Trypsinogens and Trypsin Inhibitor (PSTI/TATI) -Expression in Urogenital Organs and Tumors

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Academic Dissertation

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Helsinki 2001

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ISBN 952-91-3377-4
ISBN 951-45-9944-6 (PDF)
http://ethesis.helsinki.fi

Helsinki 2001
Yliopistopaino

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List of original publications

This thesis is based on the following original publications which are referred to in the text by their roman numerals. Some unpublished data is also presented.

I Annukka Lukkonen, Susanna Lintula, Kristina von Boguslawski, Olli Carpén, Börje Ljungberg, Göran Landberg, and Ulf-Håkan Stenman. Tumor-associated trypsin inhibitor in normal and malignant renal tissue and in serum of renal-cell carcinoma patients. *Int J Cancer,* 83, 486-490 (1999).

II Annukka Paju, Jan Jacobsen, Torgny Rasmuson, Ulf-Håkan Stenman, and Börje Ljungberg. Tumor associated trypsin inhibitor as a prognostic factor in renal cell carcinoma. *J Urol,* 165, 959-962 (2001).

III Annukka Lukkonen, Timo Sorsa, Tuula Salo, Taina Tervahartiala, Erkki Koivunen, Lorne Golub, Sanford Simon, and Ulf-Håkan Stenman. Down-regulation of trypsinogen-2 expression by chemically modified tetracyclines: association with reduced cancer cell migration. *Int J Cancer,* 86, 577-581 (2000).

IV Annukka Paju, Timo Sorsa, Taina Tervahartiala, Erkki Koivunen, Caj Haglund, Arto Leminen, Torsten Wahlström, Tuula Salo, and Ulf-Håkan Stenman. The levels of trypsinogen isoenzymes in ovarian tumour cyst fluids are associated with promatrix metalloproteinase-9 but not promatrix metalloproteinase-2 activation. *Br J Cancer,* in press.

V Annukka Paju, Anders Bjartell, Wan-Ming Zhang, Stig Nordling, Anders Borgström, Jens Hansson, and Ulf-Håkan Stenman. Expression and characterization of trypsinogen produced in the male genital tract. *Am J Pathol,* 157, 2011-2021 (2000).

Abbreviations

A2M	α_2-macroglobulin
AP-1	activator protein-1
API	α_1-protease inhibitor
BM	basement membrane
bp	base pair
cDNA	complementary deoxyribonucleic acid
CMT	chemically modified tetracycline
DNA	deoxyribonucleic acid
DOXY	doxycycline
ECM	extracellular matrix
hK2	human kallikrein-2
IFMA	immunofluorometric assay
kDa	kilodalton
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type metalloproteinase
OVCA	ovarian carcinoma
PAI	plasminogen activator inhibitor
PAR	proteinase-activated receptor
PEA3	polyomavirus enhancer A-binding protein-3
pI	isoelectric point
PSA	prostate-specific antigen
PSTI	pancreatic secretory trypsin inhibitor
RCC	renal cell carcinoma
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAT	tumor-associated trypsin(ogen)
TATI	tumor-associated trypsin inhibitor
TIMP	tissue inhibitor of metalloproteinases
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator

Introduction

Trypsinogens and Kazal-type trypsin inhibitor, which is also called pancreatic secretory trypsin inhibitor (PSTI) and tumor-associated trypsin inhibitor (TATI), have long been considered pancreas-specific. The main physiological function of pancreatic trypsin is participation in digestion by degradation of dietary proteins and by activation of other digestive enzymes such as chymotrypsinogen, proelastase, procarboxypeptidase, and prophospholipase in the small intestine. PSTI is thought to prevent the proteolytic destruction of the pancreatic gland by inhibiting prematurely activated trypsins within the pancreas. However, it has been shown that trypsinogens and PSTI/TATI are widely expressed also outside the gastrointestinal tract which suggests that they may also have other biological functions. Furthermore, tumors originating from various tissues in- and outside the gastrointestinal tract express trypsinogens and TATI, and the serum and urine levels of TATI often increase in patients with various types of cancer. Increased proteolytic activity is one of the major factors needed for tumor invasion and metastasis. Matrix metalloproteinases and plasminogen activator-plasminogen system have been the subject of the most intensive research but less is known about the role of trypsins in the proteolysis associated with tumor invasiveness. This work was undertaken to study the expression of trypsinogens and PSTI/TATI in urogenital organs and their tumors and to further elucidate their role in the proteolytic activity of tumors.

Review of the literature

1. PROTEOLYSIS AND TUMOR INVASION

A group of coordinated cellular processes is responsible for invasion and metastasis, the most life-threatening aspect of cancer. Genetic changes causing imbalance of growth regulation lead to uncontrolled proliferation necessary for both primary tumor growth and metastatic expansion. However, unrestrained growth does not, by itself, cause invasion and metastasis (Chambers and Matrisian, 1997). To succesfully create a metastatic colony, a cell or group of cells must be able to leave the primary tumor, invade the local host tissue, enter circulation, arrest at the distant vascular bed, extravasate into the target organ interstitium or parenchyma, and proliferate as a secondary colony (Chambers and Matrisian, 1997).

The mammalian organism is divided into a series of compartments separated by the extracellular matrix (ECM) consisting of the basement membrane (BM) and its underlying interstitial stroma (Liotta *et al*., 1983; Yurchenco and Schittny, 1990). During the development of invasive tumors, tumor cells disobey the social order of organ boundaries and cross into tissues where they do not belong. BMs are thin continuous extracellular structures which are present practically everywhere in the body separating organ cells, epithelia, and endothelia from interstitial connective tissue. The BMs consist primarily of type IV collagen, laminin-1 and heparan sulfate proteoglycan. Other components are fibronectin, nidogen, laminin-

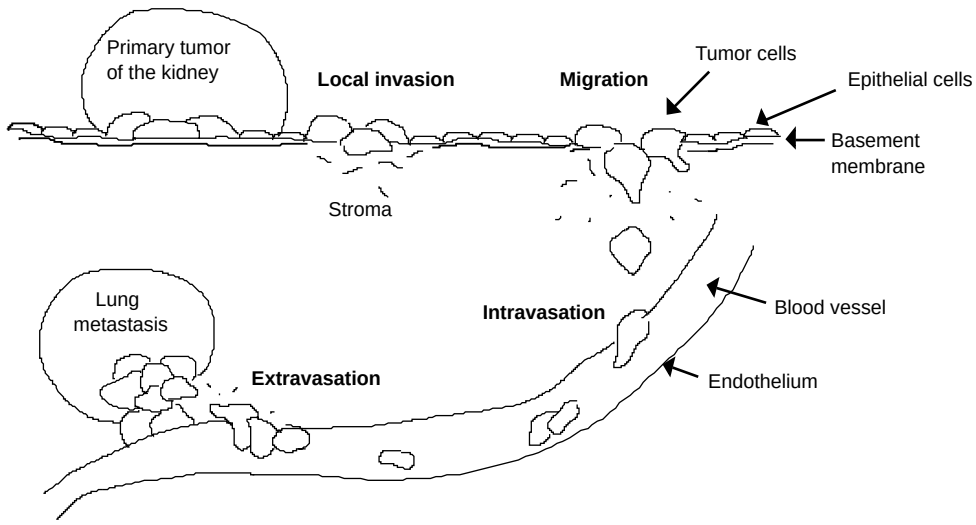

Figure 1. Schematic picture of the multistep process of tumor invasion and metastasis.

Table I. Proteinases expressed by tumors.

Proteinase class	pH-optimum	Natural inhibitors
Matrix metalloproteinases	7-9	TIMPs, α_2-macroglobulin
Collagenases		
Gelatinases		
Stromelysins		
Matrilysins		
Membrane-type MMPs		
Others		
Serine proteinases	7-9	
uPA		PAI-1, PAI-2, protease nexin-1, α_2-macroglobulin
tPA		PAI-1, PAI-2, α_2-macroglobulin
Trypsin		PSTI/TATI, α_2-macroglobulin, α_1-proteinase inhibitor
Cysteine proteinases	3-5	Cystatins
Cathepsin B		
Cathepsin L		
Aspartic proteinases	3-5	α_2-macroglobulin
Cathepsin D		

Modified from Koivunen, 1991a. Additional references, Thomas *et al.,* 1989; Roberts *et al.,* 1995.

5, type V collagen, fibrillins, chondroitin sulfate proteoglycan, and SPARC (secreted protein acidic and rich in cysteine) (Timpl and Brown, 1996). Interstitial stroma is composed primarily of type I, II, and III collagen, glycoproteins, elastin, fibrillins, and proteoglycans. The exact constitution of interstitial stroma depends on the tissue type (Tryggvason *et al*., 1987; Prockop and Kivirikko, 1995).

Increased proteolysis is one of the major cellular events necessery for invasion and metastasis (Liotta *et al*., 1983; Saksela, 1985). Tumor cells and host cells induced by tumor cells secrete proteolytic enzymes capable of degrading various components of the ECM. Proteolytic enzymes are thought to play a role in several steps of the metastatic process. They are involved in the expansion of the primary tumor and in the dissolution of the local BM and interstitial stroma (Figure 1). They are needed for intra- and extravasation of lymphatics and blood vessels, in which the subendothelial BM and endothelial cell layer have to be penetrated. Finally, to be able to form a metastatic colony in the distant organ, tumor cells have to invade the interstitial stroma and parenchyma of the target tissue (Chambers and Matrisian, 1997). Proteolytic enzymes involved in invasion and metastasis belong to four classes of proteinases: 1) Matrix metalloproteinases (MMPs), 2) serine proteinases, 3) cysteine proteinases, including the cathepsins, and 4) aspartic proteinases (Table I). The uPA-plasminogen system and MMPs have been the subject of the most intensive research.

1.1 Matrix metalloproteinases (MMPs)

MMPs are a family of highly conserved Zn^{2+}-dependent endopeptidases, which collectively are capable of degrading most components of the BM and ECM (Birkedal-Hansen *et al*., 1993). At present there are at least 21 known human MMPs which participate in many normal biological processes (*e. g.*, embryonic development, blastocyst implantation, ovulation, bone re-

modeling, and angiogenesis) and pathological processes (tumor invasion and metastasis, arthritis, and periodontal disease) (Nagase and Woessner, 1999). The members of the MMP family can be divided into six subgroups according to their domain structure and substrate specificity (Table II) (Birkedal-Hansen *et al.*, 1993; Birkedal-Hansen, 1995). Despite of some overlapping, MMPs display clear substrate specificity: collagenases including fibroblast collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13) are the principal proteinases capable of initiating degradation of native fibrillar interstitial collagens, *i. e.*, collagen I, II, and III (Kähäri and Saarialho-Kere, 1997) (Table II). The 72 kDa gelatinase A and 92 kDa gelatinase B (MMP-2 and MMP-9, respectively) are thought to play an important role in the degradation of BM type IV collagen and in the final cleavage of denatured collagen fibrils, *i. e.*, gelatin. Stromelysins have a broad substrate specificity, being capable of degrading fibronectins, laminin, elastin, proteoglycans and collagens (Chandler *et al.*, 1997; Kähäri and Saarialho-Kere, 1997). Of the recently identified membrane-type MMPs (MT-MMPs), the substrate specificity of MMP-14 is best known (Table II).

The basic 5-domain prototype structure of MMPs (Birkedal-Hansen *et al.*, 1993; Birkedal-Hansen, 1995) comprises 1) a 17-29 amino acid signal peptide, 2) a 77-87 residue propeptide that contains a free cysteine residue, 3) an about 165 residue catalytic domain which contains the Zn^{2+}-binding site, 4) a 5-50 amino acid proline-rich hinge region, and 5) an about 200 residue carboxyterminal hemopexin domain which determines the substrate specificity. MT-MMPs have an additional transmembrane domain which anchors them to the cell surface (Sato *et al.*, 1994). MMP-7 (matrilysin-1) and MMP-26 (matrilysin-2) lack the hemopexin-like domain (Birkedal-Hansen, 1995; Uría and Lopéz-Otín, 2000), and MMP-2 and MMP-9 contain a fibronectin-like domain which facilitates their binding to collagen, laminin, and gelatin (Allan *et al.*, 1995).

1.1.1 Regulation of MMP expression and activity

Regulation of MMP expression and activity is exerted at several levels involving transcriptional regulation, intra- and extracellular activation of proMMPs, and inhibition of MMPs by endogenous inhibitors (Birkedal-Hansen *et al.*, 1993; Birkedal-Hansen, 1995; Murphy and Knäuper, 1997).

A) Transcriptional regulation

Many different factors influence the transcription of MMPs, including cytokines, growth factors, hormones, oncogenic cellular transformation, and chemical agents, *e. g.*, phorbol esters. In general, the gene expression of MMPs is up-regulated by interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), transforming growth factor-α (TGF-α), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), parathyroid hormone, and endotoxin whereas interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), retinoids, and glucocorticoids downregulate the expression (Birkedal-Hansen *et al.*, 1993; Borden and Heller, 1997). The expression of MMP-1, -3, -9, -13, and -14 is readily inducible by cytokines, hormones and growth factors whereas expression of MMP-2 is barely affected by these regulatory factors (Birkedal-Hansen *et al.*, 1993; Borden and Heller, 1997). Furthermore, there is evidence to suggest that individual members or groups of the MMP family are independently regulated (Brown *et al.*, 1990; Uría *et al.*, 1998). The mechanism of transcriptional regulation has been extensively studied and the activator protein-1

Table II. Human matrix metalloproteinases, their activating capacity, activators, and substrates in the ECM.

Enzyme	M_r (kDa)	Activator of	Activated by	Substrates
Collagenases				
Fibroblast collagenase (MMP-1)	52/57	MMP-2	MMP-3, -10, plasmin, kallikrein, chymase	Collagen I, II, III, VII, VIII, X, gelatin, aggrecan, nidogen, tenascin
Neutrophil collagenase (MMP-8)	75	ND	MMP-3, -10, plasmin	Collagen I, II, III, VII, VIII, X, gelatin, aggrecan, fibronectin, laminin
Collagenase-3 (MMP-13)	53	MMP-2, -9	MMP-2, -3, -10, -14, -15, plasmin	Collagen I, II, III, IV, IX, X, XIV, gelatin, aggrecan, perlecan, fibronectin, laminin, tenascin, fibrillin
Gelatinases				
72 kDa gelatinase A (MMP-2)	72	MMP-9, -13, -17	MMP-1, -7, -13, -14, -15, -16, -24, plasmin, human trypsin	Gelatin, collagen I, II, IV, V, VII, X, XI, XIV, aggrecan, fibronectin, laminin, elastin, vitronectin, fibrillin
92 kDa gelatinase B (MMP-9)	92	ND	MMP-2, -3, -13, plasmin, elastase, human trypsin	Gelatin, collagen IV, V, VII, X, XIV, aggrecan, nidogen, fibronectin, elastin, vitronectin, fibrillin
Stromelysins				
Stromelysin-1 (MMP-3)	60/55	MMP-1, -8, -9, -13	Plasmin, kallikrein, chymase, tryptase, elastase, cathepsin G	Collagen III, IV, V, IX, X, gelatin, nidogen, aggrecan, perlecan, fibronectin, laminin, tenascin, elastin, fibrillin
Stromelysin-2 (MMP-10)	60/55	MMP-1, -7, -8, -9, -13	As stromelysin-1	Collagen III, IV, V, gelatin, nidogen, aggrecan, fibronectin, elastin
Stromelysin-3 (MMP-11)	58	ND	Furin	Fibronectin, laminin
Metalloelastase (MMP-12)	54	ND	ND	Collagen IV, gelatin, nidogen, aggrecan, fibronectin, laminin, elastin, vitronectin, fibrillin
Matrilysins				
Matrilysin-1 (MMP-7)	28	MMP-2	MMP-3, plasmin	Collagen IV, gelatin, fibronectin, laminin, nidogen, aggrecan, fibronectin, tenascin, elastin, vitronectin, versican
Matrilysin-2 (MMP-26)	29	MMP-9	ND	Collagen IV, gelatin, fibronectin
Membrane-type MMPs				
MT1-MMP (MMP-14)	63	MMP-2, -13	Plasmin, furin	Collagen I, II, III, gelatin, nidogen, aggrecan, perlecan, fibronectin, laminin, tenascin, vitronectin, fibrillin
MT2-MMP (MMP-15)	72	MMP-2, -13	ND	Fibronectin, laminin, nidogen, tenascin, aggrecan, perlecan
MT3-MMP (MMP-16)	64	MMP-2	ND	Gelatin
MT4-MMP (MMP-17)	70	MMP-2	ND	Gelatin
MT5-MMP (MMP-24)	73*	MMP-2	ND	ND
MT6-MMP (MMP-25)	34	MMP-2	ND	Collagen IV, gelatin, fibronectin
Other MMPs				
MMP-19	57*	ND	ND	Gelatin, aggrecan
Enamelysin (MMP-20)	53*	ND	ND	Amelogenin, aggrecan
MMP-23	44*	ND	ND	ND
Epilysin (MMP-28)	56*	ND	ND	ND

Modified from Ding, 1998 and Vaalamo, 2000. ND, not determined. Additional references Sorsa *et al.,* 1997; Ferry *et al.,* 1997; Mazzieri *et al.,* 1997; Nagase and Woessner, 1999; Llano *et al.,* 1999; Kajita *et al.,* 1999; Pei, 1999; Stracke *et al.,* 2000; Uría and López-Otín, 2000; Velasco *et al.,* 2000; English *et al.,* 2001; Lohi *et al*., 2001. *Predicted on the basis of the amino acid sequence.

(AP-1) binding site and polyomavirus enhancer A-binding protein-3 (PEA3) motif present in most MMP genes have been the focus of intense research (Birkedal-Hansen *et al*., 1993). Interaction of these two elements appears to confer responsiveness to growth factors, oncogene products and tumor promoters (Gutman and Wasylyk, 1990; Benbow and Brinckerhoff, 1997). It has been suggested that lack of the regulatory elements AP-1 and PEA3 in the promoter region of the MMP-2 gene is responsible for its lack of transcriptional regulation (Huhtala *et al*., 1990; Tryggvason *et al*., 1990; Benbow and Brinckerhoff, 1997). Regulation of MMP expression in polymorphonuclear neutrophils (PMNs) is uniquely different from that of the other cells. Synthesis of MMP-8 and MMP-9 is already completed by the time the PMNs enter the vasculature and any further regulation is mediated by granule release rather than transcriptional events (Birkedal-Hansen *et al*., 1993).

In addition to soluble factors, cell-matrix and cell-cell interactions are considered to be key factors in the regulation of MMP gene expression. Examples include a) induction of MMP-2 and MMP-14 expression in endothelial cells, fibroblasts, and neoplastic cells when cultured in type I collagen gel (Gilles *et al*., 1997; Haas *et al*., 1998), b) induction of MMP-1, -2, and -3 expression in fibroblasts by a transmembrane glycoprotein extracellular matrix metalloproteinase inducer (EMMPRIN) expressed on tumor cell surface (Guo *et al*., 1997), c) induction of MMP-2 expression in T cells through very late antigen (VLA-4) - vascular cell adhesion molecule-1 (VCAM-1) - mediated adhesion to endothelial cells (Romanic and Madri, 1994).

B) Activation

Apart from membrane-type MMPs and MMP-11 (stromelysin-3), which are activated intracellularly by furin (Pei and Weiss, 1995; Pei and Weiss, 1996), MMPs are secreted from cells as inactive zymogens (Nagase and Woessner, 1999). Binding of the unpaired cysteine residue in the propeptide domain with the conserved Zn^{2+} ion in the catalytic domain (cysteine switch) is thought to be responsible for maintening the latency of the MMPs (Springman *et al*., 1990; Van Wart and Birkedal-Hansen, 1990; Birkedal-Hansen, 1995; Murphy and Knäuper, 1997). The catalytic site can be exposed by agents that are able to dissociate the bond between cysteine and zinc. Several non-proteolytic agents such as sulfhydryl-reactive agents, mercurial compounds, reactive oxygen, and denaturants can break the bond *in vitro*. *In vivo*, most proMMPs are likely to be activated by tissue or plasma proteinases or opportunistic bacterial proteinases (Nagase and Woessner, 1999). Proteinases such as trypsin, plasmin, and kallikrein can process the proMMP into an active intermediate which autocatalytically cleaves itself to a fully active form. MMP-3 and MMP-10 (stromelysin-1 and –2) are able to "superactivate" procollagenases to generate enzymes with higher specific activity than the autocatalytically processed forms (Birkedal-Hansen, 1995).

Although many *in vitro* models for proMMP activation exist, the *in vivo* activation mechanisms are still poorly understood. In lung carcinomas (Tokuraku *et al*., 1995) and brain tumors (Yamamoto *et al*., 1996) MMP-14 (MT1-MMP) expression has been found to correlate with the activation of proMMP-2, suggesting that MMP-14 is a physiological activator of proMMP-2. In an *in vivo* model of acute lung injury, on the other hand, neutrophil elastase was found to be a potential physiological activator of proMMP-9 (Ferry *et al*., 1997). Several studies emphasize the importance of tightly regulated cell surface-associated activation which is represented by 1) urokinase-type plasminogen activator (uPA)/ uPA-receptor/plasminogen cascade and ac-

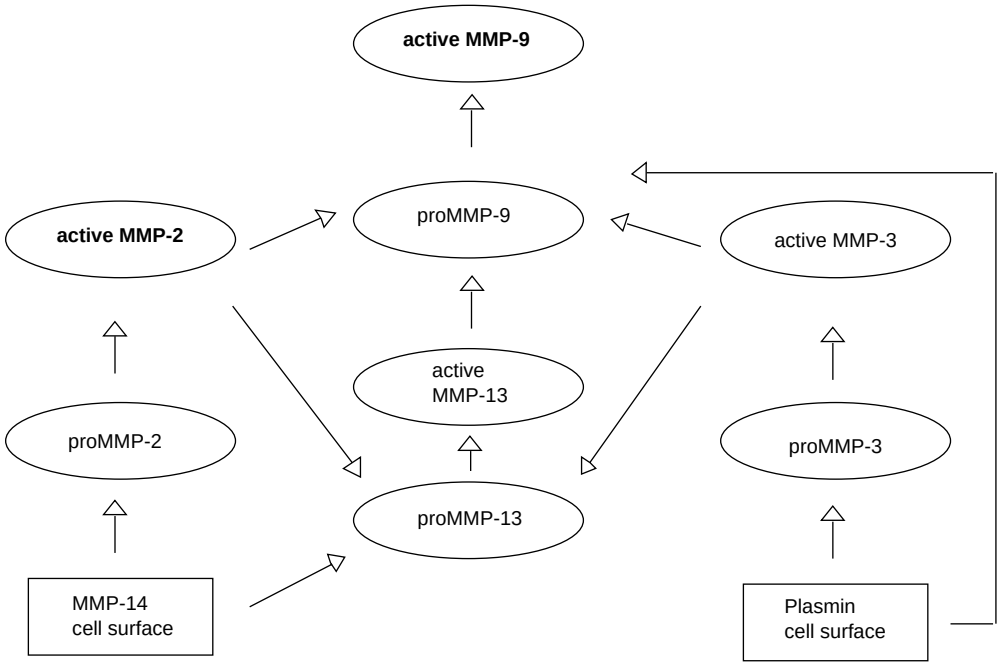

Figure 2. Matrix metalloproteinase activation cascades mediated by cell surface-bound MMP-14 and plasmin. Modified from Murphy *et al.,* 1999.

tivation of MMPs by plasmin (Mignatti *et al*., 1986; Murphy *et al*., 1992; Andreasen *et al*., 1997; Mazzieri *et al*., 1997), and 2) activation of proMMP-2 by MMP-14 (Sato *et al*., 1994) (Figure 2). These cascades are thought to be of physiological significance (reviewed by Murphy *et al*., 1999). Generation of cell surface-associated plasmin by the activity of receptor bound uPA on cell bound plasminogen, is thought to be a key initiator of MMP activation especially through the activation of proMMP-3 (prostromelysin-1) (He *et al*., 1989; Nagase *et al*., 1990; Murphy *et al*., 1999; Ramos-DeSimone *et al*., 1999). Active MMP-14 on the cell surface acts as a second focus of activation. It is thought that proMMP-2, tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), and MMP-14 are concentrated on the cell surface as a trimolecular complex (Strongin *et al*., 1995; Murphy *et al*., 1999). The initial cleavage is mediated by free MMP-14. The cleavage destabilises the structure of the propeptide domain of proMMP-2 after which autoproteolysis proceeds to release the rest of the propeptide domain and fully active MMP-2. MMP-14 may also directly activate proMMP-13 (collagenase-3) (Knäuper *et al*., 1996; Murphy *et al*., 1999). A more likely mechanism is activation of proMMP-13 by MMP-2 (Knäuper *et al*., 1996). MMP-2, MMP-3, and MMP-13 can activate proMMP-9 (Fridman *et al*., 1995; Shapiro *et al*., 1995; Knäuper *et al*., 1997). Activation of pro-MMPs is thought to be largely limited to the pericellular environment where cell-associated proteinases can function in a priviledged environment away from an excess of proteinase inhibitors. The efficiency of the activating interactions is supposed to be dependent upon mechanisms for concentration of the MMPs at the cell surface or on the ECM (Murphy *et al*., 1999).

C) Inhibition

MMP activity is controlled by α_2-macroglobulin (Sottrup-Jensen and Birkedal-Hansen, 1989) and by TIMPs, which represent a specific regulatory mechanism in extracellular milieu (Murphy and Docherty, 1992). To date four members of the TIMP family (TIMP-1, -2, -3, and -4) have been identified (Murphy and Werb, 1985; Stetler-Stevenson *et al*., 1989; Uría *et al*., 1994; Greene *et al*., 1996). Although these inhibitors have very similar inhibitory activities against most members of the MMP family, they differ in many aspects including interactions with proMMPs, solubility, regulation of expression, and tissue-specific expression (reviewed by Henriet *et al.*, 1999). TIMPs exert a two-step control on MMPs by their ability not only to inhibit active enzymes but also to regulate the activation process. TIMPs inhibit the activity of MMPs by forming tight noncovalent 1:1 stoichiometric complexes with active MMPs. With one exception, *i.e.* MMP-14, which is well inhibited by TIMP-2 and TIMP-3 but not by TIMP-1 (Will *et al*., 1996), all members of the TIMP family have a similar function toward all MMPs. TIMPs also control the autocatalytic activation of several proMMPs (DeClerck *et al*., 1991; Howard *et al*., 1991; Strongin *et al*., 1993) and have the ability to form complexes with proenzymes. TIMP-1 preferentially forms complexes with proMMP-9 (Goldberg *et al*., 1992) and TIMP-2 and TIMP-4 with proMMP-2 (Goldberg *et al*., 1989). Early experimental studies indicated an inverse correlation between TIMP-1 and metastatic potential *in vitro* and based on results of human and mouse tumor models it has been suggested that the MMP inhibitors exert anti-invasive and antimetastatic effects (Schultz *et al*., 1988; Khokha and Denhardt, 1989). However, more recent studies suggest that TIMPs are multi-functional molecules which may have paradoxical effects on tumor progression. TIMP-1 and TIMP-2 are both known to have growth factor-like properties which are separate from their MMP-inhibitory functions (reviewed by Gomez *et al*. 1997).

1.1.2 MMPs in tumor invasion and metastasis

Especially the type IV collagen-degrading enzymes MMP-2 and MMP-9 have been the focus of intensive research, because breakdown of the BM is an essential step in cancer invasion. In a pioneering study, Liotta *et al*. (1980) showed a correlation between the metastatic potential of tumor cells and their ability to degrade BM collagen. Since then, it has been shown that MMPs are overexpressed in several tumors (Westermarck and Kähäri, 1999). Transfection of human bronchial epithelial cells or rat embryo cells with Ha-ras oncogene revealed that acquisition of the metastatic phenotype is linked to secretion of type IV collagenases (Bonfil *et al*., 1989; Bernhard *et al*., 1994). On the other hand, recombinant human TIMP inhibited human amnion invasion and lung colonization of murine B16-F10 melanoma cells (Schultz *et al*., 1988) and TIMP-1 inhibited simian virus 40 T antigen-induced hepatocarcinogenesis by reduction of hepatocellular proliferation and tumor angiogenesis (Martin *et al*., 1999). Recently, Itoh and colleagues (1998; 1999) showed that tumor-induced angiogenesis, tumor growth and lung colonization were suppressed in MMP-2 and MMP-9 deficient mice, and Koivunen and colleagues (1999) showed that synthetic MMP-2- and MMP-9-inhibitory peptides prevented tumor growth and invasion in mouse models. The importance of MMP-2 in tumor angiogenesis was further evidenced by the results of Fang *et al*. (2000) which implicated MMP-2 activity as a requirement for the switch of angiogenic phenotype in a tumor model. High expression of MMPs by tumors is associated with poor prognosis, *e.*

g., MMP-1 in colorectal (Murray *et al*., 1996), MMP-2 in cervical (Davidson *et al*., 1999), and MMP-7 in esophageal cancer (Yamashita *et al*., 2000).

1.2 Plasminogen activators and plasmin

Plasmin and plasminogen activators belong to the serine proteinase family. Plasminogen is the single-polypeptide-chain zymogen form of plasmin produced mainly in the liver. It is a 90 kDa protein with a carbohydrate content of 2% (reviewed by Saksela *et al.*, 1985). Its plasma concentration is approximately 2 μM but 40% of the plasminogen is localized extravascularly (Robbins and Summaria, 1970; Mignatti and Rifkin, 1993). Active plasmin is an 85 kDa protein consisting of 2 disulfide bridge-linked polypeptide chains. The C-terminal B-chain of plasmin contains a serine proteinase domain which is responsible for catalytic activity and binding to inhibitors. The N-terminal A-chain contains 5 kringle domains (Andreasen *et al*., 1997). Angiostatin, which has been reported to inhibit angiogenesis, is a fragment of plasminogen consisting of kringles 1-4 (O'Reilly *et al*., 1994). Plasmin has a broad substrate specificity, and it is able to degrade many ECM proteins such as fibronectin, vitronectin, and laminin (Danø *et al*., 1985). It also activates several proMMPs (Eeckhout and Vaes, 1977; Werb *et al*., 1977; Murphy *et al*., 1992; Mazzieri *et al*., 1997) (Table II).

Plasminogen is activated by urokinase- and tissue-type plasminogen activators (uPA and tPA) which are 68 kDa and 54 kDa glycoproteins (Saksela, 1985). The primary role of tPA is to generate plasmin for thrombolysis, whereas uPA generates plasmin in events involving degradation of the ECM, *e. g.,* tumor invasion and tissue remodeling (Mullins and Rohrlich, 1983; Danø *et al*., 1985; Andreasen *et al*., 1997). Active uPA consists of 2 disulfide bridge-linked polypeptide chains, a C-terminal proteinase domain and an N-terminal A-chain which contains a kringle and a growth factor domain (Andreasen *et al*., 1997). Like plasminogen, uPA is secreted as an inactive proenzyme, prouPA, which can be activated by trace amounts of plasmin (Danø *et al*., 1985; Sim *et al*., 1986). Trypsin-2 (Koivunen *et al*., 1989), plasma kallikrein, and blood coagulation factor XIIa (Ichinose *et al*., 1986) activate prouPA *in vitro*. Binding of prouPA to a cell surface receptor (urokinase-type plasminogen activator receptor, uPAR) enhances plasminogen activation (Stephens *et al*., 1989; Ellis *et al*., 1991).

Plasminogen activation is controlled by plasminogen activator inhibitors (PAI) -1 and -2 and α_2-antiplasmin, which belong to a superfamily of serpins (serine proteinase inhibitors) (Ellis *et al*., 1990). Of these, PAI-1 appears to play a particularly important role in cancer invasion (Danø *et al*., 1985; Andreasen *et al*., 1997). Components of the uPA-plasminogen system are expressed in various cancers and the pattern of their expression varies between different types of cancer (reviewed by Andreasen *et al.*, 1997). In colon cancer, for example, uPA is expressed in fibroblast-like stromal cells, uPAR in cancer cells and tumor infiltrating macrophages (Pyke *et al*., 1991a), and PAI-1 in endothelial cells located in the tumor stroma (Pyke *et al*., 1991b), indicating coordinated interplay between various cell types and the proteinases and proteinase inhibitors they produce.

1.2.1 uPA-plasmin system in tumor invasion and metastasis

Several lines of evidence suggest a central role for the uPA-plasmin system in tumor invasion. It has been shown that plasminogen and plasmin activity enhance cell migration *in vitro* (Ossowski *et al*., 1973; Morimoto *et al*., 1993; Quax *et al*., 1994) and that antibodies against uPA inhibit migration (Morimoto *et al*., 1993; Quax *et*

al., 1994) and invasion through an isolated human amniotic membrane (Mignatti *et al*., 1986). In a pioneering work Ossowski and Reich (1983) showed that antibodies against human uPA inhibited lung metastasis of human tumor cells implanted onto the chorio-allantoic membrane of chicken embryos. Using a model based on dissemination of human tumors in nude mice, Ossowski *et al.* (1991) reported that antibodies against human uPA prevented local invasion of cancer cells and Quax *et al.* (1991) reported a correlation between cancer cell uPA expression and lung metastasis with a series of melanoma cell lines. Wilhelm *et al.* (1995) found that uPA antisense oligonucleotides inhibited the intraperitoneal growth of human ovarian cancer cells in nude mice, and transfection of rat PC3 prostate carcinoma cells with uPA cDNA enhanced their metastatic potential *in vivo* when inoculated into the cardiac ventricles of rats (Achbarou *et al*., 1994). The importance of the uPA-plasmin system in tumor invasion and metastasis is reflected by the relationship between the prognosis of cancer patients and the levels of uPA, uPAR, PAI-1, and PAI-2 (reviewed by Andreasen *et al.*, 1997). For example, uPA has been shown to be a prognostic marker in several cancers including colorectal (Mulcahy *et al*., 1994), ovarian (Kuhn *et al*., 1994), and breast cancer (Duffy *et al*., 1998).

2. TRYPSINOGENS

Trypsinogen is one of the first isolated and characterized enzymes which has been extensively studied and used as a model of protein structure and function. Trypsinogen was crystallized from bovine pancreas by Kunitz and Northrop in 1936, reported to occur in human pancreatic juice by Haverback *et al.* in 1960 and partially isolated from extracts of human pancreas by Buck *et al.* in 1962. Different research groups have given several names for human trypsinogens. The nomenclature is summarized in Table III.

Human pancreatic juice contains three trypsinogen isoenzymes which differ in isoelectric point (pI) (Scheele *et al*., 1981) (Table IV). The most cationic (trypsinogen-1, pI 6.2) and the most anionic (trypsinogen-2, pI 4.9) trypsinogens were purified from pancreatic juice by Figarella *et al.* in 1969. Ten years later Rinderknecht *et al.* (1979) discovered a minor trypsinogen isoenzyme, which was characterized by its intermerdiate electrophoretic mobility and a pI of 5.7 (Scheele *et al*., 1981) and therefore designated mesotrypsinogen by Rinderknecht *et al.* (1984) (trypsinogen-3 in this study). In 1993 Wiegand *et al.* (1993) characterized an additional trypsinogen (trypsinogen-4) expressed exclusively in the brain. Trypsinogen-4 has been suggested to be a splicing variant of the gene which encodes trypsinogen-3 (Wiegand *et al*., 1993). Stenman *et al.* (1988) detected and Koivunen *et al.* (1989) isolated from ovarian tumor cyst fluid two trypsinogens which probably are encoded by the same genes as the corresponding pancreatic trypsinogen-1 and -2 (Sorsa *et al*., 1997). These tumor-associated trypsinogens (TATs) are identical to their pancreatic counterparts in the amino-terminal amino acid sequence, immunoreactivity and molecular size but differ in substrate specificity, susceptibility to inhibition by protease inhibitors, and pI (Koivunen *et al*., 1989) (Table IV).

2.1 Genes, mRNAs, and proteins

By complete genomic sequencing of the human trypsinogen gene family, 8 trypsinogen genes have been found to be located in the chromosome region 7q35 and one in 9p13 but only three of them have been shown to encode a protein (Rowen *et al*., 1996). Each trypsinogen gene is composed of five exons and resides within a tandemly

Table III. Nomenclature of trypsinogen genes, cDNAs, and proteins.

Gene (Rowen *et al.,* 1996)	**cDNA**	**Reference**	**Protein**	**Reference**	**Name used in this study**
T1 (pseudogene)					
T2 (relic gene)					
T3 (pseudogene)					
T4	TRY I	Emi *et al.,* 1986	Trypsinogen-1	Figarella *et al.,* 1969	Trypsinogen-1
			Cationic	Rinderknecht *et al.,* 1979;	
				Rinderknecht *et al.,* 1984	
			Trypsinogen-3	Scheele *et al.,* 1981	
			TAT-1	Koivunen *et al.,* 1989	
T5 (pseudogene)					
T6 (no product)					
T7 (pseudogene)					
T8	TRY II	Emi *et al.,* 1986	Trypsinogen-2	Figarella *et al.,* 1969	Trypsinogen-2
			Anionic	Rinderknecht *et al.,* 1979;	
				Rinderknecht *et al.,* 1984	
			Trypsinogen-1	Scheele *et al.,* 1981	
			TAT-2	Koivunen *et al.,* 1989	
T9	TRY III	Tani *et al.,* 1990	Mesotrypsinogen	Rinderknecht *et al.,* 1979; Rinderknecht *et al.,* 1984	Trypsinogen-3
	Mesotrypsi-nogen	Nyaruhucha *et al.,* 1997	Trypsinogen-2	Scheele *et al.,* 1981	
	TRY IV	Wiegand *et al.,* 1993	Trypsinogen-4	Wiegand *et al.,* 1993	Trypsinogen-4

duplicated 10 kb segment (Rowen *et al*., 1996). The regulatory elements controlling the transcription of human trypsinogen genes have not been characterized. However, pancreas-specific regulatory elements have been shown to be involved in the transcriptional control of pancreas-specific genes including trypsinogen in rat and mouse (Walker *et al*., 1983; Cockell *et al*., 1989; Fodor *et al*., 1991). The coding region of trypsinogen-1, -2, and -3 cDNA consists of 741 basepairs (bp) (Emi *et al*., 1986; Tani *et al*., 1990; Nyaruhucha *et al*., 1997) and that of trypsinogen-4 of 780 bp (Wiegand *et al*., 1993). Trypsinogen-1, -2, and -3 are synthesized as 247 amino acid polypeptides including a 15 amino acid signal and an 8 amino acid activation peptide (Guy *et al*., 1978) (Figure 3). The a- and b-forms of trypsinogen-4, which are thought to represent allelic polymorphism, contain 260 and 259 amino acids, respectively (Wiegand *et al*., 1993). Trypsinogens contain 5 disulfide bridges (Guy *et al*., 1978). The molecular weights of trypsinogens calculated on the basis of the amino acid content and their relative molecular masses (M_r) determined by SDS-PAGE are listed in Table IV. Trypsinogen-1 and -2 share 92% and 89% identity at the cDNA and protein level, respectively (Emi *et al*., 1986). The amino acid sequence of trypsinogen-3 displays 86% and 88% sequence identity with those of trypsinogen-1 and -2, respectively (Nyaruhucha *et al*., 1997). Trypsinogen-4 cDNA shares extensive similarity with those of trypsinogen-1, -2, and –3 through exons 2-5 whereas exon 1 of trypsinogen-4 is unique. Unlike other trypsinogen isoenzymes, trypsinogen-4 has been suggested to be a

Table IV. Biochemical properties of trypsin(ogen)s.

Protein	pI	Reference	Theoretical molecular weight	Reference	Relative molecular mass (M_r) (kDa)[e]	Reference
Trypsinogen-1	6.2[a]	Scheele *et al.,* 1981	23 438[c]	Guy *et al.,* 1978	26±1	Guy *et al.,* 1978
					27	Scheele *et al.,* 1981
TAT-1	5-5.5[a]	Koivunen *et al.,* 1989			25	Koivunen *et al.,* 1989
Trypsinogen-2	4.9[a]	Scheele *et al.,* 1981	25 006[c]	Guy *et al.,* 1978	26 ±1	Guy *et al.,* 1978
					28	Scheele *et al.,* 1981
TAT-2	4[a]	Koivunen *et al.,* 1989			28	Koivunen *et al.,* 1989
Trypsinogen-3	5.7[a]	Scheele *et al.,* 1981			26	Scheele *et al.,* 1981
Trypsinogen-4						
a-form	7.5[b]	Wiegand *et al.,* 1993	24 348[d]	Wiegand *et al.,* 1993		
b-form	7.7[b]	Wiegand *et al.,* 1993	24 218[d]	Wiegand *et al.,* 1993		

[a]Isoelectric point of zymogen denatured in 8 M urea
[b]Theoretical isoelectric point of an active enzyme
[c]Theoretical molecular weight of zymogen calculated on the basis of amino acid content
[d]Theoretical molecular weight of active enzyme calculated on the basis of amino acid content
[e]Relative molecular mass of zymogen determined by SDS-PAGE

cytoplasmic protein, because a leader sequence typical of secreted proteins has not been found in its first exon (Wiegand *et al*., 1993).

2.2 Specificity and regulation of enzymatic activity

Trypsinogens belong to the serine proteinases which are neutral proteinases characterized by a catalytic triad consisting of a Ser195, His57, and Asp102 (chymotrypsin numbering) (Craik *et al*., 1985). The substrate specificity is determined by the substrate binding pocket which consists of conserved Asp189, Gly216, and Gly226. The aspartate residue (Asp189) of trypsin forms a strong electrostatic bond with arginine and lysine residues of the substrate after which trypsin hydrolyses the peptide bond on the carboxyl terminal side of either of these amino acids. The two glycines in the substrate binding pocket of trypsin permit the entry of large amino acid side chains into the hydrophobic pocket (Craik *et al*., 1985). These conserved amino acids of the human trypsinogens are shown in figure 3.

2.2.1 Activation

During the digestive process, trypsinogens entering duodenum in pancreatic secretions are activated by enterokinase (also called enteropeptidase) - a membrane-bound serine proteinase in the brush border of duodenal enterocytes (reviewed by Lu *et al*., 1999). Upon activation, trypsinogen-2 liberates an activation peptide Ala-Pro-Phe-Asp_4-Lys (Guy *et al*., 1976) whereas trypsinogen-1 liberates the same octapeptide or a pentapeptide Asp_4-Lys (Guy *et al*., 1978). During activation, the molecular mass of trypsinogens decreases by about 1 kDa. Both trypsin-1 and -2 activate trypsinogen-1 and -2 at pH 8.0 and 20 mM Ca^{2+} at the same rate but the affinity of both trypsins is higher for trypsinogen-1 than for trypsinogen-2 (Colomb and Figarella, 1979). At pH 5.6 and 1 mM Ca^{2+}, the autoactivation of trypsinogen-1 is more efficient than its activation by enterokinase. Thus under con-

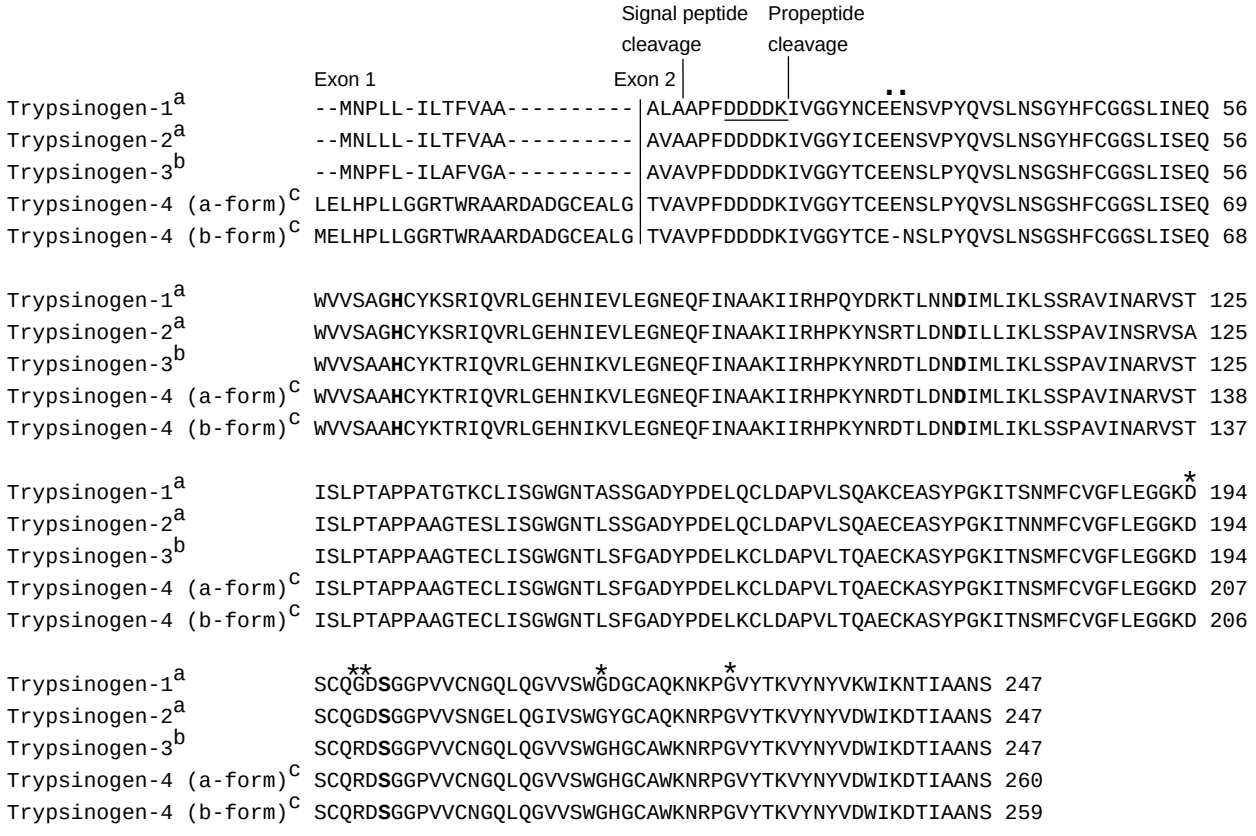

Figure 3. Amino acid sequences of human trypsinogen-1, -2, -3, and -4. [a]Emi *et al.,* 1986, [b]Nyaruhucha *et al.,* 1997, [c]Wiegand *et al.,* 1993. Residues recognized by enterokinase are underlined (Maroux *et al.,* 1971). The three amino acid residues of the catalytic triad are marked with bold type. The three amino acids determining the substrate specificity are marked with * (Craik *et al.,* 1985). The putative site of polymorphism in trypsinogen-4 is marked with two dots (Wiegand *et al.,* 1993). Position at which glycine near the active site serine in trypsinogen-1 and -2 is substituted by arginine in trypsinogen-3 and -4 is marked with ** (Nyaruhucha *et al.,* 1997).

ditions which are close to the physiological milieu in duodenum, enterokinase initiates trypsinogen activation but subsequently activation of trypsinogen by trypsin predominates (Colomb and Figarella, 1979). Furthermore, bile salts and bile acids enhance autocatalytic activation of trypsinogen (Sarkany and Moreland, 1985). Enterokinase is not known to occur in tissues other than the intestine and it is at present unclear how trypsinogens are activated in tissues lacking enterokinase. Trypsinogen can be activated *in vitro* also by cathepsin B (Greenbaum *et al*., 1959; Figarella *et al*., 1988) but the *in vivo* significance of this finding is unknown. Cathepsin B has also been suggested to participate in the premature activation of trypsinogen in pancreatitis (Halangk *et al*., 2000).

Trypsinogen-1 and -2 have two Ca^{2+} binding sites and the $pK(Ca^{2+})$ values of these sites are different for trypsinogen-1 and -2 (Colomb and Figarella, 1979). The primary Ca^{2+} binding site which is common to trypsin and trypsinogen protects the molecule against autolysis and thermal denaturation. The differences in $pK(Ca^{2+})$ values may explain why trypsin-1 is more resistant to autolysis than trypsin-2. The secondary Ca^{2+} binding site in the N-terminal activation peptide regulates the autoactivation of trypsinogens. Under physiological conditions this Ca^{2+} binding site is unsaturated in trypsinogen-2 whereas it is probably saturated in trypsinogen-1 which results in more rapid autoactivation of trypsinogen-1 (Colomb and Figarella, 1979).

2.2.2 Inhibition

The main naturally occuring inhibitors of trypsins are α_2-macroglobulin (A2M), α_1-proteinase inhibitor (API), which is also called α_1-antitrypsin (AAT), and the Kazal-type trypsin inhibitor (Kazal *et al*., 1948) which is also called pancreatic secretory trypsin inhibitor (PSTI) (Pubols *et al*., 1974) and tumor-associated trypsin inhibitor (TATI) (Huhtala *et al*., 1982). Unlike other trypsins, trypsin-3 (mesotrypsin) shows unique resistance against naturally occuring trypsin inhibitors (Rinderknecht *et al*., 1984; Nyaruhucha *et al*., 1997). It has been suggested that substitution of the amino acid Gly198 in trypsin-1 and trypsin-2, which normally participates in the interaction with trypsin inhibitors such as aprotinin (Perona *et al*., 1993), by a positively charged Arg in trypsin-3 may interfere with the trypsin-trypsin inhibitor interaction (Nyaruhucha *et al*., 1997) (Figure 3). A2M is a 720 kDa proteinase inhibitor which reacts with a large variety of proteinases (Barrett and Starkey, 1973) and is the predominant inhibitor of trypsin in serum (Ohlsson, 1988). It is synthesized mainly in the liver (Laurell and Jeppson, 1975) but also in monocytes (Hovi *et al*., 1977) and macrophages (White *et al*., 1980). In plasma, the average A2M concentration is 2 g/l (3 μM) (Ganrot, 1967; Birkenmeier and Stigbrand, 1993). API is a 51 kDa serpin synthesized in the liver (Carrell *et al*., 1982). It is quantitatively the dominating proteinase inhibitor in plasma. It inhibits several proteinases but its major physiological target is thought to be neutrophil elastase. In plasma, the average API concentration is 1.3 g/l (26 μM) (Carrell *et al*., 1982). PSTI/TATI is a very specific trypsin inhibitor which is thought to prevent inappropriate activation of trypsinogen in the pancreas (Haverback *et al*., 1960; Greene *et al*., 1976) and in other tissues expressing trypsinogen and PSTI/TATI (Stenman *et al*., 1991). PSTI/TATI is described in detail in Chapter 3.

2.3 Expression of trypsinogens

2.3.1 Occurrence of trypsinogens in various body fluids

Trypsinogen-1 and -2 represent about 19% of the total protein in pancreatic juice, and trypsinogen-1 is present at concentrations

twice those of trypsinogen-2 (Figarella *et al*., 1969; Guy *et al*., 1978). The minor isoenzyme, trypsinogen-3, occurs at very low concentrations and represents < 0.5% of the proteins in normal human pancreatic secretions (Rinderknecht *et al*., 1984). In serum of healthy subjects, average trypsinogen-1 and -2 concentrations of 15 to 26 µg/l (Borgström and Ohlsson, 1976; Geokas *et al*., 1979; Florholmen *et al*., 1984) and 5.5 µg/l (Largman *et al*., 1978), respectively, have been reported. Trypsins were found for the first time outside the gastrointestinal tract by Stenman *et al*. (1988) from cyst fluids of ovarian tumors in which they occur at high concentrations, *i. e.*, up to 2600 µg/l (Koivunen *et al*., 1990). Low concentrations of trypsinogen-1 and -2 isoenzymes with pI values similar to those of pancreatic and tumor-associated isoenzymes can be detected in serum of pancreatectomized patients, which indicates that trypsinogens in serum are not only of pancreatic origin (Itkonen *et al*., 1996). Trypsinogen-4 has recently been detected in cerebrospinal fluid of patients with aneurysms (Critchley *et al*., 2000).

2.3.2 Expression in the pancreas and extrapancreatic tissues

Pancreatic trypsinogen is produced in the acinar cells of the exocrine pancreas and stored in the secretory vesicles, from where it is secreted into the pancreatic duct in response to hormonal and neural stimuli (Eddeland and Wehlin, 1978; Dooley and Valenzuela, 1984; Owyang *et al*., 1986). Trypsinogen-1 and -2 immunoreactivity was first detected outside the pancreas in Paneth cells in the mucosa of the normal small intestine, gastric mucosa with intestinal metaplasia, and colonic metaplastic mucosa (Bohe *et al*., 1986). Recently, trypsinogen-2 expression was detected in vascular endothelial cells (Koshikawa *et al*., 1997). Koshikawa *et al*. (1998) detected trypsinogen expression at the mRNA and protein level in epithelial cells of the skin, esophagus, stomach, small intestine, lung, kidney, liver, and extrahepatic bile duct, as well as splenic and neuronal cells. The identity of the trypsinogen isoenzyme(s) was not revealed in that study. In the esophagus, trypsinogen was detected in the basal and para-basal cells of the stratified squamous epithelium whereas in the skin, trypsinogen was found to be evenly distributed from the basal cells to the stratum spinous cells. In the kidney, trypsinogen was detected in the collecting ducts and in the lung, in bronchial and bronchiolar epithelial cells. In the liver, hepatocytes were shown to be the site of trypsinogen synthesis and in the spleen trypsinogen expression was detected in macrophages, monocytes, and lymphocytes in the white pulp. In the brain, trypsinogen was detected in the nerve cells of the hippocampus and cerebral cortex (Koshikawa *et al*., 1998).

Tumor-associated trypsinogens were first demonstrated in ovarian neoplasms by Koivunen *et al*. (1989). Later, trypsinogens have been shown to be expressed in several cancers. Gastric (Fujimura *et al*., 1998) and colorectal cancer (Oyama *et al*., 2000) express trypsinogen-1. Ovarian (Hirahara *et al*., 1995; Hirahara *et al*., 1998) and pancreatic cancer (Ohta *et al*., 1994), cholangiocarcinoma (Terada *et al*., 1995), and lung neoplasms (Kawano *et al*., 1997) express trypsinogen. In these studies the identity of trypsinogen isoenzyme(s) was not specified. Cancer cell lines derived from colon (Koivunen *et al*., 1991b; Bernard-Perrone *et al*., 1998; Oyama *et al*., 2000), pancreatic (Miszczuk-Jamska *et al*., 1991; Ohta *et al*., 1998) and gastric cancer (Koshikawa *et al*., 1992; Koshikawa *et al*., 1994; Kato *et al*., 1998; Miyata *et al*., 1998) express trypsinogen-1 and -2 whereas fibrosarcoma and erythroleukemia cells mainly express trypsinogen-2 (Koivunen *et al*., 1991b). Cultured ovarian cancer cells express trypsinogen, the identity of which was not specified (Hirahara *et al*., 1995). Hirahara *et al*. (1998)

studied samples of ovarian tumors and found that the incidence of immunoreactive trypsinogen was higher in ovarian carcinomas than in borderline tumors whereas normal ovaries were negative. However, in ovarian carcinomas the incidence of trypsinogen positivity was high in early (6/11, 55%) as well as in advanced tumors (8/10, 44%) (Hirahara *et al.*, 1998). Ohta *et al.* (1994) detected trypsinogen expression immunohistochemically in 15 of 20 (75%) invasive tubular pancreatic adenocarcinomas and their metastatic lesions but not in three non-invasive intraductal papillary adenocarcinomas. They did not find correlation between trypsinogen expression and the histological differentiation of the tumor. Terada *et al*. (1995), on the other hand, found trypsinogen immunoreactivity in 26 of 37 (70%) cholangiocarcinomas and the incidence of trypsinogen immunoreactivity was higher in well-differentiated than in poorly differentiated cholangiocarcinomas. Although trypsinogen immunoreactivity was also detected in normal liver, it was not observed in any of the 36 hepatocellular carcinomas studied (Terada *et al*., 1995). Recently, trypsinogen immunoreactivity was found by Oyama *et al.* (2000) in 69 of 154 (44%) colorectal cancers preferentially at the invasive front of the cancer. They did not find statistically significant relationship between the trypsinogen expression and the clinicopathological findings.

There are few studies concerning the regulation of trypsinogen expression. Koivunen *et al.* (1991b) showed that the anti-inflammatory glucocorticoid dexamethasone downregulated trypsinogen-2 production by HT 1080 fibrosarcoma cells whereas that by COLO 205 colon carcinoma cells was not affected. The unresponsiveness of COLO 205 cells was possibly due to absence of glucocorticoid receptors. Alternatively trypsinogen expression may be differently regulated in different cell types (Koivunen *et al*., 1991b). Koshikawa *et al.* (1997) studied the expression of trypsinogen-2 in vascular endothelial cells and found that tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulated trypsinogen-2 expression and secretion whereas TNF-α, TGF-α, and IL-1β had no effect. Suppression or induction of MMP expression by glucocorticoids, tumor promoters, and cytokines is mediated by the AP-1 site in the MMP gene (Angel *et al*., 1987; Jonat *et al*., 1990; Birkedal-Hansen *et al*., 1993). Whether the AP-1 site is present in the trypsinogen gene is not known.

2.4 Biological functions of trypsins

Pancreatic trypsin mediates proteolysis of dietary proteins. Furthermore, it is the key enzyme for activation of the proenzymes chymotrypsinogen, proelastase, procarboxypeptidases A and B, and prophospholipase A.

In addition to its well known function as a digestive enzyme, trypsin probably plays a role also in other physiological and pathological processes. Both human pancreatic trypsin-1 and -2 and tumor-associated trypsin-1 and -2 activate prouPA (Koivunen *et al*., 1989). Furthermore, human pancreatic and tumor-associated trypsin-2 are very efficient activators of proMMP-9 and they also activate proMMP-2 although less efficiently (Sorsa *et al*., 1997). Bovine trypsin, which has been widely used to study the activation of proteolytic enzymes, activates several proMMPs, *e. g.*, proMMP-1 (Wilhelm *et al*., 1986), proMMP-2 (Liotta *et al*., 1981), proMMP-3 (Wilhelm *et al*., 1987), proMMP-7 (matrilysin-1) (Imai *et al*., 1995), proMMP-9 (Duncan *et al*., 1998), and the soluble domain of proMMP-14 (Will *et al*., 1996). Bovine trypsin also activates the proform of prostate-specific antigen (PSA) (Takayama *et al*., 1997).

Recently, it has been shown that bovine trypsin and human trypsin activate proteinase-activated receptor-2 (PAR-2) (Kong *et al*., 1997; Nguyen *et al*., 1999; Alm *et al*., 2000; Miyata *et al*., 2000) which is a G-

protein-coupled seven-transmembrane domain receptor widely expressed in various epithelial tissues, endothelial cells, T cells, and tumor cells lines (Nystedt *et al*., 1995; Santulli *et al*., 1995; Böhm *et al*., 1996; Dery *et al*., 1998). PARs induce G protein-mediated signal transduction by stimulating generation of inositol-1, 4, 5-triphosphate and intracellular Ca^{2+} mobilization (Santulli *et al*., 1995; Böhm *et al*., 1996; Dery *et al*., 1998). Through activation of PAR-2, trypsin stimulates integrin $\alpha_5\beta_1$-dependent adhesion to fibronectin and proliferation of human gastric carcinoma cells (Miyata *et al*., 2000) and possibly regulates enterocytes in the intestinal lumen by triggering arachidonic acid release and prostaglandin secretion (Kong *et al*., 1997). Trypsin also activates pancreatic duct epithelial cell ion channels through PAR-2 which suggests that prematurely activated trypsin in the inflamed pancreas may act in paracrine manner to regulate ion transport by ductal cells in pancreatitis (Nguyen *et al*., 1999). It has also been shown that trypsin stimulates TNF-α and IL-1β production of rat peritoneal macrophages indicating a link between increased activity of pancreatic proteinases and inflammatory cytokine production, which both are markedly associated with acute pancreatitis (Lundberg *et al*., 2000).

2.5 Clinical aspects

2.5.1 Pancreatitis

Prematurely activated trypsin is thought to play a key role in acute pancreatitis (Whitcomb, 1999). In the normal state, inactive trypsinogen is stored in cytoplasmic zymogen granules of the pancreatic acinar cells and delivered through the pancreatic duct to the small intestine where it becomes activated by enterokinase. In acute pancreatitis, intracellular activation of trypsin triggers activation of other pancreatic enzymes which leads to autodigestion of the pancreas (Steer and Meldolesi, 1988; Whitcomb, 1999). Small amounts of active trypsin is normally generated within the pancreas but it is usually rapidly inactivated by PSTI. If trypsin activity overwhelms the inhibitory potential of PSTI, trypsin is thought to be inactivated proteolytically by trypsin-3 or by autolysis (Whitcomb *et al*., 1996). Recently, hereditary pancreatitis was linked to chromosome region 7q35, and the defect was found to be caused by mutations in the trypsinogen-1 gene which may render trypsin-1 resistant to inactivation by proteolysis (Whitcomb *et al*., 1996; Gorry *et al.*, 1997). Mutations in the PSTI gene have also recently been found to be associated with chronic pancreatitis possibly by rendering PSTI a less efficient inhibitor of trypsin (Chen *et al*., 2000; Witt *et al*., 2000). The serum levels of trypsinogen-1 and -2 and their API complexes increase in patients with pancreatitis (Elias *et al*., 1977; Itkonen *et al*., 1990; Hedström *et al*., 1994), and serum trypsin-2-API, which reflects release of active trypsin into circulation, is a prognostic factor for the disease (Hedström *et al*., 1996).

2.5.2 Cancer

Several lines of evidence suggest that trypsins play a role in the proteolytic activity of tumors. Trypsinogen-1 and -2 are expressed in several cancers (Koivunen *et al*., 1989; Ohta *et al*., 1994; Hirahara *et al*., 1995; Terada *et al*., 1995; Kawano *et al*., 1997; Hirahara *et al*., 1998; Fujimura *et al*., 1998), cancer cell lines (Koivunen *et al*., 1991b; Miszczuk-Jamska *et al*., 1991; Koshikawa *et al*., 1994; Bernard-Perrone *et al*., 1998; Kato *et al*., 1998; Miyata *et al*., 1998; Ohta *et al*., 1998), and in vascular endothelial cells (Koshikawa *et al*., 1997). Trypsinogen and trypsin-API levels in serum increase in patients with pancreatic, gastric, colorectal, hepatocellular, and biliary tract cancer (Hedström *et al*., 1996; Hedström *et al*., 1999; Ichikawa *et al*., 2000;) and trypsi-

nogen-2 concentrations are significantly higher in cyst fluids of malignant than of benign ovarian tumors (Koivunen *et al*., 1990).

Human trypsin activates prouPA (Koivunen *et al*., 1989), proMMP-2, and proMMP-9 (Sorsa *et al*., 1997) which play central roles in angiogenesis, tumor invasion, and metastasis (Andreasen *et al*., 1997; Westermarck and Kähäri, 1999). Degradation of ECM and fibronectin by trypsinogen-2-producing cancer cells (COLO 205 colon carcinoma, K-562 erythroleukemia, CAPAN-1 pancreatic carcinoma, and HT 1080 fibrosarcoma) can be partially inhibited by PSTI/TATI and anti-trypsinogen antibodies and enhanced by enterokinase (Koivunen *et al*., 1991c). Overexpression of trypsinogen by gastric cancer cells is associated with increased tumorigenicity of the cells in nude mice (Kato *et al*., 1998; Miyata *et al*., 1998).

3. PSTI/TATI

The Kazal-type trypsin inhibitor was originally extracted from bovine pancreas by Kazal *et al*. (1948) and detected in human pancreatic juice by Haverback *et al*. (1960). It was characterized and renamed pancreatic secretory trypsin inhibitor (PSTI) by Pubols *et al*. (1974). Tumor-associated trypsin-inhibitor (TATI) was first found in urine of ovarian cancer patient by Stenman *et al*. (1982) and characterized by Huhtala *et al*. (1982). The human genome contains only one PSTI gene (Horii *et al*., 1987) and PSTI cDNAs isolated from the pancreas (Yamamoto *et al*., 1985) and neoplastic tissues (Tomita *et al*., 1987) are identical. Thus PSTI and TATI are encoded by the same gene. In this study the name PSTI is used when the pancreatic inhibitor is discussed whereas the name TATI is used when it is emphasized that the inhibitor originates from a tumor.

3.1 Gene, mRNA, and protein

The PSTI/TATI gene is approximately 7.5 kb long, it comprises 4 exons and is located on chromosome 5 (Horii *et al*., 1987). The gene has multiple transcription start points (Horii *et al*., 1987). It lacks the common transcription regulation sites TATA, CAAT, and GC boxes but the sequences ATAT and CAATCAAT lie at -87-84 and -149-142, respectively, in the promoter region of the gene (Horii *et al*., 1987). Yasuda *et al*. (1998) have suggested that a sequence CAATCAATAAC at -149-139 in the promoter region of PSTI gene represents a pancreas-specific regulatory element. The PSTI/TATI gene also contains an interleukin-6 responsive element (IL6RE) and an AP-1-binding site (Ohmachi *et al*., 1993; Yasuda *et al*., 1993). The PSTI/TATI gene encodes an mRNA of 237 bp (Yamamoto *et al*., 1985) which is translated to 79 amino acid peptide including a 23 amino acid signal peptide (Horii *et al*., 1987). The secreted PSTI/TATI consists of 56 amino acids, has three intramolecular disulfide bridges (Bartelt *et al*., 1977), and a molecular weight of 6240 calculated on the basis of the amino acid composition (Pubols *et al*., 1974). PSTI/TATI isolated from urine of an ovarian cancer patient was microheterogeneous in charge and the pI of the main component was 5.8 (Huhtala *et al*., 1982).

3.2 Mechanism of inhibition

PSTI/TATI inhibits trypsin in a 1:1 molar ratio (Greene *et al*., 1966; Pubols *et al*., 1974). It possesses a reactive site consisting of lysine-18 and isoleucine-19 in the mature peptide, which serves as a specific target substrate for trypsin (Bartelt *et al*., 1977). The inhibition is reversible (Fritz *et al*., 1967) due to proteolytic inactivation of PSTI/TATI by cleavage of the peptide bonds Arg42-Lys43 and Arg44-Gln45 (Kikuchi *et al*., 1989). After addition of the PSTI-trypsin complex to serum, PSTI is released

and most of the trypsin is found in complex with A2M and API (Eddeland and Ohlsson, 1978a). The mechanism by which the PSTI-trypsin complex dissociates is not clear. The three-dimensional structure of the complex between porcine PSTI and bovine trypsin has been determined by Bolognesi *et al.* (1982).

3.3 Expression of PSTI/TATI

3.3.1 Occurrence of PSTI/TATI in various body fluids

PSTI represents about 0.1 to 0.8% of the total protein in pancreatic juice (Pubols *et al.*, 1974). The mean concentration of PSTI/TATI in serum of healthy subjects as determined by radioimmunoassays was 8.1 µg/l (Eddeland and Ohlsson, 1978b) and 11.3 µg/l (Stenman *et al.*, 1982). By an immunofluorometric assay the mean concentration of PSTI/TATI in serum of healthy subjects was 6.9 µg/l and the reference range was 3.1-16 µg/l (Osman *et al.*, 1993). By a radioimmunoassay, the mean PSTI/TATI concentration in urine of healthy subjects was 14 µg/l (Eddeland and Ohlsson, 1978b) and 22 µg/g creatinine (range 7-50 µg/g creatinine) (Huhtala *et al.*, 1983). The serum PSTI/TATI level remains within the normal range after total pancreatectomy (Halila *et al.*, 1985). In second trimester amniotic fluid the median concentration was 160 µg/l (Kolho *et al.*, 1986). TATI occurs at high concentrations in cyst fluids of ovarian tumors. The median TATI concentration was 2700 µg/l in benign and 6600 µg/l in malignant mucinous cyst fluids (Koivunen *et al.*, 1990).

3.3.2 Expression in the pancreas and extrapancreatic tissues

PSTI is secreted together with trypsinogen from pancreatic acinar cells into pancreatic juice (Kazal *et al.*, 1948). Like trypsinogen, PSTI/TATI is also widely expressed in extrapancreatic tissues especially in the gastrointestinal and urinary tract. Immunoreactive PSTI/TATI has been detected in mucus-producing cells of the small intestine, colon, and stomach, as well as in the gall bladder, kidney and lung (Bohe *et al.*, 1986; Fukayama *et al.*, 1986; Shibata *et al.*, 1986; Shibata *et al.*, 1987; Bohe *et al.*, 1990; Freeman *et al.*, 1990a; Marchbank *et al.*, 1996). Expression of PSTI/TATI in the gastrointestinal tract, kidney, and in the acinar cells of the breast has been demonstrated both at the mRNA and protein level (Marchbank *et al.*, 1996). The regulatory mechanisms of PSTI expression and secretion in extrapancreatic tissues are not known.

TATI was first demonstrated in gynecological cancers, *i. e.*, ovarian, cervical, and endometrial cancer by Stenman *et al.* (1982) and Huhtala *et al.* (1982; 1983). Later, TATI has been shown to be expressed in pancreatic (Ogawa *et al.*, 1987), colorectal (Ogawa *et al.*, 1987; Higashiyama *et al.*, 1990a; Tomita *et al.*, 1990), gastric (Ogawa *et al.*, 1987; Higashiyama *et al.*, 1990b), liver (Ogawa *et al.*, 1987; Ohmachi *et al.*, 1993), lung (Ogawa *et al.*, 1987; Tomita *et al.*, 1987; Higashiyama *et al.*, 1992), breast (Ogawa *et al.*, 1987), endometrioid, cervical, and ovarian cancer (Ueda *et al.*, 1989), and in carcinoma of gall bladder (Bohe *et al.*, 1991). Cancer cells derived from pancreatic (Ogawa *et al.*, 1987; Ogata and Murata, 1988), gastric, ovarian, lung, liver (Ogawa *et al.*, 1987), and colon cancer (Ogawa *et al.*, 1987; Koivunen *et al.*, 1991b) produce TATI. In gastric carcinoma of intestinal-type, a stronger TATI immunoreactivity was observed in advanced carcinomas (90%) than in early carcinomas (63%). In gastric carcinoma of diffuse-type the incidence of immunoreactive TATI was high in both advanced (85%) and early carcinomas (93%) (Higashiyama *et al.*, 1990b). In colorectal cancer, the incidence of immunoreactive TATI was found to correlate with the depth of invasion and histological dif-

ferentiation, the incidence being higher in more invaded and better differentiated carcinomas than in less invasive and poorly differentiated carcinomas (Higashiyama *et al*., 1990a). However, the expression of TATI mRNA did not correlate with progression or histological differentiation of colorectal cancer (Tomita *et al*., 1990). Furthermore, in the study of Bohe *et al*. (1990) immunoreactive TATI was not found in neoplastic colonic mucosa whereas it was detected in normal colonic mucosa. In mucinous ovarian tumors the incidence of TATI immunoreactivity was higher in benign (80%) than in malignant tumors (43%) (Ueda *et al*., 1989). In pulmonary adenocarcinoma the TATI immunoreactivity seemed to be more frequent in early than in advanced adenocarcinomas. It was also higher in those with well or moderate histological differentiation than in those with poor histological differentiation (Higashiyama *et al*., 1992). These studies suggest that TATI expression is differently regulated in malignancies arising from different tissues.

3.4 Biological functions of PSTI/TATI

The main physiological function of PSTI is thought to be the protection of the pancreas from destruction by inadvertently activated trypsin (Haverback *et al*., 1960; Greene *et al*., 1976). Identification of trypsinogen and PSTI/TATI expression in mucus-producing cells of the gastrointestinal tract has led to a suggestion that PSTI/TATI is also important in preventing excessive digestion of the gastrointestinal mucus (Freeman *et al*., 1990b; Playford *et al*., 1991; Marchbank *et al*., 1996). It has been suggested that PSTI/TATI plays a role in maintaining mucosal integrity and in stimulating epithelial repair (Marchbank *et al*., 1996; Marchbank *et al*., 1998). It may also participate in tumor scar formation (Higashiyama *et al*., 1992). In addition to trypsin, PSTI/TATI efficiently inhibits acrosin (Huhtala, 1984) thus possibly playing a role in reproduction.

In tumors producing both TATI and trypsinogen, TATI may have a similar function as in the pancreas, *i. e.*, protection of the tumor against the destructive activity of trypsin within tumor cells (Stenman *et al*., 1991). Large amounts of proteinases may also cause degradation of ECM to an extent that is detrimental to the adhesion needed for cell migration (Andreasen *et al*., 1997). Although high concentrations of TATI may protect the ECM against trypsin-mediated proteolysis so that invasion becomes inhibited, it may at lower concentrations protect ECM to an extent which is needed for cell adhesion. This may explain the observation that patients which have non-mucinous ovarian cancer and elevated TATI levels in serum have worse prognosis than those with normal TATI levels (Venesmaa *et al*., 1998).

PSTI/TATI has been proposed to exert growth factor-like properties. This possibility was raised when it was discovered that PSTI/TATI and epidermal growth factor (EGF) are of similar size, share significant amino acid sequence homology and have three disulfide bridges (Hunt *et al*., 1974; Scheving, 1983). In spite of the homology at cDNA and amino acid sequence level, the structures of PSTI/TATI and EGF genes are so different that they are not thought to be derived from a common ancestral gene (Horii *et al*., 1987). PSTI/TATI binds to a cell surface receptor, which is distinct from EGF receptor (Niinobu *et al*., 1986; Niinobu *et al*., 1990) and it has been shown to stimulate the growth of human fibroblasts (Ogawa *et al*., 1985a) and endothelial cells (McKeehan *et al*., 1986) and rat pancreatic cancer cells (Freeman *et al*., 1990c). However, in the study of Ohmachi *et al*. (1994), PSTI/TATI did not have an effect on the growth of fibroblasts or pancreatic cancer cells. Furthermore, growth stimulation of endothelial cells has also been shown to be induced by other serine proteinase inihibitors, *e. g.*, aprotinin and API

(McKeehan *et al*., 1986), suggesting that stimulation of cell growth may be brought about by general inhibition of proteinase activity and possibly by inhibition of degradation of other growth factors (Freeman *et al*., 1990c). These observations suggest that PSTI/TATI may have direct or indirect growth promoting activity on non-neoplastic and neoplastic cells but its mechanism of action and significance *in vivo* remains to be clarified.

The serum levels of PSTI/TATI increase in connection with severe inflammatory diseases (Ogawa *et al*., 1980; Lasson *et al*., 1986), tissue destruction (Ogawa *et al*., 1985b) and major surgery (Matsuda *et al*., 1985) which suggests that PSTI/TATI plays a role in the acute phase reaction. Many acute phase proteins are proteinase inhibitors produced by the liver in response to inflammatory cytokines. They are thought to suppress nonspecific tissue damage caused by proteinases released from activated immune and phagocytic cells (Roberts *et al*., 1995). Being a source of acute phase proteins, the liver is also a potential source of PSTI/TATI. Although PSTI/TATI mRNA has not been detected in normal human liver tissue (Yamamoto *et al*., 1985) the possibility that it originates from the liver is supported by the finding that it is produced in hepatoblastoma cells (Yasuda *et al*., 1990) and hepatocellular cancer (Ohmachi *et al*., 1993; Jönsson *et al*., 1996). Furthermore, its synthesis by hepatoblastoma cells is stimulated by IL-6 (Yasuda *et al*., 1990) which is a major proinflammatory cytokine induced by TNF-α and IL-1 (Roberts *et al*., 1995). An IL-6 responsive element (IL6RE) has been identified in the PSTI/TATI gene (Yasuda *et al*., 1993). The role as well as the origin of PSTI/TATI in acute phase reactions is presently unknown. Whereas PSTI/TATI expression in the pancreas and tumors seems to be associated with production of trypsin, a proteinase reacting with PSTI/TATI has not yet been identified in inflammatory cells (Stenman *et al*., 1991). However, it has recently been shown that trypsin stimulates TNF-α and IL-1 production by rat peritoneal macrophages (Lundberg *et al*., 2000) which may indicate a link between trypsin and PSTI in the acute phase response.

3.5 Clinical aspects

The serum and urine levels of TATI are elevated in patients with various types of cancer (reviewed by Stenman *et al.* 1993), *e. g.*, in patients with advanced gynecological (Huhtala *et al*., 1982; Stenman *et al*., 1982; Halila *et al*., 1988; Mogensen *et al*., 1990), pancreatic (Matsuda *et al*., 1983; Haglund *et al*., 1986), and renal cancer (Meria *et al*., 1995). The mechanisms causing the elevation of TATI in cancer patients may be production of TATI by the tumor or reaction against tissue destruction caused by invasive cancer (Stenman *et al*., 1991). As a tumor marker TATI is most useful in the management of mucinous ovarian cancer (Halila *et al*., 1988) in which it complements CA 125 whereas in patients with non-mucinous ovarian cancer it does not improve sensitivity (Mogensen *et al*., 1990). High serum levels of TATI have been observed in patients with stage I mucinous ovarian cancer (Halila *et al*., 1988) who generally have a good prognosis (Vergote *et al*., 1993). TATI has been shown to be an independent prognostic marker in advanced non-mucinous epithelial ovarian cancer (Venesmaa *et al*., 1994; Venesmaa *et al*., 1998) and in bladder cancer (Kelloniemi *et al.*, unpublished).

Aims of the study

The aim of this work was to study the expression of trypsinogens and PSTI/TATI in normal and malignant urogenital tissues and to elucidate their role in the proteolytic activity of tumors. The specific aims listed below were formed during the course of the study.

1) It has been shown that serum TATI levels are increased in patients with renal cell cancer but the explanation for this phenomenon and its usefulness in the monitoring of the disease is lacking. Therefore we studied whether PSTI/TATI is expressed in normal and malignant renal tissue and in renal cancer cell lines (I) and analysed the value of TATI as a prognostic factor in renal cell carcinoma (II).

2) Little is known about the mechanisms which regulate the expression of trypsinogen. Therefore we studied the effect of chemically modified tetracyclines and doxycycline, which are inhibitors of MMP expression, on trypsinogen expression in cancer cells (III).

3) Human trypsin-2 activates proMMP-2 and proMMP-9 *in vitro*. To elucidate the role of trypsins in the activation of proMMPs *in vivo*, we studied the association of trypsinogen-1, trypsinogen-2, their API complexes, and TATI with activation of proMMP-2 and proMMP-9 in ovarian tumor cyst fluids (IV).

4) Human seminal plasma contains several proteinases, *e. g.*, prostate-specific antigen (PSA) and human kallikrein-2 (hK2) which regulate sperm motility. The physiological activators of proPSA are not known. Because bovine trypsin activates proPSA *in vitro*, we studied whether trypsinogens occur in human seminal fluid and are expressed in the male genital tract (V).

Materials and methods

1. SAMPLES

The use of samples of human tissues and body fluids for this study was approved by the ethical committees of Helsinki University Central Hospital, Finland and University Hospital in Umeå and Malmö University Hospital, Sweden.

Serum of patients with renal cell carcinoma (I, II) were obtained from the serum bank of the Department of Urology and Andrology, University Hospital in Umeå (Table I). Control sera were obtained from healthy laboratory personnel from Helsinki University Central Hospital. Specimens of normal and malignant renal tissue and pancreatic tissue (I) were obtained from the Department of Pathology, the Haartman Institute, Helsinki University Central Hospital and the Department of Urology and Andrology, University Hospital in Umeå. Ovarian adenocarcinoma tissues were obtained from the Department of Obstetrics and Gynecology, Helsinki University Central Hospital (IV). Tissue specimens of human prostate, seminal vesicles, ductus (vas) deferens, epididymis, testis, and pancreas (V) were obtained from the Department of Surgery, Malmö University Hospital. Ovarian tumor cyst fluid samples (IV) were obtained from the Department of Obstetrics and Gynecology, Helsinki University Central Hospital. Seminal plasma samples (V) were obtained from the Department of Obstetrics and Gynecology, Helsinki University Central Hospital. Urine from patients with pancreatitis used for purification of trypsinogen and PSTI/TATI (III, V) were obtained from the Department of Surgery, Helsinki University Central Hospital.

2. SPECIAL REAGENTS

The antimicrobial tetracycline, doxycycline, and the chemically modified tetracyclines lacking antimicrobial activity, CMT-1, CMT-3, CMT-5 and CMT-8, were provided by Collagenex Pharmaceuticals, Inc., Newtown, PA, USA. Chromogenic peptide substrates N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide (S-2222) and 3-carbomethoxy-propionyl-L-arginyl-L-prolyl-L-tyrosine-p-nitroanilide (S-2586) were purchased from Kabi-Vitrum, Stockholm, Sweden. Bovine enterokinase (E. C. 3.4.21.9) was purchased from Boehringer-Mannheim, Mannheim, Germany. Antibodies used are listed in Table II.

3.METHODS

The methods used are listed in Table III and briefly described below. Details can be found in the original publications (I-V).

3.1 Purification of trypsinogen, PSTI/TATI, and PSA

Trypsinogen-1, trypsinogen-2 and PSTI/TATI were purified from urine of pancreatitis patient and from seminal fluid by affinity chromatography using monoclonal anti-trypsinogen-1 (3E8), anti-trypsinogen-2 (14F10 and 14D4) and anti-PSTI/TATI

Table I. Samples of patients and controls.

Sample	Subject	n	Used in
Serum samples	RCC patients	21	I
	RCC patients	188	II
	Healthy volunteers	84	II
Tissue samples			
Normal renal tissue	RCC patients	20	I
RCC tissue	RCC patients	32	I
Normal pancreatic tissue	Patients with pancreatic cancer	2	I, V
OVCA tissue	Patients with mucinous OVCA	10	IV
	Patients with serous OVCA	10	IV
Normal tissues of the male genital tract			
Testis	Patients with CAP	6	V
Epididymis	Patients with CAP	6	V
Ductus (vas) deferens	Patients with CAP	6	V
Ampulla of ductus (vas) deferens	Patients with CAP	6	V
Prostate	Patients with CAP, CAB or BPH	8	V
Seminal vesicles	Patients with CAP or CAB	8	V
Ejaculatory duct	Patients with CAP	6	V
Prostatic utricle	Patients with CAP	6	V
Urethra	Patients with CAP	6	V
Cyst fluids			
Cyst fluid of ovarian tumor	Patients with malignant ovarian tumor	20	IV
	Patients with benign ovarian tumor	41	IV
Seminal plasma	Men participating in an IVF program	24	V

Abbreviations: RCC, renal cell carcinoma; OVCA, ovarian carcinoma; IVF, *in vitro* fertilization; CAP, prostate cancer; CAB, cancer of urinary bladder; BPH, benign prostatic hyperplasia.

(6E8) columns. Further fractionation was performed by ion exchange (trypsinogens) and reverse phase (PSTI/TATI) chromatography. ProPSA was purified from LNCap cell medium (Corey *et al*., 1998) and mature PSA from seminal fluid as described (Zhang *et al*., 1995).

3.2 Cell culture

All cells were obtained from American Type Culture Collection (Rockville, MD, USA) (Table IV) and were grown at 37 °C in a humified atmosphere with 5% CO_2. Human renal cancer cell lines A-498 and ACHN were maintained in Eagle's Minimum Essential Medium supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 90% Earle's BSS, 10 % fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Renal cancer cell lines 786-O, A 704, Hs 126.T, Hs 227.T, 796-P, the colon adenocarcinoma cell line COLO 205, and the prostatic adenocarcinoma cell line LNCap were maintained in

Table II. Antibodies used.

Antibody	Description	Source/reference	Used in
Immunohistochemistry			
EC8	MAb anti-TATI, IgG	Osman *et al.,* 1993	I, IV
7401	PAb anti-try-1, sheep serum	Borgström and Ohlsson, 1976	V
3E8	MAb anti-try-1, IgG	Itkonen *et al.,* 1990	V
6D11	MAb anti-try-1, IgG	Itkonen *et al.,* 1990	V
Fahat	PAb anti-try-2, sheep serum	Kimland *et al.,* 1989	V
8336	PAb anti-try-2, rabbit serum	Kimland *et al.,* 1989	V
8F7	MAb anti-try-2, IgG	Itkonen *et al.,* 1990	IV, V
14D4	MAb anti-try-2, IgG	Itkonen *et al.,* 1990	V
14F10	MAb anti-try-2, IgG	Itkonen *et al.,* 1990	V
MAB 1482	MAb anti-try, IgG	Chemicon international, Temecula, CA, USA	V
MMP-2	PAb anti-MMP-2, IgG	Turpeenniemi-Hujanen *et al.,* 1992	IV
MMP-9	PAb anti-MMP-9, IgG	Kjeldsen *et al.,* 1993	IV
Immunofluorometric assays			
11B3 (Eu) & 6E8	TATI	Osman *et al.,* 1993	I, II, IV
6D11 (Eu) & 3E8	Trypsinogen-1	Itkonen *et al.,* 1990	IV, V
14F10 (Eu) & 14D4	Trypsinogen-2	Itkonen *et al.,* 1990	IV, V
PAb anti-API (Eu) & 3E8	Trypsin-1-API	Hedström *et al.,* 1999	IV
PAb anti-API (Eu)& 14F10	Trypsin-2-API	Hedström *et al.,* 1994	IV
MAb anti-ACT (Eu) & H117	PSA-ACT	Leinonen *et al.,* 1996	V
Immunoaffinity chromatography			
3E8	MAb anti-try-1, IgG	Itkonen *et al.,* 1990	V
14F10	MAb anti-try-2, IgG	Itkonen *et al.,* 1990	V
14D4	MAb anti-try-2, IgG	Itkonen *et al.,* 1990	V
6E8	Mab anti-PSTI/TATI, IgG	Osman *et al.,* 1993	III, V
6C11	MAb anti-PSA, IgG	Leinonen *et al.,* unpublished	V
Western blotting			
Rabbit 13	PAb anti-try, rabbit serum	Koivunen *et al.,* 1989	IV, V
14F10	MAb anti-try-2, IgG	Itkonen *et al.,* 1990	III
MMP-2	PAb anti-MMP-2, IgG	Turpeenniemi-Hujanen *et al.,* 1992	IV
MMP-9	PAb anti-MMP-9, IgG	Kjeldsen *et al.,* 1993	III, IV

Abbreviations: MAb, monoclonal antibody; PAb, polyclonal antibody; TATI, tumor-associated trypsin inhibitor; try, trypsinogen; MMP, matrix metalloproteinase; API, α_1-proteinase inhibitor; PSA, prostate-specific antigen; ACT, α_1-antichymotrypsin; IgG, purified IgG fraction; Eu, europium-labelled Ab

RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. LNCap medium additionally contained 0.1 mM non-essential amino acids and 5 mM testosterone.

3.2.1 Cell growth assay

Proliferation of COLO 205 cells was determined using the MTT Based Cell Growth Determination Kit (Sigma Biosciences, St. Louis, MO, USA) according to the manufacturer´s instructions with minor

Table III. Methods used in this study.

Method	Used in
Protein purification	III, V
Cell culture	I, III, V
Cell growth assay	III
In vitro cell migration assay	III
RNA and DNA methods	
RNA isolation	I, III, V
RT-PCR	I, III, V
Cloning	I, V
Sequencing of DNA	III, V
In situ hybridization	I, V
Northern blotting	V
Southern blotting	I, III
Immunological methods	
Time-resolved immuno-fluorometric assay (IFMA)	I, II, III, IV, V
Immunohistochemistry	I, IV, V
Western blotting	III, IV, V
Zymography	III, IV
Measurement of enzyme activity	III, V
Statistical analyses	II, III, IV

modifications. Briefly, cells were removed in PBS by a cell scraper and MTT solution was added. The dye metabolized by the cells was dissolved and the absorbance was read at 564-690 nm on a microplate reader (Labsystems Multiskan R Bichromatic, Labsystems, Helsinki, Finland).

3.2.2 In vitro cell migration assay

The *in vitro* migration of COLO 205 cells was measured using Transwell migration chambers (Corning Costar Corporation, Cambridge, MA, USA) coated with 10 μg/ml laminin from human placenta (Sigma). COLO 205 cells were seeded in the complete serum-containing medium in the presence or absence of enterokinase, TATI, CMTs, or DOXY into the upper wells of the chamber. After culture for 24 h, the number of cells that had migrated to the bottom of the lower wells were counted microscopically.

3.3 RNA and DNA methods

3.3.1 RNA isolation

Total RNA was extracted from exponentially growing cultured cells and from fresh tissue specimens according to the method of Chomczynski and Sacchi (1987) using Heavy Phase Lock Gel tubes (5 Prime-> 3 Prime, Inc., Boulder, CO, USA).

3.3.2 RT-PCR

One μg of total RNA was transcribed into cDNA using SuperScript™ II –reverse tran-

Table IV. Cell lines used.

Cell line	Description	Source	Used in
A-498	Renal cancer, epithelial	ATCC	I
ACHN	Renal cancer, epithelial	ATCC	I
786-O	Renal cancer, epithelial	ATCC	I
A 704	Renal cancer, epithelial	ATCC	I
Hs 126.T	Renal cancer, epithelial	ATCC	I
Hs 227.T	Renal cancer, fibroblast	ATCC	I
796-P	Renal cancer, fibroblast	ATCC	I
COLO 205	Colon adenocarcinoma	ATCC	III
LNCap	Prostate adenocarcinoma	ATCC	V

Abbreviation: ATCC, American Type Culture Collection

scriptase (Gibco BRL, Paisley, Scotland) according to the manufacturer's instructions. Contamination of RNA samples with cDNA was excluded with control reactions without RT. One µl of reverse transcription product was amplified in a 40-µl reaction volume in 1 X PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100; Finnzymes, Espoo, Finland), 0.25 mM of each dNTP, 20 pmol of antisense and sense primers, and 1.6 U of Dynazyme DNA polymerase (Finnzymes). The primers and amplification conditions for PSTI/TATI are found in publication I and those for trypsinogens in publications III and V. The RT-PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. The intensities of the PCR bands were estimated with a UMAX Powerlook II digital scanner (UMAX Technologies, Inc., Fremont, CA, USA) using the NIH Image program for Macintosh (III).

3.3.3 Cloning of RT-PCR products

PSTI/TATI and trypsinogen cDNAs prepared by RT-PCR from COLO 205 total RNA were cloned using the TA Cloning Kit (Invitrogen, San Diego, CA, USA) to generate templates for *in situ* hybridization riboprobe synthesis.

3.3.4 DNA sequencing

The identity of RT-PCR products and the sequence of the templates used for *in situ* hybridization riboprobe synthesis was confirmed by sequencing using an ABI Prism™ Dye Terminator Cycle Sequencing Core Kit with AmpliTaq DNA Polymerase and the ABI Prism 310 Genetic Analyser (PE Biosystems, Foster City, CA, USA).

3.3.5 In situ hybridzation

In vitro transcriptions of digoxigenin-labeled sense and antisense probes for PSTI/TATI (I) were done using the DIG RNA-labeling kit (Boehringer-Mannheim) and a 243-bp long PSTI/TATI cDNA fragment as a template. Fluorescein-labeled trypsinogen probes (V) were prepared by fluorescein-UTP riboprobe synthesis using the RNA color kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and a 627-bp long trypsinogen-2 cDNA fragment as a template. *In situ* hybridization was performed on 4- or 5-µm tissue sections which were deparaffinized, treated with proteinase K and fixed in 4% paraformaldehyde. Sections were covered with hybridization buffer containing 200 or 250 ng/ml antisense or sense probe. After overnight incubation at 42 °C or 55 °C in a humified chamber, the slides were washed at stringent conditions. The digoxigenin-labeled probe for PSTI/TATI was detected using anti-digoxigenin-alkaline phosphatase Fab fragments (Boehringer-Mannheim) and Fast Red tablets. Fluorescein-labeled probe for trypsinogen was detected using anti-fluorescein-alkaline phosphatase conjugate (Amersham Pharmacia Biotech), nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Pancreatic tissue was used as a positive control in each experiment.

3.3.6 Northern blotting

Nylon membrane blotted with 2 µg of poly A RNA (Clontech Laboratories, Inc., Palo Alto, CA, USA) was hybridized with a 627-bp long ^{32}P-labeled trypsinogen-2 cDNA. The ^{32}P-cDNA-mRNA hybrids were visualized by autoradiography. A glyceraldehyde-3-posphate dehydrogenase (GAPDH) cDNA probe was used to quantify the amount and control the quality of loaded poly A RNA.

3.3.7 Southern blotting

Aliquots of the RT-PCR products were separated in 1.5% agarose gel, stained with ethidium bromide and blotted onto nylon membranes. The membranes were hybrid-

ized with 20-bp (PSTI/TATI) and 25-bp (trypsinogen-2) long ^{32}P-labeled cDNA probes. The ^{32}P-cDNA-cDNA hybrids were visualized by autoradiography.

3.4 Immunological methods

3.4.1 Time-resolved immunofluorometric assay (IFMA)

Immunofluorometric assays for TATI (Osman *et al.*, 1993), trypsinogen-1 (Itkonen *et al.*, 1990), trypsinogen-2 (Itkonen *et al.*, 1990), trypsin-1-API (Hedström *et al.*, 1999), trypsin-2-API (Hedström *et al.*, 1994), and PSA-ACT (Leinonen *et al.*, 1996) were based on monoclonal catcher antibodies and europium (Eu) labeled monoclonal or polyclonal tracer antibodies as shown in Table II. The assays were performed in microtitration plates in two steps. Twenty-five µl of sample and 200 µl of assay buffer were pipetted into the wells. After incubation, the wells were emptied, washed, and filled with 200 µl of assay buffer containing 50 ng of Eu-labeled antibody. After incubation and washing, 200 µl of enhancement solution was added and the fluorescence was measured in a 1234 DELFIA research fluorometer (Wallac, Turku, Finland). When necessary, an increase in detection sensitivity was achieved by using a sample volume of 200 µl or by concentrating the samples with Centricon-3 concentrators (Amicon Inc., Beverly, MA, USA). The concentration of PSA was determined by the Delfia EQM PSA kit (Wallac, Turku, Finland) which recognizes free PSA and total PSA equally.

3.4.2 Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded tissues sections using immunoperoxidase staining with the Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) for TATI, trypsinogen-2, MMP-2, and MMP-9 (I, IV) and DAKO ChemMate™ Detection Kit and a staining machine (DAKO TechMate™ 500/1000, BioTek Solutions, USA) for trypsinogen-1 and –2 (V). The primary antibodies used are listed in Table II. The tissue sections were deparaffinized and rehydrated after which they were treated with 0.5% hydrogen peroxide to quench endogenous peroxidase activity. To enhance antigen retrieval, the tissue sections were pretreated either with pepsin, trypsin or with microwave heat depending on the antibody. Non-specific staining was blocked by treatment with normal serum. The sections were first incubated with primary and after washing with secondary antibodies. Bound antigen-antibody complexes were visualised by the avidin-biotin complex immunoperoxidase technique (ABC) (Elite ABC Kit) (I, IV) or with peroxidase/3-amino-9-ethylcarbazole reagent of the ChemMate kit (V). For each tissue section, a negative control was stained by replacing the monoclonal and polyclonal primary antibodies with non-immune mouse or rabbit IgGs, respectively. Pancreatic tissue was used as a positive control.

3.4.3 Western blotting

Cell culture medium, cyst fluid, or purified protein samples were separated by SDS-polyacrylamide gel electrophoresis on 7.5% or 3-16% gels under non-reducing or reducing conditions and transferred to nitrocellulose filters electrophoretically. The filters were incubated with polyclonal or monoclonal antibodies against trypsinogen, MMP-2, or MMP-9 as listed in Table II. The antigen-antibody complexes were visualized with peroxidase-conjugated secondary antibodies and diaminobenzidine tetrahydrochloride.

3.5 Zymography

Cell culture medium or cyst fluid samples were run under non-reducing conditions on

7.5-10% gradient SDS-polyacrylamide gels impregnated with 1 mg/ml gelatin. After washing, the gels were incubated in 50 mM Tris-HCl, pH 7.8, containing 150 mM NaCl, 1 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% NaN_3 for 48 h at 37 °C and stained with Coomassie Brilliant Blue. The total MMP content and the proportions of proforms and active forms of MMP-2 and MMP-9 were estimated with the Bio-Rad Model GS-700 Imaging Densitometer using the Molecular AnalystTM/PC program.

3.6 Measurement of enzyme activity

3.6.1 Measurement of trypsin-2 activity

The activity of trypsin-2 preincubated in the presence or absence of CMTs, DOXY or TATI was determined by using β-casein as a substrate essentially as described (Sorsa *et al.*, 1997) or by using a chromogenic peptide S-2222 as a substrate and by measuring the absorbance at 405 nm on a microplate reader (III). The activity of trypsinogen fractions purified by immunoaffinity and anion exchange chromatography from seminal fluid were determined using S-2222 as a substrate (V).

3.6.2 Measurement of proPSA activation

Activation of proPSA purified from LNCap cell medium by trypsin-1 and trypsin-2 purified from seminal fluid was measured by analysing the formation of PSA-α_1-antichymotrypsin complex (PSA-ACT) using PSA and PSA-ACT immunoassays (V).

Activation of recombinant proPSA (kindly provided by Dr. Janita Lövgren, University of Turku, Finland) by pancreatic trypsin-2 was studied using S-2586 as a substrate and monitoring the absorbance at 405 nm on a microplate reader.

3.7 Statistical analyses

The Mann-Whitney U test was used to compare the differences between groups (II, III, IV). The survival curves were constructed using the Kaplan-Meier method, and comparison of survival times was performed with the log-rank test (II). The independence of various factors as predictors of survival was analysed by the stratified log-rank test (II). All tests were two-sided and the significance level was set to a p-value ≤ 0.05.

Results and discussion

1. EXPRESSION AND PROGNOSTIC VALUE OF TATI IN RENAL CELL CARCINOMA (I, II)

Renal cell carcinoma (RCC), which originates from the epitelium of the renal parenchyma, is the third most common genitourinary tumor, accounting for more than 2% of cancer incidence in the United States and an estimated 12 000 deaths in 1998 (Tsui *et al*., 2000). There are currently no clinically useful serum markers for this disease. According to several studies, the serum levels of TATI are elevated in patients with RCC and the clinical sensitivity of TATI has been found to be between 37% and 69% (Larbre *et al*., 1990; Taccone *et al*., 1991; Meria *et al*., 1995). The specificity of TATI is rather low (65%) rendering it unsuitable for primary diagnostics, but Meria *et al.* (1995) suggested that it may be useful for post-operative follow-up. It has been unclear whether the increase of serum TATI in patients with RCC is caused by an acute phase reaction frequently associated with cancer, impaired renal function, or by production of TATI by malignant and benign renal tissue. To answer this question, we studied the expression of TATI in histologically normal and malignant renal tissue and in seven RCC cell lines by immunohistochemistry, RT-PCR, *in situ* hybridization, and IFMA. TATI concentrations were also determined in serum of 21 RCC patients by IFMA (I). By immunohistochemistry, TATI was detected in the histologically normal renal tissue in which it localized to the distal convoluted tubules whereas the glomeruli and proximal tubules were negative. TATI was not detected in RCC tissue by immunohistochemistry, but by RT-PCR TATI transcripts were detected both in normal and malignant renal tissue. However, TATI mRNA was not detectable by *in situ* hybridization in either tissue under conditions in which PSTI/TATI mRNA was demonstrated in the acini of the normal pancreas. RT-PCR and IFMA showed that TATI mRNA and protein were expressed also by RCC cell lines (Table V). The serum concentrations of TATI were elevated in 57% of all RCC patients. Only one patient had a clearly elevated serum creatinine indicating impared renal function, which could explain the TATI elevation in that patient.

Expression of PSTI/TATI in the kidney has earlier been studied by Fukayama *et al.* (1986) and by Marchbank *et al.* (1996), who detected PSTI/TATI mRNA and protein expression in the transitional epithelium of the renal pelvis. In the study of Fukyama *et al.* PSTI/TATI immunoreactivity was also

Table V. Expression of TATI protein and mRNA in renal cancer cell lines.

Cell line	Morphology	TATI (ng/l)	TATI RT-PCR
A-498	Epithelial	7.7	+
ACHN	Epithelial	ND	+
786-O	Epithelial	10.0	+
A 704	Epithelial	1.8	+
769-P	Fibroblast	1.5	+
Hs 126.T	Epithelial	-	-
Hs 227.T	Fibroblast	-	+

Abbreviation: ND, not determined

detected in the collecting ducts of the fetal kidney. However, the staining in the fetal collecting ducts decreased with increasing gestational age, until no staining was observed in the adult kidney. The authors suggested that this phenomenon might reflect differentiation of the collecting ducts. Marchbank *et al*. detected PSTI/TATI peptide, but not mRNA in the collecting ducts of adult kidney suggesting renal reabsorption of the filtered peptide by the tubules. This observation differs from our findings. We detected TATI immunoreactivity in the distal tubules which do not participate in protein reabsorption (I). Because TATI mRNA also was detected by RT-PCR, this suggests that TATI is expressed by tubular cells. A rapid turnover of TATI mRNA may explain why we failed to detect TATI transcripts in renal tissue by *in situ* hybridization. The negative result of the immunohistochemical staining of malignant renal tissue may, on the other hand, be caused by rapid leakage or secretion of TATI, probably produced at low levels, into circulation.

In RCC, the extent of tumor spread (stage) and nuclear grade are considered to be the main prognostic factors (Fuhrman *et al*., 1982; Medeiros *et al*., 1988). However, in subsets of patients within a given disease stage, there are large variations in the clinical course and survival time. Therefore, there is a need for prognostic factors that could identify patients with an aggressive disease. Although there are no specific serum markers for RCC, some general markers, *e. g.*, CA-125 (Grankvist *et al*., 1997), tumor necrosis factor-α (Dosquet *et al*., 1997), interleukin-10 (Wittke *et al*., 1999), and neuron-specific enolase (Rasmuson *et al*., 1999) provide some prognostic information.

We studied the prognostic value of preoperative serum TATI in relation to stage and grade in 158 patients with RCC and a normal serum creatinine (II). Elevated preoperative serum TATI levels (cut-off 16 μg/l) were detected in 48% of the RCC patients (in 39% of those with stage I or II and in 56% of those with stage III or IV tumors) (Table VI). The concentration of TATI in cancer patients (median 15 μg/l) was significantly higher than in controls (median 11 μg/l) ($p < 0.0001$). Serum TATI correlated with clinical stage and nuclear grade (Table VI). In univariate analysis, survival was strongly associated with clinical stage and nuclear grade ($p < 0.0001$), and patients with elevated TATI levels had significantly shorter survival time than those with normal TATI levels ($p = 0.005$) (Fig-

Table VI. Serum TATI concentration in relation to grade and stage of the tumor.

			TATI (μg/l)		
	Cases	**Elevated (%)**	**Range**	**Median**	**Inter-quartile range**
Stage					
I, II	70	27 (39%)	6.2-493	13.8*	11.1-21.0
III	41	22 (54%)	8.2-155	17.0	10.2-39.5
IV	47	27 (57%)	6.1-1140	22.4	12.2-38.1
Grade					
1, 2	40	15 (38%)	8.5-493	13.1**	9.9-20.4
3	80	37 (46%)	8.5-1440	14.1	11.1-24.5
4	38	24 (63%)	6.2-1140	23.2	11.7-67.2

*Mann-Whitney U test p = 0.5 and 0.01 versus III and IV, respectively.
**Mann-Whitney U test p = 0.06 and 0.003 versus III and IV, respectively.

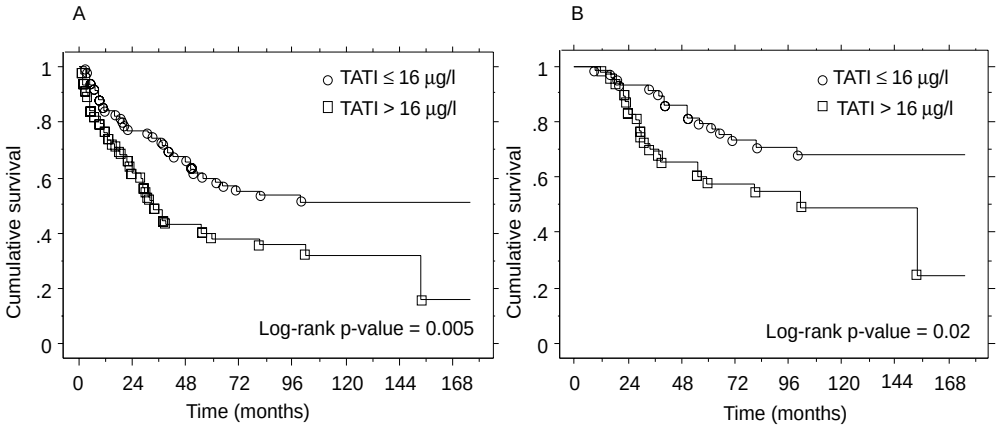

Figure 1. Kaplan-Meier cancer specific survival according to preperative TATI concentration in patients with RCC. A, all stages. B, stages I-III. Cut-off 16 µg/l.

Table VII. Univariate analysis of variables predicting survival by the log-rank test.

	All stages	Stages I-III	Stage IV
TATI cut-off (µg/l)			
16.0	0.005	0.02	0.18
30.0-50.0	< 0.0005	<0.006	<0.004
Grade	< 0.0001		
Stage	< 0.0001		

ure 1, Table VII). In a stratified log-rank test, TATI was a prognostic factor independent of stage ($p = 0.03$) and grade ($p \leq 0.04$) among all patients and also among those with non-metastatic disease.

TATI is cleared from the circulation by renal excretion and is partially reabsorbed by the renal tubules. Thus serum TATI increases in patients with markedly impaired renal function (Tramonti *et al*., 1996). Elevated serum concentrations of creatinine indicate impaired renal function, and patients with serum creatinine above 125 µmol/l were therefore excluded from the analysis. In the study of Meria *et al*. (1995), the sensitivity of TATI was 69%, which is higher than in our studies (57%, study I and 48%, study II). Exclusion of patients with elevated serum creatinine from our analysis may explain the difference.

It is seemingly paradoxical that a high concentration of TATI, an inhibitor of trypsin, is associated with poor prognosis. Trypsinogen mRNA and protein are expressed in the kidney (Koshikawa *et al*., 1998), and it is possible that, like in the pancreas, the role of TATI in some tumors is to inhibit intracellular trypsin activity and hence protect the tissue producing it.

2. ASSOCIATION OF TRYPSINOGEN EXPRESSION IN TUMOR CELLS WITH ACTIVATION OF PROMMP-2 AND -9 AND WITH CELL MIGRATION (III, IV)

Tetracyclines are broad-spectrum antibiotics which act by blocking bacterial protein synthesis. Golub *et al.* (1983) found that they are also anti-collagenolytic, an effect which is unrelated to their antibacterial properties and possibly of therapeutical importance *per se*. In order to prevent side effects caused by emergence of antibiotic-resistant micro-organisms, chemically modified tetracyclines (CMT) lacking antimicrobial activity have been developed (Golub *et al*., 1987). Both tetracyclines and

CMTs are capable of inhibiting MMP activity and expression (Golub *et al*., 1983; Golub *et al*., 1987; Uitto *et al*., 1994) and are therefore potential therapeutic agents for treatment of cancer. The inhibition of MMP activity has been suggested to be dependent on the ability of tetracyclines to chelate Ca^{2+} and Zn^{2+} ions (Golub *et al*., 1983). This is supported by the finding that CMT-5, which lacks the β-diketone moiety at carbon-11 and carbon-12 required for chelating of multivalent metal cations, does not inhibit MMP activity (Sorsa *et al*., 1998) (Figure 2). The mechanism by which tetracyclines and CMTs inhibit MMP expression (Uitto *et al*., 1994; Jonat *et al*., 1996; Hanemaaijer *et al*., 1998) is not clear. Tetracyclines inhibit protein kinase C activity (Webster *et al*., 1994) which plays a pivotal role in the transcriptional activation of certain MMP genes (Case *et al*., 1990; Takahashi *et al*., 1993). Protein kinase C is part of a signal pathway comprising to the AP-1 complex, which is supposed to be a transcription-stimulating factor binding to the MMP promoter (Angel *et al*., 1987).

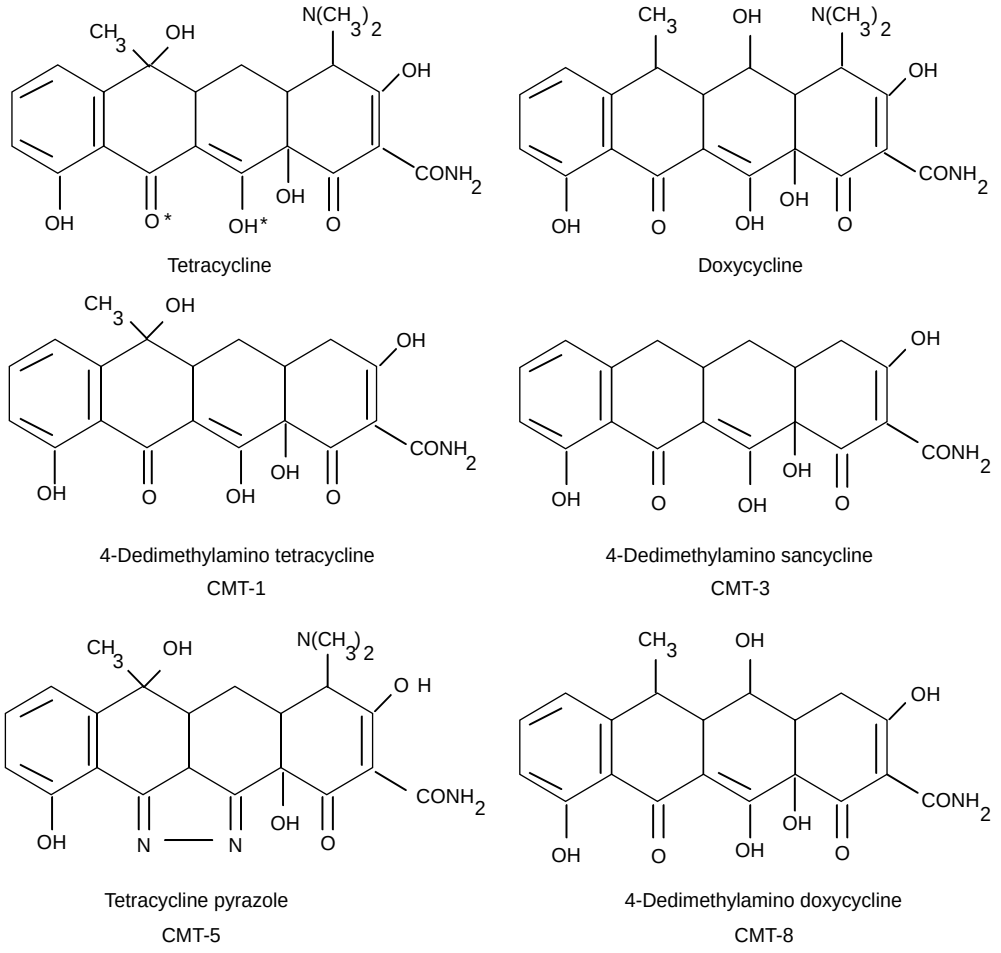

Figure 2. Chemical structures of tetracycline, doxycycline, and the chemically modified tetracyclines (CMTs). The carbonyl group at C_{11} and the OH group at C_{12} which form the Ca^{++} and Zn^{++} binding site in the molecules excluding CMT-5 are marked with * in tetracycline.

However, Jonat *et al.* (1996) suggested that the transcriptional inhibition of stromelysin by tetracycline is not due to a blocking of the activity of AP-1 but is mediated by yet unidentified sequences upstream of the AP-1 binding site in the stromelysin gene.

We aimed at investigating whether the synthetic tetracycline doxycycline and CMTs affect trypsinogen-2 expression and activity (III). We found that DOXY and several CMTs (CMT-1, -3, 5, and -8) downregulated the expression of trypsinogen-2 in COLO 205 colon adenocarcinoma cells by 20-70% at non-cytotoxic concentrations (0.1 to 1 μM) (Figure 3). The mechanism causing the inhibition of trypsinogen-2 expression remains to be elucidated. Trypsin-2 activity was not affected by doxycycline or CMTs. This is in line with the fact that the catalytic activity of trypsin is not dependent of divalent cations. Although Ca^{2+} is essential in stabilazing trypsin and the absence of Ca^{2+} causes rapid autolysis of especially trypsin-2 (Colomb *et al.*, 1978), it is likely that the CMT and DOXY concentrations used in the inhibition experiments (50 μM) were not high enough to cause Ca^{2+} deprivation and subsequent autodegradation and loss of activity of trypsin-2.

The role of trypsin in cancer cell migration was studied using DOXY and CMTs together with enterokinase and TATI. By zymography we first found that addition of enterokinase to the cell culture medium enhanced proMMP-9 activation. This effect was inhibited by TATI and DOXY, but not by CMTs. Furthermore, DOXY and CMTs showed no detectable effect on MMP-9 expression which may be explained by the use of concentrations lower than those generally used to inhibit MMPs (Uitto *et al.*, 1994; Hanemaaijer *et al.*, 1997; Seftor *et al.*, 1998; Gu *et al.*, 2001). We also found that, when added to the cell culture, enterokinase enhanced migration of COLO 205 cells and TATI abolished this effect (Figure 4). DOXY was most effective in inhibiting the migration of enterokinase-treated cells, which could be explained by simultaneous

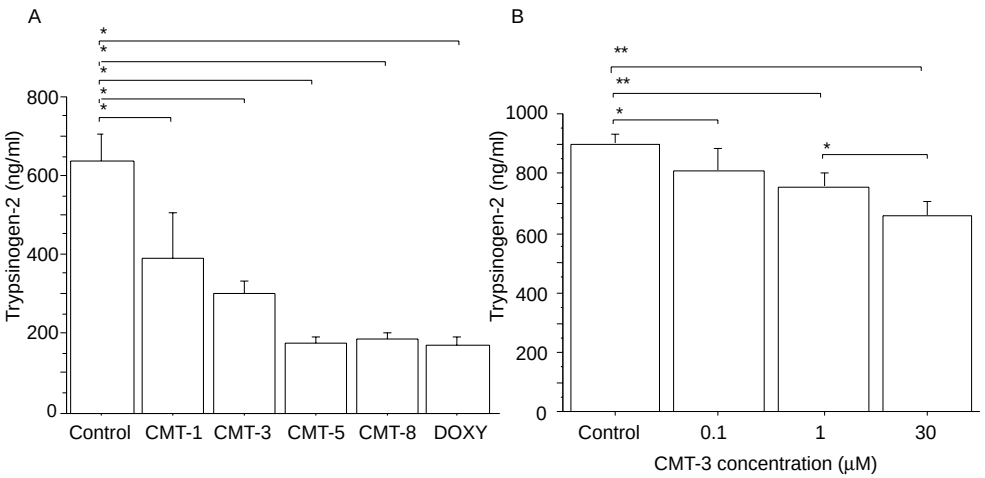

Figure 3. Effects of CMTs and DOXY on the release of trypsinogen-2 protein into culture medium of COLO 205 cells. A, Cells were cultured in the presence or absence of 1 μM CMT-1, CMT-3, CMT-5, CMT-8, or DOXY. B, Cells were cultured in the presence or absence of 0.1, 1.0, and 30 μM CMT-3. After 48 h, trypsinogen-2 immunoreactivity was measured by an immunofluorometric assay. Trypsinogen-2 concentrations were corrected for the cell number determined using a cell proliferation assay based on the activity of mitochondrial dehydrogenases. The results represent mean values and standard deviations from 4 (A) and 6 (B) parallel wells. * $p < 0.03$, ** $p < 0.005$, Mann-Whitney U test.

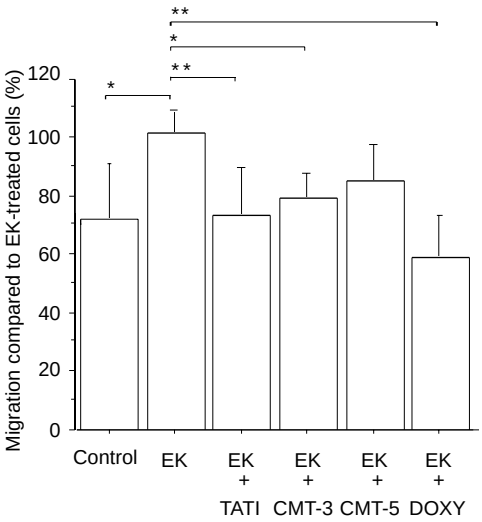

Figure 4. *In vitro* migration of COLO 205 cells treated with enterokinase (EK) through laminin-coated Transwell chambers over 24 h was normalized to 100% and compared with the migration of cells cultured in the presence or absence of 3 nM enterokinase and 1.5 μM TATI, 1 μM CMT-3, CMT-5, or DOXY. Cells having migrated on the bottom of the wells were counted. The results represent mean values and standard deviations from 6 parallel wells from 2 representative experiments. *p < 0.03, **p < 0.003, Mann-Whitney U test.

inhibition by DOXY of trypsinogen-2 expression and proMMP-9 activation. CMT-3 and CMT-5, which only affected trypsinogen-2 expression, but not enterokinase-induced proMMP-9 activation, inhibited migration of enterokinase-treated cells to a lesser extent than TATI and DOXY. Our results are in line with earlier studies showing that tetracyclines and CMTs inhibit migration of cancer cells (Fife and Sledge, 1995; Seftor *et al*., 1998; Lokeshwar, 1999) and they further show that part of this effect may be mediated by trypsin inhibition. The ability of TATI to inhibit COLO 205 cell migration induced by enterokinase further supports the notion that trypsin plays a role in cell migration. However, general conclusions can not be drawn on the basis of these results because only one cell line was studied.

At the concentrations used, up to 100 μM, CMTs and DOXY were not cytotoxic. On the contrary, culture of the cells in the presence of 1 μM DOXY or CMTs resulted in a 2- to 4-fold increase in cell number. CMT-3 and CMT-8 have been reported to decrease the proliferation of keratinocytes (Mäkelä *et al*., 1998) and CMT-3 and DOXY to cause death of cultured monocytes and macrophages but not fibroblasts possibly by an apoptotic mechanism (Bettany and Wolowacz, 1998). In the study of Seftor *et al.* (1998), on the other hand, CMT-3 and CMT-8 caused proliferation of human melanoma cells. The effect of CMTs and DOXY on cell proliferation may be specific to certain cell lines and dependent on the CMT concentrations used. The increased cell proliferation may also be caused by suppression of general protease activity (McKeehan *et al*., 1986; Freeman *et al*., 1990c). This is supported by our finding that TATI also enhanced cell proliferation whereas enterokinase decreased it (III).

Epithelial ovarian cancers arising from the germinal epithelium constitute 80-90% of all malignant ovarian tumors. Of these, serous cystadenocarcinomas constitute 60-70% and mucinous cystadenocarcinomas 10-20%. Trypsinogen (Hirahara *et al*., 1995; Hirahara *et al*., 1998), TATI (Huhtala *et al*., 1982; Ueda *et al*., 1989), MMP-2 and MMP-9 (Autio-Harmainen *et al*., 1993; Naylor *et al*., 1994) are expressed in ovarian cancer and they are found at high concentrations in tumor cyst fluids (Koivunen *et al*., 1990; Furuya *et al*., 2000). A recent study has shown that human trypsin-2 activates proMMP-2 and proMMP-9 *in vitro* (Sorsa *et al*., 1997). Therefore we wanted to elucidate the role of trypsin in the activation of proMMP-2 and -9 *in vivo* by comparing the concentrations of trypsinogen-1 and -2, their α_1-protease inhibitor (API) complexes, and TATI in ovarian tumor cyst fluids with the degree of activation of proMMP-2 and -9. We furthermore studied the immunohistochemical expression and localization of

trypsinogen-2, TATI, MMP-2, and MMP-9 in ovarian tumors. We found that the proportion of active MMP-9 was significantly greater in cyst fluids with high (≥ 87 μg/l) than in those with low trypsinogen-2 concentrations (p = 0.05). The proportion of active MMP-9 was also greater in cyst fluids with trypsin-1-API and trypsin-2-API concentrations higher than their median concentrations (≥ 12 and 42 μg/l, respectively) (p = 0.4 and 0.1, respectively). The differences were statistically significant at cut-off levels of 30 μg/l (trypsin-1-API) and 100 μg/l (trypsin-2-API) (p = 0.003 and 0.03, respectively) (Figure 5A). Contrary to MMP-9, the activation of MMP-2 was inversely associated with the concentrations of trypsinogen-1, trypsinogen-2, trypsin-2-API, and TATI. The proportion of active MMP-2 was smaller when trypsinogen-1 and trypsin-2-API concentrations exceeded their median concentrations (20 and 42 μg/l, respectively) (p = 0.02). Similarly, less active MMP-2 occured in cyst fluids with trypsinogen-2 and TATI concentrations above their median concentrations (87 and 25 μg/l, respectively) (p = 0.1 and 0.2, respectively). The results were statistically significant when cut-off concentrations of 50 μg/l (for trypsinogen-2) and 1000 μg/l (for TATI) were used (p = 0.01 and 0.04, respectively) (Figure 5B).

These results suggest that trypsin-1 and trypsin-2 are involved in the activation of proMMP-9 but not proMMP-2 *in vivo*. Trypsin-2 activates proMMP-2 to a 65 kDa species *in vitro* (Sorsa *et al*., 1997) but after prolonged incubation further fragmentation to a 40 kDa species occures. The N-terminal amino acid sequence of the 40 kDa product was the same as that of the 65 kDa form, indicating additional trypsin-2-mediated processing in the C-terminal region (Sorsa *et al*., 1997). It is possible that high concentrations of trypsin, a very efficient proteinase, may result in inactivation of MMP-

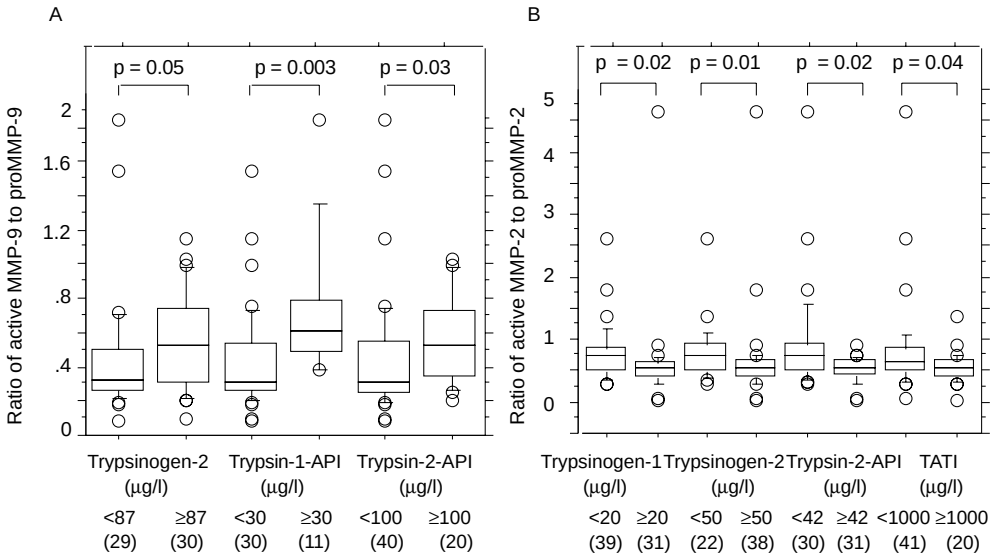

Figure 5. A, Ratio of active MMP-9 to proMMP-9 in ovarian tumor cyst fluids in relation to trypsinogen-2, trypsin-1-α_1-proteinase inhibitor (trypsin-1-API), and trypsin-2-API concentrations. B, Ratio of active MMP-2 to proMMP-2 in ovarian tumor cyst fluids in relation to trypsinogen-1, trypsinogen-2, trypsin-2-API, and TATI concentrations. The box denotes the 25th, 50th, and 75th percentiles while the whiskers represent the 10th and 90th percentiles. Values outside these limits are indicated by circles. The number of samples analysed is shown in parenthesis.

2 or its activator MMP-14 *in vivo*.

We found that the concentrations of trypsinogen-1 and trypsinogen-2 were significantly higher in malignant than in benign cyst fluids (Figure 6). This is in line with the results of Koivunen *et al.* (1990). In addition, the concentrations of trypsin-API complexes, which reflect the proportion of active trypsin, were significantly higher in malignant than in benign fluids. Interestingly, the molar ratio of trypsinogen to TATI was significantly higher in serous than in mucinous cyst fluids (Figure 7). The excess of protease in relation to its inhibitor may be related to the poorer prognosis of serous than of mucinous ovarian carcinomas (Vergote *et al*., 1993).

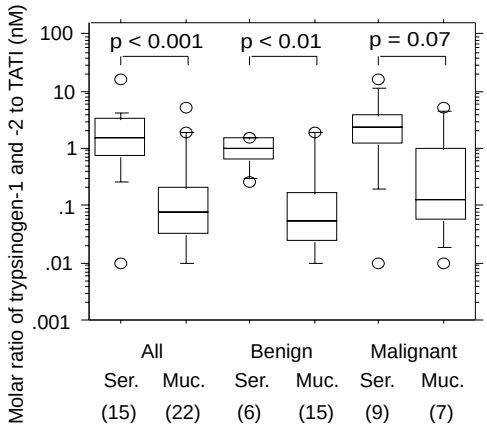

Figure 7. Molar ratio of trypsinogen-1 and -2 to TATI in cyst fluids of serous and mucinous ovarian tumors. The symbols are as in figure 5.

By immunohistochemistry, we detected trypsinogen-2 and TATI in the secretory epithelium of ovarian carcinomas as has been earlier described (Ueda *et al*., 1989; Hirahara *et al*., 1995; Hirahara *et al*., 1998). Trypsinogen-2 and TATI were detected more frequently in mucinous than in serous tumors and in agreement with this, the trypsinogen and TATI concentrations were significantly higher in mucinous than in serous cyst fluids. In ovarian carcinomas, MMP-2 mRNA expression has been detected mainly in stromal fibroblasts and endothelial cells but only rarely in epithelial cells (Autio-Harmainen *et al*., 1993; Naylor *et al*., 1994; Afzal *et al*., 1998) whereas immunoreactive MMP-2 has mainly been detected in the cytoplasm and at the surface of epithelial tumor cells (Autio-Harmainen *et al*., 1993; Höyhtyä *et*

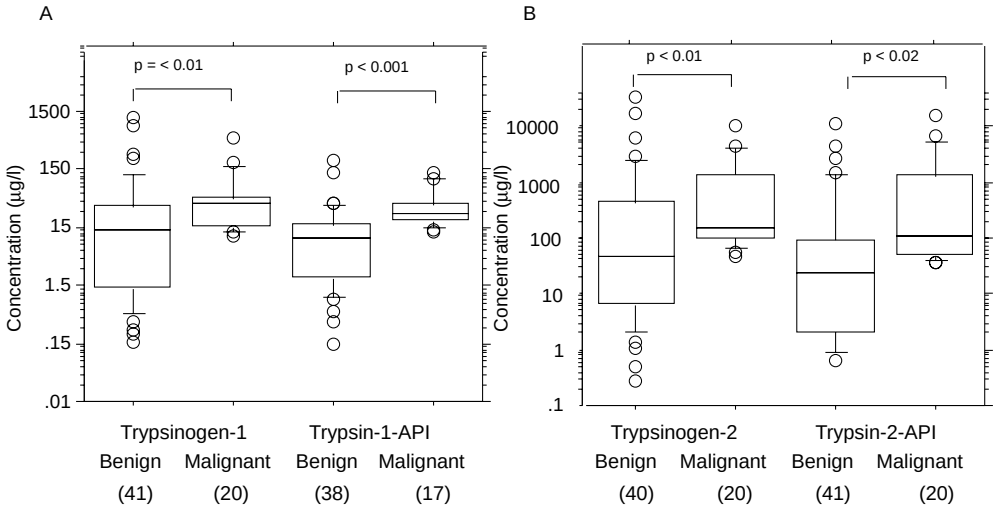

Figure 6. Box plot showing the concentrations of (A) trypsinogen-1 and trypsin-1-API and (B) trypsinogen-2 and trypsin-2-API in cyst fluids of malignant and benign ovarian tumors. The symbols are as in figure 5.

al., 1994; Afzal *et al*., 1996; De Nictolis *et al*., 1996). Several cultured ovarian carcinoma cells produce MMP-2 and MMP-9 (Moser *et al*., 1994; Fishman *et al*., 1997; Westerlund *et al*., 1997; Boyd and Balkwill, 1999). In agreement with earlier studies (Autio-Harmainen *et al*., 1993; Höyhtyä *et al*., 1994; Afzal *et al*., 1996; De Nictolis *et al*., 1996) we found by immunohistochemistry that MMP-2 localized to the vascular endothelial, epithelial, and stromal cells. Furthermore, in line with several studies suggesting stromal macrophages and tumor-infiltrating neutrophils as major sources of MMP-9 in invasive cancers, *e. g.*, in the colon (Pyke *et al*., 1993; Nielsen *et al*., 1996), bladder (Davies *et al*., 1993a), and breast cancer (Davies *et al*., 1993b) and ovarian carcinoma (Naylor *et al*., 1994), we detected MMP-9 immunoreactivity in neutrophils and monocyte-macrophage-like cells both in stromal and epithelial areas. This is in contrast to the findings of Huang *et al*. (2000), who recently demonstrated MMP-9 mRNA and protein both in stromal and neoplastic cells of ovarian carcinomas. MMP-9 expression has also been detected in squamous cell carcinoma cells of the lung (Canete-Soler *et al*., 1994), vulva (Johansson *et al*., 1999), and esophagus (Ohashi *et al*., 2000). The immunohistochemical findings support the notion that stromal cells, inflammatory cells, and cancer cells co-operatively produce and induce tissue-destructive serine proteinases and MMPs that facilitate tumor invasion and metastatic spread (Pyke *et al*., 1993).

3. EXPRESSION AND CHARACTERIZATION OF TRYPSINOGENS PRODUCED IN THE MALE GENITAL TRACT (V)

Human semen consists primarily of the secretions of the accessory genital glands including the prostate, seminal vesicles, and Cowper's glands. It contains several proteolytic enzymes such as human kallikrein-2 (hK2) (Deperthes *et al*., 1995) and prostate-specific antigen (PSA) (Wang *et al*., 1979). PSA is a chymotrypsin-like serine proteinase (Lilja, 1985), whereas hK2 has trypsin-like enzymatic activity (Clements, 1994). Recent studies have shown that a recombinant proform of PSA is activated by bovine trypsin and recombinant hK2 (Lövgren *et al*., 1997; Takayama *et al*., 1997), but the physiological activators of proPSA and prohK2 are not known. It is likely that they are activated by an enzyme with trypsin-like specificity, since the cleavage of the activation peptide occurs at the carboxyterminal side of an arginine (Lövgren *et al*., 1997). Therefore, it was of interest to find out whether trypsin is present in human seminal fluid.

By an immunofluorometric assay we found that trypsinogen-1 and -2 are present at high concentrations (medians 0.4 and 0.5 mg/l, respectively) in human seminal fluid. We purified both isoenzymes to homogeneity by immunoaffinity and anion exchange chromatography (Figure 8). The size of the purified trypsinogen isoenzymes was 25-28 kDa as indicated by SDS-PAGE and Western blotting. Most of the trypsinogen-1 purified from seminal fluid was enzymatically active whereas trypsinogen-2 occured as the proform, which could be activated by enterokinase *in vitro*. In addition, nicked forms of both isoenzymes were detected by Western blotting. These were enzymatically inactive and could be separated by ion exchange chromatography from trypsinogen and active trypsin.

To study the origin of trypsins found in seminal plasma, we performed immunohistochemical analysis of the various tissues of the male genital tract. Trypsinogen protein was detected in the human testis, epididymal glands, deferent duct, seminal vesicles, prostate, the ejaculatory duct, urethra, and prostatic utricle. Expression of trypsinogen mRNA in the same organs was further dem-

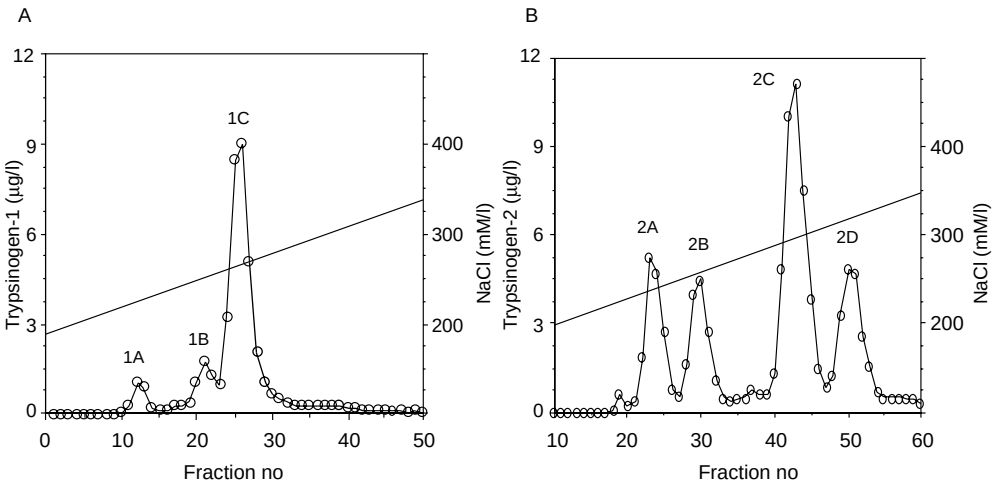

Figure 8. Anion exchange chromatography fractionation of trypsinogen-1 and -2 purified by immunoaffinity chromatography from seminal fluid. Trypsinogen-1 (A) and trypsinogen-2 (B) peaks were determined by immunofluorometric assays. The major trypsinogen-1 peak (1C) was enzymatically active whereas the two minor peaks were inactive and the activity was not induced by treatment with enterokinase. The four trypsinogen-2 peaks did not show enzymatic activity, but the major peak (2C) was activated after treatment with enterokinase.

onstrated by *in situ* hybridization. Trypsinogen mRNA was also detected in the prostate and seminal vesicles by RT-PCR and Northern blotting. Sequencing of the RT-PCR products revealed that trypsinogen-1 and -2 expressed in the prostate and trypsinogen-1 expressed in seminal vesicles were encoded by the same genes as the corresponding pancreatic ones.

To investigate the function of trypsin in the male genital tract, we studied whether it can activate the proform of PSA, and whether its activity is affected by Zn^{2+} ions present at high concentrations in the prostate and seminal plasma (Kavanagh, 1985). We found that trypsin isolated from seminal plasma activated proPSA isolated from LNCap cells (Figure 9A). This result was confirmed by activation experiments using human pancreatic trypsin-2 and recombinant proPSA (kindly provided by Dr. Janita Lövgren, University of Turku, Finland) (Figure 9B). Zn^{2+} inhibited trypsin-2 activity in a dose-dependent manner. However, the inhibition of trypsin was less efficient than that of hK2: 100 µM Zn^{2+} inhibited hK2 activity by 90% (Lövgren *et al.*, 1999) whereas trypsin activity was inhibited only by 18% (V).

This is the first study to show that trypsinogens occur at high concentrations in seminal plasma, and that they appear to be derived from the auxiliary sex glands as evidenced by immunostaining. *In situ* hybridization and RT-PCR analysis confirmed that this was due to local production rather than to uptake. The widespread distribution of trypsinogen in the male genital tract suggests that this proteinase may play a physiological role in reproduction. It is tempting to speculate that this function is related to activation of other proteinases like PSA and hK2 that are present in seminal plasma at high concentrations. The main function of PSA is thought to be the dissolution of the sperm-entrapping gel formed immediately after ejaculation by cleavage of the gel forming proteins semenogelin I and II (Lilja, 1985). It is likely that an enzyme with trypsin-like specificity activates proPSA and prohK2. Indeed, we found that human trypsin activates proPSA. HK2, another po-

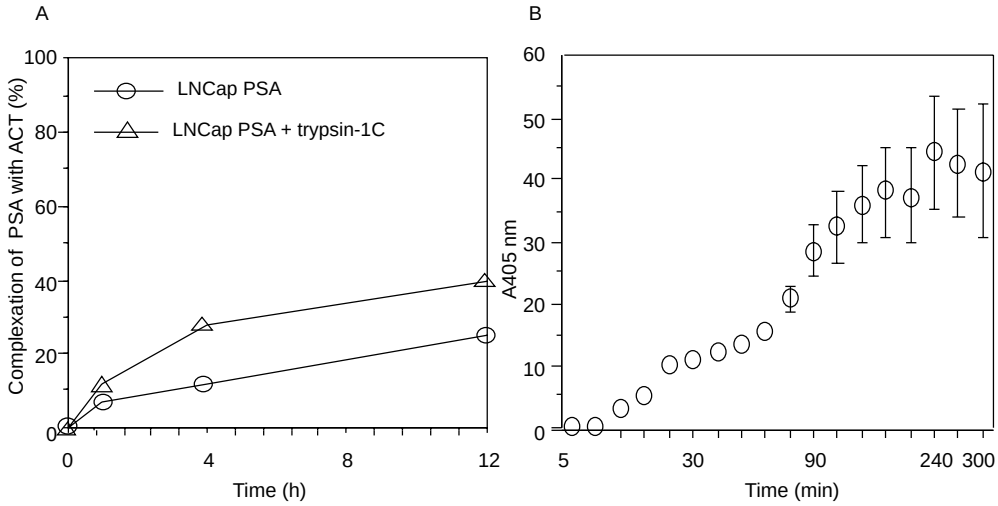

Figure 9. A, Activation of proform of prostate-specific antigen (PSA) produced by prostatic adenocarcinoma (LNCap) cells by trypsin-1 (1C) purified from seminal fluid. Activation of proPSA was monitored by analysing the complex formation between PSA and α_1-antichymotrypsin (ACT) by PSA-ACT immunoassay and recovery of PSA immunoreactivity. B, Activation of recombinant proPSA by trypsin-2 purified from urine of pancreatitis patient. Activation of proPSA was determined by measuring the cleavage of a chromogenic peptide substrate (S-2586) by reading the absorbance at 405 nm.

tential physiological activator of proPSA (Lövgren *et al.*, 1997), occurs in seminal plasma at an avarage concentration of 6 μg/ml, which is about six times higher than that of trypsin. However, Zn^{2+} inhibits more efficiently hK2 than trypsin activity. Therefore, trypsin may activate proPSA under circumstances where hK2 is inhibited.

Whereas trypsinogen is widely expressed in the various organs of the male genital tract, PSA and hK2 are expressed only in the prostate. In the prostate the distribution of trypsinogen is different from that of hK2 and PSA. Whereas the latter mainly are expressed in the secretory cells of the acini, trypsinogen is mainly found in the luminal cells of the prostatic excretory ducts. This suggests that trypsin and PSA get into contact only when they are admixed in the prostatic fluid during ejaculation. PSA is active also in patients with aplasia of the seminal vesicles and the deferent duct (Lilja, 1985). This suggests that trypsin produced in the prostate may be sufficient for initiation of the proteinase cascade leading to activation of both prohK2 and proPSA. On the other hand, partially cleaved isoforms of both PSA and hK2 occur in seminal plasma, and the cleavage sites that have been identified are typical of trypsin (Watt *et al.*, 1986; Christensson *et al.*, 1990; Zhang *et al.*, 1995; Deperthes *et al.*, 1995; Lövgren *et al.*, 1997; Lövgren *et al.*, 1999). Thus trypsin may contribute not only to the activation of prohK2 and proPSA, but also to their degradation in seminal plasma.

Adenocarcinoma of the prostate is the most commonly diagnosed invasive neoplasm among males in industrialised countries (Parkin *et al.*, 1997). Considering the expression of trypsinogen in other types of cancer, it will be important to investigate whether trypsinogen expression is retained in malignant prostatic epithelial cells. In preliminary studies we have found that prostate cancer tissue and prostate cancer cell lines LNCap, DU-145, and PC-3 express trypsinogen at the mRNA and protein level (unpublished data).

Summary and conclusions

Trypsinogen is the most abundant protein synthesized by the pancreas, reflecting its pivotal role in protein digestion and in the activation of other digestive proenzymes. Trypsinogen and its specific inhibitor PSTI/TATI have long been considered pancreas-specific but recent studies have demonstrated their broad distribution in human tissues which suggests that they possess several biological functions. Trypsinogen and PSTI/TATI have gained renewed attention by the identification of mutations in their genes which have been linked to hereditary and chronic pancreatitis. Several lines of evidence also suggest that trypsin may play a role in tumor invasion by directly degrading the ECM or by activating other tumor-associated proteinases, e. g., prouPA and proMMPs. A role of trypsin and PSTI/TATI in cancer is also supported by the finding that PSTI/TATI is a prognostic factor in ovarian and bladder cancer. The aim of this work was to study further the expression of trypsinogen and PSTI/TATI in human tissues and especially in urogenital organs and their tumors. We also wished to elucidate the role of trypsin and PSTI/TATI in the proteolytic activity of tumors.

We first studied the expression of PSTI/TATI in the kidneys, and found that PSTI/TATI is expressed in the distal tubules of normal renal tissue, in renal cell carcinoma (RCC), and in RCC cell lines which probably provides an explanation for the elevation of serum TATI levels in patients with RCC. We then studied the prognostic value of TATI in RCC and found that the preoperative serum TATI concentration is an independent prognostic factor in RCC among all patients and among those with non-metastatic disease. Therefore, PSTI/TATI may be used to identify patients with increased risk of aggressive disease that might benefit from additional treatment modalities.

Little is known about the mechanisms which control trypsinogen expression in cancer cells. Doxycycline and chemically modified tetracyclines (CMTs) are agents that inhibit cancer cell migration and invasion apparently by inhibiting the expression and activity of MMPs. Therefore we studied whether these agents also have an effect on trypsinogen-2 expression and activity. We found that they downregulated trypsinogen-2 expression in COLO 205 colon adenocarcinoma cells without affecting trypsin-2 activity. We furthermore found that DOXY, CMTs and TATI inhibited the migration of COLO 205 cells, which could be induced by the trypsinogen-activating enzyme enterokinase. These results suggest that the ability of DOXY and CMTs to inhibit tumor invasion may be mediated by effects on both MMPs and trypsin.

The gelatinases MMP-2 and MMP-9 are believed to play a key role in tumor invasion by their ability to degrade type IV collagen, which is the main component of the basement membrane. Because human trypsin-2 activates proMMP-2 and pro-MMP-9 *in vitro* we aimed at elucidating the function of trypsins *in vivo* by studying whether the concentrations of trypsinogen-1 and -2, their α_1-proteinase inhibitor (API) complexes, and TATI are related to proMMP-2 and -9 activation in cyst fluids

of ovarian tumors. We found that high trypsinogen and trypsin-API concentrations in ovarian tumor cyst fluids are associated with proMMP-9 but not proMMP-2 activation. We also found that trypsinogen and trypsin-API concentrations were significantly higher in cyst fluids of malignant than of benign ovarian tumors. These results suggest that trypsin may play a role in ovarian tumor invasiveness.

Human seminal plasma contains several proteolytic enzymes including prostate-specific antigen (PSA) which dissolves the sperm-entrapping gel formed immediately after ejaculation and thus releases motile sperm. Bovine trypsin and recombinant human kallikrein-2 (hK2) activate the proform of PSA *in vitro* but the physiological activators of proPSA are not known. In the present study we show for the first time that trypsinogen-1 and -2 are widely expressed in the male genital tract and that they occur at high concentrations in seminal fluid. Furthermore, we show that trypsin-1 and -2 purified from human seminal fluid activate proPSA *in vitro*. These findings suggest that trypsins may play a role in reproduction.

Acknowledgements

This study was carried out at the department of Clinical Chemistry in the University of Helsinki during the years 1996-2001. I thank professor Ulf-Håkan Stenman, the head of the department, for providing excellent working facilities. I want warmly thank Uffe also for supervising this work. I admire his profound and wide knowledge in science, his way of analysing the questions and problems from all sides, and his ability to come up with new ideas over and over again. I am most grateful for his constant encouragement and the way he has supported me to learn to work more independently.

I want to thank docent Erkki Koivunen and professor Börje Ljungberg for co-supervising this work. Erkki´s vast expertise has been extreamly valuable for my study and I always could have relied on his constructive criticism and advice when needed.

I wish to thank professor Carl G. Gahmberg, the head of the Division of Biochemistry, University of Helsinki, for his positive attitude towards my thesis.

Docent Ulpu Saarialho-Kere and docent Olli Saksela were the official referees of this study. I am grateful for their efficient review and for their constructive criticism and comments.

I wish to express my gratitude to my co-authors Timo Sorsa, DDS, PhD, Taina Tervahartiala, MD, Susanna Lintula, MSc, Anders Bjartell, MD, PhD, Caj Haglund, MD, PhD, Wan-Ming Zhang, MD, PhD, Olli Carpén, MD, PhD, Kristiina von Boguslawski, MSc, Tuula Salo, DDS, PhD, Arto Leminen, MD, PhD, Torsten Wahlström, MD, PhD, Stig Nordling, MD, PhD, Torgny Rasmuson, MD, PhD, Anders Borgström, MD, PhD, Göran Landberg, MD, PhD, Jan Jacobsen, MD, PhD, Jens Hansson, MD, PhD, Lorne Golub, MD, PhD, and Sanford Simon, MD, PhD. Their cooperation has been most valuable and I am grateful for having had the opportunity to work with them.

I owe my thanks to Mrs Sanna Kihlberg, Mrs Anne Ahmanheimo, Mrs Taina Grönholm, Ms Maarit Leinimaa, Mr Oso Rissanen, Mrs Elina Laitinen, and Mrs Sari Nieminen for expert technical assistance and for friendship throughout this study. Ms Marianne Niemelä is acknowledged for her efficient way of taking care of all practical things in the laboratory.

I want warmly thank my friends and colleagues Susanna Lintula, MSc, Meerit Kämäräinen, PhD, and Mrs Kristiina Nokelainen for support and advice during my very first steps as a junior scientist. Thanks are due to docent Riitta Koistinen from whom I have received help when in trouble with protein chemistry and to Patrik Finne, MD, PhD, to whom I have turned for advice in statistics and for help in making the layout of this book. I also wish to thank Jari Leinonen, PhD, for his support, his patience for answering all my question no matter what is the issue, and for companionship in congresses.

I owe my thanks to my friends and colleagues Pia Vahteristo, Kristina Hotakainen, Piia Vuorela, Laura Sarantaus, Heini Lassus, Hannu Koistinen, Jakob Stenman, Henrik Alfthan, Erik Mandelin, Johanna Tapper, Anitta Tamminen, Merja Lindfors, Liisa Airas, Jan Andersén, Can

Hekim, Ping Wu, Zhu Lei, Outi Itkonen, Gynel Arifdsan, Annikki Löfhjelm, and all the others in the research laboratory. You have made of our lab a working place where a good sense of humor and amusing in- and outdoor activities are almost as important as getting results.

Many thanks are directed to all my friends with whom I have had fun far away from work. Especially, I want to thank Sole who first struggled with me trough the biochemistry studies and with whom I since have shared many joys and sorrows. Warm thanks are also directed to all my relatives and my parents in-law, Helena and Hannu, and their whole family.

I wish to thank, from the bottom of my heart, my parents Tuulia and Tapio and my sister Taru and her family for their everlasting love and support.

My warmest thoughts are devoted to Marko. I thank you for your constant love, patience, and encouragement during all these years.

This study was financially supported by the Finnish Cancer Foundation, the Maud Kuistila Foundation, the Alfred Kordelin Foundation, Helsinki University Central Hospital, the Sigrid Juselius Foundation, the Finnish Academy of Sciences, and University of Helsinki.

Helsinki, April 2001

Annukka Paju

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