fresh medium supplemented with 5% FBS and different concentrations (0, 1, 5, 10 and 20 μg/ml) of various C-terminal fragments of collagen XVIII in triplicate wells. After 72 h, the cells were dispersed in 0.05% trypsin and counted in a Bürker-Türk chamber. In part IV of this study the proliferation assay was done as follows: suspension of primary MLEC (7,000 cells/well, passage 4-5) were plated to 96-well plates precoated with 0.1% gelatin and 10 μg/ml fibronectin. After the cells had attached, they were serum starved (2% FBS) for 24 h. The cells were stimulated with media containing VEGF (10 ng/ml) with 0, 5, 50 or 500 nM of recombinant arresten or T7 peptide. 5 μg/ml polymyxin B (Sigma) was used to inactivate the effects of endotoxins (Liu *et al.* 1997). After 48-h incubation, methylene blue staining was performed: the cells were washed with 1 X PBS, fixed with 10% formalin in neutral buffered saline (Sigma) for 30 min at room temperature. After formalin removal the cells were stained with 1% solution of methylene blue (Sigma) in 0.01 M borate buffer (pH 8.5) for 30 min at room temperature. Cells were washed with borate buffer, and methylene blue was extracted from the cells with 0.1 N HCl/ethanol (1:1). After 1 h, the plates were read on a microplate reader (Bio-Rad) using light absorbance at 655 nm.

4.9 In vitro cell migration assay (III)

HSC-3 cell migration was studied using 8.0-µm pore size and 6.5-mm diameter Transwell inserts (Costar, Cambridge, MA) that were coated with type I collagen (BD Biosciences, Bedford, MA) (1 µg/cm^2 in 10 mM acetic acid for 1 day), and equilibrated in serum-containing medium for 2 h before use. Cells were preincubated at 37°C in a humidified 5% CO_2 atmosphere for 30 min in the presence of 5 or 20 µg/ml endostatin. For migration assay, 600 µl of the serum-containing medium was added to the lower compartment of the migration apparatus and 20,000 cells in a volume of 100 µl of serum-containing medium were plated on a type I collagen coated Transwell filter. After culturing for 6 h, the cells were fixed in methanol, washed and stained in toluidine blue. The cells were removed from the upper surface of the membrane with a cotton swab, and the cells that migrated to the underside of the membrane were counted under the microscope (Koivunen *et al.* 1999, Heikkilä *et al.* 2002).

4.10 Cell viability assay (IV)

Cell viability was measured by MTT (3-(4,5-dimethylthiatzol-2-yl)-2,5-diphenyl tetrasodium bromide, Chemicon, Temecula, CA, USA) assay. Integrin α1 knockout and wild type MLECs (4000 cell/well) were plated in a 96-well plate in MLEC media. The cells were incubated with 0, 5, 50, 500 nM of arresten for 24 h, and the MTT reagent was added for 6 hours. Polymyxin B (5 µg/ml) was used in all wells to remove the effects of possible endotoxin contamination of the bacterial purified arresten. TNF-α (Clontech, Palo Alto, CA) (80 ng/ml) was used as a control for cell death. Live cells produced black crystals in the presence of MTT, and the crystals were dissolved in isopropanol with 0.04 N HCl. The purple color was measured on ELISA reader (BioRad) with a wavelength of 570 nm.

4.11 Cell attachment assay (IV)

96-well plates were coated with 10 µg/ml arresten or 0.5 µg/ml vitronectin (BD Biosciences, San Jose, CA). Plates were blocked with 30 mg/ml bovine serum albumin (Sigma) for an hour. HUVECs or primary MLECs from wild type or integrin α1 knockout mice (100 000 cells/ml) were incubated with 10 µg/ml integrin α1β1 antibody (a generous gift from Humphrey Gardner) for 15 min. In some assays, the cells were incubated with 1 or 5 µg/ml of RGD (Arg-Gly-Asp) peptide or corresponding control peptide RAD (Arg-Ala-Asp) (Peptides International, Louisville, KY). Then 100 µl of cell suspension/well was added onto plates and incubated for 45 min at 37ºC. After washing, the cells were incubated overnight in full media, and the number of attached cells was then determined with methylene blue staining.

4.12 Assays for gelatinase and collagenase activity

4.12.1 Zymography (I, II, III)

Gelatinases were studied by gelatin zymography. It was performed in 10% SDS-PAGE gel that had been cast in the presence of 1 mg/ml fluorescently (2-methoxy-2,4-diphenyl-3-[^{2}H]furanone; Fluka, Ronkonkoma, NY) labeled gelatin (O'Grady *et al* 1984). After electrophoresis, SDS was removed by 2.5% Triton X-100 to renature the gelatinases. Gels were then incubated in 50 mM Tris-HCl buffer (pH 7.8, 150 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) overnight at 37ºC. The degradation of gelatin was visualized under long-wave UV light. Gels were also stained with 0.5% Coomassie blue R-250. The intensities of the separate bands from at least four separate experiments were measured quantitatively by ScionImage software (Scion Corporation, USA).

4.12.2 Gelatin degradation assay (III)

The degradation of gelatin was also assayed using ^{125}I-labeled gelatin as a substrate (Risteli & Risteli 1987). 50 ng of recombinant MMP-9 (Oncogene Research Products, Boston, MA), MMP-2 purified from human gingival fibroblast cultures or MMP-9 purified from human gingival keratinocyte culture media (Mäkelä *et al.* 1994) was incubated with 0, 0.05, 5, 10 or 20 µg of recombinant endostatin in 20 µl of buffer (50 mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM $CaCl_2$) for 1 h at 37ºC. The samples were then treated with or without 1.3 mM APMA at 37ºC for 1 h and incubated with soluble ^{125}I-labeled gelatin (1.5 µM) for 1 h at 37ºC. Undegraded gelatin was precipitated with 20% trichloroacetic acid. The radioactivity in the supernatants, containing the degraded gelatin and reflecting the gelatinase activity, was counted with a γ-counter (Clinigamma, LKB Wallac, Turku, Finland). The radioactivity reflected gelatinase activity (Mäkelä *et al.* 1994).

4.12.3 Collagen degradation assay (III)

The degradation of native type I collagen by MMP-8 and -13 was determined by a collagen degradation assay. 50 ng of recombinant human MMP-8 or -13 (Invitrotek GmHb, Berlin, Germany) was incubated with 600 ng of recombinant endostatin at 37ºC for 45 min with or without APMA treatment (1 mM at 37ºC for 1 h), after which 1.5 µM type I collagen in substrate buffer (Sorsa *et al.* 1994) was added and incubated for 5 h at room temperature. The proteins were electrophoresed by 8-10% SDS-PAGE and stained with Coomassie Brilliant Blue.

4.13 Immunological assays

4.13.1 Western blot (I, II, III)

Western blotting was performed to confirm the results of gelatin zymography used in part I of the study. A monoclonal antibody against active MMP-9 only (Duncan *et al.* 1998) was used. The intensities of the bands from triplicate experiments were analyzed by ScionImage software. In part III of the study, various amounts of recombinant endostatin (0, 120, 360, 600 or 960 ng) were incubated with 50 ng of recombinant MMP-2 and -9 at 37ºC for 45 min with or without APMA treatment (1 mM at 37ºC for 1 h). In addition to APMA activation, proMMP-9 was also activated using 10 ng TAT-2 (purified from serum-free conditioned medium of COLO 205 colon carcinoma cells or cyst fluid of ovarian tumors as described in Koivunen *et al.* 1989 and Koivunen *et al.* 1991) for 45 min at 37ºC (Sorsa *et al.* 1997), and the reaction was stopped with 80 ng of the specific inhibitor TATI (tumor-associated trypsinogen inhibitor, purified from urine of cancer or pancreatitis patients as described in Koivunen *et al.* 1990 and 1991). Protein samples were separated on 10% SDS-polyacrylamide gels under reducing conditions, and electrotransferred to nitrocellulose membranes (Scleiher & Schüll, Dassel, Germany). Non-specific binding was blocked with 5% non-fat dry milk for 90 min at 37ºC. The membranes were incubated with the monoclonal anti-MMP-2 (pretested dilution of 1:400, Chemicon) and polyclonal anti-MMP-9 (pretested dilution of 1:500, Kjeldsen *et al.* 1993) antibodies for 3 h at 37ºC and followed by incubation with peroxidase-conjugated goat anti-rabbit (for polyclonal antibodies) or anti-mouse (for monoclonal antibodies) immunoglobulins (pretested dilution of 1:200, DAKO A/S, Glostrup, Denmark) for 1 h at room temperature. After extensive washing, the immunoreactive proteins were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK) or with 60 mg/ml diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl, pH 8.0, and 0.003% H_2O_2 (Sorsa *et al.* 1994).

4.13.2 Immunomagnetic precipitation (III)

Various concentrations (0, 0.5 and 5 µg/ml) of recombinant endostatin carrying a histidine-tag were preincubated with 200 ng of MMP-9 (purified from human gingival keratinocyte culture media (Mäkelä et al 1994) or with 200 ng of recombinant MMP-9 (Oncogene Research Products, Boston, MA) for 2 hours at room temperature in PBS. 50 µl of IgG-coated magnetic beads (Dynabeads M-280 Sheep anti-Mouse IgG, Dynal, Oslo, Norway) were conjugated with 5 µg of a monoclonal penta-His antibody (Qiagen, Hilden, Germany) for 2 hours in 0.1% BSA-PBS (pH 7.4) at room temperature. The beads were washed twice with 500 µl of 0.1% BSA-PBS, suspended with the preincubated MMP-9-endostatin mix and incubated 2 hours at RT. Unbound proteins were removed by washing the beads three times for 5 minutes with 1% Triton X-100-0.1% SDS-PBS. The bound protein precipitates were removed from the beads by boiling

in loading buffer for 5 min, fractionated on a 12% SDS-PAGE and identified by Western blotting using penta-His antibody and polyclonal MMP-9 antibody.

4.13.3 CD31 staining of tumor blood vessels (IV)

The CD31 immunohistochemical staining was performed as previously described (Hamano *et al.* 2003). Briefly, frozen sections were fixed in cold 100% acetone for 3 minutes and air-dried. They were incubated with rat anti-mouse CD31 antibody (Pharmingen) at room temperature for 2 h, washed three times in PBS and incubated with FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) at room temperature for 1 h. After four PBS washes, Vectashiels (VectorLaboratories, App Imaging) anti-fade mounting medium was applied and the sections were cover-slipped and imaged. For controls, sections were directly incubated with secondary antibody. In each group, the numbers of CD31 positive blood vessels were counted in 10-30 fields at 200X or 400X magnification in a blinded fashion.

4.14 N-terminal microsequence analysis (II)

MMP cleavage products separated on a 12% SDS-PAGE under reducing conditions were blotted onto a PVDF membrane and Coomassie blue-stained bands were excised from the membrane. The N-terminal ends of the cleavage products were determined by automated Edman degradation with a 492 Procise protein sequencer (Applied Biosystems Inc.).

4.15 Protein purification

4.15.1 Purification of human recombinant endostatin (II, III)

The cloning of the recombinant human endostatin used in this work has been described earlier (Rehn *et al.* 2001). A fragment of human collagen XVIII that corresponds to mouse endostatin sequence (O'Reilly *et al.* 1997) was cloned to a pQE-31 expression vector and transformed into the Escherichia coli strain M15(pRep4). Recombinant N-terminal His-tagged protein was expressed according to the protocol suggested by Qiagen (Hilden, Germany). The recombinant endostatin was purified according to a previously described protocol (Rehn *et al.* 2001). Briefly, bacterial pellets were lysed, sonicated and applied to ProBond column (Invitrogen). Bound protein was eluted with imidazole gradient, and eluted endostatin was refolded *in vitro* by dialyzing against buffer with decreasing concentration of urea and finally against PBS. Soluble refolded endostatin was applied to HiTrapSP cation exchange column (Amersham Pharmacia), eluted by NaCl gradient, dialyzed, applied to heparin-Sepharose CL-6B column (Amersham Pharmacia),

eluted by NaCl gradient, dialyzed, and finally passed through a Polymyxin agarose column (Sigma).

4.15.2 Expression and purification of C-terminal fragments of human collagen XVIII (II)

The cDNAs encoding the C-terminal NC1 domain of human collagen XVIII (rhNC1) and the 28-, 25- and 24-kDa endostatin-containing fragments starting at residues Trp^{89}, Tyr^{107} and Tyr^{116}, respectively, were amplified by PCR from a human α1(XVIII) cDNA clone HP19.3 (Saarela *et al.* 1998). The PCR fragments were cloned into the KpnI/HindIII (rhNC1) or BamHI/HindIII (Trp^{89}, Tyr^{107} and Tyr^{116}) site of the expression vector pQE-30 (Qiagen), which contains an N-terminal His-tag. The identity of the PCR-amplified fragments was verified by sequencing. The resulting clones were transformed into the *E. coli* strain M15(pRep4), and recombinant proteins were expressed according to the protocol suggested by Qiagen. For proliferation assay, the three His-tagged recombinant proteins were purified according to a previously described protocol (Rehn *et al.* 2001). When purifying rhNC1 for N-terminal microsequence analysis, the heparin-Sepharose and Polymyxin columns were omitted.

4.15.3 Isolation of human collagen XVIII from cell culture media (II)

Conditioned SFCM from HepG2 cell cultures was collected after 72 h of incubation and subjected to heparin Sepharose purification (Amersham Pharmacia Biotech). Collagen XVIII was eluted from the column with 20 mM Tris buffer, pH 7.5, containing 1 M NaCl. The heparin affinity-purified collagen XVIII was concentrated by ultracentrifugation (Ultrafree, MWCO 30 kDa, Millipore), and the buffer was changed to 50 mM Tris-HCl buffer, pH 7.8, 0.2 M NaCl, 1 mM $CaCl_2$ using a desalting chromatography column (Bio-Rad). The total protein concentration was measured using the Roti®-Quant protein assay (Carl Roth GmbH+Co, Karlsruhe, Germany). Complete™ EDTA-free protease inhibitor cocktail (Boehringer Mannheim) was used throughout the collagen XVIII isolation to prevent proteolysis of the sample.

4.15.4 Purification of human recombinant arresten (IV)

Human recombinant arresten was cloned as described previously (Colorado *et al.* 2000). Briefly, the sequence encoding arresten was amplified by PCR from the α1 NC1 (IV)/pDS vector (Neilson *et al.* 1993), the resulting cDNA was ligated into pET22b(+) expression vector (Novagen, Madison, WI). The plasmid constructs were transformed to BL21 expression vector (Novagen). Overnight culture was inoculated into 500 ml of LB media and grown until OD600 was 0.6. Protein expression was induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. After a 3-hour

induction, the cells were harvested, lysed in lysis buffer (6 M guanidine, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0), sonicated and centrifuged. The supernatant was passed through a Ni-nitriloacetic acid agarose column (Qiagen, Chatsworth, CA) four times at a speed of 1 ml/min. Arresten was eluted with an increasing concentration of imidazole (10, 25, 40, 125 and 250 mM) in 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0, and dialyzed twice against PBS. Arresten concentration and purity was assayed with the bicichoninic acid assay (Pierce, Rockford, IL) and SDS-PAGE.

4.16 Synthesis of T7 peptide (IV)

Synthetic T7 peptide (TMPF LFCN VNDC NFAS RNDY SYWL) derived from tumstatin (Maeshima *et al.* 2001) was synthesized at Tufts University Core Facility and analyzed by mass spectrophotometric analysis and purified by analytical HPLC.

4.17 Processing of human collagen XVIII and the rhNC1 domain by MMPs (II)

Aliquots of heparin Sepharose-enriched human collagen XVIII from HepG2-cells (total protein 8 µg) or rhNC1 (3-5 µg) were incubated with various MMPs (molar enzyme/substrate ratios between 1:3 and 1:1000 as indicated in II) from 30 minutes up to 72 hours at 37ºC in 50 mM Tris-HCl, pH 7.8, containing 0.2 M NaCl and 1 mM $CaCl_2$. When available, MMPs from two different sources were used for the digestions. Latent forms of MMPs were activated with 0.5 mM APMA, and MMP cleavage was inhibited with 20 mM EDTA. The catalytic activities of the pure human and rat MMPs used were confirmed by assaying their degradative action against native type I collagen, gelatin, β-casein and laminin-5 γ2-chain as described (Sorsa *et al.* 1997, Hanemaaijer *et al.* 1997, Teronen *et al*. 1999, Blezinger *et al.* 1999, Pirilä *et al.* 2003). The digestions were terminated by SDS sample buffer containing β-mercaptoethanol. The cleavage products were analyzed by Western blotting with antibodies against human endostatin.

4.18 CAM assay (I, III)

The CAM (chorioallantoic membrane) assay was done according to Kim *et al.* (1998) except for a few modifications. Chick embryos were maintained at 37ºC in a humidified incubator and turned manually at least three times a day. Cells used for the invasion experiment (HSC-3 and HSC-3+TAT-2) were detached from the culture dish, counted and resuspended in PBS++ solution with Ca^{2+} and Mg^{2+} (13.7 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 700 µM $CaCl_2$ $2H_2O$, 490 µM $MgCl_2$ $6H_2O$). 2 x 10^6 cells in 50 µL of PBS++ with or without 48 ng enterokinase (Boehring Mannheim, Germany) and 600 ng TATI (Koivunen *et al.* 1991) were inoculated onto a CAM of 10-day-old chick embryos (part I), in which an artificial air sack was created. In part III the same amount

of HSC-3 cells was inoculated with different amounts of endostatin (0, 0.5, 5, 20 and 50 µg/CAM). After 50 hr of incubation, eggs were cut along the long circumference, and the upper halves (with the inoculum) and the contents of the lower halves of the eggs were discarded. The CAMs lining the cavity of the eggshell was removed, snap-frozen and used for extraction of genomic DNA.

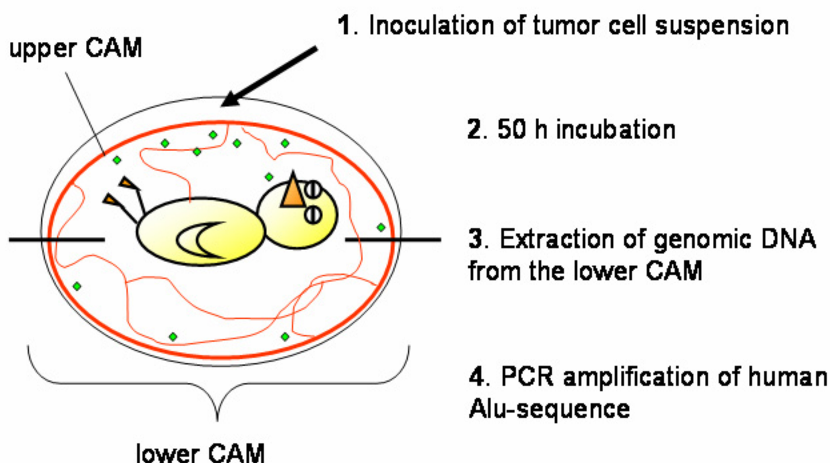

Fig. 7. Schematic illustration of the chicken chorioallantoic membrane assay.

The frozen CAMs were crushed to fine powder, suspended in digestion buffer (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS, 0.1 mg/ml proteinase K) and incubated at 50ºC for 18 h. The samples were extracted with phenol/chloroform/isoamylalcohol (25:24:1) and centrifuged 10 min at 1,700 x g. The DNA in the aqueous phase was precipitated with 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of ethanol, centrifuged 2 min at 1,700 x g, washed, dried and resuspended in sterile water. The radioactive PCR, which produced an Alu-band of 224 base pairs, was done according to Kim *et al.* (1998), except that the enzyme used in PCR was Dynazyme (Finnzymes). PCR products were electrophoresed on a 6% polyacrylamide gel at 1500 V for 1.5 hr and exposed to film at -70ºC. The bands from four separate experiments were quantitated by densitometric scanning using ScionImage.

4.19 Matrigel plug assay (IV)

Matrigel (BD Biosciences) was thawed at 4ºC. Before injection to the integrin α1 knockout and wild type balb/c mice, it was mixed with 20 U/ml heparin (Pierce,

Rockford, IL), 50 ng/ml VEGF (R&D Systems, Minneapolis, MN) and 100 ng/ml bFGF (R&D Systems) with either PBS (control group, n=10 for wild type or n=9 for knockout), 200µM T7 peptide (T7 group, n=9) or 200 µM recombinant human arresten (arresten group, n=10 for wild type or n=9 for knockout). All groups had 3% DMSO in the Matrigel. The Matrigel mixture was injected subcutaneously. After 6 days the mice were sacrificed, the plugs removed, fixed in 10% formalin in neutral buffered saline (Sigma Diagnostics), embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). Sections were examined by light microscopy, and the number of blood vessels was counted and averaged (200X magnification). All mouse experiments were performed according to institutional animal care guidelines.

4.20 In vivo tumor studies (IV)

Mouse colon tumor cells (CT26) that were tumorigenic in syngeneic wild type and integrin α1 knockout balb/c mice (Xiang *et al.* 1998, Pozzi *et al.* 2002) were harvested. Cell viability was estimated with thymidine blue staining, and 0.5 million viable cells were injected subcutaneously into 3-month-old wild type and integrin α1 knockout littermates. The tumors were allowed to grow to around 40 mm^3. Arresten, T7 or PBS with 4% DMSO was injected i.v. daily at an equimolar dosage of 10 mg/kg (arresten) or 1 mg/kg (T7) for 16 days. The tumor volume was measured using the standard formula length x $width^2$ x 0.52. Each group contained 6 or 7 mice.

4.21 Statistical analysis (I, IV)

Scheffe's test (I) or Student's t-test (IV) was performed to estimate the statistical significance of differences. P values less than 0.05 were considered significant.

5 Results

5.1 The effect of TAT-2 on gelatinases and invasion (I)

5.1.1 Generation of stably transfected cells secreting TAT-2

To investigate the role of TAT-2 in carcinoma cell invasion, the full-length TAT-2 cDNA in the pCR3.1 eukaryotic expression vector was transfected into two oral squamous cell carcinoma cell lines, the more malignant HSC-3 and the less malignant SCC-25, and to one premalignant immortalized human gingival mucosal keratinocyte cell line, IHGK. Normally these cell lines do not express TAT-2 or only do so at very low levels as analyzed by RT-PCR. The TAT-2 transfected cells were selected from untransfected ones with geneticin selection antibiotic and as a result, only a few clones originating from single cells were growing per cell culture well. Stably transfected clones were examined for TAT-2 expression, first with RT-PCR and then immunofluorometrically. After the transfection, the amount of TAT-2 mRNA was elevated in all of the clones of the three cell lines examined by RT-PCR. More importantly, the immunofluorometric measurements showed that the amounts of secreted TAT-2 protein in the media (48 hr) of the selected TAT-2 transfected clones were significantly higher than in control cells transfected only with the empty vector. In SCC-25 cells the increase of TAT-2 protein after the transfection was highest; control cells produced no TAT-2 protein at all, but in the transfected clones the mean production was 35 ng/ml. In IHGK cells the TAT-2 production increased from less than 1 ng/ml to 22 ng/ml. Control HSC-3 cells secreted about 2 ng/ml TAT-2, whereas TAT-2 transfected HSC-3 clones produced 7 ng/ml TAT-2. In order to create model cell lines for gelatinase activation and intravasation studies, clones with the highest increase in TAT-2 production were chosen for future experiments. Tumor-associated trypsinogen also has a specific inhibitor, TATI (tumor-associated trypsin inhibitor) (Halila *et al.* 1988). By measuring the amounts of endogenous TATI levels in all the culture supernatants we excluded the possibility that the endogenous TATI might inhibit TAT-2. In all three cell lines studied, the TATI levels were very low –

in fact, below detection limit – and thus endogenous TATI levels did not interfere with the experiments.

5.1.2 The effect of TAT-2 transfection on progelatinase activation

Trypsinogen-2, the latent form, was first activated to catalytically competent trypsin-2 by adding enterokinase to the cell culture medium. MMP-2 and -9 secreted into the culture media by the three lines of control oral epithelial cells and the selected clones of TAT-2-transfected cells described in the previous chapter were analyzed by gelatin zymography and Western blotting. MMP-9 was partially in the converted form already in the media of the HSC-3 control cells, but the proportion of active MMP-9 was significantly increased in the TAT-2 transfected HSC-3 clones. We selected two clones with the highest increase in the TAT-2 protein production for zymography. The addition of TATI, a specific naturally existing inhibitor of TAT-2, prevented the increased conversion of the 92-kDa proMMP-9 to the 77-kDa active form. The ratio of active MMP-9 *vs.* latent MMP-9 increased up to 4.4-fold, the average increase being 2.2-fold. In the presence of TATI, the active MMP-9 *vs.* proMMP-9 ratio was reduced back to about the level of controls. The results were from four separate experiments. The differences in active MMP-9 *vs.* proMMP-9 ratios between control and TAT-2 transfected HSC-3 conditioned cell culture media are statistically different ($p \leq 0.01$) as well as differences in ratios between TAT-2 transfected and TATI treated media ($p \leq 0.01$). Western blotting experiments confirmed the results of zymography. The use of a monoclonal antibody specific for only the active form of MMP-9 in the Western blot (Duncan *et al.* 1998) revealed that the amount of active MMP-9 in TAT-2 transfected HSC-3 cell media increased about 80% compared to the control HSC-3 cell media. However, the proMMP-9 secreted by the IHGK and SCC-25 cells was not affected by TAT-2 overproduction. Also, the increased TAT-2 production had no effect on proMMP-2 in any of the cell lines. It remained in the latent form in the control HSC-3 cells and in TAT-2 transfected HSC-3 clones as well as in SCC-25 and IHGK cells before and after the TAT-2 transfection.

5.1.3 The effect of TAT-2 on carcinoma cell intravasation

To study how the TAT-2 transfection would affect intravasation, the first step of invasion, CAM (chicken embryo chorioallantoic membrane) assay was used. The intravasation of the TAT-2 transfected HSC-3 cells was enhanced up to 80% compared to the control HSC-3 cells. As the TAT-2 activator enterokinase is not only specific to TAT-2, the addition of enterokinase along with the cells was alone sufficient to increase the intravasation by 24% (SD ± 11.0 %) when compared to untreated HSC-3 control cells. However, the increase in the intravasation of TAT-2 transfected HSC-3 cells in the absence of enterokinase was higher, 43% (SD ± 12.4%). Further, the intravasation of TAT-2 transfected HSC-3 cells in the presence of enterokinase was even more efficient, 66% (SD ± 19.4%) higher compared to the untreated control cells. In order to investigate further whether TAT-2 actually was the reason for the increased intravasation, the activity

of TAT-2 was inhibited by a specific inhibitor, TATI, which was inoculated onto the upper CAMs together with the TAT-2-transfected or control cells with or without enterokinase. In the presence of TATI, the intravasation of the TAT-2 transfected cells was only 15% (SD ± 2.5%) higher than in untreated control cells. In other words, the TAT-2 transfected HCS-3 cells intravasated about 65% less efficiently in the presence of TATI than in the absence of it ($p \leq 0.05$). When also enterokinase was present, the addition of TATI reduced the intravasation of TAT-2 transfected cells almost to the level of untreated controls. In the absence of TATI, the intravasation efficiency of enterokinase treated and TAT-2 transfected HSC-3 cells was as high as 12-fold. The difference was statistically very significant ($p \leq 0.001$).

5.2 Processing of collagen XVIII by MMPs (II)

5.2.1 Processing of HepG2-derived human collagen XVIII by MMPs

Human collagen XVIII enriched by heparin affinity chromatography from conditioned serum-free HepG2 hepatoblastoma cell culture media was incubated with various MMPs (molar enzyme/substrate ratio between 1:3 and 1:15 as indicated in II) for 24 h at 37°C. The cleavage products were separated with SDS-PAGE under reducing conditions and identified by Western blotting with polyclonal antibodies against the N-terminal (anti-all huXVIII) and C-terminal (HES.6) portions of the collagen XVIII molecule. The over 200-kDa full-length glycosylated collagen XVIII molecule formed on a 7% polyacrylamide gel a smear-like band, which was detected with both the anti-all huXVIII and the HES.6 antibodies. In 12% SDS-PAGE it migrated as a sharp band near the border of the stacking and separating gels. Although a protease inhibitor cocktail was used throughout the purification procedure, certain endogenous proteases from the HepG2 cells digested the protease sensitive hinge region of the collagen XVIII, producing faint bands between 24 and 38 kDa that were reactive with the HES.6 antibody. However, degradation of the full-length collagen XVIII and a significant increase in the occurrence of small endostatin-containing peptides in the interval 24-30 kDa was clearly detected after the incubation of collagen XVIII with MMP-3, -7, -9, -13 and -20 for 24 h at 37ºC, whereas MMP-1, -2, -8 and -12 resulted in only very little, if any, degradation of collagen XVIII as compared with a control incubation without MMPs. Even the use of increased amounts of these MMPs or prolonged incubations of up to 72 h did not result in the generation of endostatin fragments. Next, a detailed time course study and series of enzyme dilutions for each MMP were performed. With MMP-7, -9 and -20, only a 30-minute incubation at 37°C was sufficient to yield cleavage products between 24-38 kDa reactive to endostatin antibody, and with time, the proportion of smaller endostatin-containing peptides between 24-30 kDa increased. During longer incubations the dominant cleavage products of 30, 28 and 24 kDa accumulated by MMP-7, -9 and -20, respectively. MMP-3 and MMP-13 were considerably less active against collagen XVIII,

and an efficient cleavage was only seen after several hours of incubation at 37ºC. Molar enzyme/substrate ratios lower than 1:10-1:15 proved to be fairly inactive in generating endostatin-containing fragments from HepG2-derived collagen XVIII; only MMP-9 showed a slight effect with a ratio of 1:50.

5.2.2 Inhibition of MMPs

The addition of EDTA to the incubation mixtures yielding a final concentration of 20 mM completely abolished the proteolytic cleavage, confirming that it is in fact MMP-dependent. To study more closely the endogenous MMP cleavage of human collagen XVIII by HepG2 cells we used the broad-spectrum MMP inhibitor marimastat (BB-2516) to block the activity of major metalloproteinase subtypes. The addition of marimastat (1-50 μM) into the cell culture medium impaired the formation of endostatin-containing fragments between 24-30 kDa, indicating that their production is due to MMP action on type XVIII collagen. When the intensities of the endostatin-containing peptides were quantified, the results showed that the addition of 1-50 μM marimastat into the culture media decreased the formation of endostatin-containing fragments from 25 to 60%. The more specific synthetic inhibitor called MMP Inhibitor III, which inhibits the activity of MMPs -1, -2, -3, -7 and -13, reduced the formation of these fragments. TIMP-1 (30-50 nM), an endogenous inhibitor of all the MMPs used in this study (Baker *et al.* 2002), also had the same inhibitory effect. The quantification of the endostatin-containing peptides showed that TIMP-1 decreased the formation of the fragments 20-30%.

5.2.3 Production of MMPs by HepG2 cells

To see whether the ability of these five MMPs to generate endostatin-containing fragments might have any significance *in vivo*, the synthesis of these MMPs by the HepG2 cells was analyzed. The mRNA expression of the five MMPs that were able to degrade collagen XVIII and produce endostatin fragments was studied by RT-PCR. As expected, MMP-3, -7 and -13 were clearly expressed by HepG2 cells, but in contrast to earlier publications (Giambernardi *et al.* 1998, Grant *et al.* 1999), MMP-9 and -20 mRNAs were also detected. The identities of their PCR products were confirmed by DNA sequencing. Furthermore, the active forms of these MMPs were also present in the HepG2 culture media. The presence of collagenolytic activity in the HepG2 conditioned media was verified by the collagen degradation assay that showed the processing of native type I collagen to characteristic ¾ αA-cleavage products. Media pretreated with TIMP-1 failed to generate these fragments. Zymography analysis revealed one 92-kDa gelatinolytic band representing the latent form of MMP-9 and a 82-kDa band representing the active form. In addition, antibodies against MMP-3, -7, -13 and -20 recognized several immunoreactive species that corresponded to the reported molecular weights of latent and active forms of these MMPs.

5.2.4 Processing of the recombinant NC1 domain of human collagen XVIII by MMPs

To get a better insight into the degradative activity of MMPs on the NC1 domain of collagen XVIII, we cloned and expressed the recombinant human NC1 (rhNC1) in *E. coli* and purified it with the aid of a HIS-tag inserted into the N-terminus of the recombinant protein. 3-5 μg of the purified rhNC1 was incubated with the MMPs, molar enzyme/substrate ratios being 1:10 or 1:15, for 24 h at 37°C. The resulting cleavage products were analyzed by Western blotting with a polyclonal peptide antibody ES2a (antibody produced as described in II). This antibody detects the C-terminal fragments of NC1, but not the N-terminal ones like HES.6 antibody. The results of these digestions confirmed the results obtained with the endogenous HepG2-derived collagen XVIII. Again, MMP-3, -7, -9, -13 and -20 clearly degraded the rhNC1, yielding endostatin-containing fragments of 20-30 kDa, whereas MMP-1, -2, -8, and -12 did not show any activity against rhNC1, even when used in molar excess amounts. The lowest functional molar enzyme/substrate ratios for MMP-3, -7, -9, -13 and -20 were 1:100, 1:1000, 1000, 1:100 and 1:1000, respectively. MMP-7 formed three cleavage products reactive with the ES2a antibody, the major bands having molecular masses of 30 kDa and 27 kDa. MMP-20 cleaved NC1 into a 24-kDa peptide detectable with ES2a, and MMP-13 generated two fragments of approximately 24 and 28 kDa. Incubation with MMP-3 and MMP-9 resulted only in a partial digestion of NC1 in the 24-h period. MMP-3 produced several ES2a-positive peptides between 20 kDa and 30 kDa, and MMP-9 generated two endostatin-related fragments with molecular masses of 28 kDa and 24 kDa. The calculated sizes of the major cleavage products corresponded to the endostatin-containing fragments released from HepG2-derived collagen XVIII by MMPs. There was one difference in cleavage of endostatin fragments between full-length collagen XVIII and recombinant NC1 domain: the cleavage of the 20-kDa endostatin fragment by MMP-3 was detected only when recombinant NC1, but not HepG2-derived native collagen XVIII, was used as a substrate, which may reflect defects in the folding of the rhNC1. The addition of a metal chelator, 20 mM EDTA, inhibited the degradative action of the MMPs on the recombinant NC1.

5.2.5 Sites of cleavage of the human collagen XVIII NC1 domain by MMPs

To determine the specific cleavage sites generated in the NC1 domain by MMP-3, -7, -9, -13 and -20, 10-15 μg of recombinant human NC1 was incubated with these MMPs for 24 h at 37°C. The cleavage products excised from a Coomassie blue-stained PVDF membrane were sequenced by automated Edman degradation. When available, MMPs from two different sources were used for these experiments, and each cleavage product was sequenced at least twice. The results are summarized in Table 6.

Table 6. Endostatin fragments generated from human collagen XVIII NC1 domain by distinct MMPs.

MMP	Molar enzyme substrate ratio	Size (kDa)	N-terminal sequence	Cleavage site
MMP-3	1:10, 1:20, 1:30	24	YVHLRPARXT	Ser115 - Tyr116
		20	SHRXFQPVL	His130-Ser131
MMP-7	1:20, 1:30	30	LHDSNPYPXR	Gln69 - Leu70
		27	ILASPPXL	Asp93 - Ile94
		24	XVHXRPARXT	Ser115 - Tyr116
MMP-9	1:10	28	WRADDILASP	Pro88 - Trp89
		25	YPGAXXX	Pro106-Tyr107
MMP-13	1:15, 1:25, 1:30, 1:50	28	XRADDILASP	Pro88 - Trp89
		24	YVHLRPARPT	Ser115 - Tyr116
		24	SSYVHLRPART	His113 - Ser114
MMP-20	1:40, 1:100	24	YVHLRPARPT	Ser115 - Tyr116

5.2.6 Endothelial cell proliferation assay

The anti-angiogenic activities of the native 20-kDa endostatin, three C-terminal fragments from human collagen XVIII, the 24-, 25- and 28-kDa endostatin-containing peptides starting at residues Tyr^{116} and, Tyr^{107} and Trp^{89}, respectively, and the full-length NC1 domain of 38 kDa, were measured using bFGF-stimulated HUVEC cells. Depending on the experiment, 11,500 - 12,500 cells were plated on each well in 24-well plates. After 24 h incubation the culture medium was replaced with fresh medium containing different concentrations (0-1 μM) of various endostatin-containing fragments. After 72 h incubation, the cells were counted and the proliferation of the cells was compared to control samples (% of control). The native endostatin and all four longer endostatin-containing peptides inhibited the proliferation of HUVEC cells *in vitro* in a dose-dependent fashion. The inhibition with increasing concentrations of the fragments ranged between 25-80% of the controls. The smaller 25- and 24-kDa fragments followed nicely the inhibition profile of recombinant human endostatin, whereas the two longer fragments had more effect on proliferation of HUVECs at high concentrations. Some of the peptides (native 20-kDa endostatin, the 28- and 25-kDa fragments) were also tested in 24 h proliferation assay, and they showed essentially similar inhibition curves as in the 72 h assay. During the proliferation assays the longer endostatin-containing fragments started to degrade to the stable 20-kDa endostatin form. However, the two longer fragments appeared to inhibit HUVEC cell proliferation slightly more efficiently, suggesting that the anti-proliferative effects caused by the longer fragments could not only be a consequence of their processing to endostatin. but also indicated activity of the longer fragments themselves. We also tested some of these fragments in an *in vitro* VEGF-induced endothelial cell migration assay and saw that endostatin was more potent in preventing HUV-EC-C cell migration than the 24-kDa peptide, whereas the endostatin

and 28-kDa fragments inhibited human hybrid endothelial cell (EA.hy926) migration with the same efficiency.

5.3 The effect of endostatin on gelatinases and collagenases (III)

5.3.1 Endostatin inhibits the activation and activity of proMMP-9

In order to determine whether the anti-tumor activity of endostatin is associated with MMPs, and particularly with the inhibition of MMP-9, the effect of endostatin on the levels of and degree of activation of the MMP-9 secreted by the cultured HSC-3 tongue carcinoma cells was studied. Gelatin zymography of the serum-free culture medium revealed that endostatin clearly blocked the APMA-mediated proMMP-9 activation at endostatin concentrations from 12.5 μg/ml to 40 μg/ml (0.625 to 2 μM) in a dose-dependent manner. The molecular sizes of proMMP-9 and active MMP-9 were 92 kDa and 77 kDa, respectively. Without APMA activation endostatin had no effect on MMP-9, so endostatin interferes somehow with this activation process. The ratios of proMMP-9 *vs.* active MMP-9 increased slightly even with lower concentrations of endostatin (0.05-0.5 μM). With endostatin concentrations of 0.625 and 0.75 μM the ratio was 1.5-fold, and at endostatin concentrations of 0.875, 1, 1.5, and 2 μM the ratios were 2-, 2-, 2.5- and 3-fold, respectively, in relation to the control. The effect of endostatin on the catalytic activities of gelatinases was studied by measuring the degradation of radioactively labeled gelatin. Endostatin decreased the activity of APMA-activated MMP-9 (50 ng, purified from human gingival keratinocyte culture media) from 100% to 92%, 85%, 76% and 61% with molar ratios of 0, 5:1, 500:1, 1000:1 and 2000:1 to MMP-9, respectively. Endostatin also inhibited the activity of MMP-9 without the treatment with APMA, but it was slightly less efficient: gelatin degradation was decreased from 100% to 98%, 97%, 82% and 70% with molar ratios of 0, 5:1, 500:1, 1000:1 and 2000:1 to MMP-9 (endostatin amounts of 0, 0.05, 5, 10 and 20 μg), respectively.

5.3.2 Cell density affects the ability of endostatin to inhibit MMP-9

The density of HSC-3 cells per well was found to be rather critical for the inhibitory effect of endostatin in the cell culture experiments. The ideal cell density was found to be 28,000 cells/well in 24-well plates. At lower cell densities (18,000 cells/well), the effect of endostatin was much less clear, and at higher densities (38,000 cells/well), the effect on proMMP-9 activation disappeared almost completely.

5.3.3 Endostatin inhibits APMA and TAT-2 activation of recombinant human proMMP-9

In addition to studying how endostatin affected gelatinases in the HSC-3 cell culture system, recombinant purified gelatinases were also used. Recombinant human endostatin was incubated with recombinant proMMP–9 that was activated with APMA or TAT-2. The processed forms of MMP-9 were then separated by SDS-PAGE and identified by Western blotting. As was expected based on the results of zymography, endostatin decreased the APMA-mediated activation of MMP-9 in a dose-dependent manner. The endostatin concentrations used were a little lower than in cell culture experiment: 0, 120, 360, 600 and 960 ng (molar ratios of 12:1, 36:1, 60:1 and 96:1). The ratios of pro/active MMP-9 increased 1.6-fold when endostatin was present compared to the control treated only with APMA. Although APMA activation is quite an efficient way to activate MMPs and study the activation mechanisms, APMA is a chemical agent that does not occur in the nature. We also activated proMMP-9 with its naturally occurring activator, TAT-2, and studied the effects of different amounts of endostatin (0, 200, 400, 600, 800 and 1000 ng, endostatin/MMP-9 molar ratios of 20:1, 40:1, 60:1, 80:1 and 100:1). Endostatin clearly decreased TAT-2 mediated conversion of the 92-kDa proMMP-9 to the 77-kDa active form. The ratio of the pro/active MMP-9 band intensity increased up to 7-fold with the highest amount of endostatin compared to the control treated with only TAT-2, and even with 200 ng of endostatin the ratio was 2.7-fold higher than the pro/active MMP-9 ratio with only TAT-2 and without endostatin.

5.3.4 Endostatin binds to proMMP-9 in vitro

Since endostatin was able to inhibit the activation of proMMP-9 by both APMA and TAT-2, it seemed possible that endostatin would form a complex with MMP-9. To examine this, recombinant MMP-9 and MMP-9 purified from human gingival keratinocyte culture media was incubated with increasing concentrations of HIS-tagged endostatin. The complex was immunoprecipitated with the anti-HIS antibody. A Western blot shows that proMMP-9 was coprecipitated in the presence of HIS-tagged endostatin in a dose-dependent manner. However, a trace amount of MMP-9 was detectable even in the absence of endostatin.

5.3.5 The effect of endostatin on recombinant human proMMP-2

In HSC-3 cell media endostatin had no apparent effect on the 72-kDa proMMP-2 levels and activation or on the levels of the barely detectable 62-kDa active form, but it very clearly inhibited the formation of smaller (approximately 40 kDa) activation products of MMP-2. The effect of endostatin on recombinant proMMP-2 was identified by Western blotting. Again endostatin reduced the fragmentation of APMA-treated 72-kDa proMMP-2 to approximately 40-kDa species, but it did not affect the conversion to the 62-kDa

form. A gelatin degradation assay with MMP-2 purified from human gingival fibroblast cell media showed that endostatin inhibited APMA activation of MMP-2, and in high concentrations also reduced the MMP-2 activity in the absence of APMA. Gelatin degradation was decreased from 100% to 82, 60, 41 and 47% in the presence of APMA and from 100% to 95, 97, 63 and 74% in the absence of APMA with endostatin concentrations of 0, 0.05, 5, 10 and 20 μg, respectively.

5.3.6 The effect of endostatin on MMP-8 and -13

The activities of human interstitial collagenases MMP-8 and -13 were studied by assaying type I collagen degradation by SDS-PAGE. Recombinant endostatin (20:1 molar ratio to MMP-13) inhibited the MMP-13 mediated formation of α1A-, α2A-, α1B- and α2B-degradation products from α1 and α2 chains of native type I collagen, when MMP-13 was treated with APMA. However, endostatin had no effect on MMP-8 (20:1 molar ratio) mediated type I collagen degradation in the presence or absence of APMA.

5.4 Endostatin inhibits human carcinoma cell migration *in vitro*

Endostatin is an efficient inhibitor of the migration of endothelial cells (Sudhakar *et al.* 2003), but its effect on the migration of human oral squamous cell carcinoma HSC-3 cells had not been known thus far. It was studied here using an *in vitro* Transwell assay measuring random cell migration. Endostatin concentrations of 0-1 μM prevented migration of HSC-3 cells in a concentration-dependent manner. Migration was 89, 80 and 55% of controls (100%) with endostatin concentrations of 0.15, 0.25 and 1 μM, respectively. With the highest concentration of endostatin (2 μM), the inhibitory effect on migration was partially lost (66%).

5.5 Endostatin inhibits human carcinoma cell intravasation in the CAM model

During intravasation the tumor cells invade the bloodstream through blood vessel walls. It is one of the first key steps of the carcinoma process leading to metastasis. We studied the effect of recombinant endostatin on the intravasating capacity of the HCS-3 carcinoma cells. In the *in vivo* CAM assay the intravasation decreased in a dose-dependent manner to half with low concentrations of endostatin (0.5 and 5 μg/CAM), but with higher concentrations of endostatin (20 and 50 μg/CAM) the inhibitory effect on intravasation efficiency was partially lost and dose-dependency was no longer observed. The intravasation efficiencies were 66, 55, 63 and 71% compared to untreated controls with endostatin concentrations of 0.5, 5, 20 and 50 μg/CAM, respectively.

5.6 The anti-angiogenic and anti-tumorigenic mechanism of arresten (IV)

5.6.1 Arresten binds to integrin α1

Human arresten inhibits tumor angiogenesis in mice by targeting vascular endothelial cells in the tumors (Colorado *et al.* 2000). Human recombinant his-tagged arresten was purified in *E. coli* resulting in mainly soluble 29-kDa protein consisting of 26-kDa native arresten and 3-kDa polylinker and 6-histidine tag sequences. It has been previously shown that arresten binds to integrin α1β1 as well as heparan sulphate protoglycan on the endothelial cell surface, but it was not known whether the binding to the α1β1 integrin was sufficient for the anti-angiogenic and anti-tumorigenic function (Colorado *et al.* 2000). The attachment of primary mouse lung endothelial cells from knockout mice lacking integrin α1 (Gardner *et al.* 1996) or wild type balb/c mice were studied. On arresten coated plates the attachment of wild type endothelial cells was significantly reduced ($p < 0.01$) in the presence of integrin α1 blocking antibody (56% of control), but the integrin α1β1 blocking antibody had no effect on the attachment of integrin α1 knockout cells (107% of control). IgG was used as control, and it did not inhibit the attachment of the wild type cells to arresten (90%), and it even slightly increased the integrin α1 knock out MLEC attachment (129%). As another control, cell attachment onto plates coated with fibronectin was examined, as it is known that integrin α1β1 does not bind fibronectin. As expected, the attachment of wild type or integrin α1 endothelial cells to fibronectin was not affected by integrin α1β1 or control IgG blocking antibodies.

5.6.2 Arresten functions RGD-independently

The RGD (Arg-Gly-Asp) sequence present in many matrix molecules is important for the function for the binding of many integrins, especially integrin αvβ3 and α5β1 (Plow *et al.* 2000). To elucidate whether the binding of integrin α1β1 on endothelial cells was dependent of the RGD sequence, synthetic cyclic RGD peptide and negative control peptide RAD (Arg-Ala-Asp) were used in attachment assays. Neither the RGD peptide nor the negative control peptide had any effect on the endothelial cell attachment on the arresten-coated surface. Vitronectin coating was used as positive control, since binding between vitronectin and integrin αvβ3 is known to be RGD-dependent (Plow *et al.* 2000): as expected, in the presence of the RGD peptide cell attachment to vitronectin was reduced almost 50% ($p < 0.05$).

5.6.3 Integrin α1 is required for the anti-proliferative and anti-apoptotic effect of arresten

If the anti-angiogenic action of arresten was in fact mediated through integrin α1β1, the cells lacking that receptor should not respond to it. T7 peptide corresponding to the active site of tumstatin inhibits angiogenesis via binding to integrin αvβ3, and thus the lack of integrin α1 should not have any effect on its anti-angiogenic properties (Maeshima *et al.* 2000a). Both arresten and T7 peptide inhibited the proliferation of wild type primary mouse lung endothelial cells in response to VEGF stimulation. The inhibition was 50, 52 and 72% of control with arresten (5, 50 and 500 nM) and 58, 56 and 51% with equimolar amounts of T7 peptide. As hypothesized, the proliferation of MLEC from integrin null mice was inhibited only with T7 (58, 44 and 25% of control). There was no clear concentration-dependent effect with arresten (proliferation 97, 139 and 91% of control). The effects of arresten and T7 on the survival and apoptosis of wild type and integrin α1 knockout endothelial cells was measured using the MMT assay that is based on the detection of functioning mitochondria in living cells. Again, arresten inhibited cell survival of only the wild type cells, whereas T7 had an inhibitory effect on both wild type and integrin α1 knock out cells. Endothelial cell survival was 34% and 23% of control when wild type cells were treated with 500 nM arresten or T7 peptide, respectively. When integrin α1 null cells were treated with 500 nM T7, cell survival was 26% of control, but the equimolar arresten treatment had almost no effect on the endothelial cell survival, which was 88% of control.

5.6.4 The effect of arresten in the matrigel plug assay

In order to study the *in vivo* effect of arresten on the formation of new capillaries in wild type and integrin α1 knockout mice, a Matrigel plug assay was performed (Passaniti *et al* 1992). Matrigel was injected subcutaneously into the mice in the presence of VEGF, bFGF, heparin and 3% DMSO, with or without 200 μM arresten or T7 peptide. In the wild type mice the number of blood vessels decreased to 60% of control (100%) with arresten and to 56% of control with T7 peptide. In the integrin α1 knockout mice, the T7 peptide caused the number of blood vessels to decrease to 66% of control, but arresten slightly increased the formation of blood vessels to 115% of control. These results suggest that integrin α1 is indeed important for the anti-angiogenic function of arresten.

5.6.5 Arresten does not inhibit tumor growth in integrin α1 knockout mice

Colon and renal carcinoma cells that are implanted subcutaneously into integrin α1 knockout mice produce smaller and less vascularized tumors than the same cells in wild type mice (Pozzi *et al.* 2000 and 2002). To determine how arresten treatment would affect

tumor growth in wild type and knockout mice colon carcinoma, CT26 cells (0.5 million viable cells) were injected subcutaneously into the backs of littermate mice. The tumors become detectable in about 3 days. The treatment with arresten and T7 peptide was started when the tumors had reached about 50 mm^3 of tumor volume that was measured with the formula length x $width^2$ x 0.52. Arresten and T7 peptide was injected intravenously to the mice daily. On the 16th day of treatment the mice were sacrificed. As expected the tumors grew more slowly in the knockout mice. Arresten treatment did not inhibit the growth of tumors in the integrin $\alpha 1$ knockout mice; the tumors even grew a little faster and were slightly larger in the arresten-treated knockout mice. T7 peptide had an inhibitory effect on tumor growth in both the wild types and the knockouts. Arresten inhibited the growth of tumors in the wild type mice. The average tumor size at the end of the experiment in wild type control mice was 631 mm^3. Both arresten and T7 reduced the tumors to about 375 mm^3. The average tumor size in integrin $\alpha 1$ null mice was 400 mm^3 The treatment with arresten did not reduce the size; the tumors were in fact larger (549 mm^3), but T7 was an efficient tumor growth inhibitor in null mice reducing the tumor size to 290 mm^3. To determine if the vasculature in the tumors of the knockout mice was altered, the number of CD31 positive vasculature was counted. The wild type mice demonstrated a statistically significant decrease in the number of tumor blood vessels upon treatment of arresten and T7-peptide. The amount of vessels in the tumors in the integrin $\alpha 1$ null mice was slightly smaller, and only treatment with T7 decreased the amount of vessels. The arresten treatment of the null mice had no effect on vessel density.

6 Discussion

6.1 Why is cancer such a sinister disease?

The life expectancy of humans has constantly been increasing as researchers have discovered novel treatments for diseases that used to be fatal. The longer people live, the more likely it is that mutations occur in the genome leading to genetic alterations that finally lead to a malignant phenotype (Hanahan and Weinberg 2000). Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis, leading to uncontrolled growth of a population of cells of the cancer patient. For example a bacterial infection is easy to treat, as prokaryotic and eukaryotic cells exert major differences in their biology, and thus treatments can be engineered accordingly (Alberts *et al.* 1994). Although tumor cells do have several differences as compared to normal cells, essentially it is much harder to target the treatment specifically to the tumor cells without critically harming normal cells. Novel evidence has recently been presented suggesting that not only do the tumor cells differ from normal cells, but that the tissues and cells surrounding the tumor have also changed. Although cancer research has generated an enormously complex and vast body of knowledge, Hanahan and Weinberg (2000) are foreseeing that cancer research will develop into a more logical science, where the complexities of the disease described in the laboratory and the clinic will become understandable in terms of a small number of underlying principles. It will be possible to understand with precision how and why treatment regimens and specific anti-tumor drugs succeed or fail. In the future, anticancer drugs will target each of the hallmark alterations of cancer that are hopefully used in concert with diagnostic techniques to detect and identify all stages of the disease progression.

6.2 The importance of protease activation cascades involving TAT-2

As proteases participate in many events in both physiological conditions and pathological events, it is crucially important that their activities are strictly regulated. TAT-2 can activate proMMP-9 *in vitro* at the lowest molar ratio reported so far, and it can also partially, although less efficiently, activate proMMP-2 *in vitro* (Sorsa *et al.* 1997). However, *in vivo* the situation is not so straightforward, as proteases form complex networks and cascades with their activators, inhibitors and other regulators. The efficiency of TAT-2 as an activator of gelatinases *in vivo* had not been clarified. Therefore, the full-length TAT-2 gene was transfected into premalignant and malignant oral epithelial cell lines with varying capacity to metastasize. The aim was to create model cell lines with elevated TAT-2 protein production *in vivo*. The results show that TAT-2 overexpression by highly malignant HSC-3 tongue squamous cell carcinoma cells resulted in significantly elevated proMMP-9 activation. This TAT-2-associated MMP-9 activation resulted in increased intravasation of the HSC-3 cells in the CAM invasion and intravasation model. These findings are in line with the recent data on the *in vivo* relationship between TAT-2 and gelatinases. In ovarian tumor cyst fluids TAT-2 levels are significantly associated with activation of proMMP-9, but not proMMP-2 activation (Paju *et al.* 2001). Furthermore, the reduction of TAT-2 secretion by cultured COLO-205 cells with chemically modified tetracyclines reduced significantly MMP-9, but not MMP-2 activation (Lukkonen *et al.* 2000). The downregulation of TAT-2 expression by chemically modified tetracyclines as well as inhibition of TAT-2 by TATI decreased significantly malignant COLO-205 cell migration (Lukkonen *et al.* 2000). *In vitro* TAT-2 only very moderately activates proMMP-2 (Sorsa *et al.* 1997). These previous results support the findings in this study, where TAT-2 had no effect on the activity of proMMP-2 in any of the cell lines analyzed. It suggests that the activities of MMP-9 and MMP-2 are regulated by distinct cascades, even though they are structurally closely related. Indeed, in addition to human trypsin-2, proMMP-9 can be activated by several other MMPs. In addition, proMMP-9 activity can be affected by downregulation of TIMP-1 by TNF-α and simultaneous proteolytic activation by chymotrypsin-like proteinase (Han *et al.* 2002). ProMMP-2 activation on the other hand requires the presence of active MT1-MMP and the binding of TIMP-2 (Strongin *et al.* 1995). Alternatively, it can happen via an autolytic method (Bergmann *et al.* 1995), through the uPA/plasmin system on the cell surface (Mazzieri *et al.* 1997, Monea *et al.* 2002) or mast cell tryptase (Lohi *et al.* 1992).

The overproduction of TAT-2 efficiently activated MMP-9 only in the HSC-3 cells, in which the increase in TAT-2 production after transfection was lower than in the other two transfected cell lines. TAT-2 is secreted as inactive trypsinogen-2 form that needs to be activated before it is capable of activating other proteinases. Enterokinase is a trypsinogen activator (Miyata *et al.* 1998, Lu *et al.* 1999) that is known to interfere with the immunofluorometric assay by digesting part of the latent TAT-2 molecule essential for antibody recognition (Itkonen *et al.* 1990). Since only the HSC-3 cell line naturally produces TAT-2, it might be possible that this cell line had also naturally existing enterokinase-like activity, absent from the two less malignant cell lines, interfering with the immunofluorometric assay. Thus, the actual amount of TAT-2 protein in the cell

media of TAT-2 transfected HSC-3 cells might be higher than measured. The fact that the addition of enterokinase increased intravasation even in control HSC-3 cells seems to be partly due to enterokinase activating the naturally existing TAT-2 in HSC-3 cells. Enterokinase was also previously found to be capable of activating proMMP-9 *in vitro* to some extent (Lukkonen *et al.* 2000).

Significant amounts of progelatinases occur *in vivo* in complex with TIMPs (tissue inhibitor of metalloproteinase), which is likely to prevent accidental or premature activation of proMMPs (Wilhelm *et al.* 1989). It was previously found that TAT-2 also activated proMMP-9 complexed with TIMP-1, but the activation was clearly slower and less efficient. Trypsin-2 was further shown to degrade TIMP-1 (Sorsa *et al.* 1997). In addition to gelatinases, TAT-2 can eventually activate other proMMPs *in vitro*, such as proMMP-1, -3, -8 and -13, and proteinases and directly degrade distinct extracellular matrix and basement membrane components, such as growth factor receptors and integrins (Koivunen *et al.* 1991b, Miyata *et al.* 1998, Moilanen *et al.* 2003). Therefore, TAT-2 can contribute to matrix degradation and remodeling both directly and indirectly via activation of proteinases. Different cell lines utilize various eventually compensatory mechanisms to activate their secreted and cell surface-associated proteinases. SCC-25 cells that normally do not produce TAT-2 most likely use different routes to activate progelatinases, and therefore no clear effect on gelatinases was observed after TAT-2-transfection. HSC-3 cells are known to be more invasive than SCC-25 cells (Ramos *et al.* 1997). In fact, the lack of TAT-2 in normal untransfected SCC-25 cells can be one explanation for that phenomenon. As the concentrations of trypsinogen-2 correlates with the malignancy of tumors (Koivunen *et al.* 1990, Paju *et al.* 2001), it might be a good clinical candidate for a clinical prognostic marker that could identify patients with an aggressive disease. TATI levels also seem to correlate with patient survival in renal cell carcinoma. Paradoxically, patients with elevated TATI levels had significantly shorter survival time that those with normal TATI levels (Paju *et al.* 2001a). However, a higher molar ratio of trypsinogen to TATI suggests poorer prognosis (Vergote *et al.* 1993, Paju *et al.* 2001b).

6.3 The dual role of MMPs in cancer

For quite a while the scientific community was convinced that the activity of MMPs was mostly a bad thing in cancer, and as soon as specific and efficient enough MMP-inhibitors were discovered, it would be an efficient cancer treatment. Unfortunately, the story proved out to be more complicated than that. MMPs can affect multiple steps during the tumor growth. Originally, the MMPs were considered to be important almost exclusively in invasion and metastasis. However, recent studies have revealed that MMPs are involved in several steps of cancer progression. Not only do they degrade basement membranes and other physical barriers, enabling metastatic cells to migrate and spread, but they also affect cellular and immune processes. Many angiogenesis inhibitors are stored as cryptic fragments within larger precursor matrix molecules that are not themselves antiangiogenic, and the regulation of proteolytic processing plays an important role in the vascularization of tumors (Folkman 1995, Hanahan and Folkman

1996). It is also important to note that the activity of MMPs on non-matrix substrates, such as chemokines, growth factors, growth factor receptors, adhesion molecules and apoptosis mediators, is essential for the rapid and critical cellular responses required for tumor growth and progression (Coussens *et al.* 2002). A good example of the complex network regulating cancer development is the fact that MMP-9 can be an initial modulator of the angiogenic switch by promoting the release of VEGF (Bergers *et al.* 2000) and an inhibitor of the angiogenic switch by releasing endostatin, angiostatin and tumstatin from their parent matrix molecules, as mentioned before. Increased MMP-9 expression reduced tumor growth and vasculature (Pozzi *et al.* 2002). In addition, metastasis and carcinogenesis have been shown to decrease in MMP-9 knockout mice using many experimental mouse models (Egeblad and Werb 2002). In part I of this study it was found that the enhanced activation of MMP-9 increased squamous cell carcinoma intravasation.

Multiple studies have implicated elevated levels of MMPs in various cancers. In mouse transplantation assays, relatively benign cancers acquire malignant properties when MMP expression is upregulated. Highly malignant cells become less aggressive when MMP expression or activity is reduced (Coussens and Werb 1996). Furthermore, the expression and activity of MMPs is increased in almost every type of human cancer, and this correlates with advanced tumor stage, increased invasion and metastasis, as well as shortened survival. Although several studies find high levels of TIMPs correlating with poor prognosis, it probably reflects the fact that the balance between MMPs and TIMPs, although still favoring MMPs, is overall higher (Egeblad and Werb 2002).

The majority of MMPs and other proteases are expressed and released by surrounding stromal or inflammatory cells in response to factors released during normal tissue repair or by tumors. Cancer cells evidently stimulate tumor stromal cells to synthesize MMPs in a paracrine manner through secretion of interleukins, interferons and growth factors (Werb 1997, Shapiro 1998). Nevertheless, certain MMPs such as MMP-2, -7, -12 and -13 are expressed *in vivo* by tumor cells themselves or by the endothelial cells of tumor capillaries (Hanemaaijer *et al.* 1993, Jackson and Nguyen 1997, Nagashima *et al.* 1997).

MMPs generate growth-promoting signals. They can release precursors of growth factors (Manes *et al.* 1999), and through their effects on the extracellular matrix composition and indirectly regulating proliferative signals through integrins (Agrez *et al.* 1994). Evasion of apoptosis permits survival in the presence of genetic instability, and MMPs have both apoptotic and anti-apoptotic actions (Egeblad and Werb 2002). One way how MMPs regulate apoptosis is by the cleavage of adhesion molecules, such as VE-cadherin, thus facilitating the shedding of these adhesion molecules and resulting in the typical rounding up of apoptotic cells (Herren *et al.* 1998). The cleavage of cell adhesion molecules by MMPs and the liberation of TGF-β play a role in the epithelial-mesenchymal transition associated with cancer. On the other hand, MMPs are also involved in cell differentiation (Egeblad and Werb 2002). MMPs seem to have dual or even opposite effects on tumor angiogenesis as well, on one hand by facilitating extracellular matrix degradation, enabling endothelial cells to invade the stroma and facilitating neovascularization (Hiraoka *et al.* 1998) and on the other hand by blocking pathological angiogenesis by releasing cryptic inhibitors of endothelial cell growth, such as endostatin (O'Reilly *et al.* 1997, Ferreras *et al.* 2000), angiostatin derived from plasminogen (Dong *et al.* 1997, Cornelius *et al.* 1998, O'Reilly *et al.* 1999) and tumstatin

derived from the type IV collagen α3 chain (Hamano *et al.* 2003). This is supported by the findings that MMP-9 deficient mice have accelerated growth of tumors, at least partially because the mice cannot cleave tumstatin or other cryptic angiogenesis inhibitors from the parent molecules (Hamano *et al.* 2003). Interestingly, this regulation seems to be reciprocal, as this study (parts II and III), in line with others, demonstrated that endostatin is able to regulate the activity of certain MMPs (Kim *et al.* 2000, Lee *et al.* 2002). On the other hand, MMP-2 deficient mice show reduced tumor angiogenesis and growth (Itoh *et al.* 1998). The immune system is capable of recognizing and attacking cancer cells, but the cells have developed ways to escape immune surveillance. MMPs are involved in the escape mechanism, for example via activating chemokines (Egeblad and Werb 2002). Finally, as shown by this study and others, MMPs are important in tumor intravasation, invasion and metastasis.

6.4 Why does endostatin inhibit tumor growth – or does it always?

The discovery of endostatin in 1997 (O'Reilly *et al.* 1997) raised high hopes for cancer cure and enormous hype in the media as well. The idea of the crucial importance of angiogenesis in cancer growth proposed by Judah Folkman more than 30 years ago was indeed revolutionary (Folkman 1971). A lot of focus has been addressed to study endostatin as well as other basement membrane-derived endogenous inhibitors of angiogenesis. The number of publications on endostatin in the PubMed database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) from 1997 to January of 2005 exceeds 670. Yet the fundamental mechanisms of endostatin have remained fairly unclear, as endostatin seems to affect more or less everything. Clinical trials with endostatin have so far been quite unsuccessful.

The evidence available indicates that different types of tumor cells use distinct molecular strategies to activate the angiogenic switch. This raises the obvious question of whether a single antiangiogenic therapeutic will be sufficient enough to treat all tumor types or whether an ensemble of such therapeutics needs to be developed, each responding to a distinct program of angiogenesis (Hanahan and Weinberg 2000). It has been shown that the phenotype of tumor-associated microvessels is different from both normal and non-tumor-associated angiogenic vessels. The expression of several adhesion molecules, such as E-selectin and VE-cadherin, is lost or diminished in tumor vessels (McDonald and Foss 2000), while others are overexpressed, such as integrin αvβ3 (MacDonald and Foss 2000) and heparin sulphate proteoglycan CD44 family members (Foster-Horvath *et al.* 2004). Thus, it is possible to target the anti-angiogenic therapy to the tumor vasculature without any harmful effects on the normal vasculature. The basement membrane in tumor vessels is abnormal, making the vessels dynamic. Although the vessels are almost completely covered by basement membranes, the membrane has conspicuous structural abnormalities, including a loose association with endothelial cells and pericytes, broad extensions away from the vessel wall, and multiple layers visible by electron microscopy (Baluk *et al.* 2003). Interestingly, basement membranes are also structurally altered in collagen XVIII deficient mice. The basement membrane of these mice is broadened, leading to hydrocephalus and kidney problems (Utriainen *et al.* 2004).

The amounts of endostatin (up to 2 μM) that have been used in parts II and III of this study are higher than the reported physiological levels. The levels of circulating endostatin in the serum of mice are around 40-100 ng/ml (2-5 nM) (Fukai et al. 2002), although levels as high as 100-300 ng/ml (5-15 nM) have been reported (Marneros & Olsen 2001). It is quite low compared to the endostatin concentrations that are effective in the inhibition of tumor growth, and thus it has been proposed that the anti-angiogenic and anti-tumor effects of endostatin might in fact be pharmacological effects at high doses, and not necessarily related to the physiological function of endostatin (Olsen 2002). Indeed, the lack of the physiological levels of endostatin has no effect on the growth of fibrosarcomas and melanomas in collagen XVIII/endostatin deficient mice (Fukai et al. 2002). Instead, the mice had ocular abnormalities that include delayed regression of the vessels on the retina surface and abnormal outgrowth of retinal vasculature (Fukai *et al.* 2002). Patients with Knobloch syndrome caused by mutation in the collagen XVIII gene have eye abnormalities as well (Sertie *et al.*2000). In a surrogate model of human angiogenesis, systemic delivery of recombinant endostatin (serum levels only 30-35 ng/ml, 1.5-1.75 nM) dramatically inhibited migration, vessel formation and maturation (Skovseth *et al.* 2004). The physiological function of endostatin seems to have more to do with eye development than tumor angiogenesis, but this does not make the work done with higher endostatin concentrations in cancer treatments in any way less important. However, Joki *et al.* (2001) treated human glioblastoma xenografts by encapsulated cells secreting human endostatin. An inhibition of tumor growth was observed even by concentrations as low as 151 ng/ml (7.5 nM) (Joki *et al.* 2001). On the other hand, the physiological levels of tumstatin seem to protect from pathological angiogenesis and cancer growth. Mice deficient in tumstatin exhibit accelerated tumor growth with enhanced pathological angiogenesis, while angiogenesis associated with development, wound healing and liver regeneration is unaffected (Hamano *et al.* 2003).

It is not entirely clear which cells and mechanisms are responsible for and the most important in endostatin release. Collagen XVIII mRNAs are expressed by epithelial and endothelial cells and liver hepatocytes (Saarela *et al.* 1998, Musso *et al.* 1998, Schuppan *et al.* 1998) in placenta (Pollheimer *et al.* 2004) and in cartilage (Pufe *et al.* 2004), and there is a lot of collagen XVIII protein in the vascular and epithelial BM zones (Muragaki *et al.* 1995, Musso *et al.* 1998, Saarela *et al.* 1998, Sasaki *et al.* 1998, Miosge *et al.* 1999). A recent study suggests that soluble full-length type XVIII collagen is also present in human plasma (Musso *et al.* 2001). In part II of this work, it was found that cultured hepatoma-derived HepG2 cells, which retain the biosynthetic capabilities of normal liver parenchymal cells (Knowles *et al.* 1980), secreted full-length collagen XVIII into the culture media as well as several endostatin-containing peptides. HepG2 cells were also shown to express mRNAs for MMPs -3, -7, -9, -13 and -20, which are all able to cleave endostatins from collagen XVIII *in vitro*. Furthermore, we find here that the HepG2 cells produce active MMP-9 and collagenases as well, probably representing MMP-13. The formation of endostatin-containing peptides between 24-30 kDa was reduced by three different MMP inhibitors, supporting the direct role for the MMPs in generating endostatin fragments from collagen XVIII in the HepG2 cell system, and possibly in tissues as well (Ständker *et al.* 1997, Sasaki *et al.* 1998, John *et al.* 1999). One of the endostatin-containing fragments that were released from collagen XVIII by MMPs *in*

vitro had the N-terminal sequence YVHLRPARPT, and interestingly, this fragment has also been detected in human plasma (Sasaki *et al.* 1998).

The biological activity and physiological significance of the endostatin-related peptides in serum and tissues are not fully understood. Originally, only the 20-kDa endostatin molecule was reported to possess antiangiogenic activity as measured using an *in vitro* proliferation assay of bFGF-stimulated bovine capillary endothelial cells (O'Reilly *et al.* 1997). It was therefore suggested that proteolytic activation and exposure of a new N-terminal sequence are necessary for endostatin action (Sasaki *et al.* 1998). In other studies, the recombinant full-length NC1 domain of human collagen XVIII and the endostatin and NC1 fragments with flag-tag modified N- or C-termini also prevented VEGF-induced migration of human umbilical vein endothelial cells *in vitro* (Yamaguchi *et al.* 1999). Short peptides derived from human endostatin have been shown to possess potent antiangiogenic properties *in vitro* and *in vivo* (Cattaneo *et al.* 2003, Chillemi *et al.* 2003, Wickström *et al.* 2004).

Studies with competitive ligand binding with RGD peptide suggest that endostatin binding to integrin $\alpha5\beta1$ replaces pro-angiogenic ligands from that site (positive regulator of angiogenesis) (Rehn *et al.* 2001, Wickström *et al.* 2002, Sudhakar *et al.* 2003). That is a different mechanism compared to tumstatin, as the binding of tumstatin to integrin $\alpha v\beta3$ functions as an active anti-angiogenic signal (negative regulator of angiogenesis) (Sudhakar *et al.* 2003). It should also be noted that several ligands can bind to the same integrins and they can thus have dual or opposite effects on cell behavior (Geiger *et al.* 2001). Sudhakar *et al.* (2003) have recently reported that human endostatin is a potent inhibitor of HUVEC cell migration with no effect on proliferation. The inhibitory effect on cell migration is mediated by binding to $\alpha5\beta1$ integrin, which leads to blocking of the ERK1/p38 MAPK pathway (Sudhakar *et al.* 2003). However, in another study endostatin did not affect the phosphorylation of a series of signal transduction components including p38 MAPK. Endostatin does not affect the proliferation of bFGF-induced human dermal microvascular endothelial cells, indicating that the effects on endothelial cells might be cell-type specific (Eriksson *et al.* 2003), and that even different types of endothelial cells might respond to endostatin in a completely different fashion. Indeed, human endostatin inhibited the proliferation of bovine capillary endothelial cells, and endostatin also inhibited HUVEC proliferation in the experimental system used in part II of this study. Furthermore, it seems to depend on the inductive cytokine whether endostatin has an effect on angiogenesis (Sasaki *et al.* 2000) as well as on the matrix environment the cells reside on (Geiger *et al.* 2001). Functional human and mouse endostatins may differ, as cathepsin L cleaving human recombinant endostatin results in an endostatin-like fragment that is 11 amino acids longer than the murine endostatin (Felbor *et al.* 2000). The cell density might also be very critical, as was discovered in part III of this study. It might also be possible that the expression of integrins and other cell surface receptors fluctuates depending on the cell type and growth phase of endothelial cells. We observed also the biphasic nature of endostatin, as higher concentrations of endostatin started to be less effective (part III).

Binding to MMPs is one mechanism for the anti-tumor effect of endostatin. Previous reports on the effects of endostatin on proMMP-2 activation have been somewhat conflicting. Kim *et al.* (2000) reported that endostatin inhibited the activation and activity of proMMP-2 in endothelial cells, but there is also a study reporting no changes in MMP-

2 activation events in response to endostatin treatment (Wickström *et al.* 2001). Two approximately 40-kDa fragments of purified MMP-2 formed in the presence of endostatin and the MMP-activator APMA, and endostatin reduced the formation of the smaller fragment in a dose-dependent manner that was associated with reduction in the gelatinolytic activity of MMP-2 (part III). In MMP-2 autoactivation, multiple smaller activation products are formed in addition to the 62-kDa active enzyme (Bergmann *et al.* 1995). Furthermore, proMMP-2 lacking the hinge region and hemopexin-like domain has the molecular size of 48 kDa, which is converted to the 42-kDa form upon APMA activation. The 42-kDa form has an activity comparable with the native 62-kDa active form (Lee *et al.* 2002). Therefore, our results seem to support the report by Kim *et al.* (2000). Endostatin did not inhibit all MMPs in this study. It inhibited the activity of MMP-13, but not MMP-8. As observed here, the MMPs that were able to digest endostatin-containing fragments appeared to be the same that were also inhibited by endostatin, suggesting product inhibition in regulation of these MMP activities. Although MMP-2 was not found to cleave endostatin-containing fragments in this study, others have reported that MMP-2 is able to generate endostatin-containing fragments after prolonged incubation, although much less efficiently than the other MMPs (Ferreras *et al.* 2000). MMP-2 and endostatin may have an interactive relationship similar to other MMPs, but the effects on both directions might be weaker. APMA, used as an MMP activator, is a chemical compound that does not exist *in* vivo. Therefore it was interesting to evidence that in addition to APMA-mediated activation, endostatin inhibited the activation of MMP-9 mediated by the naturally occurring and tumor-associated TAT-2 (Koivunen *et al.* 1990, Sorsa *et al.* 1997, Miyata *et al.* 1998, Paju *et al.* 2001). Previous reports have suggested that endostatin can directly interact with proMMP-2 (Kim *et al.* 2000) or the catalytic domain of active MMP-2 (Lee *et al.* 2002). Since MMPs share a highly homologous catalytic domain (Bode *et al.* 1999), the binding of endostatin solely to this region would result in inactivation of all MMPs. Here it was proven not to be the case, suggesting that additional interactions are required to accomplish the inhibitory effect of endostatin on certain MMPs. In this study it was found that also MMP-9 and endostatin form a complex *in vitro*. Interestingly, it has only recently been found that also tumstatin downregulates the activity of certain MMPs, namely MMP-2, -9, -13 and -14, as well as interfering with the plasminogen activator system (Pasco *et al.* 2004).

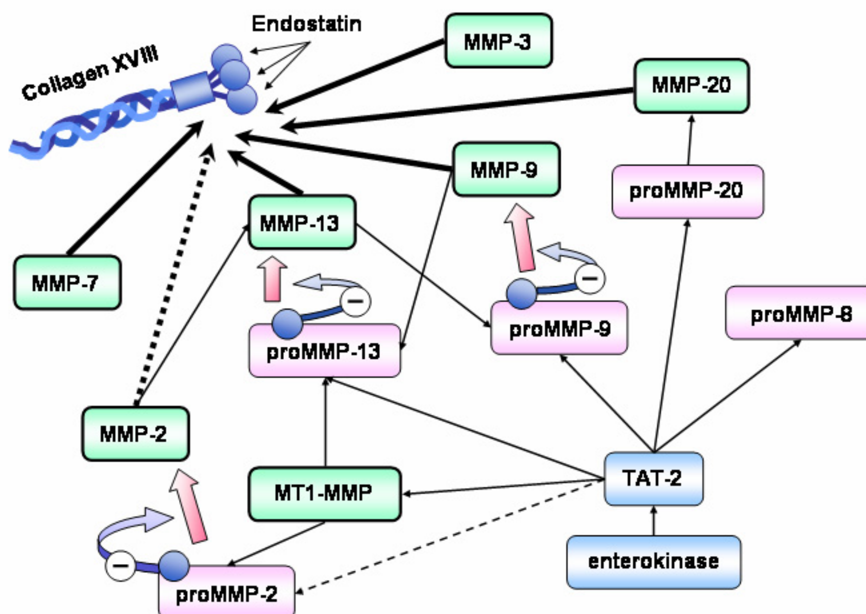

Fig. 8. The complex network of MMP activation cascades involving TAT-2 and endostatin. In this study it was found that MMP-3, -7, 9, -13 and -20 cleave endostatin-containing fragments from collagen XVIII (thick black arrows). Others have also found MMP-2 to be able to release endostatin. The activation of proMMP-2, -9 and -13 (thick grey arrows) is inhibited by endostatin binding to the proform (endostatin monomers are shown as grey circles). This study and others have elucidated that TAT-2 can activate several proMMPs. (Adapted from Nagase 1997, Sorsa et al. 1997, Ferreras et al. 2000, Kim et al. 2000, Väänänen et al. 2001, Overall 2002, Moilanen et al. 2003.)

Endostatin has gone through phase I clinical trials, and on the basis of these it has been concluded that recombinant endostatin can be administered to patients at high concentrations with no signs of toxicity (Herbst *et al.* 2002). Currently it is in phase II trials. Endostatin has not become the miracle cancer drug it was hoped when its anti-cancer properties were first discovered. In fact, it has recently been discovered in some clinical studies that overexpression of collagen XVIII, and thus elevated levels of circulating serum endostatin, is actually associated with poor outcome in non-small cell lung cancer (Iizasa *et al.* 2004). On the other hand, some studies have found that circulating endostatin levels are normal in patients with head and neck squamous cell carcinoma (Homer *et al.* 2002), and that serum endostatin levels had no prognostic significance in patients with hepatocellular carcinoma (Poon *et al.* 2004). Strikingly, during embryonic development endostatin enhances endothelial cell proliferation and migration (Schmidt *et al.* 2004). All these recent new data about endostatin emphasize

how the actions of endostatin seem to be more complex than originally thought, and how a lot of work still needs to be done.

6.5 The mechanism of arresten

Arresten is one of the recently found endogenous inhibitors of angiogenesis. It is derived from the α1 chain of collagen IV (Colorado *et al.* 2000). It should be noted that even though all the collagen derived anti-angiogenic molecules are of about the same size, come from similar sources and have amino acid sequence similarities (O'Reilly *et al.* 1997, Maeshima *et al.* 2000, Kamphaus *et al.* 2000, Colorado *et al.* 2000), they can function via distinct mechanisms, bind different cell surface receptors and affect different parts of the angiogenic process. As mentioned earlier, endostatin binds to integrin α5β1 and inhibits migration, possibly by interfering with the signaling pathways via ERK1 and p38. Tumstatin on the other hand binds to integrin αvβ3 and inhibits the PI3-kinase/Akt/mTOR/4EBP1 signaling pathway, resulting in decreased endothelial cell proliferation (Sudhakar *et al.* 2003). The receptor of canstatin is not known yet, but canstatin has been shown to inhibit Akt activation and to induce Fas-dependent apoptosis in endothelial cells (Panka and Mier 2003). In part IV of this study the earlier finding that arresten binds to integrin α1β1 was confirmed (Colorado *et al.* 2000), and it was found that integrin α1β1 is indeed a functionally relevant receptor of the anti-angiogenic function of arresten.

Studies with canstatin and tumstatin show that even within the same molecule, different parts can participate in the inhibition of angiogenesis in distinct ways. The C-terminal part of canstatin is the domain mainly associated with the specific inhibition of proliferation of endothelial cells, whereas the N-terminal part of canstatin is associated with the potential apoptosis-inducing activity on endothelial cells (He *et al.* 2003 and 2004). Tumstatin has two separate binding sites to integrin αvβ3, the N-terminal one being associated with anti-angiogenic properties and the C-terminal one with anti-tumor activity (Shahan *et al.* 1999, Maeshima *et al.* 2000a). It is therefore quite possible that there might be more than one receptor or binding site within arresten as well. The other possible receptors in addition to integrin α1β1can be capable of affecting the anti-angiogenic and anti-tumor properties of arresten. In fact, it has previously been shown that arresten possess two binding sites on endothelial cell (CPAE) surface, a high-affinity one and a low-affinity one. In addition to the high affinity binding to integrin α1β1, arresten also binds to heparan sulphate proteoglycans (HSPGs) on the endothelial cells, but it is not yet known whether this binding is of functional significance (Colorado *et al.* 2000). In addition to the NC1 domain, the central triple helical domain also interacts with cells via α1β1 and α2β1 integrins (Eble *et al.* 1993, Setty *et al.* 1998). Interestingly, collagen IV contains cryptic integrin binding sites. During angiogenesis these sites are exposed and induce a switch in integrin recognition, with a loss of α1β1 binding sites and gain of αvβ3 binding (Xu *et al.* 2001).

When tumors were implanted into integrin α1 knockout mice, it was unexpectedly discovered that the tumors were growing more slowly in the null mice. It was suggested that this might be due to the upregulation of MMPs leading to an increased amount of

angiostatin, another inhibitor of angiogenesis that is proteolytically derived from plasminogen (Pozzi *et al.* 2000). Integrin α1β1 is known to promote cell migration, proliferation and matrix reorganization, and thus it is important in non-quiescent cells during dynamic situations, such as angiogenesis. VEGF significantly induces the expression of integrin α1β1 on the endothelial cell surface. Inhibiting the function of integrin α1β1 by antibodies leads to selective inhibition in VEGF-driven angiogenesis *in vivo* without any effects on the pre-existing vasculature. Therefore, it has been suggested that integrin α1β1 is of particular importance in pathological angiogenesis (Senger *et al.* 1997). On the other hand, the integrin α1β1 gene is downregulated in many types of cancers. The loss of α1β1 integrin was related to metastasis in breast carcinomas (Gui *et al.* 1995). It is the only collagen receptor known to activate the Ras-Shc-mitogen activated protein kinase pathway, thus being capable of promoting cell proliferation (Pozzi *et al.* 1998).

Recent studies have shown that heparan sulphate proteoglycans are important in the regulation of angiogenesis. They are large molecules that are associated either with cell membranes or with extracellular matrix, and they can have multiple functions. Particularly perlecan has been shown to have both angiogenic and anti-angiogenic effects (Segev *et al.* 2004). The bioavailability of many growth factors is regulated by the balance of their binding capacity onto HSPGs and the action of extracellular matrix degrading enzymes, such as MMPs, which can release heparin-binding growth factors from the matrix to exert their effect (Vlodavsky *et al.* 1996). Interestingly, there were more blood vessels in the Matrigel plugs with arresten in the integrin α1 null mice, the proliferation of the VEGF stimulated MLECs was increased in the presence of arresten, and furthermore, the tumors in the knockout mice treated with arresten were growing faster. Even though the differences were not statistically significant, they are very consistent in all the different angiogenesis assays. The most obvious reason for this accelerated growth of blood vessels and tumors upon arresten treatment in the integrin α1 null mice would be the upregulation of some other receptor on endothelial cells that would compensate for the lack of integrin α1β1. Integrin α2β1 has similar substrate specificity as integrin α1β1 and would therefore be a likely candidate for compensation, but no upregulation of integrin α2β1 in the α1 null tissues was observed (Gardner *et al.* 1996). As mentioned earlier, arresten also binds to heparin sulphate proteoglycans, but with a lower affinity than to integrin α1β1 (Colorado *et al.* 2000). Therefore, in the absence of integrin α1β1, more arresten may be available to bind to HSPGs despite the low affinity. The increased amount of arresten might block the binding of VEGF or other growth factors or cytokines to HSPGs, thus resulting in increased amounts of bioavailable unbound growth factors that are able to bind to their receptors that have pro-angiogenic effects on the endothelial cells.

The ligands of integrins can in many cases be reduced to minimal bioactive recognition sequences, of which the RGD sequence is the prototypical example (Ruoslahti *et al.* 1996). The RGD sequence was originally identified as the sequence in fibronectin that engages integrin α5β1. It recognizes several different integrins, such as α3β1, α8β1, αvβ1, αvβ3, αvβ5, αvβ6 and αIIbβ3 (Plow *et al.* 2000). Even though there is an RGD sequence in a macromolecule, it does not mean that the integrin will bind to that particular sequence or that it will have biological significance, as in the case of tumstatin. The RGD sequence in the N-terminal portion of tumstatin is not involved in the binding

of tumstatin to integrin αvβ3 or in the anti-angiogenic activity of tumstatin (Maeshima *et al.* 2000a). However, integrin α1β1 does not have the RGD sequence as its recognition sequence. The minimal recognition sequence of integrin α1β1 is GFOGER (Gly-Phe-HydroxyPro-Gly-Glu-Arg), which needs to be in the triple helical conformation (Knight *et al.* 1998, 2000). Recently, it was found that in the triple helical collagen IV molecule the critical amino acids for integrin α1β1 binding were Asp461 in the α1 chains and Arg 461 in the α2 chain (Renner *et al.* 2004). As the arresten molecule is cleaved from collagen IV, it loses its triple helical form, and thus this GFOGER recognition sequence cannot be relevant for the binding of arresten. Interestingly, α1β1 integrin was able to mediate cell spreading significantly better on monomeric than on triple helical type I collagen matrix, suggesting that it might prefer monomeric forms of collagen (Jokinen *et al.* 2004), possibly also collagen IV. As expected, in this study the binding of arresten to integrin α1β1 is not dependent of the RGD sequence. The actual minimal recognition sequence for integrin α1β1 within arresten remains to be determined.

7 Conclusions

The progression of cancer is a multi-step process. During this work two important aspects of this process, namely the proteolysis of the extracellular matrix and the formation of new blood vessels in the tumor, have been assessed. Several protease families are capable of digesting ECM components, thus facilitating e.g. tumor invasion. In addition to directly breaking down the ECM, MMPs and TAT-2 can also proteolytically activate other proteases by forming proteolytic cascades. Proteases are also involved in angiogenesis in several ways. One important mechanism is the release of cryptic anti-angiogenic molecules from larger ECM components, which do no inhibit angiogenesis when intact. All these processes need to be strictly regulated. In pathological conditions such as cancer, the delicate balance of regulation is disturbed.

1. We found that the overproduction of TAT-2 in malignant oral squamous cell carcinoma line HSC-3 was associated with elevated MMP-9, but not MMP-2 activation, as well as with enhanced intravasation in the *in vivo* CAM model. These results indicate that the TAT-2 and MMP-9 activation cascade plays a role in the malignant invasive growth of oral carcinomas.
2. Certain, but not all MMPs are able to cleave endostatin and endostatin-containing larger fragments from collagen XVIII. The generation of endostatin-containing fragments differing in molecular size and in N-terminal sequence was identified in the cases of MMP-3, -7, -9, -13 and -20, but not with MMP-1, -2, -8 and -12. The native 20-kDa endostatin, three longer MMP-generated endostatin-containing fragments and the entire NC1 domain were shown to inhibit endothelial cell proliferation and migration in a fairly similar fashion.
3. It seems that MMPs and endostatin are reciprocally regulated, as endostatin is able to inhibit the activation and activities of MMP-2, -9 and -13, but not MMP-8. Endostatin was found to prevent the activation of proMMPs by TAT-2. The inhibition of tumor-associated MMPs could at least in part explain the anti-tumor effect of endostatin. Although most of the endostatin research is focusing on endothelial cells, we found here that endostatin also inhibits the migration of tumor cells. Interestingly, endostatin starts to lose its effect at higher concentrations. In addition, cell density was also found to be critical for the efficient MMP-inhibition of endostatin.

4. The mechanism of arresten, a type IV collagen derived endogenous inhibitor of angiogenesis and tumor growth, was characterized. The earlier finding that arresten binds to integrin α1β1 was confirmed. More importantly, we found that integrin α1β1 is a functionally relevant receptor for the anti-angiogenic functions of arresten, as integrin α1 null endothelial cells did not respond to arresten treatment, and we observed no reduction in tumor growth or neoangiogenesis in the integrin α1β1 deficient mice. The RGD peptide, a common minimal recognition motif for integrins, was not important in the binding of arresten to integrin α1β1.

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