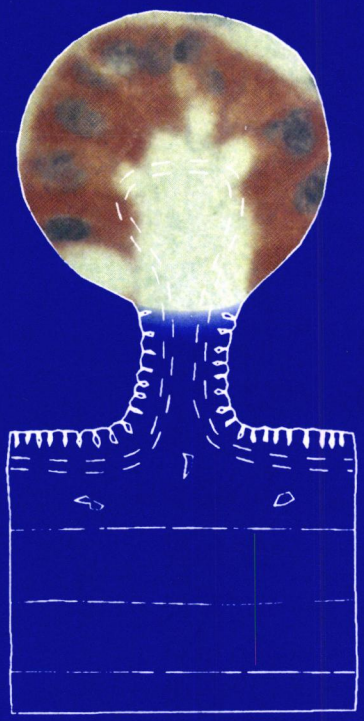
REGULATION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR EXPRESSION IN GASTROINTESTINAL NEOPLASIA



Kees Sier

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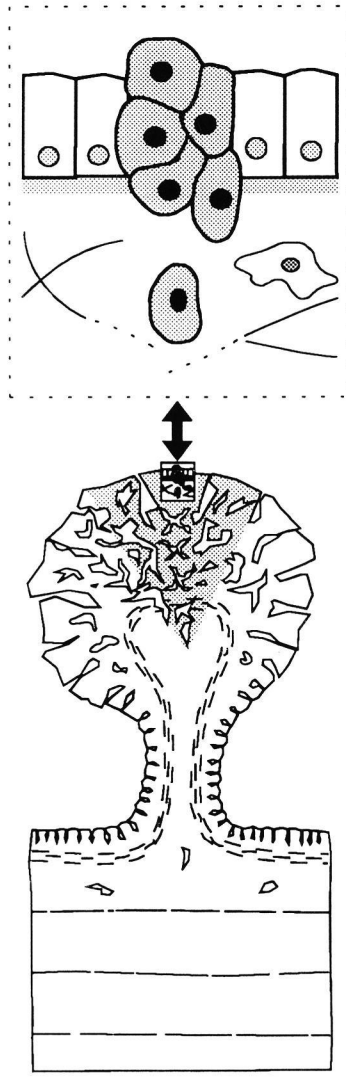

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REGULATION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR EXPRESSION IN GASTROINTESTINAL NEOPLASIA

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het college van dekanen te verdedigen
op dinsdag 30 november 1993
te klokke 15.15 uur

door

Cornelis Frederikus Maria Sier

geboren te Amsterdam in 1959

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PROMOTIECOMMISSIE

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STELLINGEN

1. Bestudering van plasminogeen activatie in dikke darmweefsel afkomstig van patiënten met familiaire adenomateuze polyposis kan een belangrijke bijdrage leveren aan het onderzoek van colorectale carcinogenesis.

   dit proefschrift

2. De ratio tussen u-PA en t-PA antigeen heeft een hoger onderscheidend vermogen tussen normaal en tumor weefsel van het spijsverteringskanaal dan u-PA of t-PA afzonderlijk.

   P.A.F. de Bruin et al. Cancer Res 1988; 48: 4520-45524, dit proefschrift

3. Het feit dat 'knock-out muizen', waarvan het u-PA gen volledig uitgeschakeld is en die dus zelf geen u-PA kunnen produceren, niet vroegtijdig het leven laten verhoogt de kans op therapeutische mogelijkheden voor patienten met een carcinoom door middel van interventie in het urokinase-afhankelijke plasminogeen activatie systeem.

4. Het afwezig zijn van u-PA in tumorcellen en op tumorcelmembranen in de immunohistologische studie van Pyke et al. duidt op het niet aanwezig zijn van de u-PA receptor op deze cellen, wat tegenstrijdig is met andere bevindingen van deze auteur.

   C. Pyke et al. Cancer Res 1993; 53: 1911-1915

5. De ontdekking van insuline-gerelateerde peptiden in de poelslak *Lymnaea stagnalis* bevestigt het evolutie-model voor de insuline superfamilie.

   A.B. Smit et al. Nature 1988; 331: 535-538

6. De titel van het artikel 'Invasion of the trophoblasts' van Strickland en Richards is ook best te gebruiken als naam voor een computerspel of voor een nieuwe film van S. Spielberg.

   S. Strickland en W.G. Richards. Cell 1992; 71: 355-357

7. Op niet iedere i hoort een puntje.

8. Darmwier, *Enteromorpha intestinalis*, is geen darmflora.

9. Onhandig gebruik van wildcards (*) bij een gecomputeriseerd literatuur-onderzoek naar colorectale tumoren kan een gekleurd beeld opleveren.

10. Fuzzy logic is niet alleen handig voor huishoudelijke apparatuur en zijn gebruikers, maar kan ook zeer nuttig zijn voor de medische wetenschap(per).

11. Een echte Griekse held neemt geen genoegen met een transferium, maar kiest voor het Olympisch stadion.

12. Wij zullen die Belgen toch eens serieus moeten gaan nemen nu ze zelfs met zoiets simpels als het benummeren van autoweg-afslagen kilometers op ons voorliggen.

13. Homo sapiens non urinat in ventum.

14. De toestand van de vlaggen in de senaatskamer van de Leidse Universiteit doen afbreuk aan de gehele ruimte en zij zouden, indien mogelijk, gerestaureerd moeten worden.

15. Golf is ook voor 'gewone mensen'; een golf-vereniging vaak niet.

16. Everything should be made as simple as possible, but not simpler.

A. Einstein

17. Liever 4 jaar Leiden dan 4 dagen Nijmegen.

18. De snip houdt van water, de uil niet.

19. Wie dit proefschrift echt goed gelezen heeft zou bij benadering moeten kunnen zeggen hoe vaak het woord plasminogeen (geheel, in samenstelling of in afkorting Eng./Ned.) er in voorkomt.

20. Wie woorden in een proefschrift gaat zitten tellen heeft ze niet allemaal meer op een rijtje.

C.F.M. Sier
Leiden, 30 november 1993

voor mijn ouders

CONTENTS

Chapter I

GENERAL INTRODUCTION AND AIM OF THE STUDIES

CARCINOGENESIS

Introduction

Cancer is after the cardiovascular diseases the second cause of death in the Netherlands and in other Western countries (1). Development of common human cancers, particularly solid tumours, is a highly complex process with many potential causes including environmental factors, genetic predisposition, and somatic mutations. In contrast to benign tumours, malignant carcinomas are able to invade foreign tissues and form metastatic foci at distant locations in the body. During the process of cancer invasion and metastasis a number of natural barriers have to be surpassed by the migrating tumour cells (Figure 1). At the early stage of invasion, epithelial cancer cells detach from the primary lesion, penetrate the subepithelial basement membrane, and migrate into the stroma of adjacent interstitial connective tissue. Eventually the tumour(cells) will reach a lymphatic or a capillary vessel and intravasate by traversing the lymphatic endothelium, or the subendothelial basement membrane and the endothelial layer of the capillary. Then the tumour cells spread through the circulatory system. A small percentage of the circulating malignant cells is able to attach to the vessel wall at a distant site, arrest and proliferate there or even penetrate the endothelial layer and the underlying basement membrane, and migrate into the host tissue parenchyma. Ultimately these metastatic cells have to be able to proliferate in the foreign tissue (2-5).

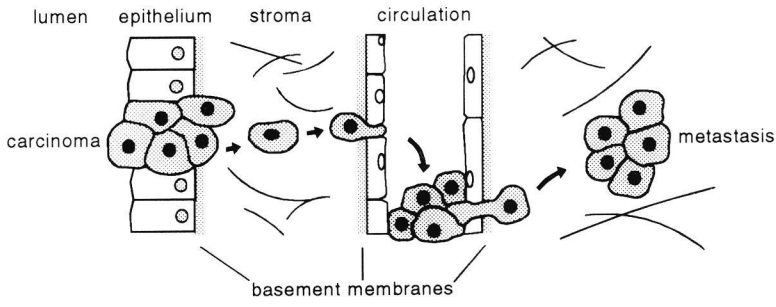

Figure 1. Model of the sequential steps in the process of gastrointestinal carcinogenesis.

Gastrointestinal carcinomas

Carcinoma of the oesophagus is a relatively uncommon tumour, and it has a poor prognosis with only four of 100 patients being alive at 5 year after presentation. About 2% to 5% of the carcinomas are originating from the columnar epithelium present at the distal part of the oesophagus, the remaining cancers are of squamous epithelial origin (6-8). Heavy usage of cigarettes and alcohol are considered to be the most important risk factors for oesophageal carcinoma. The presence of epithelial dysplasia is considered to be the most frequent precancerous lesion of the oesophagus (8,9).

As for oesophageal carcinomas the prognosis of stomach cancer is poor with a 5 year survival of 5% to 20%. Gastric carcinomas form a heterogenous group of cancers which are difficult to classify. A model for the origin of gastric carcinoma has been proposed going from normal mucosa via gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia to gastric cancer (8,9).

Cancer of the large bowel and the rectum, colorectal carcinoma, is next to carcinoma of the lung, the most lethal form of cancer with more than 4000 deaths a year in the Netherlands only (1). The majority of colorectal cancers arise from benign adenomatous polyps or adenomas (9-13). Adenomatous polyps are proliferations of the intestinal mucosa which project into the lumen of the gut. They arise through the loss of growth control of cells in the colonic crypts (Figure 2). Based on their microscopic appearance adenomas are divided into tubular, villous, and tubulovillous types, but they all represent variants of the same neoplastic process (14-16). The hypothesis of the origin of colorectal carcinomas from benign neoplasia, the adenoma-carcinoma sequence, is supported by various lines of evidence including histological, biochemical, and epidemiological studies, as well as experimental colorectal tumours induced in animals (12-20). The study of familial adenomatous polyposis coli (FAP) has made an important contribution to the understanding of carcinogenesis in colorectal tissue. Familial adenomatous polyposis coli is a dominantly inherited autosomal disorder characterized by an early onset of multiple adenomatous polyps in the colorectum, which will inevitably lead to colorectal carcinoma when untreated (21-23). Colorectal tumourigenesis is thought to proceed through a series of genetic alterations involving oncogenes

(e.g. Ras) and tumour suppressor genes (e.g. p53) (23-27). The accumulation of genetic alteration is recognized in the phenotype of the neoplastic cells and in the formation of tumour tissue.

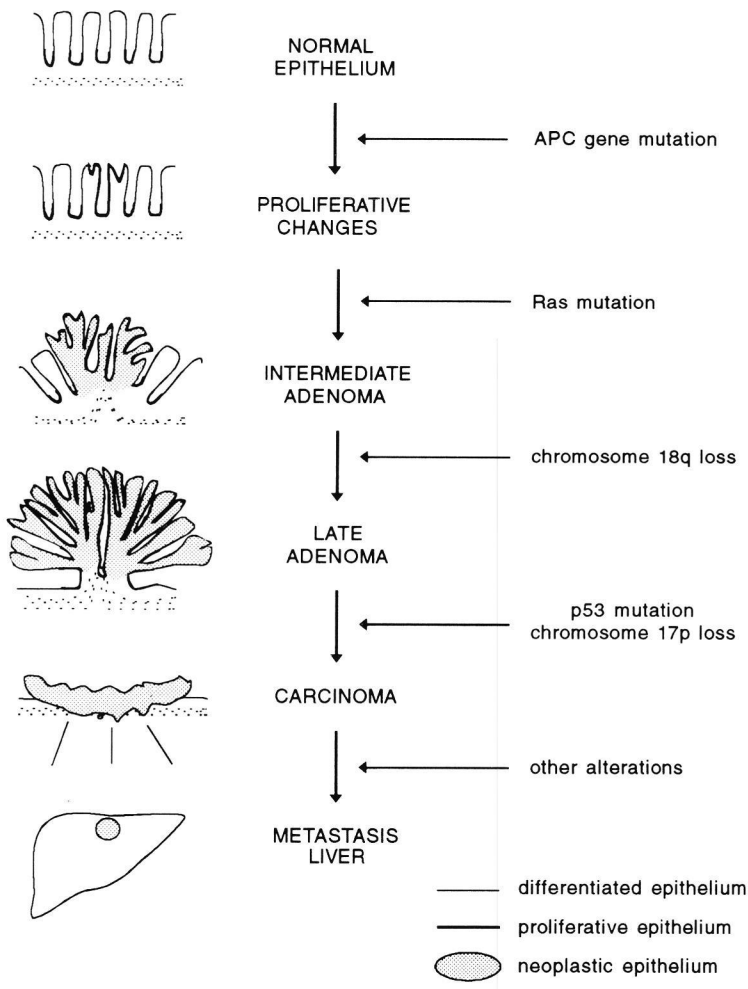

Figure 2. Schematic representation of the genetic model for colorectal tumourigenesis according to Fearon and Vogelstein (24,25) showing sequential steps in the process of neoplastic growth, cancer invasion, and metastasis.

Metastasis

Clinical observation of cancer patients and studies with human tumours in animals, e.g. xenografts by tumour cell injection or orthotopical implantation, have revealed that tumour cell types may metastasize to specific organs independent of vascular anatomy. The site specificity of tumours was shown to be dependent not only on the properties of metastasizing cells, e.g. motility, proteolytic capacity, growth factor production, presence of cell surface receptors, and adhesion molecules, but also of specific organ factors of the metastasized host tissue. For colorectal cancer and other gastrointestinal carcinomas the preferable site of metastasis is the liver (28-34). Metastases are the commonest tumours seen in the liver. The majority of patients with a liver metastasis dies within 5 years (9).

EXTRACELLULAR PROTEOLYSIS

Invasiveness is the ability of (tumour)cells to cross anatomic barriers that separate tissue compartments, e.g. basement membranes, interstitial stroma, and intercellular junctions. Basement membranes do not contain openings large enough to allow the penetration of invading tumour cells and the same is true for the interstitial stroma (3). Therefore, to invade this extracellular matrix (ECM), the components of these structures have to be proteolyzed. The ECM is composed of a large number of components that interact with each other and with the different cell types which are present (Figure 3). The major constituents of the extracellular matrix, and in fact the most abundant proteins in mammalian tissues, are collagens (35,36). Thus far 12 types of collagens have been found, of which types I, II, III, and V are the most common in interstitial stroma and collagen type IV is abundant in basement membranes. In intercellular spaces collagens assemble into ordered polymers to form fibrils. Aggregation of these fibrils form collagen fibers, a major structural component of extracellular matrix (37-40). Other structural components of the basement membrane and interstitial matrix are adhesive glycoproteins like laminin, fibronectin, elastin, entactin/nidogen, vitronectin, osteonectin, and proteoglycans/glycosamino-glycans like hyaluronic acid and heparan sulphate (41-44). From this brief enumeration of structural components of the ECM it is clear, that migration of

cells through these structures requires a large variety of different proteolytic activities.

Invasion takes place in numerous physiological processes in the human body, in which migration of cells is involved, like angiogenesis, ovulation, spermatogenesis, embryogenesis, corneal re-epithelization, woundhealing, and inflammation (45-54). Migrating cells are capable of temporarily degrading the extracellular matrix and basement membranes by means of proteinases. Numerous studies have shown that breakdown of extracellular matrix proteins by proteinases is also executed by migrating cells of malignant origin. These cells are able to degrade the surrounding matrix which enables them to invade the host tissue and ultimately form metastasis at distant locations in the human body.

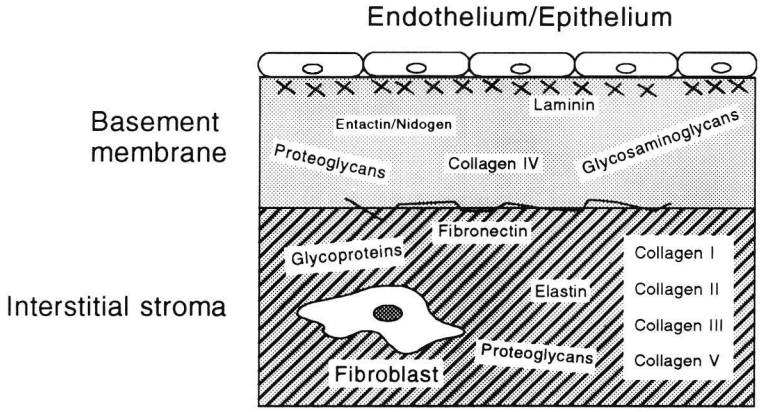

Figure 3. Schematic model of composition and structure of the extracellular matrix (ECM).

PROTEINASES

Proteinases are enzymes which hydrolyze peptide bonds. Several proteolytic enzyme systems have been found and they are divided in four major classes: aspartyl proteinases (e.g. cathepsin D), cysteine proteinases (e.g. cathepsin B, cathepsin H, cathepsin L, cathepsin N), metalloproteinases (e.g. collagenases, gelatinases, stromelysins), and serine proteinases (e.g. cathepsin G, cathepsin E, elastase, kallikreine, thrombin, trypsin, plasmin, plasminogen

activators). Proteinases have been shown to be involved in cell migration in malignant invasion and metastasis. A number of proteinases, which are reported to have elevated levels in human malignancies are briefly discussed. The plasminogen activation system, which is the subject of this thesis, is described in more detail.

Cathepsins

Cathepsins (Gr: to digest) are a diverse group of acidic proteinases present in most mammalian cells. They are usually found in lysosomes, but enhanced presence and excretion into the extracellular space has been described in pathological conditions. Association of cathepsins with malignancy, particularly cathepsins B and D, has been suggested by several investigators. Enhanced levels were found in malignant tissues of colorectum, stomach, ovarium and bladder (55-58). Increased amounts of cathepsin D have proven to be of prognostic importance in breast cancer (59-65). Cathepsins are able to degrade matrix proteins such as collagens, laminin, fibronectin, and proteoglycans (Figure 3). Next to the fact that cathepsins are proteolytic themselves, cathepsins have been shown to activate other proteinases, which are present in a zymogen form in the extracellular matrix (for example pro-urokinase and pro-metalloproteinases) (66-70).

Matrix metalloproteinases

The family of matrix metalloproteinases (MMPs) form a group of hydrolytic enzymes, which share the following features: 1) functioning at neutral pH, 2) a zinc ion at their active site, 3) consistent sequence homologies, 4) secreted in a latent pro-enzyme form and activated after proteolytic cleavage, 5) inhibited by tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2). They are roughly divided into three subgroups based on their (main) substrate: the interstitial collagenases, the type IV collagenases or gelatinases, and the stromelysins (66,71-74). There is a large body of literature correlating the expression of MMPs with tumour invasion and metastasis (75). Especially the type IV collagenases (MMP-2 and MMP-9) and the broad substrate specific stromelysins (MMP-3, MMP-7, MMP-10) are reported to be enhanced in plasma

and in malignant tissues of patients with carcinomas (76-79). Several types of proteinases, e.g. plasmin, stromelysin, cathepsin B, L, and G, and elastase, are known to be involved in the activation of pro-metalloproteinases (66).

Plasmin

Plasmin, a serine proteinase, is produced as a single-chain glycoprotein consisting of 791 amino acids, which has no proteolytic activity: plasminogen. Plasminogen is found in plasma and tissues. The liver is the principal site of synthesis but other tissues have been shown to produce plasminogen also (80). The zymogen plasminogen is converted to active plasmin by cleavage of the single-chain molecule. After cleavage the two chains stay linked together by disulfide bonds. Several proteinases have been reported to be able to perform this activation, but specific plasminogen activators are considered to be the most important *in vivo*. Plasmin contains several domains with specific characteristics; e.g. a proteolytic domain, and five kringle domains (81). Plasmin is best known for its fibrinolytic activity in the lysis of blood clots. Next to the breakdown of fibrin, its 'classical' substrate in the circulation, plasmin is also able to degrade many matrix proteins, for example fibronectin, laminin, proteoglycans, and gelatins. Plasminogen and plasmin have been found to bind to several surface components on different cell types (82-84). The existence of specific plasminogen receptors has been reported but the significance of those structures for plasminogen activation has not been completely established yet (82,85-88), although cell-bound plasmin is found to be resistant to inhibition by alpha-2-antiplasmin (89). Besides degradation of matrix components and the activation of MMPs, plasmin has also been found to activate latent forms of growth factors, which on their turn could play a role in the regulation of plasminogen activation (90).

This preceding short introduction on proteinases makes clear, that the proteolysis of complicated structures as basement membranes and interstitial stroma, which are composed of numerous components, cannot be done by one 'almighty proteinase'. ECM breakdown is a concerted action involving different proteinases, receptors, and inhibitors, which even may outnumber the

components of the matrix they degrade. Despite our growing knowledge about other proteinase systems, a key role in proteolytic degradation is still assigned to the plasminogen activation system. Plasmin is a broad spectrum proteinase, it has the ability to activate pro-MMPs, a family of proteinases with high proteolytic capacity, and the plasmin mediated degrading process can take place in a strictly localized way at cell-substrate and cell-cell contacts because of the presence of specific receptors.

PLASMINOGEN ACTIVATION

History

Hippocrates (460-377 BC) was probably one of the first scientists who described invasion of malignant tumours in host tissue (2). The putative role of proteolytic enzymes in this process, however, was only recognized at the turn of the 20th century. Several studies reported that tumour explants dissolved fibrin clots, a frequently used tissue culture technique at that time (91-93). Thirty years later Fisher (94) proposed that this clot lysis by tumour explants could be due to the activation of serum plasminogen to its active form. In 1973, after the demonstration of a dramatic increase in extracellular proteolysis of cultured cells caused by oncogenic viruses, an enzyme responsible for plasminogen activation was identified (95). The name urokinase was introduced after it became clear that human urine contains large amounts of this plasminogen activator (96). Now it is clear that the plasminogen activation system is a proteolytic cascade composed of the enzyme plasmin, its precursor plasminogen, and two types of plasminogen activators. The catalytic action of plasmin and its activators is further modulated by specific inhibitors, while receptors for plasmin/plasminogen and its activators play a role in both activation and focussing the proteolytic activity at cell-cell and cell-matrix contact sites.

Plasminogen activators

Plasminogen activators are glycoproteins which convert plasminogen into active plasmin. Two distinct plasminogen activators are known, tissue-type (t-PA) and urokinase-type (u-PA) (Table 1). Both types of plasminogen activator

are the product of distinct genes and are secreted as single-chain proteins. u-PA and t-PA also exist in a two-chain form. While two-chain t-PA as well as single-chain t-PA (sc-t-PA) are both able to activate plasminogen, single-chain u-PA, in contrast, is an inactive pro-enzyme (pro-u-PA), which needs cleavage by plasmin, kallikrein, or cathepsin to become the active two-chain form (u-PA) (97-99). The two chains of cleaved t-PA and u-PA remain linked by disulfide bonds, like in plasmin. The B-chain of both activators has a proteolytic domain containing histidine, aspartic acid, and serine residues, similar to that of other known serine proteinase. A cysteine-rich growth factor domain homologous to epidermal growth factor, and a kringle structure like in plasminogen are found in the A-chain of both plasminogen activators (Figure 4). Sequential homologies within serine proteinases suggest common evolutionary ancestor gene(s) (100). Important structural differences between t-PA and u-PA are an additional kringle domain and a finger domain at the amino-terminus both located on the A-chain of the t-PA molecule. These extra structures of t-PA are involved in the binding to fibrin and hence also in the dependence of t-PA activity on the presence of fibrin. Therefore, t-PA is considered to be the major activator which participates in the blood clot dissolution. u-PA does not bind to fibrin, although some effect of fibrin on u-PA activity has been described (101). (Pro)-u-PA can bind to a specific u-PA receptor via the growth factor domain of the A-chain, which localizes the effects. Besides proteolytic activity, u-PA has been shown to possess mitogenic (102-105) and growth factor-like properties (106), probably also via the growth factor domain.

The involvement of urokinase in malignancy has been demonstrated by a number of *in vitro* experiments. Invasion and metastasis of human carcinoma cell lines in nude mice correlates with the enhanced expression of u-PA (107) and transfection of different cell lines with the u-PA gene resulted in a significant increase of invasive capacity (108-110). Moreover, the invasive and metastatic ability of several cell lines could be blocked with antibodies against u-PA (111-116). Enhanced presence of u-PA has been reported in primary tumours and metastases from different tissue origin (117-125), but also in plasma and ascites of carcinoma patients (126-128).

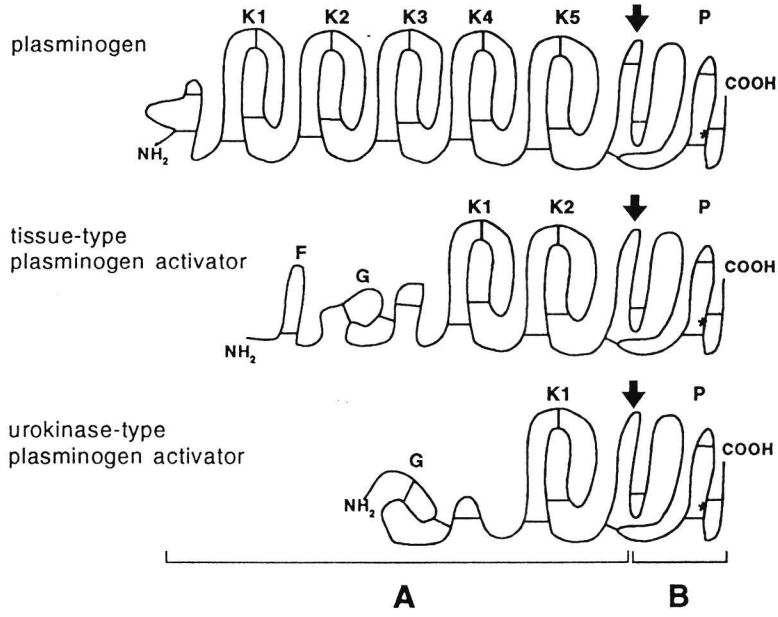

Figure 4. Schematic representation of plasminogen and its activators. Domains are labeled K for kringles, P for proteolytic, F for finger, and G for growth factor. Arrows represent cleavage sites; - are disulfide bridges; asterisks represent the active site serine residues; A and B indicate chains after cleavage.

Plasminogen activator inhibitors

Several types of inhibitors of plasminogen activators have been described: plasminogen activator inhibitor type-1 (PAI-1), type-2 (PAI-2), type-3 (PAI-3), and protease nexin-1 (129). With respect to occurrence, specificity, and reactivity in carcinomas, only PAI-1 and PAI-2 seem to be important.

PAI-1, a 50 kD glycoprotein of 379 amino acids, is synthesized by endothelial cells and is abundantly present in the circulation. It is also produced by a variety of cells in culture, including vascular smooth muscle cells, hepatocytes, fibroblasts, and tumour cells (130). At the cellular level PAI-1 is located in the cell substratum attached to the ECM (131), regulating pericellular proteolysis by forming inactive complexes with sc-t-PA, t-PA, and active u-PA, but not with inactive pro-u-PA. Uncomplexed PAI-1 is unstable and degenerates into a latent form unless it is stabilized by binding to vitronectin. The occurrence

of enhanced PAI-1 levels has not only been shown in several tumour cell lines but also in carcinoma tissues (132-134).

PAI-2 is found to be synthesized in the placenta and by monocytes (135-137). It occurs in 2 forms, unglycosylated in cytosols (47 kD) and secreted in a glycosylated form (60 kD). PAI-2 inhibits u-PA better than sc-t-PA or t-PA, but it does not interact with pro-u-PA. A role for PAI-2 in inflammatory processes is suggested (138). PAI-2 has been detected in the medium of several neoplastic cell lines and in malignant ascites (130,137). Degradation of extracellular matrix by colon cancer cell lines via the urokinase pathway of plasminogen activation was inhibited by administration of recombinant PAI-2 (139).

PAI-3 is found in urine and plasma (129,140,141), and is thought to be identical to the protein C inhibitor. Although PAI-3 has been reported to be present in occasional colonic and breast carcinoma cells (142,143), the role of this inhibitor in malignancy is unclear.

Protease nexin-1, a glycoprotein found in fibroblasts, is an unspecific serine proteinase inhibitor of trypsin, plasmin, and thrombin. Studies on the distribution of protease nexin-1 in various tissues have shown that it is primarily a brain protein (144). Although protease nexin-1 is less reactive with plasminogen activators than PAI-1 or PAI-2, inhibition of tumour cell matrix degradation by protease nexin-1 has been reported (145).

Plasminogen activator receptors

Urokinase can bind to the surface of cells via the urokinase receptor (u-PAR) (146,147). This receptor was first found on monocytes and has been detected since on a variety of cancer cell lines. It is a single-chain, cysteine-rich, highly glycosylated protein of 335 amino acids. The receptor is post-translationally processed to a protein of 313 amino acids consisting of three internal repeating domains and it is attached to the cell surface by a glycosyl-phosphatidylinositol (GPI) anchor. The u-PA receptor binds pro-u-PA and u-PA with high affinity (Kd 0.1-1.0 nM) (148). The receptor binding site of u-PA is located at the growth factor domain of the A-chain (amino acids 13-30).

Table 1. Characteristics of some important components of the plasminogen activation pathway (158).

Component	Molecular Weight	Amino Acids	Chromosome	Specificity
Plasminogen	90-92	791	6q	e.g. laminin, fibronectin, fibrin, proteoglycans, u-PA, gelatins
t-PA	72	530	8pq	plasminogen
u-PA	54	411	10q	plasminogen
PAI-1	50	379	7q	t-PA, u-PA
PAI-2	47u/60g	415	18q	u-PA, t-PA
PAI-3	43	406	14	u-PA, t-PA, thrombin
protease nexin-1	43	378	?	trypsin, thrombin, plasmin, u-PA, t-PA
u-PA receptor	55-60	313-335	19q	pro-u-PA, u-PA

p = short arm of the chromosome; q = long arm of the chromosome;
u = unglycosylated; g = glycosylated.

Receptor-bound pro-u-PA can be converted to its active form. u-PA at the cell surface is still sensitive to inhibition by PAI-1 and PAI-2 in contrast to surface bound plasmin, which escapes inhibition by alpha-2-antiplasmin. The u-PA receptor focusses the enzymatic activity of u-PA, and hence of plasmin, at focal and cell-cell contacts. On top of that, the receptor also regulates the proteolysis by internalizing u-PA/PAI complexes. Internalized receptor releases the ligands u-PA and PAI to the cell lysosomes, and recycles back to the surface (149). Recently, a role of u-PA and its receptor in signal transduction has been suggested (150,151) in addition to the proteolytic activity and the previously mentioned mitogenic role.

Several cell surface structures have been reported which bind t-PA. Although t-PA binding has been reported for human endothelial cells and liver cells *in vitro*, the nature of these structures has not been fully characterized yet. A role for liver cell t-PA receptors is suggested in the rapid clearance of t-PA from the circulation by internalization of t-PA/PAI-1 complexes (152-155). The finger domain of t-PA has been reported to be involved in binding to endothelial t-PA receptors (156).

AIM OF THE STUDIES

Plasminogen activation is a complex cascade of interactions in between active enzymes, zymogens, inhibitors, and receptors. In a number of physiological and pathological processes in which migration of cells is involved, plasminogen activation, particularly via the urokinase pathway, seems to play a key role. In previous studies carried out by our group (157), the presence of plasminogen activators in normal and neoplastic epithelial tissue of the colorectum was investigated. Those studies made clear that in the development of colorectal tumours urokinase is the most prominent plasminogen activator. In the present thesis, the role of the major components which contribute to the regulation of the function of plasminogen activators in gastrointestinal malignancies is further investigated. In order to do so, the studies comprised analyses of activators and inhibitors on protein and mRNA level, as well as their functional interactions, and the contribution of the u-PA receptor. The studies also included tissues from other parts of the gastrointestinal tract than the colorectum.

In the first place, the tissue localization of urokinase in colorectal tumours is investigated using a monoclonal antibody against u-PA in an immuno-histochemical study. Secondly, the presence of u-PA and t-PA in carcinomas of the upper part of the gastrointestinal tract is evaluated using endoscopically obtained biopsies from oesophageal and stomach origin. Uncontrolled extracellular proteolysis will eventually be catastrophic for the host tissues as well as for the tumour. Specific inhibition of a large potential of proteolytic enzymes could be a way to regulate this activity. In order to study the possible regulating role of the two fast-acting inhibitors PAI-1 and PAI-2 in the proteolytic activity of u-PA and t-PA, both inhibitors are investigated by measuring their presence and activity in normal and neoplastic colorectal tissues using specific ELISAs and zymograms. Special attention is focussed on the presence of the plasminogen activation system in colorectal adenomatous polyps and in metastases in the liver, respectively the major precursor and the final stage of colorectal carcinogenesis. Another important component in the regulation of the urokinase pathway of plasminogen activation, the urokinase receptor, is studied with a radioligand binding assay in gastrointestinal carcinomas, colonic

adenomas and liver metastasis, and in their corresponding normal tissues. In the last section of this thesis the regulation of plasminogen activation is studied in gastrointestinal neoplasia at the level of RNA expression. mRNAs of u-PA, its receptor, and the inhibitors PAI-1 and PAI-2 are evaluated in endoscopical biopsies of gastrointestinal neoplasia and adjacent normal tissues using northern blotting. The imbalance between activation and inhibition of plasminogen activators in (pre)carcinomatous tissues will be discussed in relation with the possible diagnostic and clinical value of determining plasminogen activator parameters in gastrointestinal tumours.

REFERENCES

1. Statistisch jaarboek 1993. Centraal bureau voor de statistiek: overledenen naar primaire doodsoorzaak, leeftijd en geslacht in het jaar 1992.
2. Liotta LA. Mechanisms of cancer invasion and metastasis. In: Liotta LA, Kaiser HE, eds. Cancer growth and progression. Vol. 3. Influence of tumor development on the host. Dordrecht, Boston, London: Kluwer Academic Publishers, 1989: 58-71.
3. Tryggvason K. Extracellular matrix and its enzymatic degradation in tumor invasion. In: Liotta LA, Kaiser HE. eds. Cancer growth and progression. Vol. 3. Influence of tumor development on the host. Dordrecht, Boston, London: Kluwer Academic Publishers, 1989: 72-83.
4. Fidler IJ. Critical factors in the biology of human cancer metastasis: Twenty-eight G.H.A. Clowes Memorial Award Lecture. Cancer Res 1990; 50: 6130-6138.
5. Fidler IJ. Cancer metastasis. Br Med Bull 1991; 47: 157-177.
6. Earlam R, Cunha-Melo JR. Oesophageal squamous cell carcinoma: I. A critical review of surgery. Br J Surg 1980; 67:381-385.
7. Matthews HR. Cancer of the oesophagus. Vol. 1. Clinical Cancer Monographs. Houndsmill, Basingstoke, Macmillan Press, 1987.
8. Correa P. Precursors of gastric and esophageal cancer. Cancer 1982; 50: 2554-2565.
9. Fielding JWL, Priestman TJ. Gastrointestinal oncology. Philadelphia: Lea & Febiger, 1986.
10. Morson BC. Precancerous lesions of the colon and rectum. Classification and controversial issues. JAMA 1962; 179: 316-321.
11. Muto T, Bussey HJR, Morson BC. The evolution of cancer of the colon and rectum. Cancer 1975; 36, 2251-2270.
12. Day DW. The adenoma-carcinoma sequence. Scand J Gastroenterol 1984; 19: 99-107.
13. Love RR. Adenomas are precursor lesions for malignant growth in nonpolyposis hereditary carcinoma of the colon and rectum. Surg Gynecol Obstet 1986; 162: 8-12.
14. Cole JW, McKalen A. Studies on the morphogenesis of adenomatous polyps in the human colon. Cancer 1963; 16: 998-1002.
15. Konishi F, Morson BC. Pathology of colorectal adenomas: a colonoscopic survey. J Clin Pathol 1982; 35: 830-841.
16. Kellokumpu I, Husa A. Colorectal adenomas: Morphologic features and the risk of developing metachronous adenomas and carcinomas in the colorectum. Scand J Gastroenterol 1987; 22: 833-841.

17. Griffioen G, Bosman FT, Verspaget HW, de Bruin PAF, Biemond I, Lamers CBHW. Colorectal adenomas: clinical and morphological aspects. A review of 166 polyps from 124 Dutch patients. Anticancer Res 1989; 9: 1685-1690.
18. Griffioen G, Bosman FT, Verspaget HW, de Bruin PAF, Biemond I, Lamers CBHW. Mucin profiles and potential for malignancy of human colorectal adenomatous polyps. Cancer 1989; 63: 1587-1591.
19. Griffioen G, Bosman FT, Verspaget HW, de Bruin PAF, Biemond I, Lamers CBHW. The expression of carcinoembryonic antigen and secretory component in adenomas of the human colon and rectum. Dis Colon Rectum 1989; 32: 954-959.
20. Griffioen G, Bosman FT, Verspaget HW, Sier CFM, Biemond I, Lamers CBHW. Comparative evaluation of carcinoembryonic antigen, secretory component, and mucins in index and metachronous adenomas of the colorectum. Gastroenterology; 101: 919-926.
21. Bleiberg H, Mainguet P, Galand P. Cell renewal in familial polyposis: comparison between polyps and adjacent healthy mucosa. Gastroenterology 1972; 63: 240-245.
22. Ando M, Takemura K, Maruyama M, Endo M, Iwama T, Yuasa Y. Mutations in c-K-ras gene codon 12 during colorectal tumorigenesis in familial adenomatous polyposis. Gastroenterology 1992; 103: 1725-1731.
23. Kikuchi-Yanoshita R, Konishi M, Ito S, et al. Genetic changes of both p53 alleles associated with the conversion from colorectal adenoma to early carcinoma in familial adenomatous polyposis and non-familial adenomatous polyposis patients. Cancer Res 1992; 52: 3965-3971.
24. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988; 319: 525-532.
25. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990; 61: 759-767.
26. Fearon ER, Jones PA. Progressing toward a molecular description of colorectal cancer development. FASEB J 1992; 6: 2783-2790.
27. Scott N, Bell SM, Sagar P, Blair GE, Dixon MF, Quirke P. p53 expression and K-ras mutation in colorectal adenomas. Gut 1993; 34: 621-624.
28. Hart IR. "Seed and soil" revisited: mechanism of site-specific metastasis. Cancer Metastasis Rev 1982; 1: 5-17.
29. Giavazzi R, Campbell DE, Jessup JM, Cleary K, Fidler IJ. Metastatic behavior of tumor cells isolated from primary and metastatic human colorectal carcinomas implanted into different sites in nude mice. Cancer Res 1986; 46: 1928-1933.
30. Liotta LA. Tumor invasion and metastases - Role of the extracellular matrix: Rhoads memorial award lecture. Cancer Res 1986; 46: 1-7.
31. Zetter BR. The cellular basis of site-specific tumor metastasis. N Engl J Med 1990; 322: 605-612.
32. Pauli BU, Augustin Voss HG, El Sabban ME, Johnson RC, Hammer DA. Organ-preference of metastasis. The role of endothelial cell adhesion molecules. Cancer Metastasis Rev 1990; 9: 175-189.
33. Fu X, Besterman JM, Monosov A, Hoffman RM. Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens. Proc Natl Acad Sci USA 1991; 88: 9345-9349.
34. Fidler IJ. Orthotopic implantation of human colon carcinomas into nude mice provides a valuable model for the biology and therapy of metastasis. Cancer Metastasis Rev 1991; 10: 229-243.
35. Burgeson RE. New collagens, new concepts. Ann Rev Cell Biol 1988; 4: 551-557.
36. van der Rest M, Garrone R. Collagen family of proteins. FASEB J 1991; 5: 2814-2823.

37. Engvall E, Hessle H, Klier G. Molecular assembly, secretion, and matrix deposition of type VI collagen. J Cell Biol 1986; 102: 703-710.
38. Keene DR, Sakai LY, Lunstrum GP, Morris NP, Burgeson RE. Type VII collagen forms an extended network of anchoring fibrils. J Cell Biol 1987; 104: 611-621.
39. Timpl R. Structure and biological activity of basement membrane proteins. Eur J Biochem 1989; 180: 487-502.
40. Kucharz EJ. The collagens: biochemistry and pathofysiology. Structure, heterogeneity, and distribution. Berlin Heidelberg New York: Springer Verlag, 1992.
41. Christensen L. The distribution of fibronectin, laminin and tetranectin in human breast cancer with special attention to the extracellular matrix. APMIS 1992; 100 (26 Suppl): 6-39.
42. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. FASEB J 1992; 6: 861-870.
43. Scott JE. Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues. FASEB J 1992; 6: 2639-2645.
44. Mignatti P, Rifkin DB. Biology and biochemistry of proteinases in tumor invasion. Biol Rev 1993; 73: 161-195.
45. Strickland S, Beers WH. 1976. Studies on the role of plasminogen activator in ovulation. J Biol Chem 1976; 18: 5694-5702.
46. Strickland S, Richards WG. Invasion of the trophoblasts. Cell 1992; 71: 355-357.
47. Sappino AP, Huarte J, Belin D, Vassalli JD. Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. J Cell Biol 1989; 109: 2471-2479.
48. Goldfarb RH, Ziche M, Murano G, Liotta LA. Plasminogen activators (urokinase) mediate neovascularization: Possible role in tumor angiogenesis. Semin Thromb Hemost 1986; 12: 337-338.
49. Bacharach E, Itin A, Keshet E. In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggests a concerted role in regulating physiological angiogenesis. Proc Natl Acad Sci USA 1992; 89: 10686-10690.
50. Vihko KK, Kristensen P, Danø K, Parvinen M. Immunohistochemical localization of urokinase-type plasminogen activator in sertoli cells and tissue-type plasminogen activator in spermatogenic cells in the rat seminiferous epithelium. Dev Biol 1988; 126: 150-155.
51. Dvorak HF. Tumors:wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986; 315: 1650-1659.
52. Grøndahl-Hansen J, Lund LR, Ralfkiær E, Ottevanger V, Danø K. Urokinase- and tissue-type plasminogen activators in keratinocytes during wound reepitheliazation in vivo. J Invest Dermatol 1988; 90: 790-795.
53. van Evencooren A, Leprince P, Rogister B, Lefebvre PP, Delree P, Selak I, and Moonen G. Plasminogen activators in developing peripheral nervous system cellular origin and mitogenic effect. Dev Brain Res 1987; 36: 101-108.
54. Woessner JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991; 5: 2145-2154.
55. Weiss RE, Liu BCS, Ahlering T, Dubeau L, Droller MJ. Mechanisms of human bladder tumor invasion: role of protease cathepsin B. J Urol 1990; 144: 798-804.
56. Durdey P, Cooper JC, Switala S, King RFGJ, Williams NS. The role of peptidases in cancer of the rectum and sigmoid colon. Br J Surg 1985; 72: 378-381.
57. Chung SM, Kawai K. Protease activities in gastric cancer tissues. Clin Chim Acta 1990; 189: 205-210.
58. Kobayashi H, Moniwa N, Sugimura M, Shinohara H, Ohi H, Terao T. Increased cell-surface urokinase in advanced ovarian cancer. Jpn J Cancer Res 1993; 84: 633-640.

59. Schmitt M, Janicke F, Graeff H. Tumor-associated proteases. Fibrinolysis 1992; 6 (Suppl 4): 3-26.
60. Rochefort H, Augereau P, Briozzo P, et al. Structure, function, regulation and clinical significance of the 52K pro-cathepsin D secreted by breast cancer cells. Biochimie 1988; 70: 943-949.
61. Thorpe SM, Rochefort H, Garcia M, et al. Association between high concentrations of Mr 52,000 cathepsin D and poor prognosis in primary human breast cancer. Cancer Res 1989; 49: 6008-6014.
62. Duffy MJ, Brouillet JP, Reilly D, et al. Cathepsin D concentration in breast cancer cytosols: Correlation with biochemical, histological, and clinical findings. Clin Chem 1991; 37: 101-104.
63. Foucre D, Bouchet C, Hacene K, et al. Relationship between cathepsin D, urokinase, and plasminogen activator inhibitors in malignant vs benign breast tumours. Br J Cancer 1991; 64: 926-932.
64. Kute TE, Shao ZM, Sugg NK, Long RT, Russell GB, Case LD. Cathepsin D as a prognostic indicator for node-negative breast cancer patients using both immunoassays and enzymatic assays. Cancer Res 1992; 52: 5198-5203.
65. Foekens JA, van Putten WLJ, Portengen H, et al. Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. J Clin Oncol 1993; 11: 899-908.
66. Nakajima M, Chop AM. Tumor invasion and extracellular matrix degradative enzymes: regulation of activity by organ factors. Semin Cancer Biol 1991; 2: 115-127.
67. Duffy MJ. The role of proteolytic enzymes in cancer invasion and metastasis. Clin Exp Metastasis 1992; 10: 145-155.
68. Goretzki L, Schmitt M, Mann K, et al. Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-uPA) by the cysteine protease cathepsin L. FEBS Lett 1992; 297: 112-118.
69. Kobayashi H, Schmitt M, Goretzki L, et al. Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (pro-uPA). J Biol Chem 1991; 266: 5147-5152.
70. Learmonth MP, Li W, Namiranian S, Kakkar VV, Scully MF. Modulation of the cell binding property of single chain urokinase-type plasminogen activator by neutrophil cathepsin G. Fibrinolysis 1992; 6 (Suppl 4): 113-116.
71. Matrisian LM. The matrix-degrading metalloproteinases. BioEssays 1992; 14: 455-463.
72. Murphy G, Docherty AJP. The matrix metalloproteinases and their inhibitors. Am J Respir Cell Mol Biol 1992; 7: 120-125.
73. Emonard H, Grimaud JA. Matrix metalloproteinases. A review. Cell Mol Biol 1990; 36: 131-153.
74. Mignatti P, Rifkin DB. Biology and biochemistry of proteinases in tumor invasion. Biol Rev 1993; 73: 161-195.
75. Cottam DW, Rees RC. Regulation of matrix metalloproteinases: their role in tumor invasion and metastasis (review). Int J Oncol 1993; 2: 861-872.
76. Zucker S, Lysik RM, Zarrabi MH, Moll U. Mr 92,000 type IV collagenase is increased in plasma of patients with colon and breast cancer. Cancer Res 1993; 53: 140-146.
77. Garbisa S, Scagliotti G, Masiero L, Di Francesco C, Caenazzo C, Onisto M, Micela M, Stetler-Stevenson WG, Liotta LA. Correlation of serum metalloproteinase levels with lung cancer metastasis and response to therapy. Cancer Res 1992; 52: 4548-4549.
78. McDonnell S, Navre M, Coffey RJ, Matrisian LM. Expression and localization of the matrix metalloproteinase Pump-1 (MMP-7) in human gastric and colon carcinomas. Mol Carcinog 1991; 4: 527-533.

79. van der Stappen JWJ, Hendriks T, Wobbes T. Correlation between collagenolytic activity and grade of histological differentiation in colorectal tumors. Int J Cancer 1990; 45: 1071-1078.
80. Raum D, Levey R, Taylor PO, Starzl TE. Synthesis of human plasminogen by the liver. Science 1980; 208: 1036-1037.
81. Castellino FJ. Biochemistry of human plasminogen. Semin Thromb Hemost 1984; 10: 18-23.
82. Plow EF, Freaney DE, Plescia J, Miles LA. The plasminogen system and cell surfaces: Evidence for plasminogen and urokinase receptors on the same cell type. J Cell Biol 1986; 103: 2411-2420.
83. Burtin P, Chavanel G, Andre-Bougaran J, Gentile A. The plasmin system in human adenocarcinomas and their metastases. A comparative immunofluorescence study. Int J Cancer 1987; 39: 170-178.
84. Pollanen J, Stephens RW, Vaheri A. Directed plasminogen activation at the surface of normal and malignant cells. Adv Cancer Res 1991; 57: 273-328.
85. Stephens RW, Pollanen J, Tapiovaara H, et al. Activation of pro-urokinase and plasminogen on human sarcoma cells: A proteolytic system with surface-bound reactants. J Cell Biol 1989; 108: 1987-1995.
86. Correc P, Zhang S, Komano O, Laurent M, Burtin P. Visualization of the plasmin receptor on carcinoma cells. Int J Cancer 1992; 50: 767-771.
87. Burtin P, Zhang S, Schauffler J, Komano O, Sastre X, Mathieu MC. Visualization of the plasmin receptor on sections of human mammary carcinoma cells. Int J Cancer 1993; 53: 17-21.
88. Dudani AK, Cummings C, Hashemi S, Ganz PR. Isolation of a novel 45 kDa plasminogen receptor from human endothelial cells. Thromb Res 1993; 69: 185-196.
89. Humphries JE, Vasudevan J, Gonias SL. Fibrinogenolytic and fibrinolytic activity of cell-associated plasmin. Arteriosclerosis Thromb 1993; 13: 48-55.
90. Keski-Oja J, Koli K, Lohi J, Laiho M. Growth factors in the regulation of plasminogen-plasmin system in tumor cells. Semin Thromb Hemost 1991; 17: 231-239.
91. Fleisher MS, Loeb L. On tissue fibrinolysins. J Biol Chem 1915; 21: 477-501.
92. Astrup T. Fibrinolysis: past and present, a reflection of fifty years. Semin Thromb Hemost 1991; 17: 161-174.
93. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation and cancer. Adv Cancer Res 1985; 44: 139-266.
94. Fisher A. Beitrag zur Biologie der Gewebezellen. Eine vergleichend-biologische Studie der normalen und malignen Gewebezellen in vitro. Wilhelm Roux Arch Entw Mech Org 1925; 104: 210-219.
95. Ossowski I, Unkeless JC, Tobia A, Quigley JP, Rifkin DB, Reich E. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. II Mammalian fibroblasts cultures transformed by DNA and RNA tumor viruses. J Exp Med 1973; 137: 112-115.
96. Sobel GW, Mohler SR, Jones NW, Dowdy ABC, Guest MM. Urokinase: an activator of plasma profibrinolysin extracted from urine. Am J Physiol 1952; 171: 768-769.
97. Petersen LC, Lund LR, Nielsen LS, Dano K, Skriver L. One-chain urokinase-type plasminogen activator from human sarcoma cells is a proenzyme with little or no intrinsic activity. J Biol Chem 1988; 263: 11189-11195.
98. Kobayashi H, Schmitt M, Goretzki L, et al. Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (pro-uPA). J Biol Chem 1991; 266: 5147-5152.

99. Goretzki L, Schmitt M, Mann K, et al. Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-uPA) by the cysteine protease cathepsin L. FEBS Lett 1992; 297: 112-118.
100. Patthy L. Evolutionary assembly of blood coagulation proteins. Semin Thromb Hemost 1990; 16: 245-259.
101. de Munk GAW, Rijken DC. Fibrinolytic properties of single chain urokinase-type plasminogen activator (pro-urokinase). Fibrinolysis 1990; 4: 1-9.
102. Kirchheimer JC, Wojta J, Christ G, Hienert G, Binder BR. Mitogenic effect of urokinase on malignant and unaffected adjacent human renal cells. Carcinogenesis 1988; 9: 2121-2123.
103. Kirchheimer JC, Wojta J, Christ G, Binder BR. Functional inhibition of endogenously produced urokinase decreases cell proliferation in a human melanoma cell line. Proc Natl Acad Sci USA 1989; 86: 5424-5428.
104. Binder BR. Influence of urokinase on cell proliferation and invasion. Blood Coagulation Fibrinolysis 1990; 1: 717-720.
105. Rabbani SA, Desjardins J, Bell AW, et al. An amino-terminal fragment of urokinase isolated from a prostate cancer cell line (PC-3) is mitogenic for osteoblast-like cells. Biochem Biophys Res Commun 1990; 173: 1058-1064.
106. Nusrat AR, Mazar A, Henkin J, Chapman HA. A role for urokinase in mediating phorbol ester induced macrophage-like maturation and adhesion of U937 and other myeloid cells. Fibrinolysis 1992; 6: 71-76.
107. Quax PHA, van Muijen GNP, Weening-Verhoeff EJD, et al. Metastatic behavior of human melanoma cell lines in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-mediated matrix degradation. J Cell Biol 1991; 115: 191-199.
108. Axelrod JH, Reich R, Miskin R. Expression of human recombinant plasminogen activators enhances invasion and experimental metastasis of H-ras-transformed NIH 3T3 cells. Mol Cell Biol 1989; 9: 2133-2141.
109. Cajot JF, Schleuning WD, Medcalf RL, et al. Mouse L cells expressing human prourokinase-type plasminogen activator: effects on extracellular matrix degradation and invasion. J Cell Biol 1989; 109: 915-925.
110. Yu H, Schultz RM. Relationship between secreted urokinase plasminogen activator activity and metastatic potential in murine B16 cells transfected with human urokinase sense and antisense genes. Cancer Res 1990; 50: 7623-7633.
111. Ossowski L, Reich E. Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 1983; 35: 611-619.
112. Ossowski L. Plasminogen activator dependent pathways in the dissemination of human tumor cells in the chick embryo. Cell 1988; 52: 321-328.
113. Ossowski L. In vivo invasion of modified chorioallantoic membrane by tumor cells: The role of cell surface-bound urokinase. J Cell Biol 1988; 107: 2437-2445.
114. Ossowski L, Russo-Payne H, Wilson EL. Inhibition of urokinase-type plasminogen activator by antibodies: The effect on dissemination of a human tumor in the nude mouse. Cancer Res 1991; 51: 274-281.
115. Hearing VJ, Law LW, Corti A, Appella E, Blasi F. Modulation of metastatic potential by cell surface urokinase of murine melanoma cells. Cancer Res 1988; 48: 1270-1278.
116. Mignatti P, Robbins E, Rifkin DB. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 1986; 47: 487-498.
117. Oka T, Ishida T, Nishino T, Sugimachi K. Immunohistochemical evidence of urokinase-type plasminogen activator in primary and metastatic tumors of pulmonary adenocarcinoma. Cancer Res 1991; 51: 3522-3525.

118. Kirchheimer JC, Pfluger H, Ritschl P, Hienert G, Binder BR. Plasminogen activator activity in bone metastases of prostatic carcinomas as compared to primary tumors. Invasion Metastasis 1985; 5: 344-355.
119. de Bruin PAF, Verspaget HW, Griffioen G, Nap M, Verheijen JH, Lamers CBHW. Plasminogen activator activity and composition in human colorectal carcinomas. Fibrinolysis 1987; 1: 57-62.
120. de Bruin PAF, Griffioen G, Verspaget HW, Verheijen JH, Lamers CBHW. Plasminogen activators and tumor development in the human colon: activity levels in normal mucosa, adenomatous polyps and adenocarcinomas. Cancer Res 1987; 47: 4654-4657.
121. de Bruin PAF, Griffioen G, Verspaget HW, Verheijen JH, Dooijewaard G, van den Ingh HFGM, Lamers CBHW. Plasminogen activator profiles in neoplastic tissues of the human colon. Cancer Res 1988; 48: 4520-4524.
122. Sumiyoshi K, Baba S, Sakaguchi S, Urano T, Takada Y, Takada A. Increase in levels of plasminogen activator and type-1 plasminogen activator inhibitor in human breast cancer: Possible roles in tumor progression and metastasis. Thromb Res 1991; 63: 59-71.
123. Duffy MJ, O'Grady P, Devaney D, O'Siorain L, Fenelly JJ, Lijnen HR. Urokinase-plasminogen activator, a marker for agressive breast carcinomas. Cancer 1988; 62: 531-533.
124. Grøndahl-Hansen J, Christensen IJ, Rosenquist C, et al. High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res 1993; 53: 2513-2521.
125. de Vries TJ, Quax PHA, Denijn M, et al. Plasminogen activators, their inhibitors and urokinase receptor emerge in late stages of melanocytic tumor progression. Am J Pathol 1993; In press.
126. Huber K, Wojta J, Kirchheimer JC, Ermler D, Binder BR. Plasminogen activators and plasminogen activator inhibitor in malignant and non-malignant ascitic fluid. Eur J Clin Invest 1988; 18: 595-599.
127. Kirchheimer JC, Huber K, Wagner O, Binder B.R. Pattern of fibrinolytic parameters in patients with gastrointestinal carcinomas. Br J Haematol 1987; 66: 85-89.
128. Hienert G, Kirchheimer JC, Pfluger H, Binder BR. Urokinase-type plasminogen activator as a marker for the formation of distant metastases in prostatic carcinomas. J Urol 1988; 140: 1466-1469.
129. Kruithof EKO. Plasminogen activator inhibitors - A review. Enzyme 1988; 40: 113-121.
130. Quax PHA, van Leeuwen RTJ, Verspaget HW, Verheijen JH. Protein and messenger RNA levels of plasminogen activators and inhibitors analyzed in 22 human tumor cell lines. Cancer Res 1990; 50: 1488-1494.
131. Levin EG, Santell L. Association of a plasminogen activator inhibitor with the growth substrata and membrane of human endothelial cells. J Cell Biol 1987; 105: 2543-2549.
132. Reilly D, Christensen L, Duch M, Nolan N, Duffy MJ, Andreasen P. Type-1 plasminogen activator inhibitor in human breast carcinomas. Int J Canc 1992; 50: 208-214.
133. Tanaka N, Fukao H, Ueshima S, Okada K, Yasutomi M, Matsuo O. Plasminogen activator inhibitor 1 in human carcinoma tissues. Int J Cancer 1991; 48: 481-484.
134. Nakstad B, Lyberg T. Immunohistochemical localization, fibrinolytic and antifibrinolytic markers in adenocarcinoma of the lung. APMIS 1991; 99: 981-988.
135. Chapman HA, Vavrin Z, Hibbs JB. Macrophage fibrinolytic activity: Identification of two pathways of plasmin formation by intact cells and of a plasminogen activator inhibitor. Cell 1982; 28: 653-662.
136. Kawano T, Morimoto K, Uemura Y. Urokinase inhibitor in human placenta. Nature 1968; 217: 253-254.

137. Gleeson N, Gonsalves R, Bonnar J. The plasminogen activator urokinase and its inhibitor PAI-2 in endometrial cancer. Gynecol Oncol 1992; 47: 58-61.
138. Schwartz BS, Bradshaw JD. Regulation of plasminogen activator inhibitor mRNA levels in lipopolysaccaride-stimulated human monocytes. Correlation with production of the protein. J Biol Chem 1992; 267: 7089-7094.
139. Baker MS, Bleakley P, Woodrow GC, Doe WF. Inhibition of cancer cell urokinase plasminogen activator by its specific inhibitor PAI-2 and subsequent effects on extracellular matrix degradation. Cancer Res 1990; 50: 4676-4684.
140. Heeb MJ, Espana F, Geiger M, Collen D, Stump DC, Griffin JH. Immunological identity of heparin-dependent plasma urinary protein C inhibitor and plasminogen activator inhibitor-3. J Biol Chem 1987; 262: 15813-15816.
141. Suzuki K, Deyashiki Y, Nishioka J, et al. Characterization of a cDNA for human protein C inhibitor. A new member of the plasma serine protease inhibitor superfamily. J Biol Chem 1987; 262: 611-616.
142. Wojtukiewicz MZ, Zacharski LR, Memoli VA, et al. Indirect activation of blood coagulation in colon cancer. Thromb Haemost 1989; 62: 1062-1066.
143. Costantini V, Zacharski LR, Memoli VA, Kudryk BJ, Rousseau SM, Stump DC. Occurence of components of fibrinolysis pathways in situ in neoplastic and nonneoplastic human breast tissue. Cancer Res 1991; 51: 354-358.
144. Cunningham DD, Pulliam L, Vaughan PJ. Protease nexin-1 and thrombin: injury-related processes in the brain. Thromb Haemost 1993; 70: 168-171.
145. Bergman BL, Scott RW, Bajpai A, Watts S, Baker JB. Inhibition of tumor-cell-mediated extracellular matrix destruction by a fibroblast proteinase inhibitor, protease nexin I. Proc Natl Acad Sci USA 1986; 83: 996-1000.
146. Vassalli JD, Baccino D, Belin D. A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J Cell Biol 1985; 100: 86-92.
147. Stoppelli MP, Tacchetti C, Cubellis MV, et al. Autocrine saturation of pro-urokinase receptors on human A431 cells. Cell 1986; 45: 675-684.
148. Blasi F. Surface receptors for urokinase plasminogen activator. Fibrinolysis 1988; 2: 73-84.
149. Blasi F. Urokinase and urokinase receptor: A paracrine/autocrine system regulating cell migration and invasiveness. BioEssays 1993; 15: 105-111.
150. Dumler I, Petri T, Schleuning WD. Interaction of urokinase-type plasminogen activator (u-PA) with its cellular receptor (u-PAR) induces phosphorylation on tyrosine of a 38 kDa protein. FEBS Lett 1993; 322: 37-40.
151. Del Rosso M, Anichini E, Pedersen N, et al. Urokinase-urokinase receptor interaction: non mitogenic signal transduction in human epidermal cells. Biochem Biophys Res Commun 1993; 190: 347-352.
152. Owensby DA, Morton PA, Schwartz AL. Interactions between tissue-type plasminogen activator and extracellular matrix-associated plasminogen activator inhibitor type 1 in the human hepatoma cell line HepG2. J Biol Chem 1989; 264: 18180-18187.
153. Wing LR, Bennett B, Booth NA. The receptor for tissue plasminogen activator (t-PA) in complex with its inhibitor, PAI-1, on human hepatocytes. FEBS Lett 1991; 278: 95-97.
154. Bu G, Morton PA, Schwartz AL. Identification and partial characterization by chemical cross-linking of a binding protein for tissue-type plasminogen activator (t-PA) on rat hepatoma cells. J Biol Chem 1992; 267: 15595-15602.
155. Barnathan ES, Kuo A, van der Keyl H, McCrae KR, Larsen GR, Cines DB. Tissue-type plasminogen activator binding to human endothelial cells. J Biol Chem 1988; 263: 7792-7799.

156. Beebe DP, Miles LA, Plow EF. A linear amino acid sequence involved in the interaction of t-PA with its endothelial cell receptor. Blood 1989; 74: 2034-2037.
157. de Bruin PAF. Plasminogen activators in (pre)malignant conditions of the human colon. Thesis University Leiden, The Netherlands, 1988.
158. Collen C, Lijnen HR. Molecular and cellular basis of fibrinolysis. In: Hoffman R, ed. Hematology basic principles and practice. New York: Churchill Livingstone, 1991: 1232-1242.

Chapter II

IMMUNOLOCALIZATION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN ADENOMAS AND CARCINOMAS OF THE COLORECTUM

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SUMMARY

Carcinogenesis in the human colon is associated with a marked increase in the tissue content of the urokinase-type plasminogen activator (u-PA). This study was performed to determine the type of cells responsible for the u-PA increase in carcinomas of the colon and in their precursor lesions, the adenomas, by immunohistological evaluation applying monoclonal antibody 3689 directed to the B-chain of u-PA.

Normal intestinal mucosa (n=17) showed hardly any staining of u-PA, but some lamina propria cells were faintly positive. Carcinomas (n=17) and adenomas (n=16) showed a considerable and comparable staining intensity of u-PA in neoplastic columnar epithelial cells, and this staining was found to be diffuse and cytoplasmic. In a majority of the neoplastic tissues the u-PA staining was found to be patchy and not related to known risk markers of malignancy such as dysplasia in the adenomas, or to prognostic determinants such as Dukes' classification or differentiation in the carcinomas. The observation of strong u-PA positive lamina propria cells in adenomas, but infrequently observed in normal mucosa and carcinomas, was noteworthy. u-PA staining intensity of the tissue sections was found to correlate well with the u-PA antigen level in the tissue extracts determined by ELISA ($R=0.52$, $p=0.0001$), but poorly with the u-PA activity determined enzymatically ($R=0.28$, $p=0.05$).

In conclusion, the u-PA increase in neoplasia of the human colon can be attributed to an increased diffuse cytoplasmic content of u-PA in neoplastic columnar epithelial cells.

INTRODUCTION

Malignancies, particularly solid tumours, are associated with a marked increase in the urokinase-type plasminogen activator (u-PA). This enzyme has been implicated - either directly by the activation of the plasmin system or indirectly by the activation of pro-collagenase type IV - in the processes of tumour growth, invasion, metastasis, and prognosis (1-7). In colon cancer tissue increased u-PA activity and antigen levels (8-12), and enhanced secretion of u-PA by explants (13) have been reported. Moreover, elevated levels of u-PA antigen in serum (14) and urine (15) have been observed. In addition, colon

cancer cell lines with enhanced u-PA production were found to have increased numbers of u-PA receptors (16), indicative of autocrine mechanisms, and a high level of breakdown of basement membrane components (17). Colon tumour cells *in vitro* are able concomitantly to produce u-PA and the plasminogen activator inhibitor PAI-1 (18); the inhibitor PAI-2 was found to reduce their matrix degradation (19). Analysis of adenomas and carcinomas of the human colon, however, provided evidence of *in vivo* inaccessibility of u-PA to both inhibitors (20).

Most of the studies on u-PA in colon have involved biochemical and immunological detection of the enzyme in cell or tissue homogenates, and only a few studies have focused on the localization of u-PA within tissues. Several reports have provided illustrative evidence of u-PA in cancer tissue by immunohistological methods (13,15,21), but only a few of these reports evaluated larger series of tissues. Burtin *et al.* (22) found weak positive u-PA staining by immunofluorescence in 50% of the 34 colon carcinomas studied, whereas Kohga *et al.* (23) found all of their 33 carcinomas to be u-PA positive using immunoperoxidase techniques, with apical staining of malignant cells and a correlation with the u-PA activity in tissue homogenates. A small series of eight adenomas, the colonic precursor lesion to carcinomas, were also shown to have increased staining of u-PA in the neoplastic epithelial cells.

The present study was performed to determine the localization of u-PA, using the well-defined monoclonal antibody 3689 directed to an epitope within the B-chain of u-PA, in normal mucosa and in adenomas and adenocarcinomas of the human colon. The u-PA immunohistological results were correlated with risk factors for malignancy in adenomas (dysplasia and architecture) and with prognostic determinants in adenocarcinomas (Dukes' stage and differentiation). The immunohistological results were correlated with u-PA activity and with u-PA antigen levels in the tissue extracts, determined in assays with polyclonal antibodies to u-PA.

PATIENTS, MATERIALS AND METHODS

Tissue

Adenocarcinomas (n=17) of the human colorectum were from fresh resection specimens obtained from the Department of Surgery, University Hospital Leiden. Adenomas (n=16) were freshly obtained by polypectomy at the Department of Gastroenterology in Leiden. Representative parts were selected and divided into two: one part was routinely fixed in 10% phosphate-buffered formaldehyde (pH 7.0) and embedded in paraffin wax, the other was immediately frozen and stored at -70°C. Normal colonic mucosa (n=17) was obtained from the resection margins of the surgery specimens and was processed in a similar way.

Routine histology

Tissue sections, 5 μm thick, were stained with hematoxylin and eosin for routine histological evaluation. Classified according to Dukes (24), eleven of the carcinomas were stage B and six were stage C; differentiation grading showed three to be poor, eight moderate and six well-differentiated. Seven of the adenomas showed tubular mucosal architecture and nine villous; assessment of the degree of epithelial cell dysplasia showed four to be mild, six moderate and six severe (25).

Immunohistology for u-PA

Adjacent serial sections were mounted on gelatine-coated glass slides, deparaffinized by xylene and alcohol and used for immunohistology, employing the alkaline phosphatase-anti-alkaline phosphatase (APAAP) procedure (26) as previously described (27). Ion exchange purified IgG of monoclonal antibody 3689 (1.8 mg/ml), was used at a 1:2000 dilution in Tris buffer (0.02 M Tris-HCl, 0.125 M NaCl, pH 8.5) containing 1% (w/v) bovine serum albumin for 16 hours at 4°C. Monoclonal antibody 3689 (American Diagnostica, Greenwich, CT, USA) is directed against an epitope within the B-chain of u-PA and recognizes pro-u-PA, high molecular weight (HMW) u-PA, and low molecular weight (LMW) u-PA (27). Subsequently the sections were incubated with rabbit anti-mouse IgG (1:25; Sigma, München, Germany) and mouse APAAP complex (1:50;

Dakopatts, Copenhagen, Denmark) for 20 minutes each at 23°C, with washing between steps. The alkaline phosphatase activity was visualized by incubation with the naphthol AS-MX phosphate plus Fast-Red TR substrate solution, containing 1 mM levamisole. Intrinsic alkaline phosphatase activity, particularly found in microvilli of epithelial cells from normal colonic mucosa, was found to be absent from control sections incubated with this concentration of levamisole. The nuclei were counterstained with haematoxylin. Negative controls consisted of incubations with omission of primary antibodies. Sections from normal kidney, in which tubule cells are positive, served as positive controls. Immunohistochemical staining of u-PA was evaluated for intensity and estimated percentage of positive (neoplastic) epithelial cells as follows: 1 = no positive cells; 2 = few cells with weak positivity; 3 = few cells with distinct positivity; 4 = weak positivity of all cells; 5 = weak to distinct positivity in most cells (with negative areas); 6 = weak to distinct positivity in all cells; 7 = distinct positivity in all cells.

u-PA determinations

For u-PA determination, tissue samples were homogenized in 1 ml 0.1% (v/v) Tween 80, 0.1 M Tris-HCl buffer (pH 7.5) per 60 mg wet tissue, as described previously (12). The homogenates were centrifuged twice at 8 x 10^3 *g* for 2.5 minutes, the supernatants collected and used in the assays. Protein concentrations were determined by the method of Lowry (28). u-PA antigen was determined by a sandwich ELISA with rabbit anti-u-PA as catching antibody, and affinity purified goat IgG anti-u-PA as second antibody (29). The samples were incubated overnight at 23°C in a 1:10 or 1:20 dilution before the second antibody was applied. Finally, samples were incubated with rabbit anti-goat IgG-alkaline phosphatase conjugate for 2 hours, followed by the substrate, para-nitrophenyl-phosphate. The ensuing colour reaction was measured spectrophotometrically. All steps were followed by appropriate washing. A standard curve of 0-5 ng/ml HMW u-PA was included in the assay. u-PA activity was determined in a spectrophotometric assay which consisted of incubating the tissue extract with plasminogen, fibrinogen fragments, and a chromogenic plasmin substrate (S-2251). Specific u-PA activity was calculated by adding

inhibiting antibodies to u-PA to parallel incubations and determining the inhibition kinetically by subtraction (12,30). HMW u-PA standard preparation, batch nr 66/46 (National Institute of Biological Standards and Control, London, UK) was used as reference in both assays.

Calculations and statistical analysis

u-PA activity is expressed as mIU/mg protein and u-PA antigen as ng/mg protein. The relation and differences between the different parameters studied were evaluated by the analysis of variance, linear regression statistics, Student's *t*-test or separate variance estimate, and χ^2 or Fisher's exact-test. Differences were considered significant when $p \leq 0.05$.

RESULTS

Remarkable differences were observed between the immunohistological u-PA staining intensities of normal mucosa, adenomas, and carcinomas. Normal mucosa showed hardly any u-PA positivity (Figure 1*a*). Sometimes the surface epithelium showed some weak u-PA positivity. Besides the occasional positive epithelial cell, there were no positive goblet cells, but some faint staining of incidental lamina propria cells, i.e. scattered lymphocytes, histiocytes, and granulocytes, was observed (Figure 1*b*).

Carcinomas showed a wide range of staining intensities for u-PA in neoplastic epithelial cells (Figures 1*c* & 1*d*). In general, we found diffuse cytoplasmic staining of u-PA in the tumour cells, and no particular membrane staining was observed. In the majority of the carcinoma specimens, stromal, muscle, and occasionally endothelial cells were also found to be positive for u-PA. Within the carcinomas the central part of the tumour showed the highest u-PA staining intensity in only 24% (4/17) of the cases. There was no correlation between intensity of staining and Dukes' stage or grade of differentiation. In the adenomas as well as in the carcinomas an evident patchy distribution of u-PA staining intensity was frequently observed (respectively in 12/16 = 75% and 12/17 = 71% of the specimens, Figures 1*e* & 1*f*).

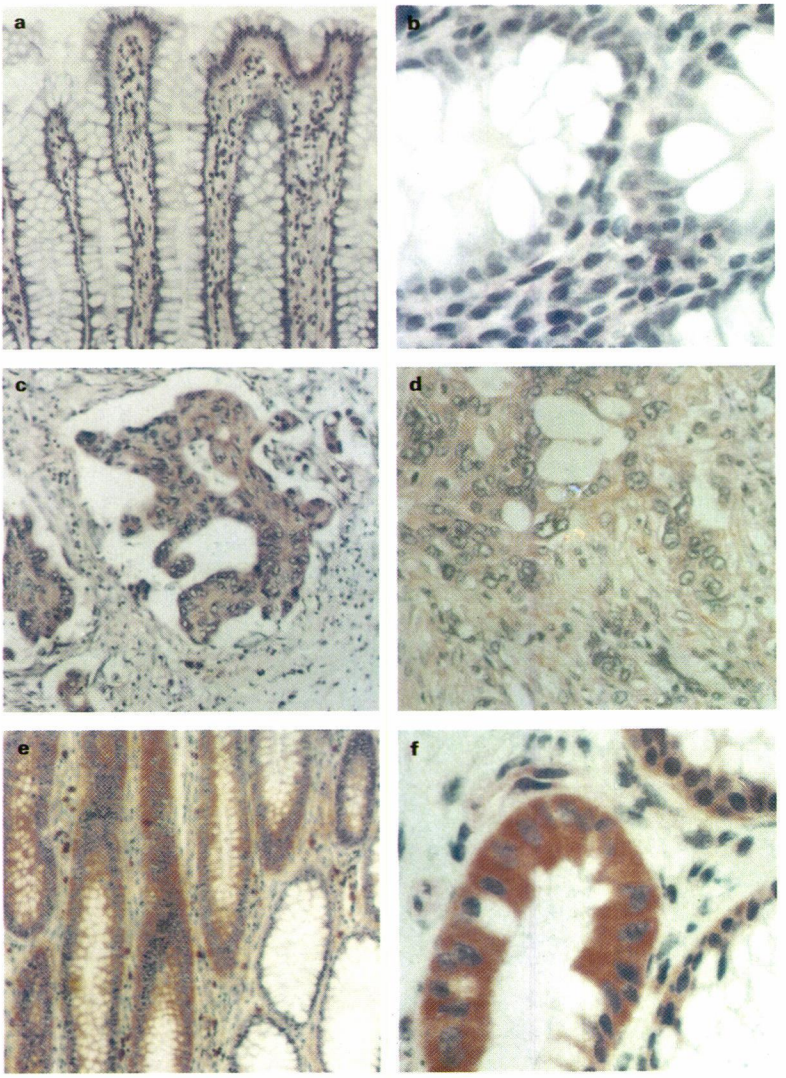

Figure 1. Immunolocalization of urokinase-type plasminogen activator (u-PA) antigen with monoclonal antibody 3689 in *a* & *b* normal mucosa, *c* & *d* carcinoma, *e* & *f* adenoma of the human colon. *a* & *b* In normal colonic mucosa, only scattered cells of the lamina propria show weak positivity, whereas epithelial cells (and goblet cells) are negative in this area (u-PA score = 1). *c* & *d* Carcinomas show distinct positive for u-PA with variable intensity of the individual tumour cells and some staining of the stroma (u-PA scores = 5). *e* & *f* Adenomas show a diffuse and patchy staining of epithelial cells. Heterogeneous intensity of u-PA staining in tubular glands is prominent with scattered positively stained cells the lamina propria (u-PA score = 6). APAAP: *a*, *c*, & *e* x160; *b*, *d*, and *f* x640.

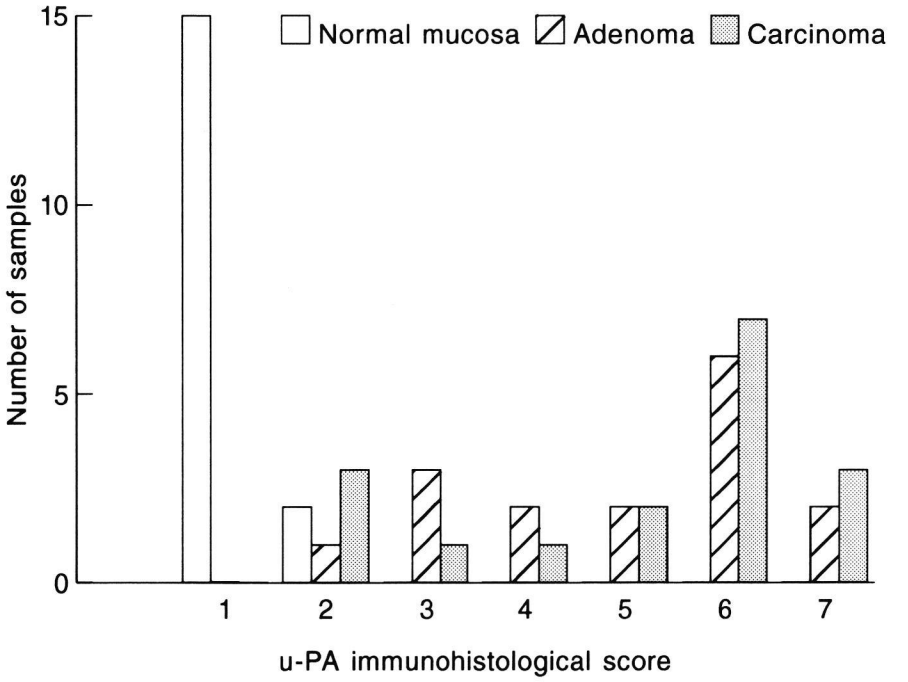

Figure 2. Distribution of the immunohistological score for urokinase-type plasminogen activator (u-PA) in normal mucosa, adenomas, and carcinomas of the human colon. Adenomas and carcinomas showed a significantly higher u-PA staining intensity than normal mucosa (χ^2=47.5, p<0.000005).

Adenomas sometimes showed areas of high staining intensity of u-PA in epithelial cells with dysplasia (Figure 1*e*). However, no significant correlation was found between the degree of dysplasia and u-PA staining intensity: for mild dysplasia the mean u-PA score was 4.8 (n=4); for moderate dysplasia the mean score was 5.7 (n=6); and for severe dysplasia the mean score was 4.3 (n=6). Moreover, no difference was found between u-PA staining in tubular and villous adenomas. In a majority of the adenomas, in addition to epithelial cell staining, cells in the lamina propria were found to contain u-PA, in particular between the crypts (Figure 1*e*). This phenomenon of u-PA positive lamina propria cells was not observed in any sample of normal mucosa, and was seen infrequently in the

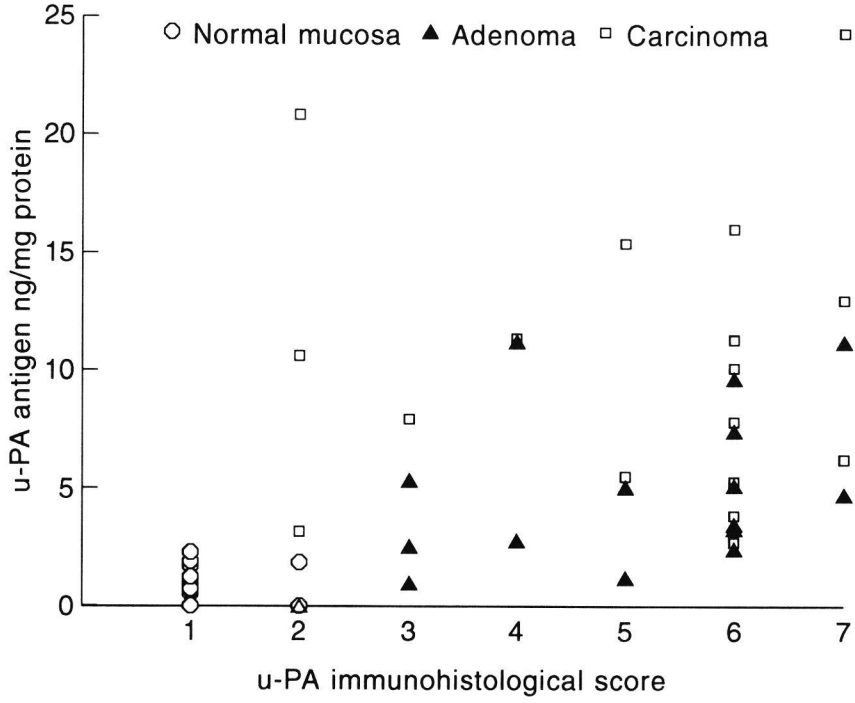

Figure 3. Correlation between the urokinase-type plasminogen activator (u-PA) immunohistological score of tissue sections, using monoclonal antibody 3689, and the u-PA antigen levels, determined by ELISA with polyclonal antibodies to u-PA, in the corresponding tissue homogenates. Normal colonic mucosa, adenomas, and carcinomas combined showed a significant correlation (R=0.52, p=0.0001).

carcinomas (3/17; 18%), but was very frequently observed in the adenomas (12/16; 75%, $p<0.002$ versus normal mucosa and carcinomas).

The immunohistological staining intensity of u-PA, as evaluated by our scoring system, was found to increase significantly from normal mucosa through adenoma to carcinoma ($\chi^2=47.5$, $p<0.000005$, Figure 2). Moreover, the mean staining intensity of u-PA increased in parallel with both the activity and the antigen levels found in the respective tissues (Table 1). In all types of tissue combined, the linear correlation coefficient of the u-PA immunohistological score with activity levels was rather low (R=0.28, p=0.05), whereas the correlation with the antigen level was found to be fairly good (R=0.52, p=0.0001, Figure 3).

Table 1. Urokinase-type plasminogen activator (u-PA) profile of human colonic tissues. Results are mean values ± SEM.

Determination	Normal mucosa (n=17)	Adenoma (n=16)	Carcinoma (n=17)
u-PA activity mIU/mg protein	43.6 ± 7.7	64.8 ± 9.8	121.1 ± 29.7[a]
u-PA antigen ng/mg protein	1.0 ± 0.2	4.8 ± 0.9[b,c]	10.3 ± 1.5[b]
u-PA immunohistology	1.1 ± 0.1	4.9 ± 0.5[b]	5.1 ± 0.4[b]

Significance of difference from normal mucosa: [a] $p<0.05$ and [b] $p<0.001$
Significance of difference from carcinoma: [c] $p<0.005$

DISCUSSION

Neoplastic tissues of the human colon were found to express elevated levels of u-PA antigen as determined by immunohistology using monoclonal antibody 3689. The u-PA staining intensity of the tissue sections corresponded well with the u-PA antigen level, determined by ELISA, and to a lesser extent with the u-PA activity level, determined enzymatically, in homogenates of the same tissue specimens. The neoplastic epithelial cells were found to be the predominant u-PA containing cell type, with u-PA identified by a diffuse cytoplasmic staining. Adenomas also showed a prominent staining of u-PA in cells of the lamina propria. No relation was found in adenomas or carcinomas of the colon between the intensity of the u-PA staining and other features of (pre)malignancy such as dysplasia and differentiation of the tumours.

Human carcinomas originating from the colon (13,15,21-23,31), oesophagus (15), stomach (15), uterus (32,33), ovary (33), breast (27), and prostate (34) have been found to be positive for u-PA by histological evaluation. These observations illustrate that malignant transformation of epithelial cells is associated with an increased intracellular content of u-PA, an antigen which is only sporadically observed in their normal tissue counterparts. In the present study we sometimes found a weak positivity of the luminal epithelium and an occasional lamina propria cell positive for u-PA in normal mucosa. The type of cell could not definitely be identified, but granulocytes (23), natural-killer cells (35), and fibroblast or macrophage-like cells present in the lamina propria (32,36) have been reported to show positive u-PA staining. In contrast to earlier reports where polyclonal antibodies to u-PA were used (23,31) we did not find

any positively stained globlet cells when monoclonal antibody 3689 was used. With respect to the subcellular localization of u-PA in the (pre)malignant epithelial cells of the colon, we found a diffuse cytoplasmic staining which is in accordance with most other studies: some workers (23,31) have shown an intense apical or luminal cell membrane staining of u-PA, but they did mention that the localization of the u-PA staining depended on the polyclonal and monoclonal antibodies used (31). The distribution of the staining within the malignant tissues in both our study and those of others was found to be patchy, and some studies even reported that the invasive part of the tumours contained the highest intensity of u-PA staining (23,34). This observation was not confirmed in the present study as a general phenomenon, but was observed in a minority of the tissues evaluated.

Adenomas were found to show a similar distribution of u-PA staining as the carcinomas, as observed previously in a preliminary study by Kohga and co-workers (23). The high frequency of u-PA positive lamina propria cells in the adenomas, which has not been reported before, was a remarkable observation in the present study. Although a definite identification of the type of cell was not established, they appeared to be eosinophilic granulocytes.

The present study showed a good and statistically significant correlation between the antigen level, determined by an ELISA, and the immunohistologically determined presence of u-PA in neoplastic tissues of the human colon. The correlation with the activity level of u-PA, however, was found to be less good. This observation can be explained by the fact that, as reported in a previous study (37), 75% of u-PA is present in its inactive pro-enzyme form (pro-u-PA), which, although not detected in the activity assay, is recognized by the antibodies used in the ELISA and immunohistology. Markus *et al.* (13) and Kohga *et al.* (23) also found an association between u-PA histology and tissue extract u-PA activity in the colon which was not further quantified. Similar to the present findings on colon cancer, was the observation of a significant association between u-PA histology and antigen level in breast cancer made by Jänicke *et al.* (27), applying another monoclonal antibody (394).

We found no relation between u-PA staining and risk factors for malignancy in adenomas (such as mucosal architecture and dysplasia) or prognostic

determinants in colorectal malignancy, such as differentiation and Dukes' stage. Although some reports have indicated an association between u-PA antigen and epithelial dysplasia in adenomas (38) and between u-PA activity and Dukes' stage in colon carcinomas (39), most studies show no statistically significant association between such parameters and u-PA expression (10-15, 23,27,37). Studies on breast cancer have revealed, however, that an increased level of u-PA antigen is a potent predictor for early relapse and survival (27). These observations further support the notion that u-PA may act as an independent prognostic determinant for solid tumours.

REFERENCES

1. Markus G. The role of hemostasis and fibrinolysis in the metastatic spread of cancer. Semin Thromb Haemost 1984; 10: 61-70.
2. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation and cancer. Adv Cancer Res 1985; 44: 139-266.
3. Saksela O. Plasminogen activation and regulation of pericellular proteolysis. Biochim Biophys Acta 1985; 823: 35-65.
4. Saksela O, Rifkin DB. Cell-associated plasminogen activation: Regulation and physiological functions. Ann Rev Cell Biol 1988; 4: 93-126.
5. Markus G. The relevance of plasminogen activators to neoplastic growth - a review of recent literature. Enzyme 1988; 40: 158-172.
6. Hart DA, Rehemtulla A. Plasminogen activators and their inhibitors: Regulators of extracellular proteolysis and cell function. Comp Biochem Physiol 1988; 90: 691-708.
7. Verspaget HW, Verheijen JH, de Bruin PAF, Griffioen G, Lamers CBHW. Plasminogen activators in (pre)malignant conditions of the colorectum. Eur J Clin Oncol 1989; 25: 565-569.
8. Corasanti JG, Celik C, Camiolo SM, et al. Plasminogen activator content of human colon tumours and normal mucosae: Separation of enzymes and partial purification. J Natl Cancer Inst 1980; 65: 345-351.
9. Tissot JD, Hauert J, Bachmann F. Characterization of plasminogen activators from normal human breast and colon and from breast and colon carcinomas. Int J Cancer 1984; 34: 295-302.
10. Gelister JSK, Mahmoud M, Lewin MR, Gaffney PJ, Boulos PB. Plasminogen activators in human colorectal neoplasia. Br Med J 1986; 293: 728-731.
11. de Bruin PAF, Verspaget HW, Griffioen G, Nap M, Verheijen JH, Lamers CBWH. Plasminogen activator activity and composition in human colorectal carcinomas. Fibrinolysis 1987; 1: 57-62.
12. de Bruin PAF, Griffioen G, Verspaget HW, Verheijen JH, Lamers CBHW. Plasminogen activators and tumour development in the human colon: activity levels in normal mucosa, adenomatous polyps, and adenocarcinomas. Cancer Res 1987; 47: 4654-4657.
13. Markus G, Camiolo SM, Kohga S, Madeja JM, Mittelman A. Plasminogen activator secretion of human tumors in short-term organ culture, including a comparison of primary and metastatic colon tumors. Cancer Res 1983; 43: 5517-5525.

14. Kirchheimer JC, Huber K, Wagner O, Binder BR. Pattern of fibrinolytic parameters in patients with gastrointestinal carcinomas. Br J Haematol 1987; 66: 85-89.
15. Nishino N, Aoki K, Tokura Y, Sakaguchi S, Takada Y, Takada A. The urokinase type of plasminogen activator in cancer of digestive tracts. Thromb Res 1988; 50: 527-535.
16. Boyd D, Florent G, Kim P, Brattain M. Determination of the levels of urokinase and its receptor in human colon carcinoma cell lines. Cancer Res 1988; 48: 3112-3116.
17. Boyd D, Ziober B, Chakrabarty S, Brattain M. Examination of urokinase protein/transcript levels and their relationship with laminin degradation in cultured colon carcinoma. Cancer Res 1989; 49: 816-820.
18. Quax PHA, van Leeuwen RTJ, Verspaget HW, Verheijen JH. Protein and messenger RNA levels of plasminogen activators and inhibitors analyzed in 22 human tumor cell lines. Cancer Res 1990; 50: 1488-1494.
19. Baker MS, Bleakley P, Woodrow GC, Doe WF. Inhibition of cancer cell urokinase plasminogen activator by its specific inhibitor PAI-2 and subsequent effects on extracellular matrix degradation. Cancer Res 1990; 50: 4676-4684.
20. Sier CFM, Verspaget HW, Griffioen G, Verheijen JH, Dooijewaard G, Lamers CBHW. Plasminogen activator inhibitors in human colonic neoplasms. Gut 1990; 31: A617.
21. Wojtukiewicz MZ, Zacharski LR, Memoli VA, et al. Indirect activation of blood coagulation in colon cancer. Thromb Haemost 1989; 62: 1062-1066.
22. Burtin P, Chavanel G, Andre J. The plasmin system in human colonic tumors: an immunofluorescence study. Int J Cancer 1985; 35: 307-314.
23. Kohga S, Harvey SR, Weaver RM, Markus G. Localization of plasminogen activators in human colon cancer by immunoperoxidase staining. Cancer Res 1985; 45: 1787-1796.
24. Dukes CE. The classification of cancer in the rectum. J Pathol 1932; 35: 323-332.
25. Konishi F, Morson BC. Pathology of colorectal adenomas: a colonoscopic survey. J Clin Pathol 1982; 35: 830-841.
26. Cordell JL, Falini B, Erber WN, et al. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J Histochem Cytochem 1984; 32: 219-229.
27. Jänicke F, Schmitt M, Hafter R, et al. Urokinase-type plasminogen activator (u-PA) antigen is a predictor of early relapse in breast cancer. Fibrinolysis 1990; 4: 69-78.
28. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
29. Binnema DJ, van Iersel JJL, Dooijewaard G. Quantitation of urokinase antigen in plasma and cultured media by use of an ELISA. Thromb Res 1986; 43: 569-577.
30. Verheijen JH, Mullaart E, Chang GTG, Kluft G, Wijngaards G. A simple sensitive spectrophotometric assay for extrinsic (tissue-type) plasminogen activator applicable to measurements in plasma. Thromb Haemost 1982; 48: 266-269.
31. Kohga S, Harvey SR, Suzumiya J, Sumiyoshi A, Markus G. Comparison of the immunohistochemical localisation of urokinase in normal and cancerous human colon tissue. Fibrinolysis 1989; 3: 17-22.
32. Larsson G, Larsson A, Åstedt B. Tissue plasminogen activator and urokinase in normal, dysplastic and cancerous squamous epithelium of the uterine cervix. Thromb Haemost 1987; 58: 822-826.
33. Saito K, Nagashima M, Iwata M, et al. The concentration of tissue plasminogen activator and urokinase in plasma and tissues of patients with ovarian and uterine tumors. Thromb Res 1990; 58: 355-366.
34. Gaylis FD, Keer HN, Wilson MJ, Kwaan HC, Sinha AA, Kozlowski JM. Plasminogen activators in human prostate cancer cell lines and tumors: correlation with the aggressive phenotype. J Urol 1989; 142: 193-198.
35. Carpén O, Saksela O, Saksela E. Identification and localization of urokinase-type plasminogen activator in human NK-cells. Int J Cancer 1986; 38: 355-360.

36. Grøndahl-Hansen J, Kirkeby Lt, Ralfkiær E, Kristensen P, Lund LR, Danø K. Urokinase-type plasminogen activator in endothelial cells during acute inflammation of the appendix. Am J Pathol 1989; 135: 631-636.
37. de Bruin PAF, Griffioen G, Verspaget HW, et al. Plasminogen activator profiles in neoplastic tissues of the human colon. Cancer Res 1988; 48: 4520-4524.
38. Suzumiya J, Hasui Y, Kohga S, Sumiyoshi A, Hashida S, Ishikawa E. Comparative study of plasminogen activator antigens in colonic carcinomas and adenomatous polyps. Int J Cancer 1988; 42: 627-632.
39. Sim PS, Stephens RW, Fayle DRH, Doe WF. Urokinase-type plasminogen activator in colorectal carcinomas and adenomatous polyps: quantitative expression of active and proenzyme. Int J Cancer 1988; 42: 483-488.

Chapter III

PLASMINOGEN ACTIVATORS IN NORMAL TISSUE AND CARCINOMAS OF THE HUMAN OESOPHAGUS AND STOMACH

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SUMMARY

Carcinogenesis in the human colon is associated with a marked increase of urokinase-type plasminogen activator and a decrease of tissue-type plasminogen activator. This study was performed to determine the concentrations of urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA) in normal tissue and carcinomas along the upper part of the gastrointestinal tract. Activity and antigen levels of both activators were determined in homogenates of endoscopically obtained biopsies from normal and carcinomatous tissues. Although the concentrations of both t-PA and u-PA in normal squamous epithelium of the oesophagus were low compared with those in columnar epithelium from the stomach, the u-PA/t-PA antigen ratio of the different locations showed hardly any difference. Significant but heterogeneous increases were found in u-PA concentrations of biopsy specimens originating from carcinomas of both epithelial cell types. A decrease in tissue-type plasminogen activator concentrations, as found in human colon carcinomas, could only be shown in carcinomas of columnar epithelium origin but not in squamous cell carcinomas of the oesophagus. The increase of urokinase-type plasminogen activator and u-PA/t-PA antigen ratio, and the decrease of tissue-type plasminogen activator in the carcinomas did not show a significant correlation with known prognostic determinants as differentiation grade, TNM classification, intestinal metaplasia, inflammation, and ulceration. The heterogeneous increase of urokinase in oesophageal and stomach carcinomas, together with the recently described association of u-PA in tissue extracts of breast carcinomas with aggressiveness and prognosis, may be of relevance to prognostic studies in oesophageal and gastric cancer.

INTRODUCTION

Plasminogen activators are involved in many protein degrading processes by converting plasminogen into the active enzyme plasmin. Tissue-type plasminogen activator (t-PA) is a key enzyme in thrombolysis, while urokinase-type plasminogen activator (u-PA) plays a major role in extracellular matrix breakdown related activities like tissue remodelling and tumour invasion (1,2). Tumour extracts of different origin such as breast, lung, prostate, stomach, and

colon were found to have raised concentrations of plasminogen activator activities. Immunological characterisation revealed that most malignant cells and tissues predominantly produce u-PA rather than t-PA depending on their origin (1-8). Little is known, however, about plasminogen activators in solid tumours of the upper gastrointestinal tract. Early studies using crude fibrinolytic activity assays and histochemical fibrin slide techniques revealed plasminogen activators to be present in cancer tissue and cell lines obtained from the oral cavity and stomach (9-11). A significant increase of urokinase-type plasminogen activator antigen in carcinomatous tissue extracts of oesophagus and stomach has recently been reported by Nishino *et al.* (12), while Takai *et al.* (13) have found a significant increase of total plasminogen activator and urokinase-type plasminogen activator activity in gastric carcinomas. Moreover, increased secretion of u-PA by gastrointestinal tumours has been shown by raised plasma and urine urokinase-type plasminogen activator concentrations in patients with carcinomas of the pancreas, stomach, and colorectum (12,14). We have previously shown that adenocarcinomas of the colon have a five to ten fold increase in u-PA content accompanied by a three to five fold decrease of t-PA, based on activity and antigen measurements (15). Comparison of plasminogen activator content between colon carcinoma resection tissue and endoscopically obtained biopsies of the same patients showed good agreement. In the same study a small group of six carcinomas of the stomach showed comparable results (16). The aim of the present study was to determine the concentrations of tissue-type plasminogen activator and urokinase-type plasminogen activator in carcinomas along the upper part of the gastrointestinal tract using endoscopical biopsies. For that purpose a comparison was made between plasminogen activator content of normal and carcinomatous biopsies of the oesophagus aligned with squamous epithelium, and of the distal oesophagus and different parts of the stomach aligned with columnar epithelium. Also the relation of plasminogen activator concentrations with histologically scored parameters such as differentiation grade, TNM classification, inflammation, ulceration, and intestinal metaplasia of the carcinomas was investigated.

PATIENTS, MATERIALS AND METHODS

Patients and biopsies

Normal mucosal biopsy specimens from three different locations of the upper gastrointestinal tract (squamous oesophagus, corpus/antrum, bulbus/duodenum) were taken during endoscopy from six dyspeptic patients without demonstrated pathology (2 men, 4 women, age 45-48 years). These biopsy specimens were confirmed endoscopically and histologically to have no signs of underlying disease. From patients with a carcinoma in the oesophagus or stomach (37 men, 10 women, age 49-88 years) biopsy specimens were obtained from macroscopically suspected tissue and from normal mucosa 5-10 cm distal and/or proximal to the tumour. One male patient had two separate oesophageal carcinomas. The biopsies were immediately frozen at -70°C. For routine diagnostic purposes and for reference, adjacent biopsies were histologically examined by the pathologist and scored for inflammation, ulceration, intestinal metaplasia, and grade of differentiation. TNM classification of the carcinomas was performed, when possible, according to Hermanek and Sobin (17) based on routine clinical evaluation. All patients included were histologically confirmed to have a carcinoma. The carcinomas were divided into two groups - that is, squamous cell carcinomas of the oesophagus and adenocarcinomas of the lower oesophagus and stomach, and for distinctness called respectively oesophageal and stomach carcinomas.

Tissue extraction and protein concentration

Biopsies were homogenized in 1 ml 0.1% (v/v) Tween 80; 0.1 M Tris-HCl (pH 7.5) per 25 mg wet tissue as described before (16). The homogenate was centrifuged twice at 8 x 10^3 *g* for 2.5 minutes, 4°C. Protein concentration of the supernatants was determined by the method of Lowry *et al.* (18).

ELISA for u-PA

The sandwich ELISA for urokinase-type plasminogen activator was carried out according to Binnema *et al.* (19). Rabbit anti-u-PA was used as catching antibody and after incubation of the samples, affinopurified goat anti-u-PA IgG (0.8 µg/ml) was added and incubated overnight. After washing, 100 µl optimal

dilution of rabbit anti-goat IgG conjugated with alkaline phosphatase was added and 100 µl para-nitrophenyl-phosphate (1 mg/ml) was used as substrate. The amount of u-PA antigen in the samples was calculated from an eight-point standard curve of u-PA (0-5 ng/ml).

ELISA for t-PA

Tissue-type plasminogen activator antigen was measured essentially as described by Rijken *et al.* (20). Rabbit anti-t-PA was used as catching antibody, an anti-t-PA horseradish peroxidase conjugate (Biopool, Sweden) as second antibody and 3,3',5,5' tetramethylbenzidine was used as substrate. Absolute quantities of t-PA antigen in the samples were calculated from an eight-point standard curve of tissue-type plasminogen activator (Biopool, Sweden, 0-4 ng/ml).

Plasminogen activator activity assay

u-PA and t-PA activities were measured by a spectrophotometric enzyme assay as described previously (15). In brief, tissue extract was incubated with plasminogen, fragments of fibrinogen and the chromogenic plasmin substrate S-2251 (Kabi, Stockholm) to detect total plasminogen activator activity. Tissue-type plasminogen activator and urokinase-type plasminogen activator activities were determined by adding specific inhibiting antibodies against t-PA and u-PA, rabbit anti-human t-PA IgG and goat anti-human u-PA IgG respectively, to parallel incubations and calculating the amount of inhibition. Urokinase-type plasminogen activator and tissue-type plasminogen activator standard preparations (National Institute of Biological Standards and Control, London, UK, batch nrs 66/46 and 83/517 respectively) were included. The inhibiting antibodies used were monospecific, showed no cross-reactivity, and blocked maximum standard u-PA and t-PA activity completely.

Calculations and statistical analysis

Activator activities were expressed as mIU urokinase-type plasminogen activator or tissue-type plasminogen activator per mg protein. Antigen concentrations were expressed as ng antigen per mg protein. Results are given

as mean ± SEM. Difference between group means were statistically tested for significance using paired and unpaired Student's *t*-test with separate variance estimate if the standard deviations were significantly different according to the F-test. Differences were considered significant when $p \leq 0.05$.

RESULTS

The antigen and activity levels of both plasminogen activators in normal tissue showed a steady increase going from squamous oesophagus to the stomach and duodenum. u-PA and t-PA in squamous oesophagus were significantly lower than in the stomach. Duodenal tissue contained more tissue-type plasminogen activator than stomach tissue, but the concentration of urokinase-type plasminogen activator in duodenal mucosa was found to be similar to that of the stomach (Table 1).

Table 1. Plasminogen activators in endoscopical biopsies from squamous oesophageal and stomach carcinomas compared with normal tissue biopsies from patients without (controls) or with (normal mucosa) carcinoma. Results shown are mean values ± SEM.

	Tissue-type plasminogen activator				Urokinase-type plasminogen activator			
	(n)	antigen ng/mg protein	(n)	activity mIU/mg protein	(n)	antigen ng/mg protein	(n)	activity mIU/mg protein
Squamous oesophagus								
controls	(5)	2.0 ± 0.5	(6)	11 ± 6	(5)	0.7 ± 0.2	(6)	4 ± 2
normal mucosa	(22)	4.1 ± 1.1	(24)	175 ± 56[b]	(23)	1.4 ± 0.4	(24)	17 ± 5[a]
carcinoma	(16)	4.3 ± 1.0	(16)	299 ± 155	(16)	18.3 ± 4.5[d]	(16)	60 ± 23
Stomach								
controls	(12)	6.8 ± 0.6[h]	(12)	1351 ± 157[h]	(12)	3.9 ± 0.7[g]	(12)	40 ± 8[g]
normal mucosa	(29)	7.4 ± 1.3[f]	(30)	1429 ± 179[h]	(30)	1.8 ± 0.3[c]	(30)	58 ± 11[g]
carcinoma	(32)	5.8 ± 1.5	(32)	442 ± 88[e]	(32)	12.4 ± 1.4[e]	(32)	78 ± 14
Duodenum								
controls	(12)	10.1 ± 1.0[h,i]	(12)	2728 ± 401[h,j]	(12)	4.2 ± 0.4[h]	(12)	30 ± 10[f]

Significance of difference from:
control tissue: [a] $p<0.05$; [b] $p<0.01$; [c] $p<0.005$
normal mucosa: [d] $p<0.005$; [e] $p<0.0005$
(comparable) oesophageal tissue: [f] $p<0.05$; [g] $p<0.001$; [h] $p<0.0005$
stomach tissue: [i] $p<0.05$; [j] $p<0.01$

In general, the normal mucosa of the patients with a carcinoma showed a similar pattern although normal mucosa of squamous carcinomas contained higher concentrations of plasminogen activators, in particular based on activity, than corresponding normal mucosa of control patients. For u-PA antigen in the stomach the opposite was observed.

Urokinase-type plasminogen activator antigen in carcinomas showed a six to seven fold increase in the stomach and a more than thirteen fold higher concentration in oesophageal tissue compared with normal mucosa. Although in both carcinomas and normal mucosae u-PA antigen and u-PA activity showed a

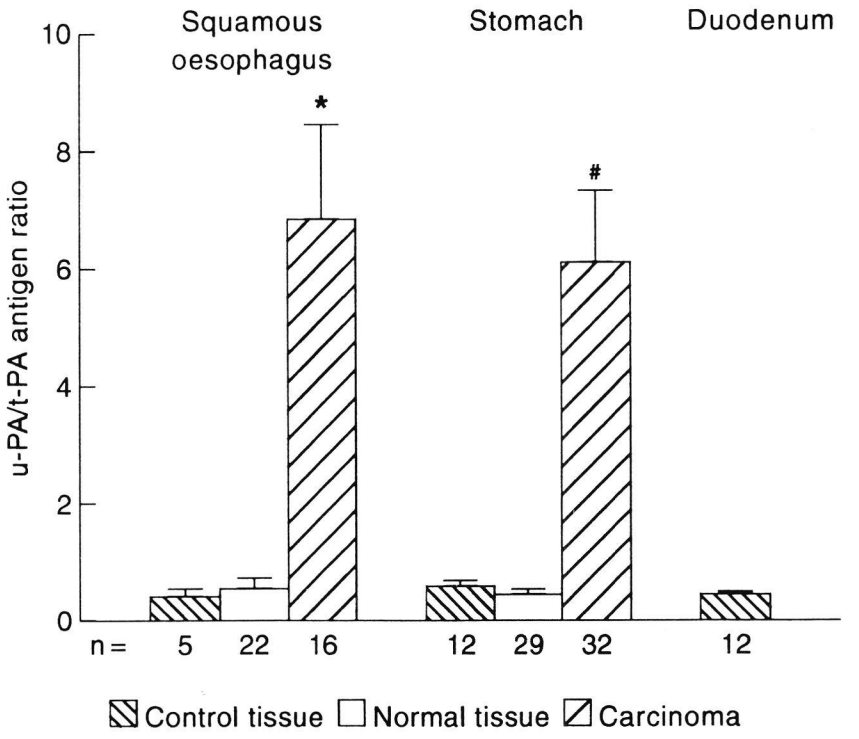

Figure 1: Ratio between urokinase-type plasminogen activator antigen and tissue-type plasminogen activator antigen in endoscopical biopsies from squamous oesophageal and stomach carcinomas compared with normal tissue from patients without (controls) or with a carcinoma. Significance of difference from normal tissue: * $p<0.01$; # $p<0.0001$.

significant correlation (respectively, R=0.38, p<0.01 and R=0.41, p<0.005) and the activity of u-PA in carcinomas was higher than in normal mucosa, this difference did not reach statistical significance (Table 1). Also with respect to t-PA, antigen and activity were in general significantly correlated in carcinomas (R=0.58, p<0.0001) and normal mucosa (R=0.32, p<0.05). Moreover, t-PA antigen as well as t-PA activity in oesophageal carcinoma tissue were not different from normal tissue concentrations. Tissue-type plasminogen activator in stomach carcinomas, however, showed a significant reduction in activity compared with normal stomach tissue, which was not seen in t-PA antigen concentration. The u-PA/t-PA antigen ratio showed no differences between normal and control mucosa independent of the origin of the tissue.

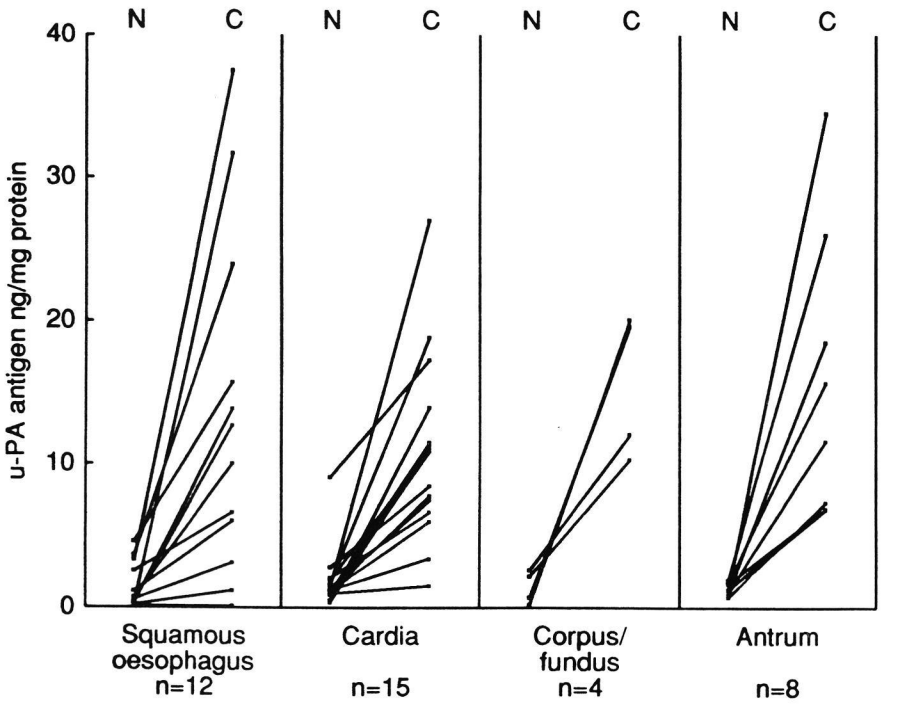

Figure 2: Urokinase-type plasminogen activator antigen in endoscopical biopsies from patients with squamous oesophageal or stomach carcinomas. N: normal mucosa, C: carcinoma tissue.

Carcinomas of the oesophagus and stomach had respectively 14 and 10 times higher ratios than normal tissues but were not different from each other (Figure 1). u-PA antigen and the u-PA/t-PA antigen ratio of carcinoma tissue and the corresponding normal mucosa of the same patient are shown in figures 2 and 3. Although all carcinomas contained more u-PA antigen than the matching normal tissues, the individual carcinoma samples showed great heterogeneity which was not associated with the localization (Figure 2). In 38 out of the 39 tissue pairs tested the u-PA/t-PA antigen ratio of the carcinomas was higher than the corresponding normal mucosa (Figure 3). Again considerable heterogeneity within the carcinomas was observed.

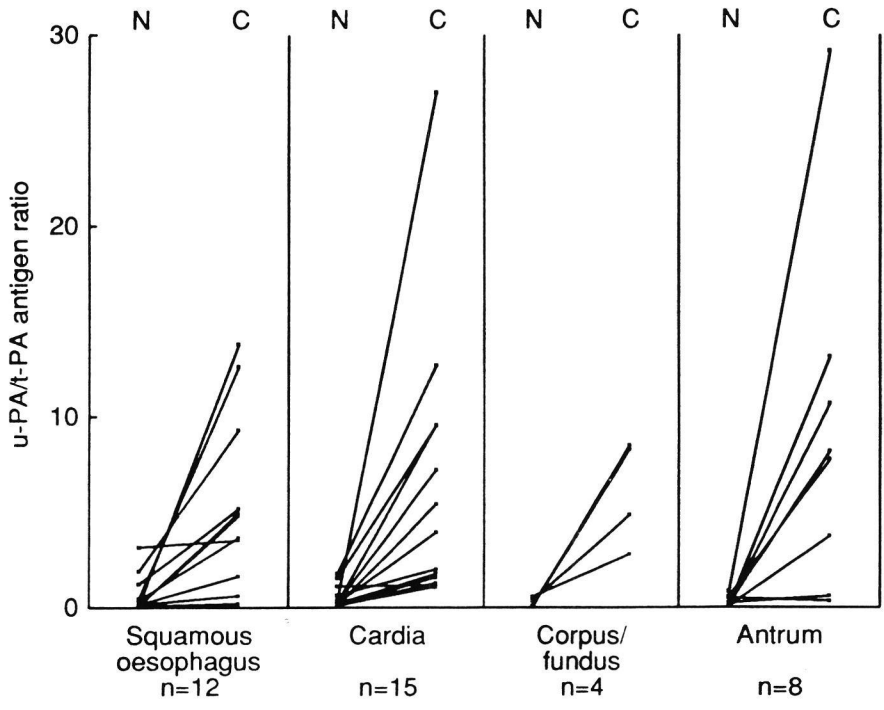

Figure 3: Ratio between urokinase-type plasminogen activator antigen and tissue-type plasminogen activator-antigen in endoscopical biopsies of patients with squamous oesophageal or stomach carcinomas. N: normal mucosa, C: carcinoma tissue.

Table 2. Urokinase-type plasminogen activator activity and antigen, and u-PA/t-PA antigen ratio in endoscopical biopsies from oesophagus and stomach carcinomas.

		u-PA activity mIU/mg protein	u-PA antigen ng/mg protein	u-PA/t-PA antigen ratio
	n			
Differentiation	47			
Well	4	43 ± 22	6.5 ± 3.3	3.5 ± 2.2
Moderate	20	58 ± 19	19.5 ± 3.7	8.7 ± 2.3
Poor	23	91 ± 18	11.1 ± 1.3[a]	4.7 ± 0.7
Inflammation	47			
No inflammation	15	73 ± 23	11.2 ± 2.6	3.2 ± 1.1
Inflammation	12	59 ± 19	12.1 ± 2.0	4.3 ± 1.0
Ulceration	20	81 ± 21	17.9 ± 3.5	9.8 ± 2.1[b,c]
Liver metastasis	48			
Absent	13	82 ± 24	13.4 ± 2.3	7.8 ± 2.0
Present	18	65 ± 21	12.5 ± 2.0	4.7 ± 1.5
Unknown	17	71 ± 19	16.9 ± 4.3	7.1 ± 2.0
Intestinal metaplasia	32	(only stomach)		
Intestinal metaplasia	8	117 ± 38	14.0 ± 3.2	8.2 ± 3.4
No intestinal metaplasia	24	65 ± 13	11.8 ± 1.5	5.4 ± 1.2

Results shown are mean values ± SEM.
Significance of difference from: [a] moderate differentiation $p<0.05$
[b] no inflammation $p<0.01$; [c] inflammation $p<0.05$
t-PA = tissue-type plasminogen activator, u-PA = urokinase-type plasminogen activator

The heterogeneity in u-PA antigen and u-PA/t-PA antigen ratio of the carcinomas, however, was not found to be related to differentiation, inflammation, metastasis formation, and the presence of intestinal metaplasia (Table 2), although some minor differences between subgroups were observed. Moreover, dividing of the carcinomas into different stages according to the TNM Clinical Classification revealed no association between antigen or activity of both plasminogen activators and the clinical staging (data not shown).

DISCUSSION

In this study a survey was made of the activity and antigen concentrations of plasminogen activators in normal mucosa biopsies obtained from three different locations of the upper gastrointestinal tract in control patients. At the same time a comparison was made of tissue-type plasminogen activator and urokinase-type plasminogen activator concentrations between normal mucosa and carcinoma tissue in endoscopical biopsies from patients with a carcinoma of the oesophagus or the stomach.

Oesophageal tissue was found to contain significantly less u-PA and t-PA, both in antigen and activity, compared with stomach tissue. This remarkable difference in expression of plasminogen activators between both normal tissues is most likely caused by differences in the type of epithelium, as squamous oesophageal tissue biopsies contain relatively more epithelium compared with columnar epithelial type tissues of the stomach because of its multilayer cell construction. The concentrations of urokinase-type plasminogen activator in the duodenum resemble the quantities found in normal stomach tissue biopsy specimens. The increase of t-PA antigen and activity in normal duodenal mucosa might be caused by a different vascularisation of the mucosa of this tissue, because tissue-type plasminogen activator is expressed mainly in endothelial cells of vessels (1,10,12). In the normal mucosa of patients with a carcinoma a similar increase in u-PA and t-PA from oesophagus to stomach was observed as in controls.

Surprisingly, plasminogen activator antigen and activity levels of oesophageal and stomach carcinomas did not show significant differences between the two epithelial tissue types. With regard to the corresponding normal tissue, u-PA antigen concentrations in both carcinoma types were significantly higher and u-PA activities were also increased but not significantly. The t-PA antigen concentrations in both oesophageal carcinomas and stomach carcinomas were similar to those of the normal tissues. In an immuno-histochemical study of squamous cervical epithelia with dysplasia and (pre)invasive squamous cell carcinoma an increase in t-PA concentration has been reported throughout the whole thickness of the epithelium (21). In the present study t-PA activity in columnar carcinomas of the stomach, however,

was significantly decreased. This decline was comparable with what we previously found in colon carcinomas (15,16). The difference in alteration between t-PA antigen and activity, and u-PA antigen and activity in columnar epithelial carcinomas has been seen before in colonic neoplasia (15) and is a consequence of the complex regulation mechanism of the activity of both proteases. In colonic carcinomas urokinase-type plasminogen activator is predominantly found in the inactive pro-enzyme form (pro-u-PA), which can be activated in the presence of plasmin. An important role in the control of (pro)-urokinase-type plasminogen activator catalyzed proteolysis is played by the u-PA receptor (22). Plasminogen activator inhibitors (PAI) also control the activity of both tissue-type plasminogen activator and urokinase-type plasminogen activator by forming inactive inhibitor-enzyme complexes (23). Increased concentrations of these inhibitors have been found in plasma and ascitic fluid of patients with malignancies of the gastrointestinal tract or breast (14,24,25). In colorectal carcinomas and in adenomatous polyps increased levels of plasminogen activator inhibitor type-1 (PAI-1) and type-2 (PAI-2) have been demonstrated concurrent with higher u-PA concentrations and inactivation of t-PA (26). Enhanced u-PA activity and PAI-1 antigen concentrations were also found by Tanaka *et al.* (27) in resected carcinomas of the colorectum and stomach, but PAI-2 antigen concentrations were not increased in carcinomas. Therefore, divergence of antigen and activity levels of the plasminogen activators is the consequence of a complex regulation cascade of pro-enzyme activation, receptor binding, and inactivation by inhibitors.

The heterogeneity, especially of the urokinase-type of plasminogen activator concentration within the carcinomas, as shown in figures 2 and 3, could not be explained by the histologically scored parameters in this study. Classification of the carcinomas based on differentiation grade, inflammation, metastasis, and intestinal metaplasia did not reveal major differences in t-PA or u-PA between subgroups. In contrast with the study of Takai *et al.* (13) the division of gastric cancer tissues into groups with different differentiation grades did not show a significant increase of plasminogen activator activities in well differentiated carcinomas. In fact, we found well differentiated carcinomas to contain significantly less u-PA antigen compared with poorly differentiated carcinomas

(Table 2). The high u-PA antigen and u-PA/t-PA antigen ratio in ulcerating carcinomas was remarkable. This increase was not detected in the activity level of urokinase-type plasminogen activator in the same carcinomas, which suggests the presence of pro-u-PA instead of active u-PA or complexes of urokinase with plasminogen activators inhibitors in this tissue.

Gastric cancer and especially oesophageal cancer are two of the most lethal gastrointestinal cancers in terms of cure rate and survival. It would be useful to be able to separate patients into good and poor prognostic groups. Many histological criteria have been analysed in resection material for their prognostic significance but were found to have little value (28,29). Recent studies on breast carcinomas showed that high u-PA concentrations in tissue extracts are positively and independently associated with aggressiveness and poor prognosis while low t-PA concentrations were found indicative for a shorter disease-free interval and survival (30-33). The heterogeneous increase of u-PA antigen concentrations in homogenates of endoscopical biopsy specimens from carcinomas of the upper gastrointestinal tract, which was not associated with several other prognostic determinants, as presented in this study, might perhaps be of value in determining prognosis in (early) oesophageal and gastric cancer.

REFERENCES

1. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 1985; 44: 139-266.
2. Markus G. The relevance of plasminogen activators to neoplastic growth - a review of recent literature. Enzyme 1988; 40: 158-172.
3. Åstedt B, Holmberg L. Immunological identity of urokinase and ovarian carcinoma plasminogen activator released in tissue culture. Nature 1976; 261: 595-597.
4. Rifkin DB, Loeb JN, Moore G, Rerdi E. Properties of plasminogen activators formed by neoplastic human cell cultures. J Exp Med 1974; 139: 1317-1328.
5. Tissot JD, Hauert J, Bachmann F. Characterization of plasminogen activators from normal human breast and colon and from breast and colon carcinomas. Int J Cancer 1984; 34: 295-302.
6. Camiolo SM, Markus G, Evers JL, et al. Plasminogen activator content of neoplastic and benign human prostate tissues; fibrin augmentation of an activator activity. Int J Cancer 1981; 27: 191-198.
7. Markus G, Takita H, Camiolo SM, Corasanti JG, Evers JL, Hobika GH. Content and characterization of plasminogen activators in human lung tumors and normal lung tissue. Cancer Res 1980; 40: 841-848.

8. Quax PHA, van Leeuwen RTJ, Verspaget HW, Verheijen JH. Protein and messenger RNA levels of plasminogen activators and inhibitors analyzed in 22 human tumor cell lines. Cancer Res 1990; 50: 1488-1494.
9. Szczepański M, Lucer C, Zawadzki J, Tolloczko T. Procoagulant and fibrinolytic activities of gastric and colorectal cancer. Int J Cancer 1982; 30: 329-333.
10. Ljungnér H, Björlin G, Åstedt B. Immunological identification of plasminogen activators in normal and malignant tissues of the oral cavity in man. Int J Oral surg 1984; 13: 334-338.
11. Baici A, Sträuli P. Release of proteinases by cultures of human cell lines derived from squamous carcinomas of the tongue and larynx. Exp Cell Biol 1985; 53: 213-219.
12. Nishino N, Aoki K, Tokura Y, Sakaguchi S, Takada Y, Takada A. The urokinase type of plasminogen activator in cancer of digestive tracts. Thromb Res 1988; 50: 527-535.
13. Takai S, Yamamura M, Tanaka K, et al. Plasminogen activators in human gastric cancers: correlation with DNA ploidy and immunohistochemical staining. Int J Cancer 1991; 48: 20-27.
14. Kirchheimer JC, Huber K, Wagner O, Binder BR. Pattern of fibrinolytic parameters in patients with gastrointestinal carcinomas. Br J Haematol 1987; 66: 85-89.
15. de Bruin PAF, Griffioen G, Verspaget HW, et al. Plasminogen activator profiles in neoplastic tissues of the human colon. Cancer Res 1988; 48: 4520-4524.
16. de Bruin PAF, Verspaget HW, Griffioen G, Verheijen JH, Dooijewaard G, Lamers CBHW. Plasminogen activators in endoscopic biopsies as indicators of gastrointestinal cancer: comparison with resection specimens. Br J Cancer 1989; 60: 397-400.
17. Hermanek P, Sobin LH. International Union Against Cancer; TNM classification of malignant tumors. Berlin: 4th ed, Springer, 1987.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
19. Binnema DJ, van Iersel JJL, Dooijewaard G. Quantitation of urokinase antigen in plasma and culture media by use of an ELISA. Thromb Res 1986; 43: 569-577.
20. Rijken DC, van Hinsbergh VWM, Sens EHC. Quantitation of tissue-type plasminogen activator in human endothelial cell cultures by use of an enzyme immunoassay. Thromb Res 1984; 33: 145-153.
21. Larsson G, Larsson Å, Åstedt B. Tissue plasminogen activator and urokinase in normal, dysplastic and cancerous squamous epithelium of the uterine cervix. Thromb Heamost 1987; 58: 822-826.
22. Blasi F. Surface receptors for urokinase plasminogen activator. Fibrinolysis 1988; 2: 73-84.
23. Sprengers ED, Kluft C. Plasminogen activator inhibitors. Blood 1987; 69: 381-387.
24. de Jong E, Knot EAR, Piket D, et al. Increased plasminogen activator inhibition levels in malignancy. Thromb Haemost 1987; 57: 140-143.
25. Huber K, Wojta J, Kirchheimer JC, Ermler D, Binder BR. Plasminogen activators and plasminogen activator inhibitor in malignant and non-malignant ascitic fluid. Eur J Clin Invest 1988; 18: 595-599.
26. Sier CFM, Verspaget HW, Griffioen G, et al. Imbalance of plasminogen activators and their inhibitors in human colorectal neoplasia; implication of urokinase in colorectal carcinogenesis. Gastroenterology 1991; 101: 1522-1528.
27. Tanaka N, Fukao H, Ueshima S, Okada K, Yasutomi M, Matsuo O. Plasminogen activator inhibitor 1 in human carcinoma tissues. Int J Cancer 1991; 48: 481-484.
28. Edwards JM, Hillier VF, Lawson RAM, Moussalli H, Hasleton PS. Squamous carcinoma of the oesophagus: histological criteria and their prognostic significance. Br J Cancer 1989; 59: 429-433.

29. Edwards JM, Jones DJ, Wilkes SJL, Hillier VF, Hasleton PS. Ploidy as a prognostic indicator in oesophageal squamous carcinoma and its relationship to various histological criteria. J Pathol 1989; 159: 35-41.
30. Jänicke F, Schmitt M, Hafter R, et al. Urokinase-type plasminogen activator (u-PA) antigen is a predictor of early relapse in breast cancer. Fibrinolysis 1990; 4: 69-78.
31. Schmitt M, Jänicke F, Graeff H. Tumour-assiocated fibrinolysis: the prognostic relevance of plasminogen activators u-PA and t-PA in human breast cancer. Blood Coagulation Fibrinolysis 1990; 1: 695-702.
32. Duffy MJ, O'Grady P, Devaney D, O'Siorain L, Fennelly JJ, Lijnen HR. Tissue-type plasminogen activator, a prognostic marker in breast cancer. Cancer Res 1988; 48: 1348-1349.
33. Duffy MJ, Reilly D, O'Sullivan C, O'Higgins N, Fennelly JJ, Andreasen P. Urokinase-plasminogen activator, a new independent prognostic marker in breast cancer. Cancer Res 1990; 50: 6827-6829.

Chapter IV

IMBALANCE OF PLASMINOGEN ACTIVATORS AND THEIR INHIBITORS IN HUMAN COLORECTAL NEOPLASIA

IMPLICATION OF UROKINASE IN COLORECTAL CARCINOGENESIS

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SUMMARY

Neoplastic growth and metastatic spread of adenocarcinomas is characterized by a marked increase of urokinase-type plasminogen activator (u-PA) and a decrease of tissue-type plasminogen activator (t-PA). In this study, the authors determined the activity and antigen levels of u-PA and t-PA, and their inhibitors, plasminogen activator inhibitors type-1 and type-2 (PAI-1 and PAI-2), in normal mucosa, adenomatous polyps, and adenocarcinomas of the human colon. The decrease in t-PA activity in the neoplastic tissues, determined enzymatically and zymographically, was significantly correlated with an increase in PAI-1 and PAI-2, in particular in carcinomas. In spite of significantly higher inhibitor levels in the neoplastic tissues, u-PA was found to be increased as well, both in antigen and in activity. The authors conclude that PAI-1 and PAI-2 are significantly increased in neoplastic tissue of the human colon and contribute considerably to the decrease of t-PA activity in carcinomas. However, the malignancy-associated increase in u-PA seems not to be affected by the plasminogen activator inhibitors. Thus, it appears that there is an imbalance between plasminogen activators and their inhibitors in colonic neoplasia in favour of u-PA, which may contribute to plasmin-mediated growth, invasiveness, and metastasis. This feature was also noticed in adenomatous polyps, supporting the malignant potency of adenomas.

INTRODUCTION

Plasminogen activation leads to the conversion of plasminogen into plasmin, a serine proteinase capable of degrading most extracellular proteins. Plasmin is not only the primary effector enzyme in thrombolysis, it also participates in the extravascular breakdown of matrix and basement membrane glycoproteins, playing a role in events such as cell migration, tissue reorganization, and invasive growth. The formation of plasmin is regulated by a complex network of molecular interactions. Two types of serine proteinases, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), have been described, which are predominantly responsible for the conversion of plasminogen to plasmin (1-4).

The dissolution of and migration through basement membrane and interstitial extracellular matrix is a critical step for malignant tumour cells. Plasminogen

activation is thought to be a major biochemical system responsible for this event (5,6). In colorectal adenomatous polyps and carcinomas, plasminogen activator activity can largely be ascribed to u-PA, whereas in normal mucosa this activity consists almost totally of t-PA (7-11). Neoplastic lesions of the colon are characterized by a strong increase of the u-PA antigen content that is found predominantly in the inactive pro-enzyme form and consequently accompanied by a less impressive increase of u-PA activity (9,12). Enhanced secretion of u-PA by tumours of the gastrointestinal (GI) tract is not only observed *in vitro* but also found in plasma and urine of patients with carcinomas of the pancreas, colorectum and stomach (13-15).

The activity of plasminogen activators is controlled by plasminogen activator inhibitors, members of the serine proteinase inhibitor (serpin) family (16). The plasminogen activator inhibitor type-1 (PAI-1) is produced by endothelial cells, hepatoma cells, fibrosarcoma cells, and hepatocytes. PAI-1 inhibits both t-PA and u-PA through the formation of a covalent inhibitor-enzyme complex (1), and it is one of the major secreted products of the vascular endothelium. Synthesis of PAI-1 is regulated by many of the same effectors that regulate the expression of t-PA (3). The second well-characterized rapid and specific plasminogen activator inhibitor (PAI-2) has been isolated from placenta and monocytes. Plasminogen activator inhibitor type-1 has a higher inhibition rate constant to both activators than PAI-2. Moreover, PAI-1 has been found to give a more rapid inhibition of t-PA; whereas for PAI-2 this has been found with u-PA (16,17).

The mechanism by which the plasminogen activators regulate the growth and invasiveness of malignant cells at the cellular level is still unclear. To investigate the role of PAIs in colonic neoplasia, we determined the antigen levels of PAI-1 and PAI-2 in homogenates of normal mucosa, adenomatous polyps, and adenocarcinomas of the human colon. Moreover, t-PA, u-PA, and complexes of PAI-1 and PAI-2 with the activators were also determined.

PATIENTS, MATERIALS AND METHODS

Patients and Tissue samples

Twenty-nine patients (aged 41-84 years; mean age 67 years; 18 men and 11 women) underwent operations for colorectal carcinoma at the University Hospital, Leiden. From each resection a fresh sample of normal colon mucosa and/or a sample of tumour was obtained. After removing fat and muscle layers, the samples were frozen at -70°C until extraction.

In another group of 27 patients (aged 32-88 years, mean age 62; 14 men and 13 women), endoscopic polypectomy was performed, providing tissue samples of 32 adenomatous polyps that were similarly stored at -70°C. For all tissue samples, adjacent fragments were evaluated by the pathologist, confirming the nature of the tissues in all cases. All neoplastic tissues were evaluated for malignancy parameters, i.e., diameter, histological type, and grade of dysplasia in the adenomatous polyps, and differentiation and Dukes' classification (A-D) in the carcinomas (18).

Tissue extraction

Extracts were prepared from 50-100 mg wet tissue samples as described before (11). Essentially, the samples were weighed and homogenized in 1 ml 0.1% (v/v) Tween 80, 0.1 M Tris-HCl (pH 7.5) per 60 mg wet tissue at 0°C. The homogenate was centrifuged twice at 8 x 10^3 *g* for 2.5 minutes, 4°C, and the supernatant was stored at -70°C until analysis.

Protein concentrations

Protein concentrations of the extracts were determined according to the method of Lowry *et al.* (19).

ELISA for PAI-1

Total PAI-1 antigen, i.e. latent, active, and complexed PAI-1, was determined using the Tintelize PAI-1 ELISA (Biopool, Umeå, Sweden) without prior denaturation of the samples. In brief, mouse monoclonal anti-human PAI-1 was used as catching antibody. After incubation with the tissue homogenates a second mouse monoclonal anti-human PAI-1, conjugated to peroxidase, was used to form

a "sandwich" enzyme-linked immunosorbent assay (ELISA) and ortho-phenylenediamine was added as substrate. The assay included the use of quenching and non specific antibodies to exclude falsely elevated results. To increase the sensitivity of the assay sample volumes of up to 80 µl were used in stead of the recommended 20 µl, resulting in a detection limit of 0.3 ng/ml.

ELISA for PAI-2

The determination of PAI-2 antigen was performed using the Tintelize PAI-2 ELISA from Biopool. The first antibody used was mouse monoclonal anti-human PAI-2 and the second was goat polyclonal anti-PAI-2 immunoglobulin (IgG) conjugated to peroxidase. Ortho-phenylenediamine was added as substrate. Unspecific response was excluded using quenching antibodies. The detection limit was decreased to 0.5 ng/ml by using 50 µl homogenate instead of 20 µl and by increasing sample incubation, conjugate incubation, and substrate incubation times.

ELISA for u-PA

The sandwich ELISA for u-PA was carried out according to Binnema *et al.* (20). Rabbit anti-u-PA was used as catching antibody. After incubation of the samples, affinopurified goat anti-u-PA IgG (0.8 µg/ml) was added and incubated overnight. After washing, 100 µl of an optimal dilution of rabbit anti-goat IgG conjugated with alkaline phosphatase was added and 100 µl para-nitrophenyl-phosphate (1 mg/ml) was used as substrate. The amount of u-PA antigen in the samples was calculated from an 8-points standard curve of u-PA (0-5 ng/ml).

ELISA for t-PA

This antigen was measured essentially as described by Rijken *et al.* (21). Rabbit anti-t-PA was used as catching antibody, an anti-t-PA-horseradish peroxidase conjugate (Biopool) as second antibody, and 3,3',5,5' tetramethylbenzidine was used as substrate. Absolute quantities of t-PA antigen in the samples were calculated from an 8-points standard curve of t-PA (Biopool; 0-4 ng/ml).

Plasminogen activator activity assay

Plasminogen activator activities were measured by a spectrophotometric enzyme assay as described previously (10,11). In brief, tissue extract was incubated with plasminogen, fragments of fibrinogen and the chromogenic plasmin substrate S-2251 (Kabi, Stockholm, Sweden) to detect total plasminogen activator activity. The activities of t-PA and u-PA were determined by adding specific protein-A affinity purified inhibiting antibodies against t-PA, rabbit anti-human t-PA IgG, and u-PA, goat anti-human u-PA IgG, to parallel incubations and calculating the amount of inhibition. Standard preparations of u-PA and t-PA (National Institute of Biological Standards and Control, London, UK, batch nrs 66/46 and 83/517, respectively) were included. The inhibiting antibodies used were monospecific, showed no cross-reactivity, and blocked maximum standard u-PA and t-PA completely.

Zymography

Tissue extracts were electrophoresed on 10% polyacrylamide gels with sodium dodecylsulphate (SDS-PAGE), and plasminogen activator activities were visualized on fibrin/plasminogen containing agarose underlay gels according to the method of Granelli-Piperno and Reich (22). Before electrophoresis, samples were incubated for 1 hour at 37°C in 2% (w/v) SDS to induce activator activity in the PA-PAI complexes (23).

Calculations and statistical analysis

Activator activities in the tissue samples were expressed as milli-international units u-PA or t-PA per milligram protein. Antigen concentrations were expressed as nanograms antigen per milligram protein. Results are given as mean ± SEM. Differences between group means were statistically tested for significance using Student's *t*-test, with separate variance estimate if the standard deviations were significantly different according to the F-test. To correlate plasminogen activator parameters, analysis of variance was used. Differences were considered as significant below $p=0.05$.

RESULTS

The PAI-1 antigen was ten fold increased in carcinoma samples compared with the corresponding normal mucosa samples, mean 1.7 ng PAI-1/mg protein (range, 0.2-9.1) versus 0.2 ng/mg protein (range, 0.02-0.5), respectively. The adenomatous polyps showed an intermediate mean PAI-1 value, 0.5 ng/mg protein (range, 0.05-1.4), which was significantly different from both normal mucosa and carcinoma tissue (Figure 1).

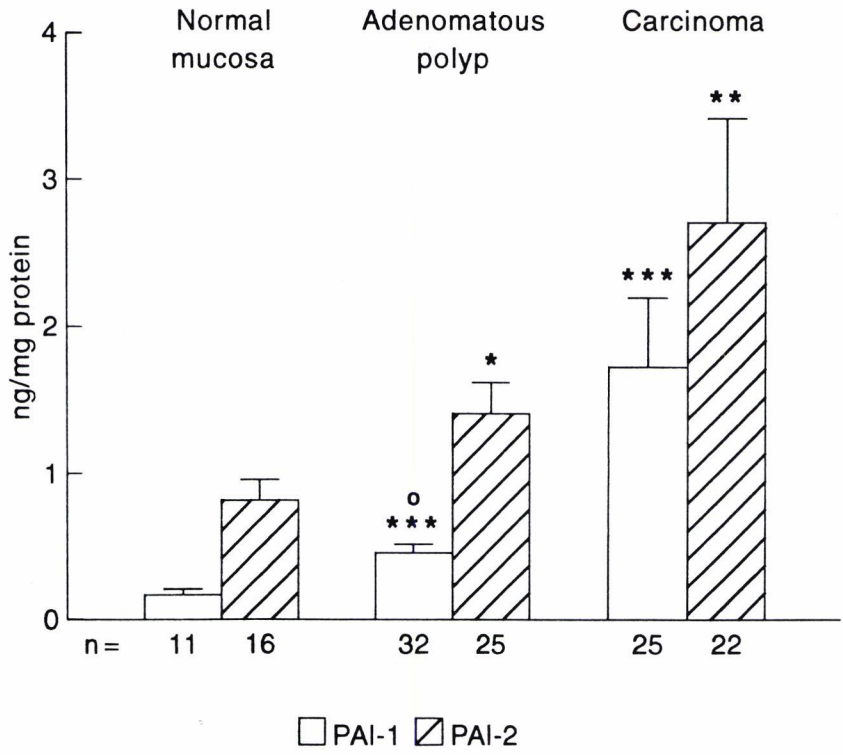

Figure 1. Quantities of plasminogen activator inhibitors PAI-1 and PAI-2 antigen in normal mucosa, adenomatous polyps, and adenocarcinomas of the human colon.
Significance of difference from normal mucosa: * $p<0.05$, ** $p<0.01$, *** $p<0.005$
Significance of difference from carcinoma: o $p<0.02$

Similar results were observed for the PAI-2 antigen. The mean antigen level of PAI-2 was two to three times higher in carcinoma tissue compared with normal mucosa tissue, 2.7 ng/mg protein (range, 0.2-12.8) versus 0.8 ng/mg protein (range, 0.1-2.2), respectively. Comparison of the adenomatous polyps, mean PAI-2 antigen level of 1.4 ng/mg protein (range, 0.04-4.9), with the adenocarcinomas showed no significant difference. Moreover, PAI-1 and PAI-2 were positively correlated in all tissues ($R=0.78$, $p<0.0005$) and predominantly in the carcinomas ($R=0.80$, $p<0.0005$).

The level of u-PA antigen was about eight times higher in the adenocarcinomas, whereas t-PA antigen showed a significant decrease. Adenomatous polyps had an intermediate value of u-PA antigen, but the t-PA antigen level was comparable to that in the carcinomas (Table 1). Activity of u-PA was about three times higher in carcinomas. In adenomatous polyps, u-PA activity showed an intermediate value between normal and carcinoma tissues, although the difference with normal mucosa was not significant. Activity of t-PA within the carcinomas was even more reduced, compared with that in normal mucosa, than t-PA antigen. However, t-PA activity in adenomatous polyps was decreased to a similar level as in carcinomas (Table 1).

Table 1. Urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA) in colonic tissue. Results are mean values ± SEM.

	Normal mucosa		Adenomatous polyp		Adenocarcinoma	
	n	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM
u-PA antigen[a]	13	1.2 ± 0.2	32	4.0 ± 0.5[f,g]	23	9.6 ± 0.8[f]
u-PA activity[b]	20	39 ± 8	32	53 ± 6[g]	26	138 ± 21[f]
t-PA antigen[a]	17	5.6 ± 0.5	32	3.6 ± 0.2[e]	26	263.9 ± 0.3[e]
t-PA activity[b]	20	1476 ± 211	32	552 ± 43[f]	26	689 ± 76[d]

[a] ng/mg protein, [b] mIU/mg protein
Significance of difference from: normal mucosa: [c] $p<0.01$, [d] $p<0.002$, [e] $p<0.001$, [f] $p<0.0005$
Significance of difference from: adenocarcinoma: [g] $p<0.0005$

The zymograms also showed an increase in u-PA activity and a decrease in t-PA activity, as determined by the lysis areas, in adenomatous polyps and

adenocarcinomas compared with normal mucosa (Figure 2). Zymographic analysis did not show any correlation between the inhibitors and u-PA specific lysis in any of the tissues studied. However, reduction of t-PA specific lysis was significantly related (all tissues $R=-0.54$, $p<0.0005$; Figure 2, Table 2) to increasing PAI-1 and PAI-2 antigen, in particular in carcinomas (respectively, $R=-0.70$, $p<0.0001$ and $R=-0.64$, $p<0.002$). The negative correlation of PAI-1 and PAI-2 with t-PA activity in the carcinomas was also recognized in the enzymatic assays (coefficients respectively, $R=-0.53$, $p<0.01$ and $R=-0.57$, $p<0.01$). In contrast, u-PA activity, which increased in the normal mucosa-adenoma-carcinoma sequence, did not show a negative correlation with PAI antigen levels. Moreover, u-PA antigen levels were found to increase in parallel with the PAI-1 and PAI-2 levels (respectively, $R=0.59$, $p<0.0005$ and $R=0.50$, $p<0.0001$). The zymogram experiments also showed a positive correlation between PAI-1 antigen and PA-PAI complex lysis areas in adenomatous polyps ($R=0.46$, $p<0.01$). Analysis of the PA-PAI complexes with regard to their electrophoretic pattern showed predominantly complexes with t-PA in both adenomas and carcinomas (Figure 2).

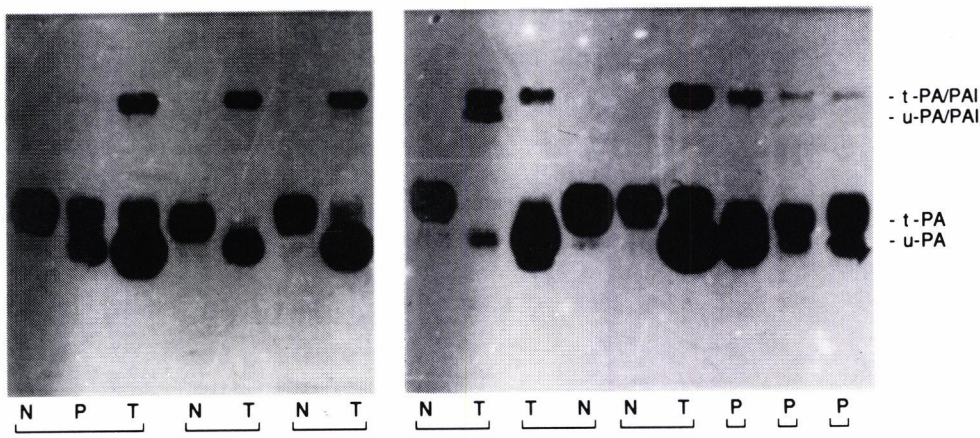

Figure 2. Zymographic analysis of human colonic tissues for plasminogen activator activity.
N: normal mucosa, P: adenomatous polyps, T: adenocarcinomas.
Brackets indicate one individual patient.
t-PA: tissue-type plasminogen activator, u-PA: urokinase-type plasminogen activator.
PA/PAI: complexes of plasminogen activators with plasminogen activator inhibitors.

Table 2. Antigen levels of plasminogen activator inhibitors PAI-1 and PAI-2 in all samples of colonic tissue compared with t-PA and u-PA activity determined by scoring specific lysis areas from zymographic analyses.

	t-PA specific lysis				u-PA specific lysis			
	PAI-1[a]		PAI-2[a]		PAI-1[a]		PAI-2[a]	
Lysis area	n	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM
not detectable	5	5.4 ± 1.4	3	7.6 ± 2.7	7	0.4 ± 0.1	13	0.9 ± 0.2
hardly visible	17	0.7 ± 0.1[b,c]	16	2.0 ± 0.4[d]	13	1.0 ± 0.6	11	2.4 ± 0.7
readily visible	36	0.5 ± 0.1[b]	31	1.3 ± 0.2	36	1.0 ± 0.3	31	1.8 ± 0.4
strong lysis	5	0.2 ± 0.1[b]	11	0.8 ± 0.2	7	1.1 ± 0.3	16	1.7 ± 0.3
		R=-0.59		R=-0.54		n.s.		n.s.
		p<0.0005		p<0.0005				

[a] ng/mg protein
Significance of difference from: not detectable: [b] p<0.05; strong lysis: [c] p<0.05, [d] p<0.02
n.s.: not significant

Comparison of the PAI antigen levels with malignancy parameters of neoplastic growth showed no relation with diameter, histological type, or grade of dysplasia in the adenomatous polyps, nor with Dukes' classification in carcinomas. However, there was a significant correlation (both R=-0.47, p<0.05) between the inhibitor antigen levels and the differentiation of the carcinomas (Table 3).

Table 3. Antigen levels of plasminogen activator inhibitors PAI-1 and PAI-2 in adenocarcinomas of the colon in relation to their differentiation.

Adenocarcinoma differentiation	PAI-1[a]		PAI-2[a]	
	n	Mean ± SEM	n	Mean ± SEM
poor	2	6.9 ± 2.2	1	12.8
moderate	14	1.4 ± 0.5[b]	12	2.4 ± 0.6
well	9	1.1 ± 0.4[c]	9	2.0 ± 0.4
		R=-0.47		R=-0.47
		p<0.02		p<0.05

[a] ng/mg protein
Significance of difference from poor differentiation: [b] p<0.005, [c] p<0.001

DISCUSSION

In the present study we surveyed the relation between plasminogen activators and their inhibitors in human colonic tissues based on antigen and activity analysis. An increased antigen level of PAI-1 and PAI-2 was found in homogenates of neoplastic tissues, i.e., adenomatous polyps and adenocarcinomas, compared with normal colon mucosa. A negative relation was found between PAI-1 and PAI-2 and t-PA activity in the zymographic analyses, in particular in the carcinomas which showed no strong t-PA bands at all, as well as in the enzymatic t-PA activity assays. In contrast, u-PA activity seemed not affected by the inhibitors, but was in fact increased in neoplastic tissues. Moreover, u-PA antigen was found to increase in parallel with PAI-1 and PAI-2 in the colonic tissues. These observations may be important with respect to the process of tumour growth and invasiveness as well as to the prognosis of these GI malignancies.

The association between plasminogen activation and neoplastic growth has been established by *in vitro* studies in animals and clinical observations (5,6). The cascade by which the plasminogen activators break down extracellular matrix is shown to be direct lysis and activation of plasmin and other proteinases like latent collagenase. The result of this proteinase activation is lysis of extracellular matrix constituents like laminin, collagen, and fibronectin (5,24,25). Subsequently, tumour cells are able to invade the basement membrane and cause metastasis formation (26,27). In most of the malignancies, u-PA is the activator involved in these dissemination processes of tumour cells. Moreover, *in vitro* studies in animal models showed that antibodies to u-PA were able to block human tumour cell invasion (28,29).

In several studies, human colorectal neoplasia was found to be associated with increased u-PA levels, both on the active enzyme level and on the antigen level in the tissue (7-11, 30-32). Moreover, these tumours showed enhanced secretion of u-PA *in vitro*, and patients with GI malignancies were found to have increased plasma and urinary levels of u-PA (13-15). Under normal physiological conditions, u-PA forms complexes with inhibitors, PAI-1 and PAI-2, when present. In patients with malignancies of the GI tract or the breast increased levels of plasminogen activator inhibitors have been found in the circulation, i.e., plasma and ascitic fluid (14,33,34). Complex formation of the inhibitors was mainly observed with t-PA and

complex formation with u-PA was either not observed or not studied. The presence of plasminogen activator inhibitors in colorectal carcinomas as shown in our study has also been observed in a previous qualitative study by Tissot *et al.* (8). Zymography showed inhibitor complexes with t-PA and u-PA in some of the carcinomas. Moreover, extracts were found to contain high concentrations of fibrinolytic inhibitors that preferentially formed complexes with u-PA. However, no quantitative data are given and specific assays for PAI-1 and PAI-2 were not available at that time. In the present study we were able, for the first time, to quantitate the amount of inhibitors in colorectal neoplasia. Moreover and more importantly, we could determine the relation and complex formation with both activators separately. The predominant presence of t-PA in the inhibitor complexes, as observed in the zymograms, confirms our previous observation that activator activity in the complexes could be inhibited by antibodies to t-PA but not to u-PA (32). As mentioned earlier, u-PA in colorectal neoplasia was enhanced both in the amount of antigen present and in activity. The difference in increase between the two parameters could be attributed to the presence of increased quantities of the inactive pro-enzyme form of u-PA as published previously (31) and seems not to be due to inactivation by either PAI-1 or PAI-2. In contrast, the decrease in t-PA activity in colonic neoplastic tissue is due to a decrease in the production of antigen and inhibition by both PAI-1 and PAI-2. The identification of the cells from which both inhibitors originated in the neoplastic lesions of the colon was not established in the present biochemical study, but they could probably be identified by immunohistological evaluation of tissue sections.

Because either active t-PA or u-PA was found to be present in our tissue homogenates it is obvious that in colonic tissues there is an excess of activators compared with their inhibitors, i.e., t-PA in normal mucosa and u-PA in neoplastic tissues. Receptor binding of active u-PA or inactive pro-u-PA, allowing the latter to convert into the active enzyme, has been found to be very important in the regulation of plasminogen activation by u-PA (35). However, studies on the accessibility of receptor-bound u-PA to PAI-1 and PAI-2 provided contradictory results. Cell-bound u-PA was readily inhibited by either one or both inhibitors using human HT-1080 fibrosarcoma cells (36), U-937 monocyte-like cells (37,38), and peripheral blood monocytes (39). In contrast, other studies with human blood

monocytes showed less inhibition of receptor bound u-PA compared to fluid phase u-PA by PAI-1 and PAI-2 (40). More importantly, studies with human colorectal cancer cell lines showed high numbers of u-PA receptors and shielding or insensitivity of receptor-bound u-PA to PAI-2 and PAI-1, respectively (41-44). These observations put more emphasis on the malignancy associated increase of u-PA in the colon which seems to escape the normal physiological control mechanisms of the body.

From recent studies on plasminogen activator expression and the prognosis of cancer, two observations with major implications emerged. Firstly, u-PA was found to be a marker that was positively associated with aggressiveness and prognosis of breast cancer (45,46). High u-PA levels in breast cancer tissue were significantly associated with a short disease-free interval after operation with a relative risk of 21.1 compared with less than 6 for hormone receptor status and lymph node involvement (47). Secondly, low t-PA levels, inverse to the u-PA levels, were indicative for a shorter disease-free interval and survival of these patients as opposed to those with high t-PA levels (48). These observations show a remarkable parallel with the increased u-PA and decreased t-PA levels in colorectal neoplasia we have found. Moreover, they are indicative for a crucial role of u-PA in the progression of malignancies.

REFERENCES

1. Hekman CM, Loskutoff DJ, Fibrinolytic pathways and the endothelium. Semin Thromb Hemost 1987; 13: 514-527.
2. Saksela O. Plasminogen activation and regulation of pericellular proteolysis. Biochim Biophys Acta 1985; 823: 35-65.
3. Saksela O, Rifkin DB. Cell-associated plasminogen activation: Regulation and physiological functions. Ann Rev Cell Biol 1988; 4: 93-126.
4. Hart DA, Rehemtulla A. Plasminogen activators and their inhibitors: Regulators of extracellular proteolysis and cell function. Comp Biochem Physiol 1988; 90: 691-708.
5. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation and cancer. Adv Cancer Res 1985; 44: 139-266.
6. Markus G. The relevance of plasminogen activators to neoplastic growth - a review of recent literature. Enzyme 1988; 40: 158-172.
7. Corasanti JG, Celik C, Camiolo SM, et al. Plasminogen activator content of human colon tumours and normal mucosaes: Separation of enzymes and partial purification. J Natl Cancer Inst 1980; 65: 345-352.
8. Tissot JD, Hauert J, Bachmann F. Characterization of plasminogen activators from normal human breast and colon and from breast and colon carcinomas. Int J Cancer 1984; 34: 295-302.

9. Gelister JSK, Mahmoud M, Lewin MR, Gaffney PJ, Boulos PB. Plasminogen activators in human colorectal neoplasia. Br Med J 1986; 293: 728-731.
10. de Bruin PAF, Verspaget HW, Griffioen G, Nap M, Verheijen JH, Lamers CBHW. Plasminogen activator activity and composition in human colorectal carcinomas. Fibrinolysis 1987; 1: 57-62.
11. de Bruin PAF, Griffioen G, Verspaget HW, Verheijen JH, Lamers CBHW. Plasminogen activators and tumour development in the human colon: activity levels in normal mucosa, adenomatous polyps, and adenocarcinomas. Cancer Res 1987; 47: 4654-4657.
12. Verspaget HW, Verheijen JH, de Bruin PAF, Griffioen G, Lamers CBHW. Plasminogen activators in (pre)malignant conditions of the colorectum. Eur J Clin Oncol 1989; 25: 565-569.
13. Markus G, Camiolo SM, Kohga S, Madeja JM, Mittelman A. Plasminogen activator secretion of human tumors in short-term organ culture, including a comparison of primary and metastatic colon tumors. Cancer Res 1983; 43: 5517-5525.
14. Kirchheimer JC, Huber K, Wagner O, Binder BR. Pattern of fibrinolytic parameters in patients with gastrointestinal carcinomas. Br J Haematol 1987; 66: 85-89.
15. Nishino N, Aoki K, Tokura Y, Sakaguchi S, Takada Y, Takada A. The urokinase type of plasminogen activator in cancer of digestive tracts. Thromb Res 1988; 50: 527-535.
16. Sprengers ED, Kluft C. Plasminogen activator inhibitors. Blood 1987; 69: 381-387.
17. Kruithof EKO. Plasminogen activator inhibitors - a review. Enzyme 1988; 40: 113-121.
18. Beart RW, Van Heerden JA, Beahrs OH. Evolution in the pathological staging of carcinoma of the colon. Surg Gynecol Obstet 1978; 146: 257-259.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
20. Binnema DJ, Van Iersel JJL, Dooijewaard G. Quantitation of urokinase antigen in plasma and ultured media by use of an ELISA. Thromb Res 1986; 43: 569-577.
21. Rijken DC, Van Hinsbergh VWM, Sens EHC. Quantitation of tissue-type plasminogen activator in human endothelial cell cultures by use of an enzyme immunoassay. Thromb Res 1984; 33: 145-153.
22. Granelli-Piperno A, Reich E. A study of proteases and protease inhibitor complexes in biological fluids. J Exp Med 1978; 148: 223-234.
23. Levin EG. Latent tissue plasminogen activator produced by human endothelial cells in culture: evidence for an enzyme-inhibitor complex. Proc Natl Acad Sci USA 1983; 80: 6804-6808.
24. Boyd D, Ziober B, Chakrabarty S, Brattain M. Examination of urokinase protein/transcript levels and their relationship with laminin degradation in cultured colon carcinoma. Cancer Res 1989; 49: 816-820.
25. Gold LI, Schwimmer R, Quigley JP. Human plasma fibronectin as a substrate for human urokinase. Biochem J 1989; 262: 529-534.
26. Markus G. The role of hemostasis and fibrinolysis in the metastatic spread of cancer. Semin Thromb Hemost 1984; 10: 61-70.
27. Reich R, Thompson EW, Iwamoto Y, et al. Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res 1988; 48: 3307-3312.
28. Ossowski L. In vivo invasion of modified chorioallantoic membrane by tumor cells: the role of cell surface-bound urokinase. J Cell Biol 1988; 107: 2437-2445.
29. Ossowski L. Plasminogen activator dependent pathways in the dissemination of human tumor cells in the chick embryo. Cell 1988; 52: 321-328.
30. Suzumiya J, Hasui Y, Kohga S, Sumiyoshi A, Hashida S, Ishikawa E. Comparative study of plasminogen activator antigens in colonic carcinomas and adenomas. Int J Cancer 1988; 42: 627-632.

31. Sim PS, Stephens RW, Fayle DRH, Doe WF. Urokinase-type plasminogen activator in colorectal carcinomas and adenomatous polyps: quantitative expression of active and proenzyme. Int J Cancer 1988; 42: 483-488.
32. de Bruin PAF, Griffioen G, Verspaget HW, et al. Plasminogen activator profiles in neoplastic tissues of the human colon. Cancer Res 1988; 48: 4520-4524.
33. de Jong E, Knot EAR, Piket D, et al. Increased plasminogen activator inhibition levels in malignancy. Thromb Haemost 1987; 57: 140-143.
34. Huber K, Wojta J, Kirchheimer JC, Ermler D, Binder BR. Plasminogen activators and plasminogen activator inhibitor in malignant and non-malignant ascitic fluid. Eur J Clin Invest 1988; 18: 595-599.
35. Blasi F, Vassalli J-D, Danø K. Urokinase-type plasminogen activator: proenzyme, receptor, and inhibitors. J Cell Biol 1987; 104: 801-804.
36. Stephens RW, Pöllänen J, Tapiovaara H, et al. Activation of pro-urokinase and plasminogen on human sarcoma cells: a proteolytic system with surface-bound reactants. J Cell Biol 1989; 108: 1987-1995.
37. Cubellis MV, Andreasen P, Ragno P, Mayer M, Danø K, Blasi F. Accessibility of receptor-bound urokinase to type-1 plasminogen activator inhibitor. Proc Natl Acad Sci USA 1989; 86: 4828-4832.
38. Ellis V, Wun T-C, Behrendt N, Rønne E, Danø K. Inhibition of receptor-bound urokinase by plasminogen-activator inhibitors. J Biol Chem 1990; 265: 9904-9908.
39. Estreicher A, Mühlhauser J, Carpentier J-L, Orci L, Vassalli J-D. The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. J Cell Biol 1990; 111: 783-792.
40. Kirchheimer C, Remold HG. Functional characteristics of receptor-bound urokinase on human monocytes: catalytic efficiency and susceptibility to inactivation by plasminogen activator inhibitors. Blood 1989; 4: 1396-1402.
41. Boyd D, Florent G, Kim P, Brattain M. Determination of the levels of urokinase and its receptor in human colon carcinoma cell lines. Cancer Res 1988; 48: 3112-3116.
42. Schlechte W, Murano G, Boyd D. Examination of the role of the urokinase receptor in human colon cancer mediated laminin degradation. Cancer Res 1989; 49: 6064-6069.
43. Baker MS, Bleakley P, Woodrow GC, Doe WF. Inhibition of cancer cell urokinase plasminogen activator by its specific inhibitor PAI-2 and subsequent effects on extracellular matrix degradation. Cancer Res 1990; 50: 4676-4684.
44. Schlechte JW, Boyd DD. Insensitivity of laminin degradation directed by receptor bound urokinase to PAI-1 in cultured colon cancer. Cancer Commun 1990; 2: 261-269.
45. Duffy MJ, O'Grady P, Devaney D, et al. Urokinase-plasminogen activator, a marker for aggressive breast carcinomas. Cancer 1988; 62: 531-533.
46. Needham GK, Nicholson S, Angus B, Farndon JR, Harris AL. Relationship of membrane-bound tissue type and urokinase type plasminogen activators in human breast cancers to estrogen and epidermal growth factor receptors. Cancer Res 1988; 48: 6603-6607.
47. Jänicke F, Schmitt M, Ulm K, Gössner W, Graeff H. Urokinase-type plasminogen activator antigen and early relapse in breast cancer. Lancet 1989; ii: 1049.
48. Duffy MJ, O'Grady P, Devaney D, O'Siorain L, Fennelly JJ, Lijnen HR. Tissue-type plasminogen activator, a prognostic marker in breast cancer. Cancer Res 1988; 48: 1348-1349.

Chapter V

PRESENCE AND ACTIVITY OF PLASMINOGEN ACTIVATORS AND INHIBITOR TYPE-1 IN NEOPLASTIC COLONIC TISSUE FROM PATIENTS WITH FAMILIAL ADENOMATOUS POLYPOSIS

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submitted

SUMMARY

Plasminogen activators are able to degrade a broad spectrum of components of the basement membrane and the extracellular matrix via the activation of plasminogen. Colorectal neoplastic tissues have previously been shown to contain high concentrations of urokinase-type plasminogen activator (u-PA) and inhibitors type-1 and type-2 (PAI-1 and PAI-2), accompanied by decreased levels of tissue-type plasminogen activator (t-PA), compared to normal colonic mucosa. In this study we evaluated plasminogen activator activity and antigen levels, and antigen levels of PAI-1 in homogenates of normal colonic mucosa, adenomatous polyps, and adenocarcinomas of 19 patients with familial adenomatous polyposis coli. Adenomas and carcinomas contained significantly more u-PA antigen than normal appearing mucosa. The increment of the u-PA antigen concentration in carcinomas was also reflected in the activity level of u-PA. The antigen and activity levels of t-PA in neoplastic tissues were significantly decreased compared to normal tissue. Moreover, there was a significant linear correlation between the decrease in t-PA, particularly t-PA activity, and the increase in size of the adenomas. The PAI-1 antigen level was found to be low in normal mucosa and adenomas, and high in carcinomas. Zymographic analyses showed that u-PA and plasminogen activator/inhibitor complex bands increased in the normal mucosa-adenoma-carcinoma sequence.

These data indicate that the conversion of normal mucosa to neoplastic tissue in familial adenomatous polyposis coli patients is associated with an increase in u-PA and the inhibitor PAI-1, accompanied by an increase in activator/inhibitor complexes. In contrast, the level of t-PA is decreased in neoplastic tissue of these patients. These observations are essentially similar to those found in sporadic adenomas and carcinomas of the colon.

INTRODUCTION

Tumourigenesis is considered to be a multistep process (1). Most human colorectal carcinomas arise from pre-existing benign adenomatous polyps or adenomas: the adenoma-carcinoma sequence (2). The existence and availability of these recognizable premalignant stadia provide an exceptional

opportunity to examine human colorectal carcinogenesis. Familial adenomatous polyposis coli (FAP) is a dominantly inherited autosomal disorder, characterized by an early onset of multiple adenomatous polyps in the colorectum, which, when untreated, will inevitably lead to colorectal carcinoma. Recently the gene which is responsible for adenomatous polyposis coli (APC) has been identified and characterized (3). Several investigators have documented the hyperproliferative state of the colonic mucosa in sporadic adenomatous polyps and in adenomas from polyposis coli patients (4-8). Cell proliferation and DNA-aneuploidy of FAP adenomas were not found to be significantly different from sporadic adenomas (5). This increased proliferation is generally associated with an increased cancer risk (1,8). Cell proliferation has also been linked to plasminogen activator expression via the phosphatidyl-inositol-diphosphate (PIP-2) pathway. Inappropriate expression of oncogenes seems to operate via the cleavage of PIP-2 and this mechanism provides a link between oncogene associated transformation, cellular proliferation, plasminogen activator expression, and tumour invasion (9).

Plasminogen activators, tissue-type (t-PA) and urokinase-type (u-PA), convert the inactive pro-enzyme plasminogen into active plasmin, a potent protease which is able to degrade several proteins of the extracellular matrix. The proteolytic activity of plasmin is controlled by a complex cascade of interactions involving receptors, (pro-)activators, and inhibitors (10,11). In addition, u-PA appears to influence the proliferation of (tumour) cell lines in an autocrine fashion (12). In tumour cell lines and in solid tumour tissues of different origin, the coordination of the balance between the components of this cascade seems to be disturbed resulting in high proteolytic activities of mainly the urokinase-type plasminogen activator (13-21).

Immunohistochemical studies have shown, that in normal colonic mucosa t-PA is present in abundant amounts, mainly in endothelial cells of capillaries, venules, small veins, and arteries, while u-PA staining was confined to occasional epithelial cells and to some cells in the lamina propria (22). t-PA staining in carcinoma tissue resembled normal mucosa, showing positive endothelial cells varying from similar to rather weak compared to normal mucosa. Staining of colonic adenomas and carcinomas for u-PA, however,

showed a dramatic difference with normal mucosa. Several studies found u-PA to be located in (pre)malignant epithelial cells and in the cytoplasm of fibroblast-like cells (15,20,22-25). The presence of u-PA mRNA in fibroblast-like cells, but not in malignant cells of colonic carcinomas was revealed by in situ hybridization (26). These data indicated, that especially the urokinase-type plasminogen activator could play a role in the genesis of adenomas and carcinomas of the colon, probably by promoting local growth and proteolysis.

In the present study normal tissue, adenomatous polyps, and carcinomas were collected from patients operated because of familial adenomatous polyposis coli. The contents of plasminogen activators and inhibitor type-1 were determined in homogenates of the normal appearing mucosa and neoplastic tissues with ELISA's, enzymatic activity assays, and a zymographic method. Adenomatous polyps were classified according to their diameter which was found, in other studies, to be correlated with the onset of malignancy (1,27), and the relationship with the presence and activity of plasminogen activators and inhibitor type-1 was investigated.

PATIENTS, MATERIALS AND METHODS

Patients

Nineteen patients (9 men, 10 women; mean age 26 years) undergoing colectomy for familial adenomatous polyposis coli were included in this study, providing us with 3 invasive carcinomas, 85 adenomatous polyps, and 31 representative parts of normal-appearing mucosa from various parts of the colon. Of all samples, adjacent fragments were histologically evaluated by the pathologist to confirm the diagnosis and origin of the tissue. One of the adenomatous polyps was found to be a carcinoma in situ and was considered carcinoma in the further study. All tissues were immediately frozen at -70°C until analysis.

Tissue extraction and protein concentration

Tissue specimens were homogenized in 1 ml 0.1% (v/v) Tween 80; 0.1 M Tris-HCl (pH 7.5) per 60 mg wet tissue as described before (28). The homogenates were centrifuged twice at 8 x 10^3 *g* for 2.5 minutes, 4°C. Protein

concentration of the supernatants was determined by the method of Lowry *et al.* (29).

Plasminogen activator activity assay

Activities of u-PA and t-PA were measured by a spectrophotometric enzyme activity assay as described previously (30). In brief, tissue extract was incubated with plasminogen, fragments of fibrinogen and S-2251 a chromogenic plasmin substrate (Kabi, Sweden) to detect total plasminogen activator activity. t-PA and u-PA activities were determined by adding specific inhibiting antibodies against t-PA and u-PA, rabbit anti-human t-PA IgG and goat anti-human u-PA IgM/IgD respectively, to parallel incubations and calculating the amount of inhibition. u-PA and t-PA standard preparations (National Institute of Biological Standards and Control, London, UK, batch nrs 66/46 and 83/517, respectively) were included. The inhibiting antibodies used were monospecific, showed no cross-reactivity, and blocked maximum standard u-PA and t-PA completely.

ELISA for u-PA

The sandwich ELISA for u-PA was carried out according to Binnema *et al.* (31). Rabbit anti-u-PA was used as catching antibody and after incubation of the samples, affinopurified goat anti-u-PA IgG (0.8 µg/ml) was added and incubated. After washing, 100 µl of an optimal dilution of donkey anti-goat IgG conjugated with alkaline phosphatase was added and 100 µl para-nitrophenyl-phosphate (1 mg/ml) was used as substrate. The amount of u-PA antigen in the samples was calculated from a 9-points standard curve of u-PA (0-3.3 ng/ml).

ELISA for t-PA

t-PA antigen was measured essentially as described by Rijken *et al.* (32). Goat anti-t-PA was used as catching antibody, an anti-t-PA-horseradish peroxidase conjugate (Biopool, Sweden) as second antibody and 3,3',5,5' tetramethylbenzidine was used as substrate. Absolute quantities of t-PA antigen in the samples were calculated from an 8-points standard curve of t-PA (Biopool, Sweden, 0-4 ng/ml).

ELISA for PAI-1

Total PAI-1 antigen, i.e. latent, active, and complexed PAI-1, was determined using the Tintelize PAI-1 ELISA (Biopool, Sweden) without prior denaturation of the samples as described previously (33). In brief, mouse monoclonal anti-human PAI-1 was used as catching antibody. After incubation with the tissue homogenates a goat polyclonal anti-human PAI-1, conjugated to peroxidase, was used to form a "sandwich"-ELISA and ortho-phenylenediamine was added as substrate. The assay included the use of quenching and non-specific antibodies to exclude falsely elevated results. In order to increase the sensitivity of the assay sample volumes of up to 80 µl were used, in stead of the recommended 20 µl, resulting in a detection limit of 0.3 ng/ml.

Zymography

Tissue extracts were incubated for 1 hour (2% (w/v) SDS, 37°C) to induce activator activity in plasminogen activator/inhibitor complexes (34). Electrophoresis of the samples took place on 10% polyacrylamide gels with sodiumdodecylsulphate (SDS-PAGE). Plasminogen activator activities were visualized on agarose underlay gels containing plasminogen and fibrin (35).

Statistical analysis

Results are given as mean ± SEM. Differences between group means were tested for significance using Student's *t*-test with separate variance estimate if the standard deviations were significantly different according to the F-test. Correlations were evaluated using linear regression statistics. Differences and correlations were considered significant when $p<0.05$.

RESULTS

Within the normal colon no significant differences in the plasminogen activators levels were detected in relation to the location of the tissue. Therefore, the mean value of the whole colon per patient is given (n=15).

The mean plasminogen activator levels in normal and neoplastic colonic tissue from patients with polyposis coli are shown in figures 1 and 2. Significant differences were found between normal mucosa and neoplastic tissues for both

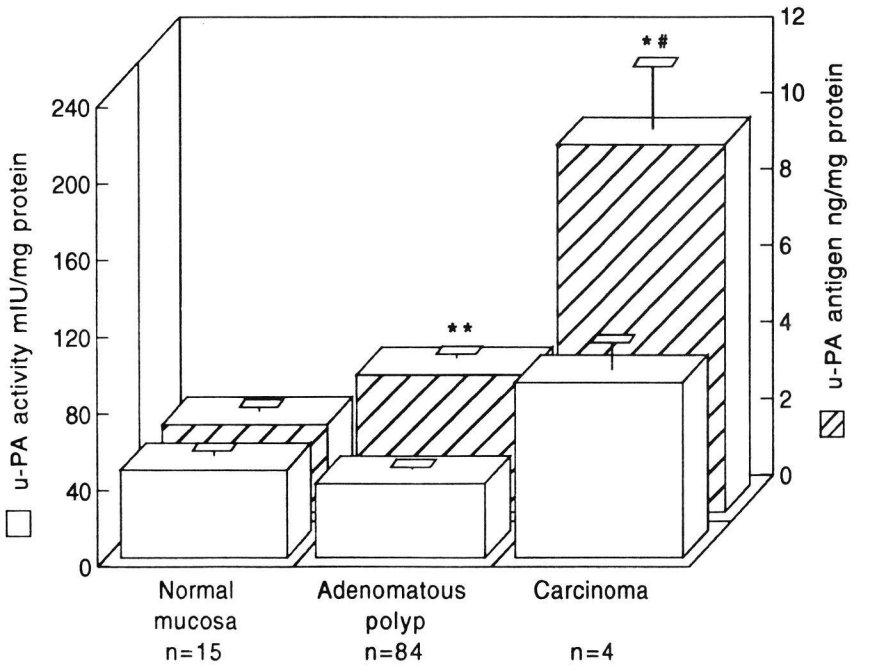

Figure 1. Activity and antigen of urokinase-type plasminogen activator (u-PA) in homogenates of normal appearing colonic mucosa and neoplastic tissue from patients with familial adenomatous polyposis coli.
Significance of difference from normal mucosa: * $p<0.05$; ** $p<0.005$.
Significance of difference from adenomatous polyp: # $p<0.05$.

plasminogen activators. u-PA levels were two to four fold increased and t-PA concentrations were approximately three fold decreased in carcinomatous tissue compared to normal mucosa. In general, plasminogen activator levels in homogenates of adenomatous polyps were in between normal and carcinomatous tissue values. As expected, the calculated ratio of the increased u-PA antigen concentration and the decreased t-PA antigen level in tumour tissue homogenates was significantly increased in comparison with normal mucosa (Table 1).

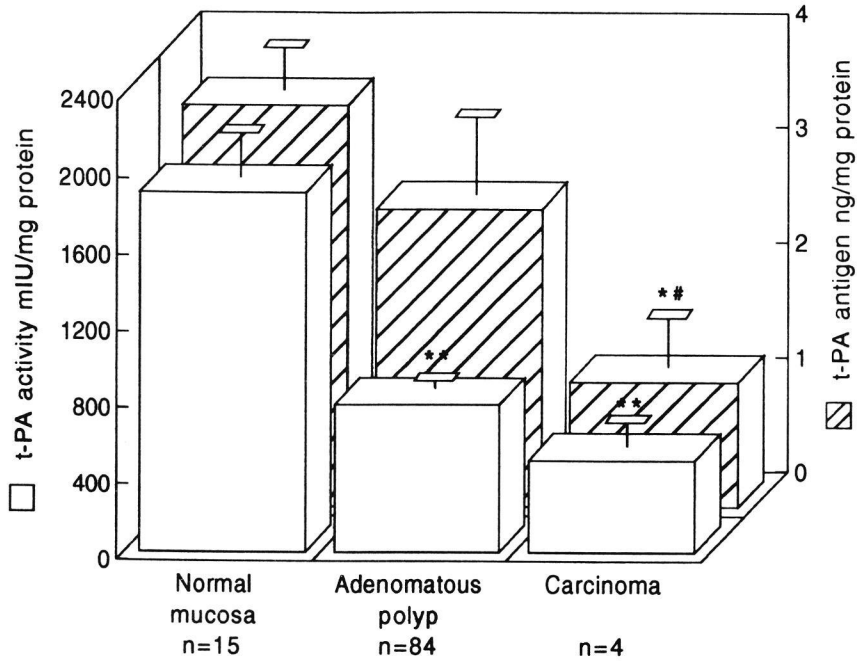

Figure 2. Activity and antigen of tissue-type plasminogen activator (t-PA) in homogenates of normal appearing colonic mucosa and neoplastic tissue from patients with familial adenomatous polyposis coli. Significance of difference from normal mucosa: * $p<0.05$; ** $p<0.005$. Significance of difference from adenomatous polyp: # $p<0.05$.

Table 1. Antigen ratio of urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), and the level of plasminogen activator inhibitor type-1 (PAI-1) in normal appearing mucosa and neoplastic tissue from patients with familial adenomatous polyposis coli.

	Normal mucosa	Adenoma	Carcinoma
u-PA/t-PA antigen ratio	0.8 ± 0.1 (n=15)	3.0 ± 0.7^{b} (n=84)	11.4 ± 3.6^{c} (n=4)
PAI-1 ng/mg protein	0.2 ± 0.1 (n=3)	0.3 ± 0.0 (n=24)	1.2 ± 0.3^{a} (n=4)

Significance of difference from normal mucosa: ^{a} $p<0.05$; ^{b} $p<0.005$.
Significance of difference from adenoma: ^{c} $p<0.05$.

With respect to PAI-1, normal mucosae and adenomas were found to have low levels of this inhibitor compared to the concentration in the carcinomas (Table 1).

Figure 3 shows the distribution of t-PA activity in adenomatous polyps, classified according to their diameter, in comparison with normal mucosa and carcinomas from the colon of patients with polyposis coli. t-PA activity was found to decrease with increasing size of the adenomas (R=-0.47, p<0.0001), in

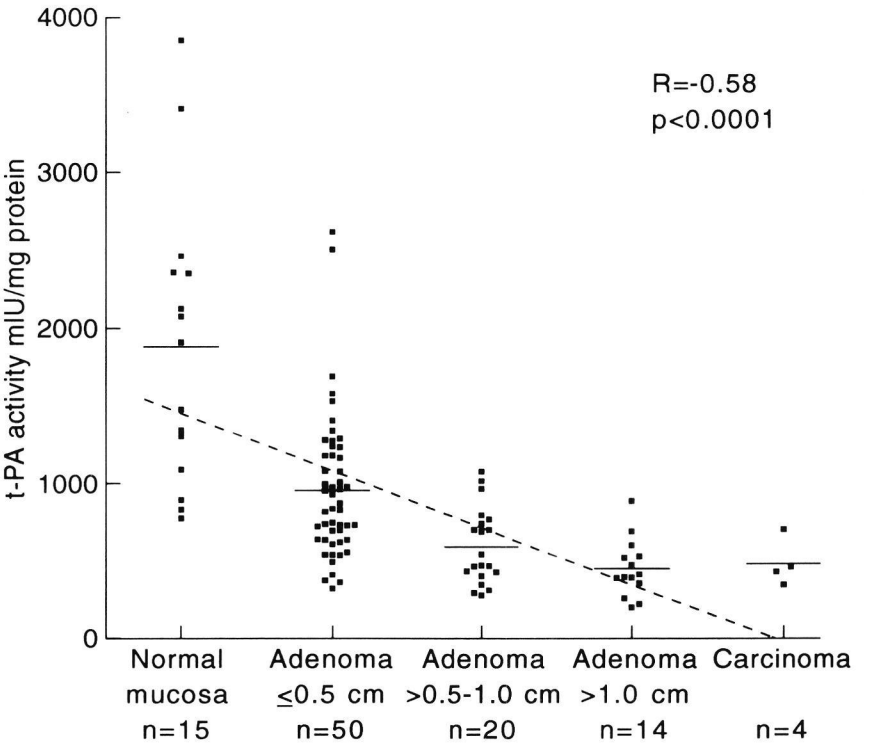

Figure 3. Distribution of the activity of tissue-type plasminogen activator (t-PA) according to size of adenomatous polyps from patients with familial adenomatous coli compared to normal appearing mucosa and carcinomas of the same patients. Bars indicate mean values of the groups.

line with the changes seen in the normal mucosa-adenoma-carcinoma sequence (R=-0.58, p<0.0001). t-PA antigen in adenomas showed a similar tendency in relation with the diameter. u-PA and PAI-1 were not found to change in parallel with the size of the adenomas. The results of the zymographic analysis of the homogenates confirmed the results of the activity and antigen assays, normal tissues and adenomas showed strong lysis in the t-PA area, whereas carcinomas and many adenomas were found to give strong u-PA lysis bands on the fibrin containing underlays (Table 2). Complexes of plasminogen activators with their specific inhibitors were hardly seen in normal tissue but regularly in the neoplastic tissues.

Table 2. Number of strong lysis bands of tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), and plasminogen activator/inhibitor complexes, scored on zymographic underlay gels of colonic tissue homogenates derived from patients with familial adenomatous polyposis coli.

Lysis band	Normal mucosa n=15		Adenoma <0.5 cm n=49		Adenoma ≥0.5-1.0 cm n=20		Adenoma ≥1.0 cm n=13		Carcinoma n=4	
		%		%		%		%		%
t-PA	15	100	49	100	20	100	13	100	3	75
u-PA	2	13	17	35	10	50	6	46	4	100
Complexes	1	7	6	12	8	40	2	15	2	50

Complexes: plasminogen activator/inhibitor complexes.

DISCUSSION

Although the existence of a colonic disorder like familial adenomatous polyposis coli offers an excellent opportunity to study the relation between plasminogen activators and the genesis of colorectal tumours, only few studies reported about the presence of these proteinases in tissue from these patients. Corasanti *et al.* (36) found low plasminogen activator activity in normal mucosa and adenocarcinoma of a single patient, while Sim *et al.* (37) reported high u-PA activity levels in adenomas but also in normal mucosa from two polyposis patients. The presence of microscopic polyps in the normal appearing tissue in the latter study, however, could not be excluded.

In the present study neoplastic tissues from patients with familial adenomatous polyposis coli were found to have high concentrations of urokinase-type plasminogen activator and inhibitor type-1, and decreased levels of tissue-type plasminogen activator compared with normal appearing colonic mucosa of the same patients. These phenomena have already been observed, by several investigators, in colonic tissue from patients with a sporadic, i.e. without a known hereditary background, adenoma or carcinoma. In fact, the changes of u-PA, t-PA, and PAI-1 levels of neoplastic polyposis tissue were comparable to those found in our previous studies on patients with sporadic adenomas and carcinomas of the colon (21,33).

Many studies, mainly *in vitro*, have been published about the role of u-PA in physiologic processes in which this enzyme could be involved, for instance promotion of migration of cells in tissue remodeling, myogenic differentiation, and wound healing (38-40), but also in the regulation of cell growth and proliferation (41,42). Cell proliferation in normal colonic epithelium takes place in the lower 1/3 zone of the crypts of Lieberkühn, and was found to be extended in adenomas of the colon (6-8). In a study with 171 polyps from 20 FAP patients, large adenomas (>0.5 cm) showed a higher level of cell proliferation than smaller adenomas, with no difference in the grade of dysplasia between the two groups (5). Immunohistochemical studies have not shown intensive staining of normal colonic epithelium cells for u-PA. Extensive u-PA positive staining has been observed, however, in colonic adenomas, especially in areas with dysplasia, and carcinomas (23,24), suggesting a role for this enzyme in the enhanced process of cell proliferation. The significant correlation between the decrease in t-PA and increasing size of the adenomas, as found in this study, was also found in sporadic colonic adenomas (21).

Neoplastic colonic cells have been shown to possess less organized cytoskeletal structures (43). Disruption of the microfilament structure of cultured cells has been shown to inhibit the production of t-PA, and to stimulate production of u-PA, inhibitor PAI-1, and other proteinases (44-46). PAI-1 levels are not only enhanced in breast cancer, but also in carcinomas and sporadic adenomatous polyps from the colon (33,47,48). Also in the present study, increased amounts of PAI-1 in neoplastic tissues were found to be

accompanied by high molecular lysis zones of these tissue homogenates on the zymograms representing complexes of activators and inhibitors. The enhancement of the urokinase-type plasminogen activator and the specific inhibitor PAI-1, which was also found *in vivo* and *in vitro* with melanoma cell lines (13), could be associated to the disruption of the cytoskeletal organization within the neoplastic cells.

Genesis of adenomatous polyps in polyposis coli is characterized by increased cell proliferation, a process which is closely related to cell migration and cell growth. The urokinase-type plasminogen activator has been shown to be involved in a great number of this kind of processes and is also found to be present in increased amounts in colorectal adenomas and carcinomas of patients with or without polyposis coli. Further studies of the plasminogen activation system in tissue from these patients, in which successive stadia of (pre)malignancy are present in one patient, could make an important contribution to the understanding of the mechanism of plasminogen activation in human colorectal carcinogenesis.

REFERENCES

1. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990; 61: 759-767.
2. Morson BC. Precancerous lesions of the colon and rectum. Classification and controversial issues. JAMA 1962; 179: 316-321.
3. Groden J, Thliveris A, Samowitz W, et al. Identification and characterization of the familial adenomatous polyposis coli gene. Cell 1991; 66: 589-600.
4. Bleiberg H, Mainguet P, Galand P. Cell renewal in familial polyposis: comparison between polyps and adjacent healthy mucosa. Gastroenterology 1972; 63: 240-245.
5. Quirke P, Dixon MF, Day DW, Fozard JBJ, Talbot IC, Bird CC. DNA aneuploidy and cell proliferation in familial adenomatous polyposis. Gut 1988; 29: 603-607.
6. Terpstra OT, van Blankenstein M, Dees J, Eilers GAM. Abnormal pattern of cell proliferation in the entire colonic mucosa of patients with colon adenoma or cancer. Gastroenterology 1987; 92: 704-708.
7. Risio M, Coverlizza S, Ferrari A, Candelaresi GL, Rossini FP. Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas, and adenocarcinomas of the large bowel. Gastroenterology 1988; 94: 899-906.
8. Gerdes H, Gillin JS, Zimbalist E, Urmacher C, Lipkin M, Winawer SJ. Expansion of the epithelial cell proliferative compartment and frequency of adenomatous polyps in the colon correlate with the strength of family history of colorectal cancer. Cancer Res 1993; 53: 279-282.
9. Gelister JSK, Boulos PB, Gaffney PJ, Mahmoud M, Lewin MR. The relationship of plasminogen activators and oncogenes to tumour invasion. Eur J Surg Oncol 1990; 16: 54-59.

10. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 1985; 44: 139-266.
11. Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. J Clin Invest 1991; 88: 1067-1072.
12. Kirchheimer JC, Wojta J, Christ G, Binder BR. Proliferation of a human epidermal tumor cell line stimulated by urokinase. FASEB J 1987; 1: 125-128.
13. Quax PHA, van Muijen GNP, Weening-Verhoeff EJD, et al. Metastatic behavior of human melanoma cell lines in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-mediated matrix. J Cell Biol 1991; 115: 191-199.
14. Hasui Y, Suzumiya J, Marutsuka K, Sumiyoshi A, Hashida S, Ishikawa E. Comparative study of plasminogen activators in cancers and normal mucosae of human urinary bladder. Cancer Res 1989; 49: 1067-1070.
15. Markus G, Camiolo SM, Kohga S, Madeja JM, Mittelman A. Plasminogen activator secretion of human tumors in short-term organ culture including a comparison of primary and metastatic colon tumors. Cancer Res 1983; 43: 5517-5525.
16. Saito K, Nagashima M, Iwata M, et al. The concentration of tissue plasminogen activator and urokinase in plasma and tissues of patients with ovarian and uterine tumors. Thromb Res 1990; 58: 355-366.
17. Schmitt M, Jänicke F, Graeff H. Tumour-associated fibrinolysis: the prognostic relevance of plasminogen activators uPA and tPA in human breast cancer. Blood Coagulation Fibrinolysis 1990; 1: 695-702.
18. Sappino AP, Busso N, Belin D, Vassalli JD. Increase of urokinase-type plasminogen activator gene expression in human lung and breast carcinomas. Cancer Res 1987; 47: 4043-4046.
19. Sier CFM, Verspaget HW, Griffioen G, Ganesh S, Vloedgraven HJM, Lamers CBHW. Plasminogen activators in normal tissue and carcinomas of the human oesophagus and stomach. Gut 1993; 34: 80-85.
20. Nishino N, Aoki K, Tokura Y, Sakaguchi S, Takada Y, Takada A. The urokinase type of plasminogen activator in cancer of digestive tracts. Thromb Res 1988; 50: 527-535.
21. de Bruin PAF, Griffioen G, Verspaget HW, et al. Plasminogen activator profiles in neoplastic tissues of the human colon. Cancer Res 1988; 48: 4520-4524.
22. Burtin P, Chavanel G, Andre J. The plasmin system in human colonic tumors: An immunofluorescence study. Int J Cancer 1985; 35: 307-314.
23. Kohga S, Harvey SR, Suzumiya J, Sumiyoshi A, Markus G. Comparison of the immunohistochemical localisation of urokinase in normal and cancerous human colon tissue. Fibrinolysis 1989; 3: 17-22.
24. Sier CFM, Fellbaum C, Verspaget HW, et al. Immunolocalization of urokinase-type plasminogen activator in adenomas and carcinomas of the colorectum. Histopathology 1991; 19: 231-237.
25. Grøndahl-Hansen J, Ralfkiær E, Kirkeby LT, Kristensen P, Lund LR, Danø K. Localization of urokinase-type plasminogen activator in stromal cells in adenocarcinomas of the colon in humans. Am J Pathol 1991; 138: 111-117.
26. Pyke C, Kristensen P, Ralfkiær E, et al. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. Am J Pathol 1991; 138: 1059-1067.
27. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988; 319: 525-532.

28. de Bruin PAF, Verspaget HW, Griffioen G, Nap M, Verheijen JH, Lamers CBHW. Plasminogen activator activity and composition in human colorectal carcinomas. Fibrinolysis 1987; 1: 57-62.
29. Lowry OH, Rosebrough NJ, Farr AL, Randall R. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265-275.
30. Verheijen JH, Mullaart E, Chang GTG, Kluft C, Wijngaards G. A simple, sensitive spectrophotometric assay for extrinsic (tissue-type) plasminogen activator applicable to measurements in plasma. Thromb Haemost 1982; 48: 266-269.
31. Binnema DJ, van Iersel JJL, Dooijewaard G. Quantitation of urokinase antigen in plasma and culture media by use of an ELISA. Thromb Res 1986; 43: 569-577.
32. Rijken DC, van Hinsbergh VWM, Sens EHC. Quantitation of tissue-type plasminogen activator in human endothelial cell cultures by use of an enzyme immunoassay. Thromb Res 1984; 33: 145-153.
33. Sier CFM, Verspaget HW, Griffioen G, et al. Imbalance of plasminogen activators and their inhibitors in human colorectal neoplasia. Implication of urokinase in colorectal carcinogenesis. Gastroenterology 1991; 101: 1522-1528.
34. Levin EG. Latent tissue plasminogen activator produced by human endothelial cells in culture: Evidence for an enzyme-inhibitor complex. Proc Natl Acad Sci USA 1983; 80: 6804-6808.
35. Granelli-Piperno A, Reich E. A study of proteases and protease-inhibitor complexes in biological fluids. J Exp Med 1978; 148: 223-234.
36. Corasanti JG, Celik C, Camiolo SM, et al. Plasminogen activator content of human colon tumors and normal mucosae: Separation of enzymes and partial purification. JNatl Cancer Inst 1980; 65: 345-351.
37. Sim PS, Stephens RW, Fayle DRH, Doe WF. Urokinase-type plasminogen activator in colorectal carcinomas and adenomatous polyps: Quantitative expression of active and proenzyme. Int J Cancer 1988; 42: 483-488.
38. Sappino AP, Huarte J, Belin D, Vassalli JD. Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. J Cell Biol 1989; 109: 2471-2479.
39. Quax PHA, Frisdal E, Pedersen N, et al. Modulation of activities and RNA level of the components of the plasminogen activation system during fusion of human myogenic satellite cells in vitro. Dev Biol 1992; 151: 166-175.
40. Grøndahl-Hansen J, Lund LR, Ralfkiær E, Ottevanger V, Danø K. Urokinase- and tissue-type plasminogen activators in keratinocytes during wound reepithelization in vivo. J Invest Dermatol 1988; 90: 790-795.
41. Sumi Y, Dent MAR, Owen DE, Seeley PJ, Morris RJ. The expression of tissue and urokinase-type plasminogen activators in neural development suggests different modes of proteolytic involvement in neuronal growth. Development 1992; 116: 625-637.
42. Kirchheimer JC, Wojta J, Christ G, Hienert G, Binder BR. Mitogenic effect of urokinase on malignant and unaffected adjacent human renal cells. Carcinogenesis 1988; 9: 2121-2123.
43. Friedman E, Verderame M, Lipkin M, Pollack R. Altered actin cytoskeletal patterns in two premalignant stages in human colon carcinoma development. Cancer Res 1985; 45: 3236-3242.
44. Santell L, Marotti K, Bartfeld NS, Baynham P, Levin EG. Disruption of microtubules inhibits the stimulation of tissue plasminogen activator expression and promotes plasminogen activator inhibitor type 1 expression in human endothelial cells. Exp Cell Res 1992; 201: 358-365.

45. Botteri FM, Ballmer-Hofer K, Rajput B, Nagamine Y. Disruption of cytoskeletal structures results in the induction of the urokinase-type plasminogen activator gene expression. J Biol Chem 1990; 265: 13327-13334.
46. Unemori EN, Werb Z. Reorganization of polymerized actin: a possible trigger for induction of procollagenase in fibroblasts cultured in and on collagen gels. J Cell Biol 1986; 103: 1021-1031.
47. Sumiyoshi K, Baba S, Sakaguchi S, Urano T, Takada Y, Takada A. Increase in levels of plasminogen activator and type-1 plasminogen activator inhibitor in human breast cancer: Possible roles in tumor progression and metastasis. Thromb Res 1991; 63: 59-71.
48. Jänicke F, Schmitt M, Graeff H. Clinical relevance of the urokinase-type and tissue-type plasminogen activators and of their type 1 inhibitor in breast cancer. Semin Thromb Hemost 1991; 17: 303-312.

Chapter VI

INACTIVE UROKINASE-TYPE PLASMINOGEN ACTIVATOR AND INCREASED PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1 ARE INVOLVED IN LIVER METASTASIS FORMATION OF HUMAN COLORECTAL CANCER

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submitted

SUMMARY

Human colorectal carcinogenesis was previously found to be associated with an increased urokinase-type plasminogen activator (u-PA) expression of which the activity was not inactivated by the simultaneously enhanced levels of plasminogen activator inhibitors PAI-1 and PAI-2. The increased proteolytic activity might contribute to invasive growth and metastasis of the tumours. In the present study homogenates of liver metastases, primary colorectal carcinomas, and their respective adjacent normal tissues were evaluated regarding the level and composition of u-PA, t-PA (tissue-type plasminogen activator), PAI-1, and PAI-2. The antigen level of u-PA was significantly increased in primary carcinomas and liver metastases compared to normal colorectal and liver tissue. Concentrations of t-PA were significantly decreased in neoplastic tissues. Liver metastases showed, in contrast to the carcinomas, hardly any plasminogen activator activity, u-PA nor t-PA. The difference between primary carcinomas and liver metastases in plasminogen activator activity could be attributed to the enhanced presence of u-PA in the inactive pro-enzyme form in combination with an increased complex formation of u-PA and t-PA with inhibitors in the metastases. Liver metastases were found to have an eight fold higher content of PAI-1 antigen compared to the primary carcinomas, whereas the level of PAI-2 was found to be similar. The excess PAI antigen in the homogenates of the metastases was able to form complexes with added exogenous t-PA or u-PA, indicating the presence of uncomplexed active inhibitors. Thus, colorectal cancer metastasis in the liver is associated with an inactivation of the enhanced u-PA cascade as present in the primary carcinomas, which might cause tumour cells to reside in the liver.

INTRODUCTION

The process of tumour cell invasion and metastasis involves sequential breakdown and re-establishment of the extracellular matrix. Plasminogen activation appears to be an important mechanism in the degradation of a broad spectrum of substrates in this matrix. Conversion of plasminogen into active plasmin is regulated by a complex system containing activators, receptors, and activator inhibitors (1-3). Extracts of carcinomas of different origin contain

increased levels of mainly the urokinase-type of plasminogen activator (u-PA) compared to normal tissue extracts (4-6). Immunohistological studies have indeed shown intensive staining with antibodies against u-PA in neoplastic tissues of stomach, colon, breast, and lung, particular at the sites of invasive growth (4,6-15). Other experiments indicate that u-PA plays a key role in tumour proliferation and metastasis. Antibodies against u-PA have been found to prevent extracellular matrix degradation by RSV transformed chick fibroblasts (16) and by human melanoma cell lines (17). Ossowski and Reich (18) have shown inhibition of metastasis, but not of primary growth, of human squamous carcinoma cells in chick embryos by anti-u-PA antibodies. *In vitro* and *in vivo* studies with human tumour cell lines from melanomas, breast, lung, stomach, and colon show strong correlations of u-PA levels of the cells with invasive and metastatic potential (19-23).

Markus *et al.* (12) have compared the secretion rate of u-PA in short term organ cultures of primary and metastatic colon tumours. The secreted activities of plasminogen activators of liver metastases were lower than those of the primary colon tumours, which is in agreement with the microthrombus theory of the carcinoma-metastasis cascade. Extracts of bone metastasis from prostatic carcinomas, however, have been found to contain a significantly higher amount of u-PA activity than the primary tumours (5). Recent u-PA localization studies in adenocarcinomas of breast, colon, and lung and their corresponding lymph node metastases in general showed more u-PA positive tumour cells in the metastatic lesion than in the original carcinoma (6,24). In a preliminary study Jänicke *et al.* (7) found the u-PA content in extracts of tumour-occupied lymph nodes not to be different from primary breast carcinomas of the same patients, while the concentration of PAI-1, a specific plasminogen activator inhibitor, was twice as high in the metastases. These apparently controversial results demonstrate the complexity of the matter. The different nature of the primary tumours, the existence of two different activators and of an inactive pro-enzyme form of urokinase-type plasminogen activator, the influence of the specific inhibitors PAI-1 and PAI-2, and last but not least the presence of receptors on the tumour cells, complicate the study on the role of plasminogen activators in invasion and metastasis (25).

In this study we have evaluated the presence of plasminogen activators u-PA and t-PA, plasminogen activator inhibitors PAI-1 and PAI-2, and complexes between activators and inhibitors in homogenates of human colorectal carcinomas and liver metastases. The neoplastic tissues were compared with the corresponding normal tissues and with tissues of liver affected by other diseases. The results might contribute to a better understanding of the role of plasminogen activation in the process of tumour invasion and metastasis.

PATIENTS, MATERIALS AND METHODS

Patients

Twenty-five patients underwent a partial liver resection providing us with fourteen representative parts of colorectal metastases, three from livers with focal nodular hyperplasia, three from hepatocellular carcinomas, and five from livers with miscellaneous diseases. Representative parts of adjacent macroscopically normal tissue were selected, if possible. From four of the patients we also had frozen tissue of the former resected primary colorectal carcinoma and normal mucosa. Tumour tissue and adjacent normal mucosa of eight patients operated for colorectal carcinoma were used as extra reference tissue. All tissues were immediately frozen at -70°C until analysis and of all samples, adjacent fragments were histologically evaluated by the pathologist to confirm the diagnosis of tissue origin and disease.

Tissue extraction and protein concentration

Tissue specimens were homogenized in 1 ml 0.1% (v/v) Tween 80; 0.1 M Tris-HCl (pH 7.5) per 60 mg wet tissue as described before (26). The homogenates were centrifuged twice at 8 x 10^3 *g* for 2.5 minutes, 4°C. Protein concentration of the supernatants was determined by the method of Lowry (27).

Plasminogen activator activity assay

Activities of u-PA and t-PA were measured by a spectrophotometric enzyme assay as described previously (28). In brief, tissue extract was incubated with plasminogen, fragments of fibrinogen and the chromogenic plasmin substrate

S-2251 (Kabi, Stockholm) to detect total plasminogen activator activity. t-PA and u-PA activities were determined by adding specific inhibiting antibodies against t-PA and u-PA, rabbit anti-human t-PA IgG and goat anti-human u-PA IgM/IgD respectively, to parallel incubations and calculating the amount of inhibition. u-PA and t-PA standard preparations (National Institute of Biological Standards and Control, London, UK, batch nrs 66/46 and 83/517 respectively) were included. The inhibiting antibodies used were monospecific, showed no cross-reactivity, and blocked maximum standard u-PA and t-PA completely.

ELISA for u-PA

The sandwich ELISA for u-PA was carried out according to Binnema *et al.* (29). Rabbit anti-u-PA was used as catching antibody and after incubation of the samples, affinopurified goat anti-u-PA IgG (0.8 µg/ml) was added and incubated. After washing, 100 µl of an optimal dilution of donkey anti-goat IgG conjugated with alkaline phosphatase was added and 100 µl para-nitrophenyl-phosphate (1 mg/ml) was used as substrate. The amount of u-PA antigen in the samples was calculated from a 9-point standard curve of u-PA (0-3.3 ng/ml).

ELISA for t-PA

t-PA antigen was measured essentially as described by Rijken *et al.* (30). Goat anti-t-PA was used as catching antibody, an anti-t-PA-horseradish peroxidase conjugate (Biopool, Sweden) as second antibody and 3,3',5,5' tetramethylbenzidine was used as substrate. Absolute quantities of t-PA antigen in the samples were calculated from an 8-point standard curve of t-PA (Biopool, Sweden, 0-4 ng/ml).

ELISA for PAI-1

Total PAI-1 antigen, i.e. latent, active, and complexed PAI-1, was determined using the Tintelize PAI-1 ELISA (Biopool, Umeå, Sweden) without prior denaturation of the samples as described previously (31). In brief, mouse monoclonal anti-human PAI-1 was used as catching antibody. After incubation with the tissue homogenates a goat polyclonal anti-human PAI-1, conjugated to peroxidase, was used to form a "sandwich"-ELISA and ortho-phenylenediamine

was added as substrate. The assay included the use of quenching and non-specific antibodies to exclude falsely elevated results. In order to increase the sensitivity of the assay sample volumes of up to 80 µl were used, in stead of the recommended 20 µl, resulting in a detection limit of 0.3 ng/ml.

ELISA for PAI-2

The determination of PAI-2 antigen was performed using the Tintelize PAI-2 ELISA (Biopool) as reported before (31). The first antibody used was mouse monoclonal anti-human PAI-2 and the second was goat polyclonal anti-PAI-2 IgG conjugated to peroxidase. Ortho-phenylenediamine was added as substrate. Unspecific response was excluded using quenching antibodies. The detection limit was decreased to 0.5 ng/ml by using 50 µl homogenate instead of 20 µl and by increasing sample incubation, conjugate incubation, and substrate incubation times.

Zymography

Tissue extracts were incubated for 1 hour (2% (w/v) SDS, 37°C) to induce activator activity in PA-PAI complexes (32). Electrophoresis of the samples took place on 10% polyacrylamide gels with sodiumdodecylsulphate (SDS-PAGE). Plasminogen activator activities were visualized on agarose underlay gels containing plasminogen and fibrin (33).

Bio-immunoassay for u-PA (BIA)

This assay is a combination of the u-PA ELISA and the enzymatic determination of u-PA in the chromogenic assay and detects u-PA and pro-u-PA separately (34). Total u-PA antigen was immuno-immobilized as in the ELISA. After incubation of the samples, 4 wells of the microtiter plate were incubated with plasmin (30 minutes, 14 U/ml) to convert pro-u-PA to active u-PA and 4 wells with buffer (0.01% (v/v) Tween 80, 0.02% (v/v) NaN_3, 0.1% (w/v) BSA in PBS). After washing the activities were spectrophotometrically measured using plasminogen and S-2251 as substrate. Enzyme activities were calculated from a standard curve of u-PA (National Institute of Biological standards and control, London, UK, batch nr 66/46). Specificity was checked by

inclusion of activity inhibiting goat antihuman u-PA IgM/IgD in 4 of the wells. Pro-u-PA was calculated by subtraction of plasmin activated total u-PA activity and the non-activated u-PA activity.

Statistical analysis

Results are given as mean ± SEM. Differences between group means were tested for significance using Student's *t*-test with separate variance estimate if the standard deviations were significantly different according to the F-test. Differences were considered significant when $p<0.05$.

RESULTS

The mean amounts of urokinase plasminogen activator in homogenates of colorectal carcinomas and liver metastases, compared to normal colorectal mucosa and normal liver tissue, are shown in figure 1. The concentration of u-PA antigen in colorectal carcinomas and liver metastases was significantly increased compared to their respective control tissues. This high antigen level was accompanied by a high u-PA activity in the primary carcinomas but, in contrast, liver metastases showed hardly any u-PA activity (Figure 1). The concentration of t-PA antigen was significantly lower in neoplastic tissues compared with their respective normal tissues, which was associated with a decrease or absence of t-PA activity in the carcinomas and metastases respectively (Figure 2).

Zymography of the homogenates from neoplastic and normal control tissues showed good agreement with the results of the enzymatic activity assay, i.e. increased u-PA activity and decreased t-PA activity in colon carcinomas compared to normal colon tissue, and virtually no t-PA or u-PA activity in liver metastases or normal liver tissue (Figure 3*a*). Especially colon carcinomas and metastases, and to some extent normal livers, showed plasminogen activator activity in high molecular weight regions (95-110 kd) of the zymogram, representing complexes of either t-PA or u-PA with plasminogen activator inhibitors (Table 1). After prolonged exposure of the underlay to the gel, eventually weak u-PA lysis bands appeared in most of the metastasis homogenates, suggesting the presence of u-PA in the pro-enzyme form.

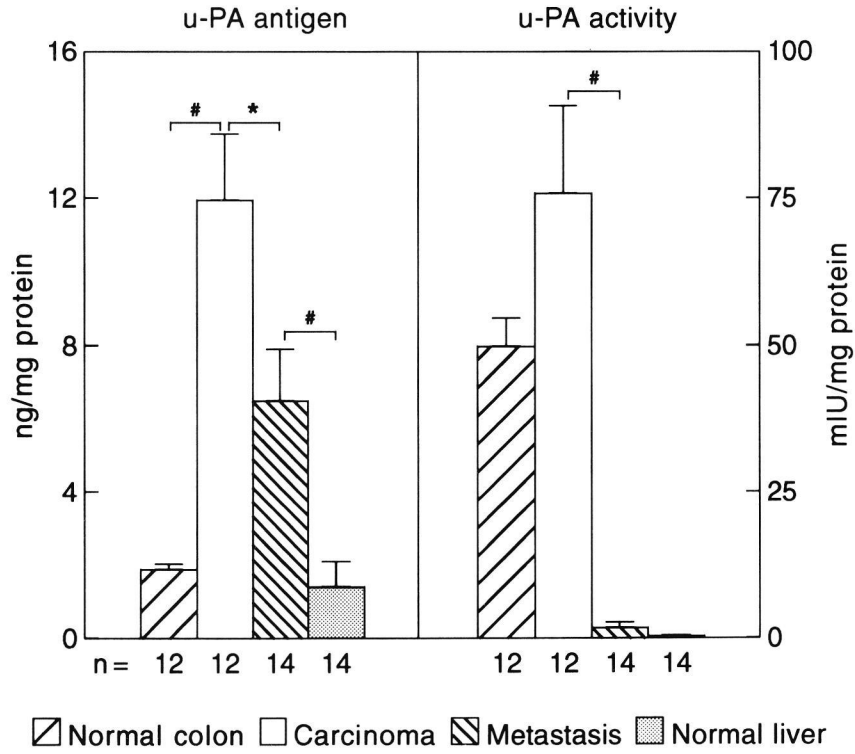

Figure 1. Concentration and activity of urokinase-type plasminogen activator (u-PA) in homogenates of human colorectal carcinoma and liver metastasis compared with adjacent normal tissues (mean ± SEM). Significance of differences: * $p<0.05$; # $p<0.005$.

Table 1. Number of homogenates in which specific lysis bands appeared after zymography of tissue homogenates of normal colon, colorectal carcinoma, liver metastasis, and normal liver. Homogenates were incubated with SDS and electrophorized on SDS-PAGE gels. Plasminogen activator activity lysis bands were scored on underlay gels containing plasminogen and fibrin.

Tissue	n	u-PA lysis bands	t-PA lysis bands	complex lysis bands	t-PA complex	u-PA complex
normal colon	11	5	11	3	3[a]	1[a]
carcinoma colon	12	12	8	11	10	5
liver metastasis	14	13[b]	0[a]	13	13	9
normal liver	14	4[b]	1[a]	10	10	1[a]

[a] very poor lysis, [b] lysis appeared after prolonged exposure.
u-PA = urokinase-type plasminogen activator, t-PA = tissue-type plasminogen activator.

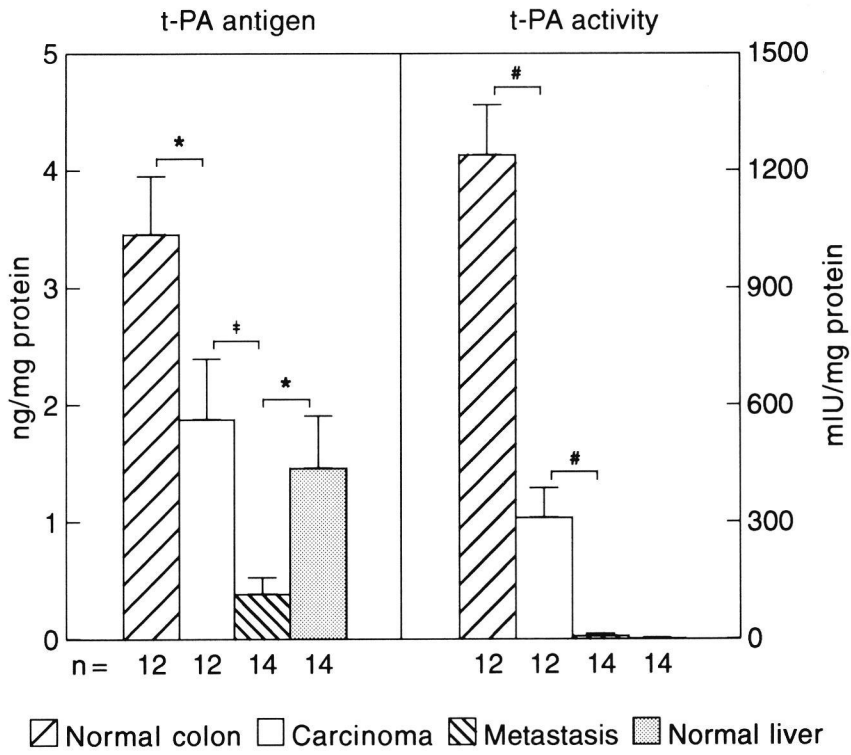

Figure 2. Concentration and activity of tissue-type plasminogen activator (t-PA) in homogenates of human colorectal carcinoma and liver metastasis compared with adjacent normal tissues (mean ± SEM). Significance of differences: * $p<0.05$; ‡ $p<0.02$; # $p<0.005$.

The portion of u-PA which was present in the active form or plasmin activatable pro-enzyme form was determined with a (BIA) for u-PA. It was found that the percentages for the colorectal carcinomas (n=12) were 29% versus 71% and for the metastases (n=14) 3% versus 97%, respectively (Table 2).

Regarding the inhibitors we found the mean antigen concentration of PAI-1 in liver metastases to be significantly higher than in all of the other tissues, even compared to the primary carcinomas. Less pronounced differences were found with PAI-2. Normal liver tissue contained the lowest level of PAI-2 antigen, while colon carcinomas showed the highest concentration (Figure 4). These differences were found to be not significant.

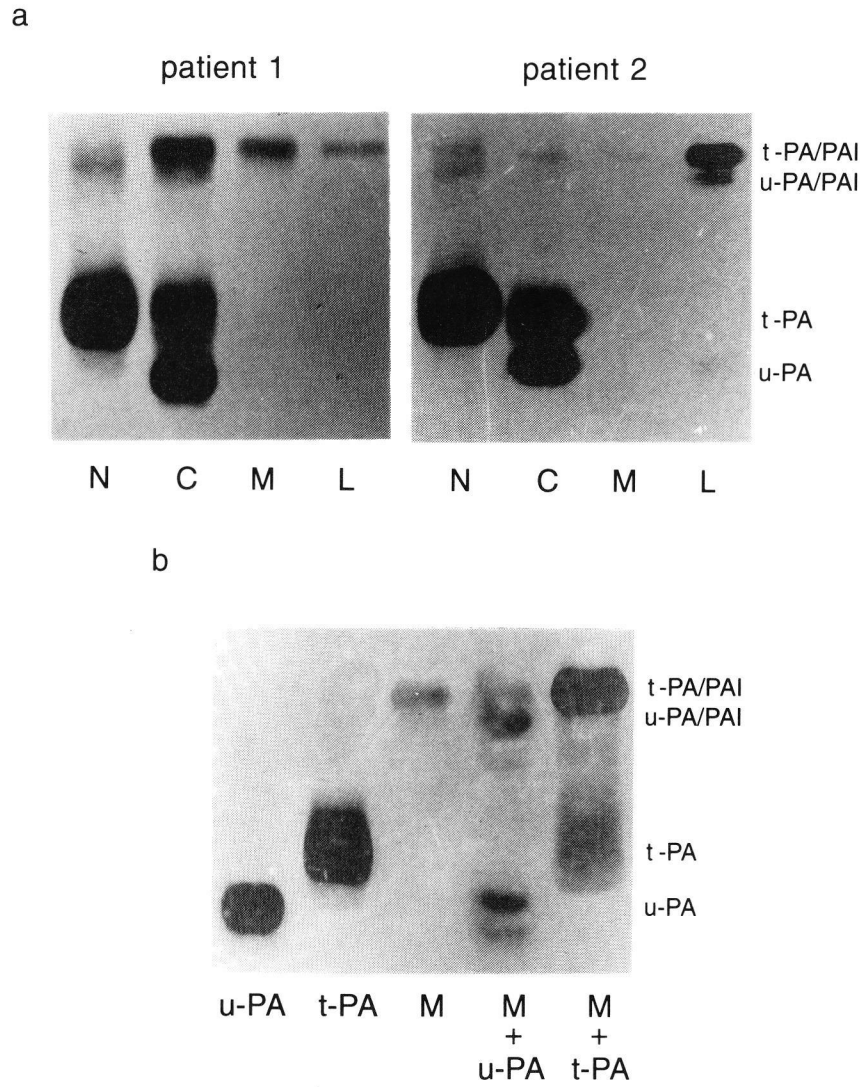

Figure 3. Zymographic analysis of plasminogen activator activity in homogenates of human colorectal and liver tissues of two patients (*a*). Zymographic analysis of liver metastasis tissue of patient 1 with/without exogenous u-PA or t-PA added to the homogenate (*b*).
N: normal colon mucosa, C: colorectal carcinoma.
M: liver metastasis of colorectal carcinoma, L: normal liver.
u-PA = urokinase-type plasminogen activator, t-PA = tissue-type plasminogen activator.
u-PA/PAI and t-PA/PAI = complexes of plasminogen activator inhibitors with u-PA or t-PA.

At least part of the PAI-antigen in the homogenates of metastases appeared to be present in an active form, since addition of external t-PA or u-PA to the extracts resulted in increased lysis bands on zymograms in (high molecular weight) complex regions at the cost of free uncomplexed regular t-PA and u-PA lysis zones (Figure 3*b*).

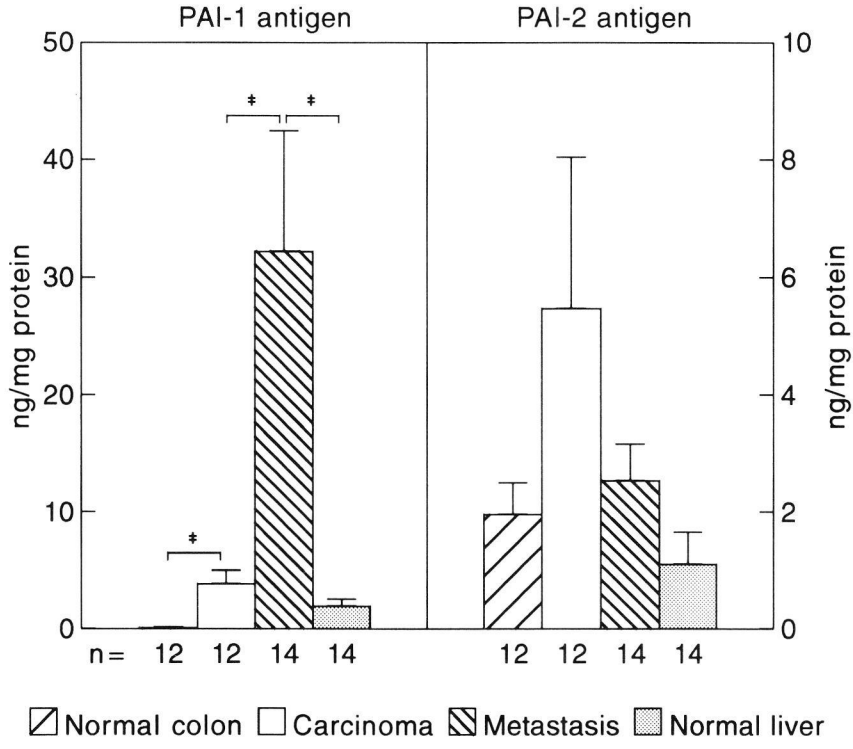

Figure 4. Concentration of plasminogen activator inhibitors PAI-1 and PAI-2 in homogenates of normal colon, colorectal carcinoma, liver metastasis, and normal liver (mean ± SEM). Significance of differences: ‡ $p<0.02$.

Table 2. Concentration of inactive pro-u-PA and active u-PA antigen in homogenates of primary colorectal carcinomas and liver metastases according to the bio-immunoassay. This chromogenic assay detects activity of u-PA by measuring the conversion of substrate by u-PA which was immuno-immobilized on microtiter plates. Total u-PA and pro-u-PA were computed from parallel incubations with or without plasmin, which converts pro-u-PA to the active form. Results are given as mean ± SEM.

Tissue	n	pro-u-PA ng/mg protein	u-PA ng/mg protein	% pro-u-PA
carcinoma colon	12	4.1 ± 0.6	2.2 ± 0.9	71 ± 7
metastasis liver	14	2.3 ± 0.7	0.3 ± 0.2	97 ± 2

u-PA = urokinase-type plasminogen activator.

The concentrations of u-PA and the inhibitors found in colorectal cancer liver metastases were also compared with those in normal liver, liver cell carcinomas, and liver focal nodular hyperplasia as shown in table 3. There were no significant differences in u-PA or inhibitor concentrations between normal liver tissue adjacent to colorectal cancer metastases resections and normal liver tissue obtained from resections because of other diseases. Noteworthy was the high concentration of PAI-1 in liver cell carcinomas, which was comparable to the level in liver metastases of colorectal cancer, and the absence of concomitant increase in u-PA in this tissue type.

Table 3. Antigen levels of urokinase-type plasminogen activator (u-PA) and inhibitors PAI-1 and and PAI-2 in homogenates of normal liver and liver disorders. u-PA, PAI-1, and PAI-2 antigen levels were determined with specific ELISAs. Results are given as mean ± SEM.

Tissue	n	u-PA ng/mg protein	PAI-1 ng/mg protein	PAI-2 ng/mg protein
Normal liver miscellaneous	11	0.4 ± 0.1	1.0 ± 0.2[a]	0.4 ± 0.2[a]
Normal liver colon carcinoma metastasis	14	1.4 ± 0.7	1.9 ± 0.6	1.1 ± 0.5
Focal nodular hyperplasia liver	3	1.8 ± 1.0	0.3 ± 0.2	1.2 ± 0.6
Liver cell carcinoma	3	1.7 ± 0.9	20.0 ± 13.3	0.8 ± 0.1
Liver metastasis colon carcinoma	14	6.4 ± 1.4[c]	32.2 ± 10.2[b]	2.5 ± 0.6

[a] n = 10 in stead of n = 11.
Significance of difference from normal liver colon carcinoma metastasis: [b] $p<0.02$, [c] $p<0.005$.

DISCUSSION

The association between elevated levels of plasminogen activators, particularly of u-PA, and neoplastic growth has been demonstrated in various studies. We previously showed that colorectal neoplasia is associated with an increase in u-PA antigen and u-PA activity (35), and that the latter is not inhibited by also enhanced PAI-1 and PAI-2 concentrations (31). The most interesting finding in the present study is the total absence of plasminogen activator activity in homogenates of liver metastases of colorectal carcinomas, in spite of the presence of an increased u-PA antigen concentration. This inactivity of the antigen is partly explained by the large percentage of inactive pro-u-PA. More importantly, however, zymographic analysis showed that a considerable amount of the urokinase antigen appeared to be complexed with specific inhibitors, thereby rendering inactive. Increased antigen concentrations of PAI-1 in homogenates of metastases support this observation. Exogenous plasminogen activators added to these homogenates were able to form complexes, visualized by zymography, indicating that an excess of inhibitors was present in the uncomplexed active form. It has been shown that PAI-1 can prevent the conversion of pro-u-PA into the active form in plasma (36), and that PAI-1 enables the subsequent internalization and degradation of u-PA by tumour cells *in vitro* (37). Thus the presence of large concentrations of PAI-1 in liver metastasis homogenates results in the absence of u-PA activity not only by forming complexes with already activated u-PA, but probably also by preventing the processing of pro-u-PA to its active form, as found in this study. Due to the known spatially controlled action of especially the urokinase-type of plasminogen activator (38,39), we can not definitely conclude, however, that *in vivo* no activator activity in liver metastasis is present. Particularly since the central part of stomach and colorectal carcinomas has been reported to contain higher levels of PAI-1 antigen than marginal parts of the tumours (40). Moreover, cell culture studies have shown that cells are able to secrete u-PA at the basolateral side, whereas PAI-activity was found at apical and basolateral sides of the cells (41).

During metastasis tumour cells must survive transport in the circulation, adhere to small blood vessels or capillaries, and might invade the vessel wall

and extravasate to the organ parenchyma. Fibrin deposition around the circulating cells and the formation of small thrombi, consisting of tumour cells, platelets, and fibrin, might prevent their recognition and lysis by natural killer cells, which are in general responsible for the destruction of those malignant cells (42). Apart from protection, the formation of these microthrombi could promote the successful lodgement of circulating tumour cells in small blood vessels of target organs (43). Nevertheless, less than 1% of tumour cells in the circulation eventually produce metastasis (44). Plasminogen activators could effectively participate in the process of invasion and metastasis, but too much proteolytic activity at the wrong moment could be disastrous for the survival of the metastasizing cells in a microthrombus. The balance between activators and inhibitors is essential and could therefore account for the difference in antigen levels of plasminogen activators and especially activator inhibitors between primary and metastatic tumours, as shown in this study. This difference in expression in liver metastases compared to their originating colorectal carcinomas is probably the consequence of a combination of selection and external regulation. The low expression of plasminogen activator activity in metastases could be a reflection of the properties of the cell(s) that gave rise to the metastatic focus (42) and was apparently an advantage for, respectively, surviving in the circulation and consecutive metastasizing thereby supporting the microthrombus theory (12). Local exposure of tumour cells to growth factors or other molecules excreted by the invaded organ, are believed to influence the differentiation of the tumour cells and the expression of plasminogen activators and activator inhibitors (45-48). Metastases vary with respect to size, growth pattern, and vascularity depending on the primary source of the tumour. Metastases derived from colon carcinomas are usually of the expanding and massive type and frequently have central liquefactive necrosis. Besides, in most metastatic colon carcinomas a thin collagenous pseudocapsule is often situated between the tumour margin and the compressed liver (49). The scarcity of proteolytic activity in spite of the presence of large quantities of u-PA in metastases of colorectal carcinomas could account for the difference in tumour outgrowth between expanding metastases and more infiltrative growing primary colorectal carcinomas.

Although the u-PA present in liver metastases undoubtedly will have a functional role in the tumour, for instance growth stimulating or angiogenesis promoting (50), it is unlikely on ground of this study, that u-PA in liver metastases contributes to any invasive or infiltrative process as assumed for the primary colorectal carcinomas. On the other hand, the large concentration of PAI-1 in liver metastases of colorectal carcinomas in relation to the total amount of plasminogen activator present in this tissue, gives reasons to suspect a complementary alternative function for this inhibitor besides complex forming, e.g. growth promotion as shown in regenerating liver (51,52). The relatively low levels of plasminogen activators and inhibitors which we found in other liver disorders, like focal nodular hyperplasia and liver cell carcinomas, show that apparently the system of plasminogen activation is not uniform in tumourigenic processes of the liver.

Cell surface plasminogen activation is involved in invasion and metastasis of colorectal carcinomas. Regulation of this process is provided at multiple levels. Although the expression and activation of pro-u-PA seem to be key events, it is clear that dominant regulating roles in the process are played by the inhibitors and probably also by the u-PA receptor. It is expected, that understanding of the regulation of plasminogen activation will eventually contribute to the treatment and prevention of tumour dissemination and metastasis.

REFERENCES

1. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 1985; 44: 139-266.
2. Duffy MJ. The role of proteolytic enzymes in cancer invasion and metastasis. Clin Exp Metastasis 1992; 10: 145-155.
3. Pöllänen J, Stephens RW, Vaheri A. Directed plasminogen activation at the surface of normal and malignant cells. Adv Cancer Res 1991; 57: 273-328.
4. Verspaget HW, Verheijen JH, de Bruin PAF, Griffioen G, Lamers CBHW. Plasminogen activators in (pre)malignant conditions of the colorectum. Eur J Cancer Clin Oncol 1989; 25: 565-569.
5. Kirchheimer JC, Pfluger H, Ritschl P, Hienert G, Binder BR. Plasminogen activator activity in bone metastases of prostatic carcinomas as compared to primary tumors. Invasion Metastasis 1985; 5: 344-355.
6. Oka T, Ishida T, Nishino T, Sugimachi K. Immunohistochemical evidence of urokinase-type plasminogen activator in primary and metastatic tumors of pulmonary adenocarcinoma. Cancer Res 1991; 51: 3522-3525.

7. Jänicke F, Schmitt M, Hatter R, et al. Urokinase-type plasminogen activator (u-PA) antigen is a predictor of early relapse in breast cancer. Fibrinolysis 1990; 4: 69-78.
8. Pyke C, Kristensen P, Ralfkiær E, et al. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. Am J Pathol 1991; 138: 1059-1067.
9. Ornstein DL, Zacharski LR, Memoli VA, et al. Coexisting macrophage-associated fibrin formation and tumor cell urokinase in squamous cell and adenocarcinoma of the lung tissues. Cancer 1991; 68: 1061-1067.
10. Sier CFM, Fellbaum C, Verspaget HW, et al. Immunolocalization of urokinase-type plasminogen activator in adenomas and carcinomas of the colorectum. Histopathology 1991; 19: 231-237.
11. Nishino N, Aoki K, Tokura Y, Sakaguchi S, Takada Y, Takada A. The urokinase-type of plasminogen activator in cancer of digestive tracts. Thromb Res 1988; 50: 527-535.
12. Markus G, Camiolo SM, Kohga S, Madeja JM, Mittelman A. Plasminogen activator secretion of human tumors in short-term organ culture including a comparison of primary and metastatic colon tumors. Cancer Res 1983; 43: 5517-5525.
13. Grøndahl-Hansen J, Ralfkiær E, Kirkeby LT, Kristensen P, Lund LR, Danø K. Localization of urokinase-type plasminogen activator in stromal cells in adenocarcinomas of the colon in humans. Am J Pathol 1991; 138: 111-117.
14. Sumiyoshi K, Baba S, Sakaguchi S, Urano T, Takada Y, Takada A. Increase in levels of plasminogen activator and type-1 plasminogen activator inhibitor in human breast cancer: Possible roles in tumor progression and metastasis. Thromb Res 1991; 63: 59-71.
15. Costantini V, Zacharski LR, Memoli VA, Kudryk BJ, Rousseau SM, Stump DC. Occurence of components of fibrinolysis pathways in situ in neoplastic and nonneoplastic human breast tissue. Cancer Res 1991; 51: 354-358.
16. Sullivan LM, Quigley JP. An anticatalytic monoclonal antibody to avian plasminogen activator: Its effect on behavior of RSV-transformed chick fibroblasts. Cell 1986; 45: 905-915.
17. Quax PHA, van Muijen GNP, Weening-Verhoeff EJD, et al. Metastatic behavior of human melanoma cell lines in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-mediated matrix degradation. J Cell Biol 1991; 115: 191-199.
18. Ossowski L, Reich E. Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 1983; 35: 611-619.
19. Boyd D, Florent G, Kim P, Brattain M. Determination of the levels of urokinase and its receptor in human colon carcinoma cell lines. Cancer Res 1988; 48: 3112-3116.
20. Quax PHA, van Leeuwen RTJ, Verspaget HW, Verheijen JH. Protein and messenger RNA levels of plasminogen activators and inhibitors analyzed in 22 human tumor cell lines. Cancer Res 1990; 50: 1488-1494.
21. Pereyra-Alfonso S, Haedo A, Bal de Kier Joffe E. Correlation between urokinase-type plasminogen activator production and the metastasizing ability of two murine mammary adenocarcinomas. Int J Cancer 1988; 42: 59-63.
22. Hart DA. Dysregulation of plasminogen activators in cancer - Potential role in invasion, metastasis and as a prognostic indicator. Fibrinolysis 1992; 6 (Suppl 1): 11-15.
23. Schultz RM, Yu H, Zhang JY. The role of urokinase and urokinase inhibitor in tumour cell metastasis. Fibrinolysis 1992; 6 (Suppl 1): 23-29.
24. Burtin P, Chavanel G, Andre-Bougaran J, Gentile A. The plasmin system in human adenocarcinoma and their metastases. A comparative immunofluorescence study. Int J Cancer 1987; 39: 170-178.

25. Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. J Clin Invest 1991; 88: 1067-1072.
26. de Bruin PAF, Verspaget HW, Griffioen G, Nap M, Verheijen JH, Lamers CBHW. Plasminogen activator activity and composition in human colorectal carcinomas. Fibrinolysis 1987; 1: 57-62.
27. Lowry OH, Rosebrough NJ, Farr AL, Randall R. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265-275.
28. Verheijen JH, Mullaart E, Chang GTG, Kluft C, Wijngaards G. A simple, sensitive spectrophotometric assay for extrinsic (tissue-type) plasminogen activator applicable to measurements in plasma. Thromb Haemost 1982; 48: 266-269.
29. Binnema DJ, van Iersel JJL, Dooijewaard G. Quantitation of urokinase antigen in plasma and culture media by use of an ELISA. Thromb Res 1986; 43: 569-577.
30. Rijken DC, van Hinsbergh VWM, Sens EHC. Quantitation of tissue-type plasminogen activator in human endothelial cell cultures by use of an enzyme immunoassay. Thromb Res 1984; 33: 145-153.
31. Sier CFM, Verspaget HW, Griffioen G, et al. Imbalance of plasminogen activators and inhibitors in human colorectal neoplasia. Implication of urokinase in colorectal carcinogenesis. Gastroenterology 1991; 101: 1522-1528.
32. Levin EG. Latent tissue plasminogen activator produced by human endothelial cells in culture: Evidence for an enzyme-inhibitor complex. Proc Natl Acad Sci USA 1983; 80: 6804-6808.
33. Granelli-Piperno A, Reich E. A study of proteases and protease-inhibitor complexes in biological fluids. J Exp Med 1978; 148: 223-234.
34. Dooijewaard G, van Iersel JJL, Brommer EJP. Quantitation of pro-UK and UK-inhibitor levels in plasma of patients and healthy men. Fibrinolysis 1986; 1 (Suppl 1): 142.
35. de Bruin PAF, Griffioen G, Verspaget HW, et al. Plasminogen activator profiles in neoplastic tissues of the human colon. Cancer Res 1988; 48: 4520-4524.
36. Dosne AM, Dubor F, Samama M. Importance of plasminogen activator inhibitor type-1 for preventing single chain urokinase plasminogen activator (scu-PA) conversion into two chain urokinase plasminogen activator (tcu-PA) in plasma in vitro. Thromb Res 1991; 63: 531-539.
37. Cubellis MV, Wun TC, Blasi F. Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1. EMBO J 1990; 9: 1079-1085.
38. Hebert CA, Baker JB. Linkage of extracellular plasminogen activator to the fibroblast cytoskeleton: Colocalization of cell surface urokinase with vinculin. J Cell Biol 1988; 106: 1241-1247.
39. Pöllänen J, Hedman K, Nielsen LS, Danø K, Vaheri A. Ultrastructural localization of plasma membrane-associated urokinase-type plasminogen activator at focal contacts. J Cell Biol 1988; 106: 87-95.
40. Tanaka N, Fukao H, Ueshima S, Okada K, Yasutomi M, Matsuo O. Plasminogen activator inhibitor 1 in human carcinoma tissues. Int J Cancer 1991; 48: 481-484.
41. van Hinsbergh VWM, van den Berg EA, Fiers W, Dooijewaard G. Tumor necrosis factor induces the production of urokinase-type plasminogen activator by human endothelial cells. Blood 1990; 75: 1991-1998.
42. Gorelik E. Protective effect of fibrin on tumour metastasis. Fibrinolysis 1992; 6 (Suppl 1): 35-38.
43. Malone JM, Wangensteen SL, Moore WS, Keown K. The fibrinolytic system. A key to tumor metastasis? Ann Surg 1979; 190: 342-349.
44. Fidler IJ. Cancer metastasis. Br Med Bull 1991; 47: 157-177.

45. Tienari J, Alanko T, Lehtonen E, Saksela O. The expression and localization of urokinase-type plasminogen activator and its type-1 inhibitor are regulated by retinoic acid and fibroblast growth factor in human teratocarcinoma cells. Cell Regul 1991; 2: 285-297.
46. Hopkins WE, Fujii S, Sobel BE. Synergistic induction of plasminogen activator inhibitor type-1 in HEP G2 cells by thrombin and transforming growth factor-beta. Blood 1992; 79: 75-81.
47. Lotan R. Retinoids as modulators of tumor cell invasion and metastasis. Semin Cancer Biol 1991; 2: 197-208.
48. Mizoguchi H, Komiyama S, Matsui K, et al. The response to epidermal growth factor of human maxillary tumor cells in terms of tumor growth, invasion and expression of proteinase inhibitors. Int J Cancer 1991; 49: 738-743.
49. Edmondson HA, Craig JR. Neoplasms of the liver. In: Schiff L, Schiff ER, eds. Diseases of the liver. Philadelphia, JB Lippincott Company, 1987: 1109-1158.
50. Binder BR. Influence of urokinase on cell proliferation and invasion. Blood Coagulation Fibrinolysis 1990; 1: 717-720.
51. Thornton AJ, Brudzinsky CJ, Raper SE, Gelehrter TD. Plasminogen activator inhibitor-1 is an immediate early response gene expressed during liver regeneration. Fibrinolysis 1992; 6 (Suppl 2): 19.
52. Schneiderman J, Sawdey M, Loskutoff DJ. Type-1 plasminogen activator inhibitor (PAI-1) gene expression in regenerating liver. Fibrinolysis 1992; 6 (Suppl 2): 19.

Chapter VII

EXPRESSION OF THE RECEPTOR FOR UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN HUMAN GASTROINTESTINAL NEOPLASIA AND RELATED LIVER METASTASES

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submitted

SUMMARY

Human carcinomas of squamous oesophagus, stomach, and colorectum, and liver metastases were previously shown to have significantly enhanced concentrations of the urokinase-type plasminogen activator (u-PA). The proteolytic activity of u-PA on the cell surface is thought to play a key role in invasion and metastasis of malignancies. In this study we determined the presence of specific u-PA receptors in membrane fractions of human gastrointestinal neoplastic tissues, liver metastases, and adjacent normal tissues. Cross-linking of ^{125}I-u-PA with u-PA receptors resulted in complexes which were visualized by SDS-PAGE, autoradiography, and subsequently quantified by laser densitometry. Colorectal carcinomas and liver metastases contained up to four fold higher levels of u-PA receptor protein compared to their corresponding normal tissues. The u-PA receptor content of premalignant adenomatous polyps was not different from normal colonic mucosa. Binding of ^{125}I-u-PA to the receptor was specific and could be completely quenched by addition of excess unlabeled u-PA. Moreover, acid treatment of the receptors prior to cross-linking did not enhance the u-PA/u-PA receptor complex formation indicating that the vast majority of the receptors was unoccupied by u-PA. The u-PA receptor levels in oesophageal and stomach carcinomas showed less difference compared with their normal reference tissues. The receptor ratio in neoplastic versus normal tissue was 2.0±0.3 for primary colonic carcinomas and 2.2±0.2 for metastatic tumours, with no difference between primary and metastatic tumours (ratio 1.1). Ratios in oesophageal and stomach tissues were 1.3±0.2 and 1.0±0.2, respectively. Northern blotting experiments showed also high expression of u-PA receptor mRNA in most oesophageal carcinomas, normal colonic mucosae and adenomatous polyps, and in all stomach and colonic carcinomas. Normal oesophageal and stomach tissues contained hardly any detectable u-PA receptor mRNA. The mean carcinoma/normal tissue ratio of u-PA receptor mRNA ranged from 1.8 to 6.3 for the colonic and oesophageal carcinomas, respectively. The increased presence of specific receptors for u-PA, particularly in primary colorectal carcinomas and their metastatic lesions in the liver, suggest once again the involvement of the urokinase pathway of plasminogen activation in gastrointestinal carcinogenesis.

INTRODUCTION

Local invasion and metastatic spread of carcinomas involve continuous disruption and re-establishment of the environment by the migrating malignant cells. Proteolytic degradation of the extracellular matrix and basement membranes is a complex interplay between different cell types, in which several enzyme systems participate. The urokinase mediated plasminogen activation pathway is one of the most important proteolytic cascades involved in invasion and metastasis of tumour cells. Plasmin is a broad spectrum degrading enzyme, which is also able to activate other extracellular matrix proteinases. The activation of plasminogen is regulated by the balance between activators and inhibitors (1). The urokinase-type plasminogen activator (u-PA) is inhibited by two specific plasminogen activator inhibitors, PAI-1 and PAI-2. A perhaps more important mode of regulation, however, is the presence of a specific cell surface receptor for u-PA (2-6). This receptor, first found on monocytes (7) and present in a variety of cancer cell lines, focusses u-PA at cell-cell contacts and accelerates the activation of u-PA, and hence of plasmin. The receptor recognizes the A-chain of urokinase and its inactive pro-form (pro-u-PA) (8). Receptor bound u-PA is still sensitive to specific plasminogen activator inhibitors, PAI-1 and PAI-2. Binding of inhibitors to cell bound u-PA promotes PAI/u-PA/u-PA receptor internalization (9-11).

Several studies found u-PA to be enhanced in human carcinomas of the oesophagus, stomach, and colon (12-15). The presence of the receptor for u-PA in gastrointestinal carcinomas, however, has not been as extensively studied. The urokinase receptor is shown in lung, ovarian, and breast carcinomas and in melanomas, and is found to be present at surfaces of several colon carcinoma cell lines (16-20). Cell bound u-PA is shown to be involved with invasion *in vitro* and *in vivo* (21). The u-PA receptor expression of colorectal carcinoma cell lines has been shown to be related to matrix degradation, invasiveness, and metastasis (20,22,23).

A recent immunohistochemical and in situ hybridization study of human colon carcinomas showed urokinase receptor protein and mRNA to be expressed at invasive foci, in tumour infiltrating macrophages and neutrophilic granulocytes, and in subpopulations of cancer cells (24). In the present study

we evaluated the presence of u-PA receptor protein and mRNA in gastrointestinal carcinomas. Squamous carcinomas of the oesophagus and adenocarcinomas of the stomach and colon were compared with their respective normal mucosa and with premalignant adenomatous polyps of the colon. Moreover, of some patients we also evaluated receptor expression in the liver metastasis of primary colonic carcinoma.

PATIENTS, MATERIALS AND METHODS

Patients

Carcinoma and adjacent normal tissue from resection specimens of five patients with squamous oesophageal carcinoma, ten patients with gastric carcinoma, fourteen patients with colonic carcinoma, and ten patients operated for metastasis of colorectal carcinoma in the liver was obtained from the department of Surgery, University Hospital Leiden. From four of the patients we obtained the primary colonic carcinoma as well as the metastasis in the liver. Adenomas of ten patients were polypectomized at the department of Gastroenterology, University Hospital Leiden. Endoscopical biopsies from normal and carcinoma tissue of five patients with squamous oesophageal carcinoma, five patients with stomach carcinoma, five patients with an adenomatous polyp, and seven patients with a colonic carcinoma were used for mRNA-analysis. One of the patients with a carcinoma in the colon also had an adenoma. All tissues were immediately frozen at -70°C until analysis. Of all samples, adjacent fragments were histologically evaluated by the pathologist to confirm the origin of the tissue.

u-PA receptor analysis

Extracts of tissue (100 mg/ml) were prepared by homogenization in 0.1 M Tris-HCl (pH 7.5). Subsequently, membrane fractions were purified by centrifugation of the homogenates in a Beckman airfuge at 10^5 RPM for 15 minutes. The pellet was resuspended in glycine buffer (0.05 M glycine, 0.1 M NaCl, pH 3.0 or 7.0, 3 minutes), neutralized with 0.5 M HEPES, 0.1 M NaCl pH 7.5, centrifuged for 15 minutes at 10^5 RPM, resuspended in 0.1 M Tris-HCl pH 8.1, 1% Triton X-114, 10 mM EDTA, 10 µg/ml trasylol, 1 mM PMSF, and finally

centrifuged for 10 minutes at 10^4 *g*, 4°C. The protein content of the supernatants was determined using the BCA microtiter plate assay (Pierce, Rockford USA). The presence of u-PA receptor in the supernatants was determined by cross-linking 40 µg protein with diisopropylfluoro-phosphate (DFP) treated human u-PA, which was radiolabeled using Na-^{125}I according to the Iodogen procedure (Pierce) (25,26). Samples and labeled u-PA were incubated with or without addition of a 100 fold excess of unlabeled u-PA in PBS-0.1% (v/v) Tween 80 (1 hour at 4°C), followed by incubation with cross-linking agent disuccinimydyl suberate (DSS, 2 mM, 15 minutes) and ammonium acetate (10 mM, 10 minutes) at room temperature. The samples were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and autoradiography using Kodak XAR-5 film and intensifying screens at -70°C. Bands on autoradiograms were scanned using a LKB ultroscan XL Enhanced Laser Densitometer (633 nm).

mRNA analysis

RNA was isolated by acid-guanidinium thiocyanate-phenol-chloroform extraction according to Chomczynski and Sacchi (27). The amount of isolated RNA was determined by measuring the OD_{260}, assuming that one OD_{260} unit is equivalent to 40 µg RNA. The samples were electrophoresed on 1.2% (w/v) denaturing agarose gels containing 7.5% (v/v) formaldehyde and ethidium bromide. RNA was transferred to nylon membranes (Hybond N; Amersham International, Amersham, United Kingdom) using a Vacugene System (Pharmacia, Uppsala, Sweden). mRNA on the membranes was hybridized with ^{32}P-labeled cDNA fragments in 7% (w/v) SDS, 0.5 M $NaHPO_3$, pH 7.2, 1 mM EDTA at 65°C. Blots were washed with 0.3 M NaCl, 0.03 M tri-sodium citrate (2x SSC), 1% (w/v) SDS for 1 hour at 65°C. Probe cDNA fragments were labeled with ^{32}P-dCTP using the random primer method (Megaprime, Amersham International). Autoradiograms were prepared using Kodak XAR-5 films and intensifying screens at -70°C. cDNA fragments used for hybridization with mRNA were a 0.6 kb Bam HI fragment of human u-PAR kindly provided by Dr L.R. Lund (28), a 1.0 kb Eco RI-Pst I fragment of human u-PA (29) kindly provided by Dr W.D. Schleuning, a 1.2 kb Pst I fragment of human PAI-1 (30), a

1.2 kb Eco RI fragment of human PAI-2 kindly provided by Dr E.K.O. Kruithof (31), and a 1.2 kb Pst I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (32). Specific bands on autoradiograms were scanned using a LKB ultroscan XL Enhanced Laser Densitometer (633 nm).

ELISA for u-PA

For u-PA determination, tissue samples were homogenized in 1 ml 0.1% (v/v) Tween 80, 0.1 M Tris-HCl buffer (pH 7.5) per 60 mg wet tissue, as described previously (33). Protein concentrations were determined by the method of Lowry *et al.* (34). The sandwich ELISA for u-PA was carried out according to Binnema *et al.* (35). Rabbit anti-u-PA was used as catching antibody and after incubation of the samples, affinopurified goat anti-u-PA IgG (0.8 µg/ml) was added and incubated. After washing, 100 µl of an optimal dilution of donkey anti-goat IgG conjugated with alkaline phosphatase was added and 100 µl para-nitrophenyl-phosphate (1 mg/ml) was used as substrate. The amount of u-PA antigen in the samples was calculated from a 9-points standard curve of u-PA (0-3.3 ng/ml).

Statistical analysis

Results are given as mean ± SEM. Differences between group means were tested for significance using paired and unpaired Student's *t*-tests with separate variance estimate if the standard deviations were significantly different according to the F-test. Differences were considered significant when $p<0.05$.

RESULTS

The urokinase receptor protein was detectable in all gastrointestinal tissues as shown in figure 1. Binding of ^{125}I-u-PA to the receptor was specific and could be completely quenched by addition of excess unlabeled u-PA. The receptors were detected without acid treatment of the membrane fractions. Acid treatment (pH 3.0 versus pH 7.0, 3 minutes) did not significantly alter the amount of receptor complexes in the radioligand affinity assay of colonic and liver tumours or adjacent reference tissues.

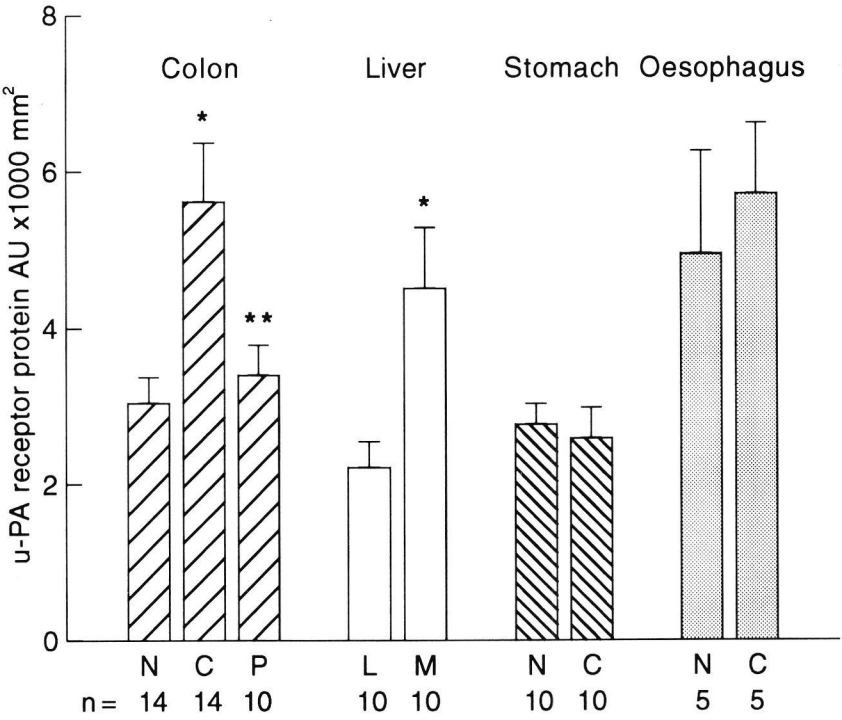

Figure 1. Results of laser densitometry scanning of autoradiograms of urokinase receptor protein in gastrointestinal neoplasia. The autoradiograms were made from SDS-PAGE gels with ^{125}I-labelled urokinase and cross-linked radioactive urokinase complexed with its receptor from tissue homogenates. Values are given in area under the curve (AU x1000 mm^2).
N: normal mucosa, C: carcinoma, P: adenomatous polyp.
L: normal liver tissue, M: liver metastasis of colorectal carcinoma.
Significance of difference from normal tissue: * $p<0.005$.
Significance of difference from carcinoma: ** $p<0.02$.

Primary colonic carcinomas and their metastases in the liver contained two times more receptor than their corresponding normal tissues. The urokinase receptor content in homogenates of premalignant adenomatous polyps did not differ from normal colonic mucosa. Although the mean u-PA receptor content of liver metastases was slightly lower than that of the primary colonic carcinomas, figure 2 shows that within individual patients virtually no differences between the receptor content of the primary and metastatic tumours exist.

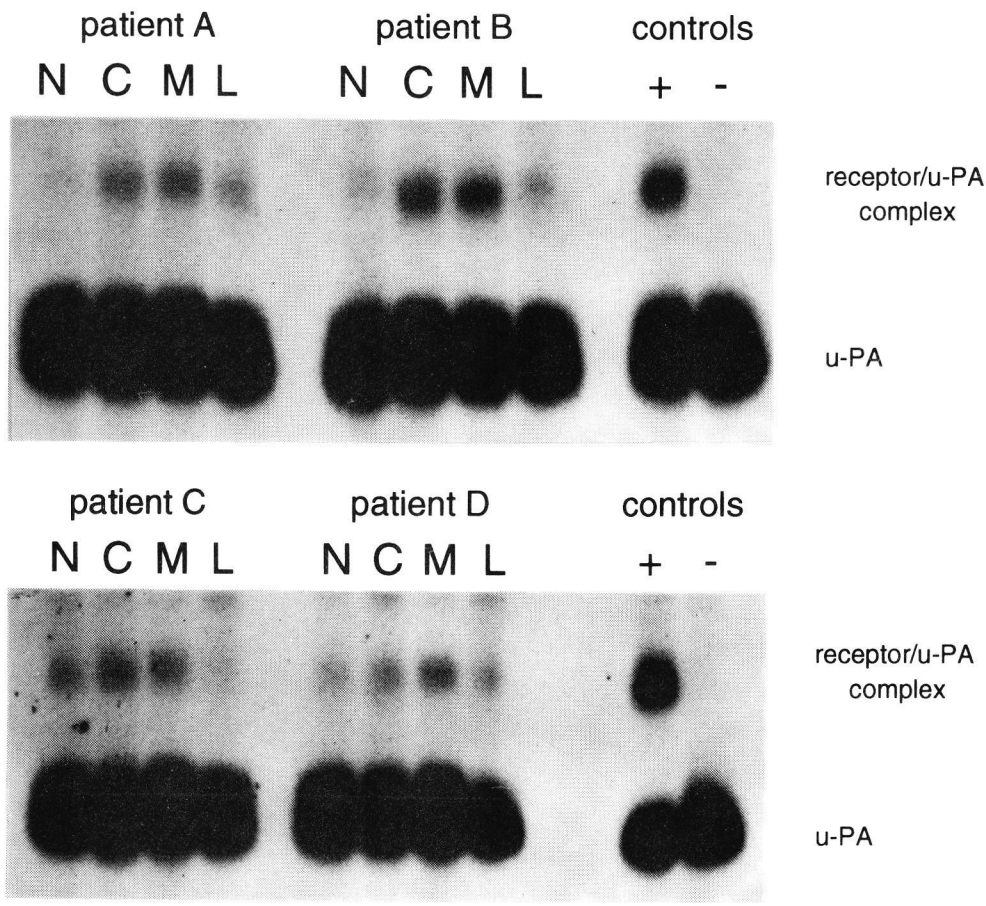

Figure 2. Autoradiogram of urokinase receptor in gastrointestinal tissue of four patients (A-D) with a primary colonic carcinoma and liver metastasis. The assay involved cell membrane isolation, ^{125}I-labelled urokinase cross-linking, gelelectrophoresis, and autoradiography.
N: normal mucosa, C: carcinoma, L: normal liver tissue, M: liver metastasis.

The level of urokinase receptor in normal gastric tissue resembled that of normal colonic mucosa. In contrast to colonic tissue, there was no difference between mean receptor content in normal and carcinoma tissue of the stomach. The similar level of the tumour and normal gastric tissue receptor content is also indicated in table 1, which shows the mean paired tumour/normal tissue ratios of the urokinase receptor of the different carcinomas. Colonic carcinomas and metastases scored ratios of approximately 2 (range 0.8-4.1 and 0.9-3.2, respectively), stomach carcinoma ratios were 1 (range 0.4-1.9).

Table 1. Ratios of urokinase receptor and urokinase protein in carcinoma/normal tissue pairs of patients with gastrointestinal malignancies. The receptor content was determined by a cross-linking assay with ^{125}I labelled urokinase and with receptors isolated from tissue homogenates. Labelled urokinase and urokinase/receptor complexes were separated using gelelectrophoresis and scanned with a laser densitometer after autoradiography. Urokinase was determined with a specific ELISA.

Tissue type	n	Urokinase receptor carcinoma/normal ratio	Urokinase carcinoma/normal ratio
Colon	14	2.0 ± 0.3	7.5 ± 2.3
Liver metastasis from colonic carcinoma	10	2.1 ± 0.2	6.3 ± 1.4
Stomach	10	1.0 ± 0.2	3.4 ± 0.6
Squamous oesophagus	5	1.3 ± 0.2	n.d.

n.d.: not done.

Squamous oesophageal carcinomas scored ratios in between (1.3, range 0.9-1.8). Especially the normal mucosa u-PA receptor content of this tissue appeared to be more heterogeneous than of the other tissue types.

The antigen concentration of urokinase, the ligand for the u-PA receptor, was found to be increased in all gastrointestinal neoplastic tissues studied (Figure 3). Only the difference between the u-PA antigen of stomach carcinomas and normal gastric mucosa did not reach statistical significance, due to an extremely high urokinase antigen level in one normal tissue sample. Comparison of the ratios of both urokinase and its receptor in carcinoma/normal tissue pairs (Table 1) showed proportionally more u-PA than u-PA receptor in the gastrointestinal carcinomas than in their matched normal tissue, although the protein levels of urokinase and its receptor showed some correlation ($R=0.28$, $p=0.01$, $n=78$) in the total group and in the carcinoma subgroup (stomach, colon, and metastasis; $R=0.33$, $p=0.05$, $n=34$).

mRNA isolation and northern blotting from the same tissues as that were used for the determination of the protein levels showed disappointing results. In many cases, especially in colonic and liver tissues, most of the mRNA, particularly the u-PA type, was degraded, probably due to the relatively long time between the operation and the tissue collection.

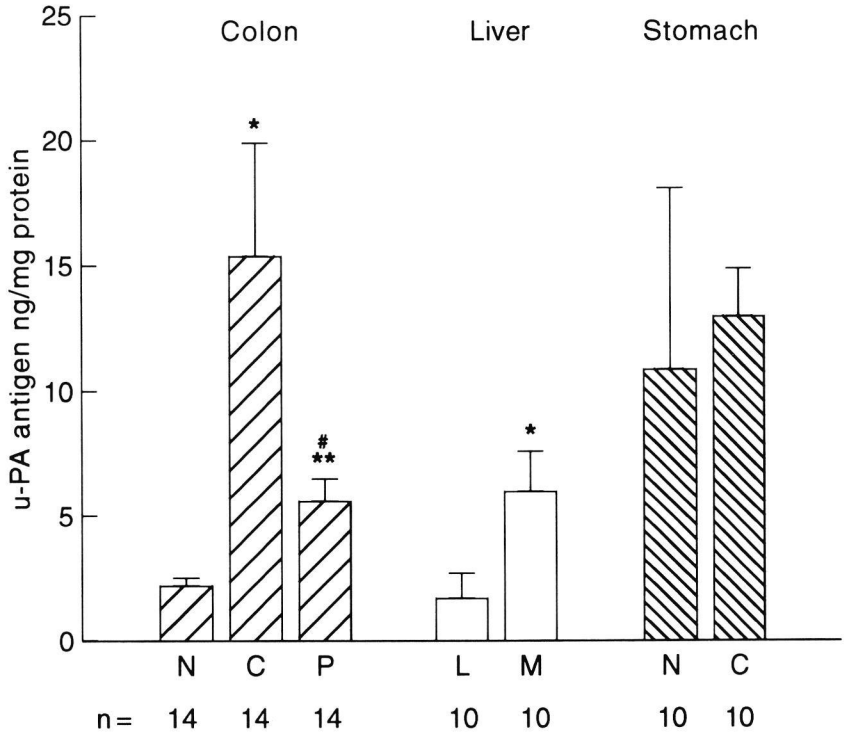

Figure 3. Urokinase (u-PA) antigen concentrations of normal and neoplastic tissues from stomach, colorectum, and liver metastasis determined with an ELISA.
N: normal mucosa, C: carcinoma, P: adenomatous polyp.
L: normal liver tissue, M: liver metastasis of colorectal carcinoma.
Significance of difference from normal tissue: * $p<0.05$, ** $p<0.005$.
Significance of difference from carcinoma: # $p<0.05$.

Therefore, the study of u-PA receptor mRNA expression was performed on quickly frozen endoscopically obtained biopsies in stead of resection derived tissue. After stripping, the nylon membranes were rehybridized with u-PA, PAI-1, and GAPDH probes (Figure 4). Of all tissues investigated, scored mRNA bands and mean calculated mRNA ratios of tumour/normal tissue, scanned by densitometer and corrected for internal standard GAPDH, are presented in table 2. Almost all colonic tissues i.e. normal mucosa, adenomatous polyps, and carcinomas showed u-PA receptor mRNA expression on northern blots (Figure 4 and Table 2).

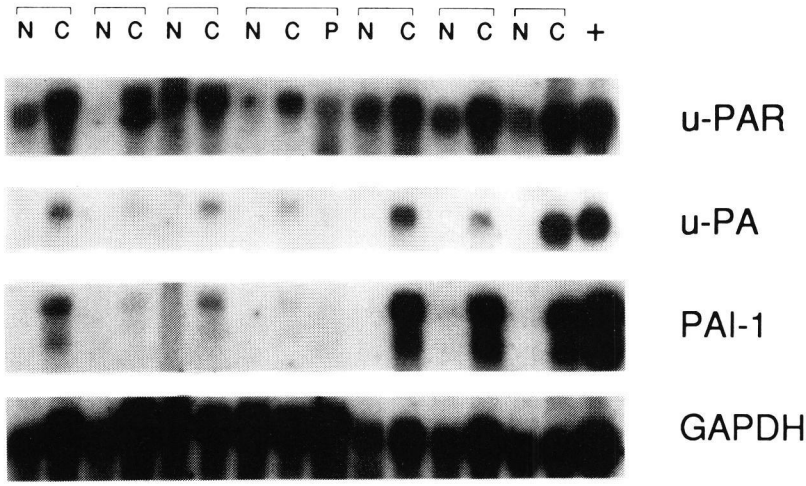

Figure 4. Northern blot of total RNA extracted from normal and neoplastic tissue the human colon. The filter was subsequently hybridized with cDNA fragments of u-PAR, u-PA, PAI-1, and GAPDH. Brackets indicate matching tissues from the same patient.
N: normal mucosa, C: carcinoma, P: adenomatous polyp.

The intensity of the bands was on average higher in carcinomas than in their corresponding normal tissue. The ligand for the u-PA receptor, urokinase, was virtually not detected in normal colonic tissue adjacent to carcinomas, but interestingly, was occasionally found in normal mucosa adjacent to adenomatous polyps (Table 2). The mRNA for u-PA was detected in all adenomas and carcinomas of the colon. Inhibitor PAI-1 mRNA was mainly seen in the carcinomas. PAI-2 mRNA was infrequently detected, but in carcinomas only (data not shown). Unlike the similar protein level of u-PA receptor found in normal gastric mucosa and carcinoma, abundant mRNA expression of the receptor was only detected in carcinomas and not in normal mucosa. Normal stomach tissue did not show mRNA for u-PA or PAI-1 either. In contrast, all gastric carcinomas contained mRNA for u-PA receptor as well as for u-PA. Inhibitor PAI-1 mRNA was present in 4 of 5 carcinomas tested. Four out of five oesophageal carcinomas contained mRNA for urokinase receptor, and all five carcinomas showed mRNA for urokinase, as well as for inhibitor PAI-1. Normal squamous mucosa did not contain detectable mRNA for u-PA receptor, u-PA, or PAI-1.

Table 2. Score of northern blot mRNA bands of receptor for urokinase-type plasminogen activator, urokinase, and plasminogen activator inhibitor type-1 in endoscopically obtained tissue pairs of patients with gastrointestinal tumours. Tumor/normal ratios were calculated from densitometrically scanned autoradiograms, corrected for internal standard GAPDH. Ratios are mean ± SEM.

Tissue type	mRNA score						Calculated ratio
	Normal mucosa			Tumour			tumour/normal
	-	+	++	-	+	++	
Colonic carcinoma (n=7)							
urokinase receptor	1	1	5	-	-	7	1.8 ± 0.3
urokinase	7	-	-	-	-	7	5.4 ± 1.7
inhibitor type-1	7	-	-	-	2	5	5.9 ± 1.6
GAPDH	-	-	7	-	-	7	
Colonic adenoma (n=5)							
urokinase receptor	1	1	3	1	1	3	0.6 ± 0.1
urokinase	3	2	-	-	1	4	2.3 ± 0.5
inhibitor type-1	5	-	-	4	1	-	3.9 ± 1.3
GAPDH	-	-	5	-	-	5	
Stomach (n=5)							
urokinase receptor	5	-	-	-	-	5	3.9 ± 1.2
urokinase	5	-	-	-	1	4	3.8 ± 1.1
inhibitor type-1	5	-	-	1	-	4	2.7 ± 0.7
GAPDH	-	3	2	-	-	5	
Oesophagus (n=5)							
urokinase receptor	5	-	-	1	3	1	6.3 ± 2.7
urokinase	4	1	-	-	2	3	3.9 ± 1.0
inhibitor type-1	4	1	-	-	1	4	4.9 ± 1.2
GAPDH	-	-	5	-	-	5	

- no expression; + limited expression; ++ abundant expression.
GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

DISCUSSION

The u-PA receptor has been extensively studied *in vitro* by cross-linking studies with tumour cell lines, and *in vivo* in carcinomas by immunohistochemistry or in situ mRNA hybridization. In contrast to other components of the plasminogen activation cascade, however, studies about the content of u-PA receptor in tissue homogenates are relatively scarce. The present work demonstrates that unoccupied receptors for urokinase are present in all homogenates of gastrointestinal tissues that were investigated. Comparison of lung, ovarian, and skin malignancies with their corresponding normal tissues already revealed higher levels of u-PA receptors in the tumours (16,17,19). In our study, binding of labelled u-PA to the receptor was particularly high in tumours of oesophagus and colon, and in liver metastasis derived from colonic carcinomas. These increased receptor levels in gastrointestinal tumour tissues emphasize the role of the u-PA pathway of plasminogen activation in carcinogenesis. Receptor bound u-PA and concomitant binding of plasminogen to the cell surface localize the proteolytic potential of the tumour cells and hence enhance their invasive properties (36).

In contrast to previous *in vitro* and *in vivo* studies (16,17,20,37-39), acid treatment of the membrane fractions prior to the ligand cross-linking did not increase binding of u-PA to the receptors in colonic, metastatic and liver tissue homogenates. This could indicate an insensitivity of these receptors to the acid treatment or an abundancy of unoccupied receptors on the membranes. Insensitivity of u-PA receptors to acid treatment has been reported for some but not all human colon carcinoma cell lines (20,38). Apparently, differences in receptor-ligand affinity occur in different cells or circumstances. In this context it is interesting, that the u-PA receptor protein content is not increased in the carcinomas of the stomach compared with normal gastric mucosa, while the mRNA for u-PA receptor is detected in all gastric carcinomas, but in none of the normal tissues. The acid environment of the stomach could play a role in the grade of occupancy of the receptors in this tissue. Other physiological ways of regulation of the u-PA receptor have been described in several studies. Phorbol esters, transforming growth factor-beta, and epidermal growth factor increase the synthesis of the receptor *in vitro* by activating gene transcription (40).

Increasing numbers of u-PA receptor have been shown to result in a decrease of its affinity (41,42). The ligand urokinase itself has been found to reduce the expression of its own binding site via an autocrine regulation of its cell surface receptor in a colonic cell line (38,43). For all tumour types in this study, the increase of u-PA in comparison with normal tissue was higher than the increase of u-PA receptor. Stimulation of u-PA receptor with u-PA has been shown to increase cell motility *in vitro* and a role for the receptor in non-mitogenic signal transduction has been suggested (44,45). Cell surface u-PA complexed with inhibitor PAI-1 is efficiently internalized and degraded, while the internalized receptor is recycled to the cell surface (5,46). A possible cell surface regulatory mechanism of urokinase by inhibitor PAI-2 has also been proposed recently (47). mRNAs involved with complicated regulatory mechanisms are known to have relatively high turnover and instability. The mRNA of u-PA has been shown to possess potential instability motifs, which suggests that u-PA mRNA undergoes post-transcriptional down regulation in certain cell types (48-50). Instability of mRNAs combined with the degrading nature of colonic tissue could be an explanation for the difficulties we met with isolation and northern blotting of especially u-PA mRNA. Instantly frozen tissue, in our study endoscopical biopsies, circumvented this problem. The mRNA levels of u-PA receptor and other components of the plasminogen activation cascade were found to be in agreement with the corresponding protein levels in this study and those of previous investigations of our group, i.e., carcinoma tissues of gastrointestinal origin contain more mRNA coding for u-PA, u-PA receptor, and inhibitors PAI-1 and PAI-2 than their adjacent normal tissues (14,51).

Determination of u-PA in homogenates of breast (52,53), bladder (54), and colon (55) has shown that high levels in carcinoma tissue are indicative of poor prognosis. Cell line studies made clear that cells expressing u-PA in combination with its receptor show the highest invasive activity (20,56). Combined with the results of our study this implicates that quantitative determination of the receptor for urokinase in carcinoma tissue on itself, or in combination with u-PA and PAI-1 antigen, could be of prognostic clinical relevance for patients with a colorectal carcinoma. The importance of the investigation of the role of u-PA receptor and u-PA in carcinomas is also

emphasized by the study of Behrendt *et al.* (57), in which suramin, a potent anticancer drug, was found to inhibit the interaction between receptor and its ligand. This effect of suramin on the urokinase pathway of plasminogen activation could contribute to the anti-invasive properties of this agent. It has also been shown that loss of the surface u-PA activity by blocking the u-PA receptor markedly inhibits metastasis of metastatic human carcinoma cells in nude mice (58). On the basis of these results it is clear that the study of the receptor for urokinase could not only make a contribution to a better understanding of the processes involved during malignant invasion and metastasis, but also offers perspectives to screen carcinoma patients. Eventually these studies may lead to an anti-cancer therapy by selectively blocking the u-PA receptor with antibodies, mutated u-PA molecules or synthetic ligands (22,58,59).

REFERENCES

1. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 1985; 44: 139-266.
2. Ellis V, Danø K. The urokinase receptor and the regulation of cell surface plasminogen activation. Fibrinolysis 1992; 6 (Suppl 4): 27-34.
3. Pöllänen J, Stephens RW, Vaheri A. Directed plasminogen activation at the surface of normal and malignant cells. Adv Cancer Res 1991; 57: 273-328.
4. Kirchheimer JC, Binder BR. Function of receptor-bound urokinase. Sem Thromb Hemost 1991; 17: 246-250.
5. Blasi F. Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. BioEssays 1993; 15: 105-111.
6. Møller LB. Structure and function of the urokinase receptor. Blood Coagulation Fibrinolysis 1993; 4: 293-303.
7. Vassalli JD, Baccino D, Belin D. A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J Cell Biol 1985; 100: 86-92.
8. Stoppelli MP, Corti A, Soffientini A, Cassani G, Blasi F, Assoian RK. Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc Natl Acad Sci USA 1985; 82: 4939-4943.
9. Ellis V, Wun TC, Behrendt N, Rønne E, Danø K. Inhibition of receptor-bound urokinase by plasminogen-activator inhibitors. J Biol Chem 1990; 265: 9904-9908.
10. Baker MS, Bleakley P, Woodrow GC, Doe WF. Inhibition of cancer cell urokinase plasminogen activator by its specific inhibitor PAI-2 and subsequent effects on extracellular matrix degradation. Cancer Res 1990; 50: 4676-4684.
11. Olson D, Pöllänen J, Høyer-Hansen G, et al. Internalization of the urokinase-plasminogen activator inhibitor type-1 complex is mediated by the urokinase receptor. J Biol Chem 1992; 267: 9129-9133.

12. Sier CFM, Verspaget HW, Griffioen G, Ganesh G, Vloedgraven HJM, Lamers CBHW. Plasminogen activators in normal tissue and carcinomas of the human oesophagus and stomach. Gut 1993; 34: 80-85.
13. Nishino N, Aoki K, Tokura Y, Sakaguchi S, Takada Y, Takada A. The urokinase type of plasminogen activator in cancer of digestive tracts. Thromb Res 1988; 50: 527-535.
14. de Bruin PAF, Verspaget HW, Griffioen G, Verheijen JH, Dooijewaard G, Lamers CBHW. Plasminogen activators in endoscopic biopsies as indicators of gastrointestinal cancer: comparison with resection specimens. Br J Cancer 1989; 60: 397-400.
15. Markus G, Camiolo SM, Kohga S, Madeja JM, Mittelman A. Plasminogen activator secretion of human tumors in short-term organ culture including a comparison of primary and metastatic colon tumors. Cancer Res 1983; 43: 5517-5525.
16. Veale D, Needham G, Harris AL. Urokinase receptors in lung cancer and normal lung. Anticancer Res 1990; 10: 417-422.
17. Casslén B, Gustavsson B, Astedt B. Cell membrane receptors for urokinase plasminogen activator are increased in malignant ovarian tumours. Eur J Cancer 1991; 27: 1445-1448.
18. Pyke C, Græm E, Ralfkiær E, et al. Receptor for urokinase is present in tumor-associated macrophages in ductal breast carcinoma. Cancer Res 1993; 53: 1911-1915.
19. de Vries TJ, Quax PHA, Denijn M, et al. Plasminogen activators, their inhibitors and urokinase receptor emerge in late stages of melanocytic tumor progression. Am J Pathol 1993; In press.
20. Boyd D, Florent G, Kim P, Brattain M. Determination of the levels of urokinase and its receptor in human colon carcinoma cell lines. Cancer Res 1988; 48: 3112-3116.
21. Ossowski L. Invasion of connective tissue by human carcinoma cell lines: requirement for urokinase, urokinase receptor, and interstitial collagenase. Cancer Res 1992; 52: 6754-6760.
22. Reiter LS, Kruithof EKO, Cajot JF, Sordat B. The role of the urokinase receptor in extracellular matrix degradation by HT29 human colon carcinoma cells. Int J Cancer 1993; 53: 444-450.
23. Takahashi K, Ikeo K, Gojobori T, Tanifuji M. Local function of urokinase receptor at the adhesion contact sites of a metastatic tumor cell. Thromb Res, Suppl X, 1990; 55-61.
24. Pyke C, Kristensen P, Ralfkiær E, et al. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. Am J Pathol 1991; 138: 1059-1067.
25. Behrendt N, Rønne E, Ploug M, et al. The human receptor for urokinase plasminogen activator. NH_2-terminal amino acid sequence and glycosylation variants. J Biol Chem 1990; 265: 6543-6469.
26. Ploug M, Rønne E, Behrendt N, Jensen AL, Blasi F, Danø K. Cellular receptor for urokinase plasminogen activator: carboxy-terminal processing and membrane anchoring by glyxosyl-phosphatidylinositol. J Biol Chem 1991; 266: 1926-1933.
27. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156-159.
28. Roldan AL, Cubellis MV, Masucci MT, et al. Cloning and expresssion of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis. EMBO J 1990; 9: 467-474.
29. Medcalf RL, van den Berg E, Schleuning WD. Glucocorticoid modulated gene expression of tissue- and urinary-type plasminogen activator and plasminogen activator inhibitor 1 and 2. J Cell Biol 1988; 106: 971-978.
30. van den Berg EA, Sprengers ED, Jaye M, Burgess W, Maciag T, van Hinsberg VWM. Regulation of plasminogen activator inhibitor-1 mRNA in human endothelial cells. Thromb Haemost 1988; 60: 63-67.

31. Schleuning WD, Medcalf RL, Hession C, Rothenbuhler R, Shaw A, Kruithof EKO. Plasminogen activator inhbitor 2: regulation of gene transcription during phorbol ester-mediated differentiation of U-937 human hystiocytic lymphoma cells. Mol Cell Biol 1987; 7: 4564-4567.
32. Fort Ph, Marty L, Piechaczyk M, et al. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucl Acid Res 1985; 13: 1431-1442.
33. de Bruin PAF, Verspaget HW, Griffioen G, Nap M, Verheijen JH, Lamers CBHW. Plasminogen activator activity and composition in human colorectal carcinomas. Fibrinolysis 1987; 1: 57-62.
34. Lowry OH, Rosebrough NJ, Farr AL, Randall R. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265-275.
35. Binnema DJ, van Iersel JJL, Dooijewaard G. Quantitation of urokinase antigen in plasma and culture media by use of an ELISA. Thromb Res 1986; 43: 569-577.
36. Cohen RL, Xi XP, Crowley CW, Lucas BK, Levinson AD, Shuman MA. Effects of urokinase receptor occupancy on plasmin generation and proteolysis of basement membrane by human tumor cells. Blood 1991; 78: 479-487.
37. Baker MS, Liang XM, Doe WF. Occupancy of the cancer cell urokinase receptor (uPAR): effects of acid elution and exogenous uPA on cell surface urokinase (uPA). Biochim Biophys Acta 1992; 1117: 143-152.
38. Hollas W, Boyd D. Regulation of the urokinase receptor by its plasminogen activator. Thromb Haemost 1991; 66: 678-683.
39. Quax PHA, van Muijen GNP, Weening-Verhoef EJD, et al. Metastatic behavior of human melanoma cell lines in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-meditated matrix degradation. J Cell Biol 1991; 115: 191-199.
40. Lund LR, Rømer J, Rønne E, Ellis V, Blasi F, Danø K. Urokinase-receptor biosynthesis, mRNA level and gene transcription are increased by transforming growth factor beta-1 in human A549 lung carcinoma cells. EMBO J 1991; 10: 3399-3407.
41. Picone R, Kajtaniak EL, Nielsen LS, et al. Regulation of urokinase receptors in monocytelike U937 cells by phorbol ester myristate acetate. J Cell Biol 1989; 108: 693-702.
42. Estreicher A, Wohlwend A, Belin D, Schleuning WD, Vassalli JD. Characterization of the cellular binding site for the urokinase-type plasminogen activator. J Biol Chem 1989; 264: 1180-1189.
43. Hollas W, Soravia E, Mazar A, Henkin J, Blasi F, Boyd D. Decreased urokinase receptor expression by overexpression of the plasminogen activator in a colon cancer cell line. Biochem J 1992; 285: 629-634.
44. Dumler I, Petri T, Schleuning WD. Interaction of urokinase-type plasminogen activator (u-PA) with its cellular receptor (u-PAR) induces phosphorylation on tyrosine of a 38 kDa protein. FEBS Lett 1993; 322: 37-40.
45. Del Rosso M, Anichini E, Pedersen N, et al. Urokinase-urokinase receptor interaction: non-mitogenic signal transduction in human epidermal cells. Biochem Biophys Res Commun 1993; 190: 347-352.
46. Cubellis MV, Wun TC, Blasi F. Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1. EMBO J 1990; 9: 1079-1085.
47. Ragno P, Montuori N, Vassalli JD, Rossi G. Processing of complex between urokinase and its type-2 inhibitor on the cell surface. A possible regulatory mechanism of urokinase activity. FEBS Lett 1993; 323: 279-284.

48. Verde P, Stoppelli MP, Galeffi P, Di Nocera P, Blasi F. Identification and primary sequence of an unspliced human urokinase poly(A)$^+$ RNA. Proc Natl Acad Sci USA 1984; 81: 4727-4731.
49. Henderson BR, Kefford RF. Conservation and potential role of RNA-instability of RNA-instability motifs in urokinase gene 3'-untranslated sequences. J Natl Cancer Inst 1991; 83: 1103-1104.
50. Henderson BR, McDonald DA, Kefford RF. Post-transcriptional regulation of urokinase plasminogen activator gene expression occurs in the nucleus of BC1 rat mammary tumor cells. Int J Cancer 1992; 50: 918-923.
51. Sier CFM, Verspaget HW, Griffioen G, et al. Imbalance of plasminogen activators and inhibitors in human colorectal neoplasia. Implication of urokinase in colorectal carcinogenesis. Gastroenterology 1991; 101: 1522-1528.
52. Schmitt M, Goretzki L, Jänicke F, et al. Biological and clinical relevance of the urokinase-type plasminogen activator (uPA) in breast cancer. Biomed Biochim Acta 1991; 50: 731-741.
53. Foekens JA, Schmitt M, van Putten WLJ, et al. Prognostic value of urokinase-type plasminogen activator in 671 primary breast cancer patients. Cancer Res 1992; 52: 6101-6105.
54. Hasui Y, Marutsuka K, Suzumiya J, Kitada S, Osada Y, Sumiyoshi A. The content of urokinase-type plasminogen activator antigen as a prognostic factor in urinary bladder cancer. Int J Cancer 1992; 50: 871-873.
55. Ganesh S, Verspaget HW, Sier CFM, et al. Prognostic relevance of mucosal plasminogen activator levels in colorectal cancer. Fibrinolysis 1992; 6 (Suppl 2): 103.
56. Quax PHA, Pedersen N, Masucci MT, et al. Complementation between urokinase-producing and receptor-producing cells in extracellular matrix degradation. Cell Regul 1991; 2: 793-803.
57. Behrendt N, Rønne E, Danø K. Binding of the urokinase-type plasminogen activator to its cell surface receptor is inhibited by low doses of suramin. J Biol Chem 1993; 268: 5985-5989.
58. Crowley CW, Cohen RL, Lucas BK, Liu G, Shuman MA, Levinson A.D. Prevention of metastasis by inhibition of the urokinase receptor. Proc Natl Acad Sci USA 1993; 90: 5021-5025.
59. Boyd D. Plasminogen dependent laminin degradation and matrigel invasion by cultured colon cancer is directed by receptor-bound urokinase. Fibrinolysis 1992; 6 (Suppl 1): 17-21.

Chapter VIII

GENERAL DISCUSSION

Since the beginning of the 20th century numerous studies have been published about a possible role of plasmin and its activators in malignancy. *In vitro* studies have indeed shown that there is an association between malignancy of cells and the expression of plasminogen activators (1,2). In general, most carcinoma cells in culture express an enhanced level of plasminogen activators, particularly of the urokinase-type. In most malignant tissue types the increase of plasminogen activation has also proven to be caused mainly by urokinase. Even in ascites, plasma, and urine samples of patients with gastrointestinal neoplasia increased levels of u-PA have been found (3-6). u-PA is known from various physiological processes to be involved in migration of cells, which suggests a role for this proteolytic enzyme in tumour invasion and metastasis. Antibodies against u-PA were found to be able to inhibit malignant invasive growth *in vitro* and *in vivo* (7-13). Moreover, various studies have shown that the production of high amounts of u-PA by primary malignant cells of human origin are correlated with the forming of metastases in nude mice (14,15).

In previous studies by our group the antigen and activity levels of u-PA and t-PA were investigated in tissue homogenates of colorectal tumours. The increased levels of u-PA in carcinomas were accompanied by a decrease in t-PA. Adenomatous polyps, precursor lesions to colorectal cancer, were shown to have intermediate levels between normal and malignant tissues of both plasminogen activators (16). On account of these observations, in chapter II of this thesis the immunolocalization of urokinase was investigated in colonic adenomas, carcinomas, and normal reference tissue, using a monoclonal antibody against u-PA. The immunohistochemical staining of u-PA in the tissue sections was compared with the u-PA antigen and activity content of homogenates of the same tissues. The immunohistological evaluation revealed a diffuse and cytoplasmic staining of neoplastic columnar epithelial cells. Most of the carcinoma specimens also showed positive u-PA staining in cells of stromal and muscular origin, and occasionally in endothelial cells. Only a minority of the carcinomas were found to have the highest intensity of u-PA staining in the invasive parts of the tumour. Adenomatous polyps showed

intense u-PA staining in dysplastic epithelial cells and in eosinophilic-like cells in the lamina propria, whereas normal intestinal mucosa contained hardly any u-PA staining cells. The intensity of u-PA staining in tissue sections correlated well with u-PA levels measured with an ELISA in homogenates of the same tissue, but there was no significant relation with known risk markers of malignancy.

The immunolocalization of u-PA in malignant colorectal tissue has been the topic of several studies. Although the majority of the investigators found enhanced staining of u-PA in carcinomas, particularly at the invasive front, there is no consensus about the cellular localization of the u-PA. In some studies urokinase has been detected in the cytoplasm and/or at the surface of tumour cells, while other investigators found no staining of carcinoma cells at all, but an intense staining of u-PA in fibroblast-like cells and macrophages (17-26). Part of these differences are probably due to the different techniques which were used to visualize the urokinase, i.e. immunofluorescence versus immunohistochemistry, different polyclonal and monoclonal antibodies, etc. A recent study using in situ hybrization revealed that mRNA for u-PA is expressed mainly in fibroblast-like cells of stromal origin and not in carcinoma cells, but that the urokinase receptor is expressed in tumour cells of colonic carcinomas, implicating that malignant cells are dependent on host tissue cells for the availability of u-PA (27). These findings, however, do not explain the total absence of u-PA protein in carcinoma cells or at their surface, as reported in some immunohistochemical studies. Independent of the origin of u-PA, the enzyme should be detectable at the surface of some carcinoma cells because of the presence of urokinase receptors on these cells. Urokinase exists in a multitude of forms in tissues. Extracellularly, u-PA can be present in the matrix or receptor bound at the cell surface. Receptor bound u-PA occurs in the inactive pro-enzyme form, as active u-PA, or complexed with inhibitors or with other extracellular matrix components (28). Intracellular existing forms of u-PA are inactive pro-u-PA and complexes of inactivated u-PA with inhibitors, internalized via the receptor. This multitude of different forms of urokinase probably plays a role in the difficulty to establish the u-PA localization in colorectal cancer tissue. The use of monoclonal antibodies which are able to

recognize all the existing forms of u-PA should give the definite answer on the exact localization of urokinase in colorectal neoplasia. The possibility exists that the regulation of extracellular proteolysis in colorectal cancer is a complex interplay between carcinoma cells and host tissue cells, and that the latter actively participate in tissue remodelling during invasion. Therefore, in future investigations extra attention should be focussed at the invasive fronts between normal and carcinoma tissues. Especially the role of plasminogen activation in the formation of fibrin depositions in and around carcinomas needs further investigation. Fibrin depositions may play a role in tumour angiogenesis, but could also function as a barrier between tumour and normal tissue (29).

Compared to colonic neoplasia, less is known about the presence and activity of plasminogen activators in malignancies along the upper part of the gastrointestinal tract. In the study described in chapter III, endoscopically obtained biopsies from oesophagus and stomach were homogenized, and plasminogen activator levels were determined in the neoplastic and normal tissue homogenates. Biopsy specimens proved to be usable in stead of resected tissues for the determination of plasminogen activator content. Although gastric columnar epithelium and squamous oesophageal epithelium do not resemble colonic mucosa in form and function, the levels of plasminogen activators found in these tissues were comparable with those in colonic tissues. Distinct differences between tumour and normal tissue were found in the levels of plasminogen activators in gastric epithelial tumours as well as in oesophageal carcinomas of squamous origin. Particularly the ratio between u-PA and t-PA antigen proved to be a reliable marker to differentiate between normal and tumour tissue in oesophageal and gastric malignancies. Also in squamous cell carcinomas of the oral cavity an increase in the presence of u-PA has recently been observed (13). Enhanced levels of u-PA and inhibitor PAI-1, but not PAI-2, have been found in stomach carcinomas (30). In contrast with our results, however, are the findings of Chung et al. (31), who reported enhanced activity of tissue-type plasminogen activator in combination with decreased urokinase-type plasminogen activator activity in gastric cancer tissues. In the latter study the amount of antigen of both plasminogen activators was not measured, which makes comparison with their findings difficult. The determination of

plasminogen activation components in tissues of the gastrointestinal tract could well be of diagnostic significance. Recent studies in breast cancers showed relations of the levels of u-PA, t-PA, and PAI-1 in tumor homogenates with disease-free interval and survival of the patients (32-34). The clinical importance of plasminogen activators and other components of the plasminogen activation cascade - i.e. inhibitors and receptors - in malignancies and precancerous conditions of the gastrointestinal tract will be the objective of further studies.

Regulation of plasminogen activation is a complex mechanism in which at least 2 types of inhibitors are involved. Early zymographic studies showed complexes of inhibitors with mainly tissue-type plasminogen activator (t-PA) to be present in homogenates of colonic carcinomas (16). Specific ELISAs for PAI-1 and PAI-2 were used in the study described in chapter IV to evaluate the antigen levels of these inhibitors in homogenates of normal, adenomatous, and carcinomatous colonic tissues. A significant increase of both inhibitors in carcinomas as well as adenomas was found to be related to the decrease of t-PA in neoplastic colorectal tissues. In contrast, the activity level of u-PA seemed not to be affected by the presence of the inhibitors; it was significantly increased compared to normal colorectal mucosa. Recently published immunohistochemical studies confirmed the enhanced presence of PAI-1 and PAI-2 in biopsy specimens of colorectal carcinomas. In those studies the inhibitors were mainly found in the central part of the tumours, whereas u-PA was located primarily at the invasive front of the tumours (25). Central parts of stomach carcinomas have also been reported to contain more PAI-1 than marginal parts of the tumours (30). The suggested difference in distribution between activator and inhibitors within carcinomas, which was also shown in melanomas (35), and in non-malignant cell migration (36), could be of crucial importance for local proteolytic activity. However, the possibility that tumour cells produce u-PA as well as inhibitors PAI-1 and/or PAI-2 cannot be ruled out. Cell culture studies have shown that malignant cells are able to secrete u-PA as well as inhibitors (37). Distinct localization in the secretion of u-PA and PAIs by cells could be an important mechanism. Polarized secretion of u-PA has been reported for epithelial cells in vitro (38,39), while TNF was found to induce

secretion of PAI-1 as well as u-PA in endothelial cells, but only the latter mainly at the basolateral site (40).

More than 95% of colorectal carcinomas originate from adenomatous polyps. These precursors of cancer have been shown to have already enhanced levels of the urokinase-type plasminogen activator (16). A lot of our present knowledge about carcinogenesis in the colorectum is based on studies of tissue from patients with familial adenomatous polyposis coli (FAP), a disease characterized by an early appearance of thousands of adenomatous polyps in the colorectum. When left untreated, these adenomas will inevitably lead to colorectal carcinoma. Surprisingly, only few studies have been performed on plasminogen activators in colonic adenomas derived from patients with FAP (41,42). The results of a study about plasminogen activators and inhibitor PAI-1 in 84 adenomatous polyps from patients with FAP are presented in chapter V. Adenomatous polyps were found to express less t-PA than normal mucosa and this decrease was more pronounced with increasing size of the adenomas. In contrast, urokinase was enhanced in adenomatous polyps, but there was no relation with the diameter of the polyps; even in most of the smallest polyps (<0.5 cm) an enhancement of u-PA was detected, indicating that increased expression of u-PA is an early event in the so-called adenoma-carcinoma sequence (43) and could therefore be of diagnostic importance. Inhibitor PAI-1 was only slightly enhanced in adenomas compared to normal appearing mucosa. The carcinomas of these FAP patients were found to have the highest u-PA and PAI-1 level with a concomitant low t-PA level. These results made clear that the presence and activity of plasminogen activators in neoplastic tissues of the colorectum of patients with familial adenomatous polyposis were similar to sporadic adenomas and carcinomas. Therefore, to study the role of plasminogen activation in colorectal carcinogenesis the use of tissue of familial adenomatous polyposis coli patients, in which different stages of neoplasia are present in one patient, could make a valuable contribution.

Metastasis is a multistep process involving numerous cell-cell and cell-matrix interactions. Probably less than 1% of the tumour cells which reach the circulation are able to form a distant metastasis (44). Circulating cells which do

not express excessive plasminogen activator activity are thought to have more chance than others to metastasize, because these cells can form aggregates of tumour cells, blood cells, and platelets. These clusters or microthrombi will get stuck more easily in capillaries than individual tumour cells and therefore will have more opportunity to adhere to the endothelium (17). Subsequently the tumour cells could then start to proliferate and form the metastasis. Eventually these cells could even invade the host tissue. Several studies have shown a relation between high u-PA expression by primary neoplastic tumour cells and their metastatic capacity. In chapter VI we compared plasminogen activator parameters in homogenates of liver metastases with primary colorectal tumours. The amount of u-PA antigen was significantly enhanced in liver metastases compared to normal liver tissue and similar to the levels found in primary tumours in the colorectum. However, in contrast to colorectal carcinomas the liver metastases showed hardly any plasminogen activator activity, u-PA nor t-PA. The absence of plasminogen activator activity in metastases could be attributed to an eight fold higher PAI-1 antigen content in metastatic tumours compared to primary carcinomas. The origin of PAI-1 in this tissue is not known. In normal tissues PAI-1 is mainly found to be produced in endothelial cells and this could also be the case in metastases. Alternatively, Quax and co-authors (37) reported extensive PAI-1 production by various carcinoma cells in culture. The high level of inhibitor PAI-1 in liver metastases could originate from the carcinoma cells and be a reflection of that small percentage of primary tumour cells which is able to survive the circulation by inhibiting proteolytic activity and forming cell clusters. Liver metastases are believed to be of clonal origin (45) and all cells like the original metastasizing cell could abundantly produce PAI-1. Another possible explanation for the extremely high PAI-1 amounts in liver metastases could be the influence of the surrounding liver tissue on the proliferating colorectal cells in the metastases. Modulating factors as hormones and growth factors are able to regulate expression of u-PA and its inhibitors *in vitro* (46). Despite the presence of PAI-1, the malignant cells in the metastases have a vast amount of activatable pro-u-PA at their disposal, thus a functional role for this enzyme and its receptor in the metastases can not be excluded. Immunohistochemistry and in situ

hybridization of plasminogen activator cascade components in liver metastasis have not been performed yet and should be able to provide us with essential information about the distribution and place of synthesis. Temporarily and localized protection of u-PA against the inhibition by PAI-1, and perhaps also PAI-2, seems to be of importance. In this context it is noteworthy that many liver metastases are surrounded by a fibrinous deposition, which could act as a separation between metastasis and liver tissue. For expansion of the metastasis within the liver, limited and localized degradation of this fibrin seems to be inevitable. Localized activity of urokinase could well be a candidate for this function.

In chapter VII the presence of another important regulator of urokinase, the urokinase receptor, was evaluated in carcinomas of the oesophagus and stomach, colorectal adenomas and carcinomas, liver metastases of colorectal tumours, and in adjacent normal tissues. All tissues investigated appeared to contain unoccupied u-PA receptor in a radio-ligand binding assay. High levels of u-PA receptor were particularly found in oesophagus and colorectal carcinomas and in liver metastases. Adenomatous polyps did not contain enhanced concentrations of the u-PA receptor. The enhanced presence of urokinase receptor in malignancies suggests once again a role for this enzyme system in localized proteolytic events. Moreover, interaction of u-PA with its receptor has recently been reported to be involved in signal transduction in *in vitro* studies (47-49), and this aspect should be considered in future studies in malignancies.

Regulation of the activation of plasminogen by urokinase is the result of the interaction of various proteins such as activators, receptors, and inhibitors. The localization and the amount of the various components are important in controlling the activity of the cascade. Influencing the synthesis of these proteins could be an important mechanism to regulate plasminogen activation. In order to study the regulating effect of synthesis levels of these proteins on the plasminogen activator cascade, messenger RNAs (mRNAs) of the most important components were determined in endoscopical biopsies of gastrointestinal neoplasms and adjacent normal tissues. Endoscopical biopsy specimens were used in stead of resected tissues because the latter proved to be unsuitable for RNA isolation from gastrointestinal tissues. Especially

colorectal tissues obtained at surgery were found to contain little intact mRNA. Tissue specific RNAses could be responsible for the rapid degradation of mRNAs. RNA isolation experiments using endoscopical biopsies taken prior to operation provided more intact mRNAs than isolations using resected tissues from the same patients. Levels of mRNA coding for u-PA, the u-PA receptor, as well as inhibitor PAI-1 were found to be higher in tumours of the oesophagus, stomach, and colorectum than in corresponding normal mucosa. These findings suggest that transcriptional activation of genes involved with plasminogen activation leads to elevated levels of their proteins, which, in turn, seems to participate in carcinogenesis of the gastrointestinal tract. The fact that tissues obtained at surgery contained little intact RNAs could also be of importance for interpreting in situ hybridization studies using resected gastrointestinal tissues (27).

From the results presented in this thesis it is clear that gastrointestinal malignancies are associated with an enhancement of the urokinase pathway of plasminogen activation in the tumour. Even the smallest benign adenomatous polyps are already found to have higher u-PA levels combined with lower t-PA levels compared with adjacent normal mucosa. Immunohistochemical staining of u-PA showed the abundant presence of urokinase in epithelial cells of adenomas and carcinomas. The balance between activators and inhibitors together with the proteolysis focussing capacity of the u-PA receptor could well be a crucial step in malignancy. Studies of plasminogen activation in malignancies of the breast, lung, ovary, skin, and other tissues revealed that different tumour types do not have to express the various components involved in the same way (50). Differences between carcinoma types are due to the differences between the malignant cells, but probably also due to differences of the surrounding normal tissue.

The role of the surrounding normal tissue in plasminogen activation of the tumours needs further investigation. On the one hand plasminogen activators are shown to be regulated by growth factors, which could be produced by malignant tissue, but also by the normal tissue as a reaction towards the presence of the tumour. On the other hand there is the intriguing controversion whether urokinase is produced by malignant colorectal cells themselves or by

stromal cells as stated earlier. Also noteworthy with respect to normal tissue is our recent finding of the prognostic value of plasminogen activators in normal tissues of patients with colorectal cancer (51). As indicated in the introduction, plasminogen activation is by no means the only proteolytic enzyme system capable of degradation of basement membrane and extracellular stroma components. Extracellular matrix degradation is a complex concerted action of several proteases and all the inhibitors and receptors that directly or indirectly regulate their activity (Figure 1).

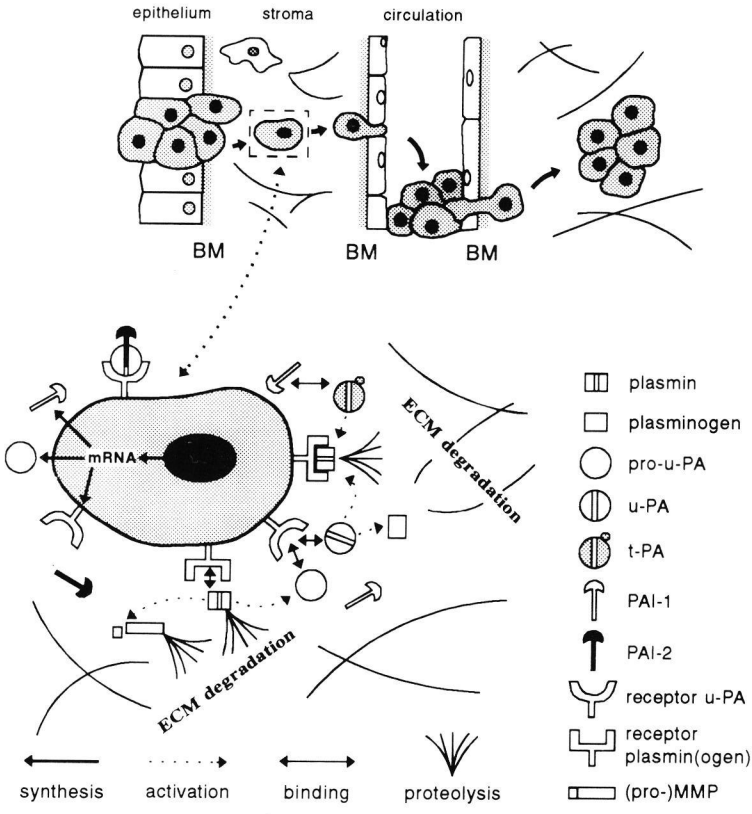

Figure 1. Magnification of the schematic carcinogenesis model of figure 1 from chapter I. The interactions between components of the plasminogen activation cascade during proteolytic activity are indicated. BM = basement membrane, MMP = matrix metalloproteinase.

Other factors besides proteinases which are thought to be of major importance in malignancy and metastasis are growth factors, components of the cytoskeleton, and adhesion molecules, but they are not discussed in this thesis. However, the urokinase type plasminogen activator has become the subject of numerous investigations concerning malignancy, and recently u-PA has been reported to have other capacities besides the activation of plasminogen. Urokinase has for instance been found to be involved in the regulation of growth factors (46). Moreover, functions of u-PA have been described which have no relation with its proteolytic capacity, for example urokinase has been demonstrated to be mitogenic, growth promoting, involved in the cytoskeleton of cells, and in signal transduction via the interaction with its receptor (28,48,49,52-55).

There has been a lot of interest in the role of plasminogen activation in cancer in the past and there will still be in the future. The use of cell lines and model systems like the nude mice have proven to be important tools in the study of plasminogen activation in malignancy. However, those techniques are not able to substitute the study of human tissues completely. Monocultures of cells do not have all of the stimuli to which cells in tissue are exposed and could therefore behave differently. Also the results of for instance metastasis formation in nude mice after injection or transplantation of human malignant cells have to be interpreted carefully because of interaction with heterogenous normal tissue and the absence of an intact immunological defense system. Knowledge about the various components of plasminogen activation, their presence, localization, interaction, and the way they influence each other is essential, not only for understanding their contribution to carcinogenesis, but also for using them as diagnostic tools. Eventually our knowledge of the process of plasminogen activation could lead to a direct interference in the interactions of the components, which could provide us with means to protect against and treat cancer of the gastrointestinal tract. Inhibiting monoclonal antibodies, recombinant constructs of plasminogen activation components, and synthetic inhibitors or receptor blockers are possible subjects for investigation in the future.

REFERENCES

1. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation and cancer. Adv Cancer Res 1985; 44: 139-266.
2. Astrup T. Fibrinolysis: past and present, a reflection of fifty years. Sem Thromb Hemost 1991; 17: 161-174.
3. Nishino N, Aoki K, Tokura Y, Sakaguchi S, Takada Y, Takada A. The urokinase type of plasminogen activator in cancer of digestive tracts. Thromb Res 1988; 50: 527-535.
4. Kirchheimer JC, Huber K, Wagner O, Binder BR. Pattern of fibrinolytic parameters in patients with gastrointestinal carcinomas. Br J Haematol 1987; 66: 85-89.
5. Huber K, Wojta J, Kirchheimer JC, Ermler D, Binder BR. Plasminogen activators and plasminogen activator inhibitor in malignant and non-malignant ascitic fluid. Eur J Clin Invest 1988; 18: 595-599.
6. Huber K, Kirchheimer JC, Sedlmayer A, Bell C, Ermler, Binder BR. Clinical value of determination of urokinase-type plasminogen activator antigen in plasma for detection of colorectal cancer: comparison with circulating tumor-associated antigens CA 19-9 and carcinoembryonic antigen. Cancer Res 1993; 53: 1788-1793.
7. Ossowski L, Reich E. Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 1983; 35: 611-619.
8. Ossowski L. Plasminogen activator dependent pathways in the dissemination of human tumor cells in the chick embryo. Cell 1988; 52: 321-328.
9. Ossowski L. In vivo invasion of modified chorioallantoic membrane by tumor cells: The role of cell surface-bound urokinase. J Cell Biol 1988; 107: 2437-2445.
10. Ossowski L, Russo-Payne H, Wilson EL. Inhibition of urokinase-type plasminogen activator by antibodies: The effect on dissemination of a human tumor in the nude mouse. Cancer Res 1991; 51: 274-281.
11. Mignatti P, Robbins E, Rifkin DB. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 1986; 47: 487-498.
12. Hearing VJ, Law LW, Corti A, Appella E, Blasi F. Modulation of metastatic potential by cell surface urokinase of murine melanoma cells. Cancer Res 1988; 48: 1270-1278.
13. Clayman G, Wang SW, Nicolson GL, et al. Regulation of urokinase-type plasminogen activator expression in squamous-cell carcinoma of the oral cavity. Int J Cancer 1993; 54: 73-80.
14. Quax PHA, van Muijen GNP, Weening-Verhoeff EJD, et al. Metastatic behavior of human melanoma cell lines in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-mediated matrix degradation. J Cell Biol 1991; 115: 191-199.
15. Marutsuka K, Suzumiya J, Kataoka H, Komada N. Correlation between urokinase-type plasminogen activator production and the metastatic ability of human rectal cancer cells. Invasion Metastasis 1991; 11: 181-191.
16. de Bruin PAF, Griffioen G, Verspaget HW, et al. Plasminogen activator profiles in neoplastic tissues of the human colon. Cancer Res 1988; 48: 4520-4524.
17. Markus G, Camiolo SM, Kohga S, Madeja JM, Mittelman A. Plasminogen activator secretion of human tumors in short-term organ culture, including a comparison of primary and metastatic colon tumors. Cancer Res 1983; 43: 5517-5525.
18. Burtin P, Chavanel G, Andre J. The plasmin system in human colonic tumors: An immunofluorescence study. Int J Cancer 1985; 35: 307-314.
19. Kohga S, Harvey SR, Weaver R, Markus G. Localization of plasminogen activators in human colon cancer by immunoperoxidase staining. Cancer Res 1985; 45: 1787-1796.

20. Kohga S, Harvey SR, Suzumiya J, Sumiyoshi A, Markus G. Comparison of the immunohistochemical localisation of urokinase in normal and cancerous human colon tissue. Fibrinolysis 1989; 3: 17-22.
21. Wojtukiewicz MZ, Zacharski LR, Memoli VA, et al. Indirect activation of blood coagulation in colon cancer. Thromb Haemost 1989; 62: 1062-1066.
22. Grøndahl-Hansen J, Ralfkiær E, Kirkeby LT, Kristensen P, Lund LR, Danø K. Localization of urokinase-type plasminogen activator in stromal cells in adenocarcinomas of the colon in humans. Am J Pathol 1991; 138: 111-117.
23. Kawanishi H, Tanaka K, Takai S, et al. Immunohistochemical analysis of plasminogen activator expression in human colorectal carcinomas: Correlation with CEA distribution and tumor cell kinetics. J Surg Oncol 1991; 46: 246-256.
24. Konno H, Tanaka T, Maruo Y, et al. Correlation between tumor proliferation and tumor tissue level of urokinase-type plasminogen activator. Eur Surg Res 1993; 25: 239-244.
25. Buø L, Lyberg T, Jørgensen L, Johansen HT, Aasen AO. Location of plasminogen activator (PA) and PA inhibitor in human colorectal adenocarcinomas. APMIS 1993; 101: 235-241.
26. Koretz K, Moller P, Schwartz-Albiez R. Plasminogen activators and plasminogen activator inhibitors in human colorectal carcinoma tissues are not expressed by the tumour cells. Eur J Cancer 1993; 29A: 1184-1189.
27. Pyke C, Kristensen P, Ralfkiær E, et al. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. Am J Pathol 1991; 138: 1059-1067.
28. Hebert CA, Baker JB. Linkage of extracellular plasminogen activator to the fibroblast cytoskeleton: Colocalization of cell surface urokinase with vinculin. J Cell Biol 1988; 106: 1241-1247.
29. Dvorak HF, Senger DR, Dvorak AM. Fibrin as a component of the tumor stroma: origins and biological significance. Cancer Metastasis Rev 1983; 2: 41-73.
30. Tanaka N, Fukao H, Ueshima S, Okada K, Yasutomi M, Matsuo O. Plasminogen activator inhibitor 1 in human carcinoma tissues. Int J Cancer 1991; 48: 481-484.
31. Chung SM, Kawai K. Protease activities in gastric cancer tissues. Clin Chim Acta 1990; 189: 205-210.
32. Jänicke F, Schmitt M, Pache L, Ulm K, Harbeck N, Høfler H, Graeff. Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer. Breast Cancer Res Treat 1993; 24: 195-208.
33. Duffy MJ, O'Grady P, Devaney D, O'Siorain L, Fenelly JJ, Lijnen HR. Urokinase-plasminogen activator, a marker for agressive breast carcinoms. Cancer 1988; 62: 531-533.
34. Grøndahl J, Christensen IJ, Rosenquist C, Brunner N, Mouridsen HT, Danø K, Blichert-Toft M. Cancer Res 1993; 53: 2513-2521.
35. de Vries TJ, Quax PHA, Denijn M, et al. Plasminogen activators, their inhibitors and urokinase receptor emerge in late stages of melanocytic tumor progression. Am J Pathol 1993; In press.
36. Sappino AP, Huarte J, Belin D, Vassalli J.D. Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. J Cell Biol 1989; 109: 2471-2479.
37. Quax PHA, van Leeuwen RTJ, Verspaget HW, Verheijen JH. Protein and messenger RNA levels of plasminogen activators and inhibitors analyzed in 22 human tumor cell lines. Cancer Res 1990; 50: 1488-1494.
38. Ragno P, Estreicher A, Gos A, Wohlwend A, Belin D, Vassalli JD. Polarized secretion of urokinase-type plasminogen activator by epithelial cells. Exp Cell Res 1992; 203: 236-243.
39. Canipari R, Zurzolo C., Polistina C, et al. Polarized secretion of plasminogen activators by epithelial cell monolayers. Biochim Biophys Acta 1992; 1175: 1-6.

40. van Hinsbergh VWM, van den Berg EA, Fiers W, Dooijewaard G. Tumor necrosis factor induces the production of urokinase-type plasminogen activator by human endothelial cells. Blood 1990; 75: 1991-1998.
41. Corasanti JG, Celik C, Camiolo SM, et al. Plasminogen activator content of human colon tumours and normal mucosae: Separation of enzymes and partial purification. J Natl Cancer Inst. 1980; 65: 345-351.
42. Sim PS, Stephens RW, Fayle DRH, Doe WF. Urokinase-type plasminogen activator in colorectal carcinomas and adenomatous polyps: quantitative expression of active and proenzyme. Int J Cancer 1988; 42: 483-488.
43. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990; 61: 759-767.
44. Weiss L. Metastatic inefficiency: Causes and consequences. Cancer Rev 1986; 3: 1-24.
45. Kerbel RS, Waghorne C, Korczak B, Lagarde A, Breitman M. Clonal dominance of primary tumors by metastatic cells: genetic analysis and biological implications. Cancer Surv 1988; 7: 597-629.
46. Laiho M, Keski-Oja J. Growth factors in the regulation of pericellular proteolysis: A review. Cancer Res 1989; 49: 2533-2553.
47. Blasi F. Urokinase and urokinase receptor: A paracrine/autocrine system regulating cell migration and invasiveness. BioEssays 1993; 15: 105-111.
48. Dumler I, Petri T, Schleuning WD. Interaction of urokinase-type plasminogen activator (u-PA) with its cellular receptor (u-PAR) induces phosphorylation on tyrosine of a 38 kDa protein. FEBS Lett 1993; 322: 37-40.
49. Del Rosso M, Anichini E, Pedersen, et al. Urokinase-urokinase receptor interaction: Non mitogenic signal transduction in human epidermal cells. Biochem Biophys Res Commun 1993; 190: 347-352.
50. Sappino AP, Belin D, Huarte J, Hirschel-Scholz S, Saurat JH, Vassalli JD. Differential protease expression by cutaneous squamous and basal carcinomas. J Clin Invest 1991; 88: 1073-1079.
51. Ganesh S, Sier CFM, Griffioen G, et al. Prognostic relevance of plasminogen activators in normal mucosa of patients with colorectal cancer. Submitted.
52. Kirchheimer JC, Wojta J, Christ G, Hienert G, Binder BR. Mitogenic effect of urokinase on malignant and unaffected adjacent human renal cells. Carcinogenesis 1988; 9: 2121-2123.
53. Nusrat AR, Mazar A, Henkin J, Chapman HA. A role for urokinase in mediating phorbol ester induced macrophage-like maturation and adhesion of U937 and other myeloid cells. Fibrinolysis 1992; 6: 71-76.
54. Botteri FM, Ballmer-Hofer K, Rajput B, Nagamine Y. Disruption of cytoskeletal structures results in the induction of the urokinase-type plasminogen activator gene expression. J Biol Chem 1990; 265: 13327-13334.
55. Frixen UH, Nagamine Y. Stimulation of urokinase-type plasminogen activator expression by blockage of E-cadherin-dependent cell-cell adhesion. Cancer Res 1993; 53: 3618-3623.

SUMMARY

In this thesis several studies are described on the regulation of the urokinase-type plasminogen activator in gastrointestinal malignancies. Plasminogen activators are enzymes which catalyze the conversion of plasminogen into plasmin. Plasminogen is commonly present in human tissues and it is the zymogen, the inactive pro-form, of plasmin. Plasmin, a serine proteinase, is a potent degrader of various proteins. The lytic capacity of plasmin is best known from the thrombolysis, the dissolution of blood thrombi in the circulation. Next to the degradation of fibrin in blood clots, plasmin is also able to breakdown proteins that are found in tissues, e.g. laminin, fibronectin, and proteoglycans. These proteins are especially found in basement membranes and interstitial stroma, two structures forming the extracellular matrix, an important constituent of tissue achitecture. Activation of plasminogen in tissues is shown to be an important mechanism for cells that are migrating through the extracellular matrix. The formation of bloodvessels for instance, the so-called angiogenesis, is a process in which the extracellular matrix is temporarily degraded by plasmin to make way for the evolving endothelium. Since the beginning of the 20^{th} century many studies reported that the activation of plasmin is also involved in malignant growth of various types of carcinomas. Malignancy is the ability of a tumour to cross anatomic barriers that separate the neoplastic cells from the normal host tissue. The degrading capacity of plasmin is considered to play a key role in these proteolytic events and it enables the tumour cells to invade the surrounding normal tissue.

The two most potent activators of plasminogen are called plasminogen activators, namely tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Previous studies in homogenates of tissues from the large bowel and rectum showed that there is more u-PA present in carcinomas than in the surrounding normal tissue. This rise in amount of u-PA was accompanied by an increase in activity of this enzyme. Remarkably, the levels of t-PA were found to be decreased in carcinoma tissue. Levels of plasminogen activators in premalignant stadia of colorectal cancer,

adenomatous polyps, were shown to be intermediate between normal tissue and carcinomas.

This thesis focusses on the regulation of the urokinase-type plasminogen activator in gastrointestinal malignancies. The major part of research in this study is done on colorectal carcinomas, because from this type of cancer it is relatively easy to obtain tissue from various stages of tumour development. The regulation of u-PA was studied in normal colorectal mucosa, in premalignant adenomatous polyps, in primary carcinomas, and in liver metastases. The study was extended with malignancies of the upper part of the gastrointestinal tract, the stomach and the oesophagus, using endoscopically obtained biopsies.

Isolation of RNA and subsequent northern blotting revealed that the increase in amount of u-PA protein in colorectal neoplasia was also seen on the mRNA level, suggesting that the u-PA which is present in adenomas and carcinomas is synthesized in the tumours and not coming from other parts of the body. In conclusion, the enhancement of u-PA antigen levels in colorectal neoplasia is regulated at the transcriptional level. This study did not definetely show which cells are responsible for the u-PA synthesis, although immunohistochemistry showed that the u-PA protein appeared to be localized primarily in the neoplastic epithelial cells of adenomas and carcinomas from the colorectum.

The amount of u-PA does not necessarily have to be correlated with the quantity of u-PA activity. Next to the presence of an inactive pro-form of u-PA (pro-u-PA), u-PA as well as t-PA can be inactivated by the formation of complexes with two specific plasminogen activator inhibitors, PAI-1 and PAI-2. Specific ELISAs for these inhibitors revealed that the levels of both PAI-1 and PAI-2 are already enhanced in adenomas and are especially high in carcinomas of the colorectum. Northern blotting showed also enhanced PAI-1 mRNA levels in colorectal neoplasia. In contrast, mRNA coding for PAI-2 was only occasionally found. Remarkably, the increased amount of inhibitors was correlated with a decrease in t-PA activity but not with the enhanced u-PA activity. Apparently, at least part of the u-PA escapes the physiological control by the inhibitors. Although homogenates of liver metastases also showed enhanced concentrations of u-PA, they did not contain detectable plasminogen

activator activity. This absence of activity was most probably caused by extremely high levels of inhibitor PAI-1. These findings suggest that regulation of the activity of u-PA in colorectal neoplasms is a delicate balance between the amount of activator and inhibitors, though more regulating factors are involved.

It is important for the invading carcinoma that the degradation of the matrix surrounding the migrating cells is restricted to certain areas, and that the proteolytic activity is specifically localized. For the urokinase-type plasminogen activator a specific receptor is known, which localizes the proteolytic activity. We evaluated the presence of u-PA receptors in homogenates of normal colorectal mucosa, adenomatous polyps, carcinomas, and liver metastases. While the levels of free u-PA receptor were enhanced in carcinomas and metastases, the adenomas did not show higher amounts of receptor than adjacent normal mucosa. The protein measurements of u-PA receptors in colorectal neoplasia were confirmed by northern blotting of u-PA receptor mRNA. These data suggest that the presence of the receptor for u-PA could be an important factor in the regulation of localized proteinolytic activity in colorectal malignancies.

Most of the studies performed on colorectal tissues were also done using biopsy specimens from the upper part of the gastrointestinal tract. Although different from colorectal mucosa in form and function, in general, normal tissues from stomach and oesophagus were not different in the content of plasminogen activation components. Stomach carcinomas and squamous carcinomas from the oesophagus showed enhanced amounts of u-PA. Decreased quantities of t-PA, as found in colorectal neoplasia, were detected only in stomach carcinomas and not in oesophageal cancers. Nevertheless the ratio between u-PA antigen and t-PA antigen, which proved to be a useful parameter in discriminating colorectal neoplasms from normal tissues, was also significantly enhanced in stomach as well as in oesophageal malignancies.

Recently, the altered levels of components of the plasminogen activation cascade in the tissues were shown to be related to the prognosis of patients with breast and colorectal carcinomas. In future investigations the role of plasminogen activation in gastrointestinal carcinogenesis has to be analysed further. Special attention should be focussed on the clinical consequences of

enhancement of this proteolytic system in malignancies. Are the increased amounts indicative for certain stages or forms of gastrointestinal neoplasia and are they useful as diagnostic tools? Which cells produce the different components of the plasminogen activation cascade? Could interference in the cascade of reactions prevent further invasive growth of primary tumours? Is metastasis formation prevented by blocking the u-PA activity or the u-PA receptor? These are some of the questions which are evoked by the studies reported in this thesis. Answers to those questions will only be obtained after extensive research. This research requires basic knowledge of the process of plasminogen activation as well as thorough evaluation of its clinical value. This can only be achieved by a joint effort of clinicians and basic scientists.

SAMENVATTING

Zoals alle levende wezens, is het menselijk lichaam opgebouwd uit cellen. Er zijn vele soorten cellen, die wat betreft uiterlijk en eigenschappen aangepast zijn aan de functie die ze vervullen. Voorbeelden van totaal verschillende celtypen zijn bloedcellen, spiercellen, zenuwcellen en huidcellen. Op een bepaald moment in hun levensfase zijn cellen in staat zich te vermenigvuldigen door middel van celdeling. Vooral de huid en de darmen zijn plaatsen in het lichaam waar een hoge mate van vernieuwing van de celpopulatie door middel van celdeling plaats vindt. Als door wat voor oorzaak dan ook de regulatie van dit celdelingsproces verstoord wordt en er ongecontroleerde deling en groei van bepaalde cellen optreedt spreken we van tumorvorming of neoplasie. Echter, pas als deze ongecontroleerde celgroei invasief gedrag vertoont, dat wil zeggen dat de cellen omliggende weefsels gaan binnendringen, spreken we van kwaadaardige nieuwvormingen of maligniteiten.

In dit proefschrift wordt verslag gedaan van een aantal studies naar de regulatie van urokinase-type plasminogeen activator in kwaadaardige aandoeningen van het spijsverteringskanaal. Plasminogeen activatoren zijn enzymen die de omzetting van plasminogeen in plasmine catalyseren. Plasminogeen komt in allerlei weefsels van het menselijk lichaam voor en is de inactieve pro-vorm van plasmine. Plasmine is het meest bekend uit de thrombolyse, het oplossen van stolsels in het bloed. Naast het oplossen van bloedstolsels is plasmine echter ook in staat om eiwitten in weefsels af te breken, b.v. laminine, fibronectine en proteoglycanen. Deze eiwitten komen vooral voor in basaal membranen en interstitieel stroma, structuren die tezamen de extracellulaire matrix vormen, een weefselcomponent met een voornamelijk ondersteunende taak. Onderzoek heeft aangetoond, dat de tijdelijke afbraak van de extracellulaire matrix door middel van de activatie van plasminogeen een belangrijk mechanisme is voor cellen om zich door weefsels te kunnen verplaatsen. De vorming van bloedvaten bijvoorbeeld, de zogenaamde angiogenese, is een proces waarin die matrix tijdelijk door plasmine wordt afgebroken, zodat de bloedvat vormende endotheelcellen kunnen uitgroeien.

Sinds het begin van de 20e eeuw hebben verschillende studies aangetoond, dat plasminogeen activatie ook een rol speelt in de kwaadaardige uitgroei van verschillende soorten kanker. Kwaadaardige kankers of carcinomen worden gekenmerkt door het vermogen om door het omringende normale weefsel te kunnen groeien. Het weefsel oplossend vermogen van plasmine wordt beschouwd als een belangrijke factor in deze invasieve groei.

De twee belangrijkste activatoren van plasminogeen worden respectievelijk weefsel-type of tissue-type plasminogeen activator (t-PA) en urokinase-type plasminogeen activator (u-PA) genoemd. Eerdere studies in homogenaten van dikke darmweefsel hebben aangetoond, dat er meer u-PA voorkomt in carcinomen dan in normaal weefsel. Deze verhoogde concentratie van u-PA ging vergezeld van een verhoogde activiteit van het enzym. Opmerkelijk was, dat het niveau van t-PA, het andere type plasminogeen activator, juist verlaagd was in het kankerweefsel. De plasminogeen activator concentraties in adenomateuze poliepen, premaligne voorstadia van dikke darmkanker, vertoonden waarden tussen het gezonde weefsel en de carcinomen in.

Dit proefschrift gaat over de regulatie van het urokinase-type plasminogeen activator in maligniteiten van het spijsverteringskanaal. Om practische redenen is het meeste onderzoek gedaan aan kanker van de dikke darm. De regulatie van u-PA werd bestudeerd in normaal darmslijmvlies, in premaligne adenomateuze poliepen, in carcinomen en in levermetastasen, uitzaaiingen afkomstig van dikke darmtumoren. De studie werd uitgebreid met maligniteiten uit hoger gelegen delen van het spijsverteringskanaal, te weten de maag en de slokdarm. Hiervoor werd echter geen gebruik gemaakt van operatief verkregen weefsel, zoals bij de dikke darm, maar van endoscopisch genomen biopten.

Northern blotting van geïsoleerd RNA toonde aan, dat de verhoogde hoeveelheid u-PA eiwit in dikke darmtumoren ook terug te vinden was op mRNA niveau. Aangenomen kan worden, dat het aanwezige u-PA eiwit in de poliepen en carcinomen dus ook daar geproduceerd is en niet van elders uit het lichaam afkomstig is. Dit onderzoek laat echter niet zien welk type cel verantwoordelijk is voor de verhoogde u-PA concentratie in de tumor, hoewel immunohistologisch onderzoek aantoonde dat het u-PA eiwit voornamelijk gelocaliseerd is in de epitheliale tumorcellen van adenomen en carcinomen van

de dikke darm en slechts incidenteel in cellen van het omliggende stroma.

De hoeveelheid u-PA eiwit hoeft niet direct gecorreleerd te zijn met de activiteit van het enzym. De enzymactiviteit wordt op verschillende manieren gereguleerd. Naast het voorkomen van een inactieve pro-vorm van u-PA (pro-u-PA), worden zowel u-PA als t-PA geinactiveerd door complexvorming met twee specifieke plasminogeen activator remmers, PAI-1 en PAI-2. Specifieke ELISA's voor deze remmers, of inhibitoren, toonden aan dat zowel PAI-1 als PAI-2 in verhoogde concentraties in poliepen voorkomen, en dat vooral in carcinomen grote hoeveelheden van beide inhibitoren aanwezig zijn. Northern blotting liet ook verhoogde PAI-1 mRNA niveaus zien in dikke darmtumoren, maar mRNA coderend voor PAI-2 werd zelden gevonden. Opmerkelijk was dat de verhoogde concentratie inhibitoren wel gecorreleerd was met de dalende t-PA activiteit, maar niet met de juist verhoogde u-PA activiteit. Waarschijnlijk is een deel van het aanwezige u-PA in staat om aan de fysiologische controle door de inhibitoren te ontsnappen. Homogenaten van levermetastasen vertoonden ook verhoogde u-PA concentraties, maar geen plasminogeen activator activiteit. De afwezigheid van activiteit werd naar alle waarschijnlijkheid veroorzaakt door de extreem hoge hoeveelheden van PAI-1. Deze bevindingen suggereren dat de regulatie van de activiteit van u-PA in dikke darmtumoren een delicaat evenwicht is tussen de hoeveelheid activator en inhibitor(en), maar waarschijnlijk spelen er meer factoren een rol.

Voor een invasief carcinoom is het van belang dat de matrix rondom de migrerende cel op het juiste moment en op de juiste plaats afgebroken wordt. Voor u-PA is een specifieke receptor beschreven, die in staat is de eiwit-afbraak van het enzymsysteem te localiseren. Wij hebben de aanwezigheid van u-PA receptoren in homogenaten van normaal dikke darmslijmvlies, adenomateuze poliepen, carcinomen en levermetastasen onderzocht. Terwijl de concentraties vrije u-PA receptor verhoogd waren in carcinomen en metastasen, bleken de poliepen niet meer van deze receptor te bevatten dan normaal darmweefsel. Deze op eiwit niveau gevonden waarnemingen werden bevestigd door northern blotting van u-PA receptor mRNA geïsoleerd uit hetzelfde darmweefsel. Deze resultaten suggereren dat de aanwezigheid van de u-PA receptor een belangrijke factor zou kunnen zijn in de regulatie van met name de

gelocaliseerde afbraak van eiwit in kwaadaardige tumoren van de dikke darm.

De meeste van de hierboven beschreven studies in dikke darmweefsels werden ook uitgevoerd op endoscopische biopten uit hoger gelegen gedeelten van het spijsverteringskanaal. Ofschoon deze weefsels in vorm en functie verschillen van die uit de dikke darm, waren het normale slijmvlies van maag en slokdarm nauwelijks verschillend in het niveau van plasminogeen activatie componenten. Kanker van de maag en plaveiselcel-carcinomen van de slokdarm vertoonden verhoogde concentraties u-PA, zowel op eiwit als mRNA niveau. Verlaging van de hoeveelheid t-PA, zoals ook aangetoond in dikke darmtumoren, werd wel waargenomen in maagcarcinomen maar niet in slokdarmkanker. Ondanks dat was de ratio tussen de hoeveelheid u-PA en t-PA, een bruikbare parameter voor het onderscheid tussen normaal en tumor in dikke darmweefsel, toch ook significant verhoogd in maligniteiten van de maag en van de slokdarm.

Onlangs werd aangetoond dat concentraties van componenten betrokken bij de activatie van plasminogeen, zoals u-PA en PAI-1, in tumoren gerelateerd zijn met de overleving van patiënten met borstkanker of dikke darmkanker. Verder onderzoek zou een completer beeld moeten geven van de rol van plasminogeen activatie in maligniteiten van het spijsverteringskanaal. Speciale aandacht zou hierbij gegeven moeten aan de klinische betekenis van deze verhoogde afbraak van eiwitten in maligniteiten. Zijn de verhoogde concentraties van activatoren, inhibitoren of receptoren bruikbaar om verschillende stadia in maag- en darmmaligniteiten te diagnostiseren? Welke cellen maken de verschillende componenten van het plasminogeen activatie systeem? Zou ingrijpen in de elkaar opvolgende reacties verdere invasieve groei kunnen voorkomen? Kan blokkeren van u-PA activiteit dan wel de u-PA receptor de vorming van metastasen voorkomen? Dit zijn een aantal vragen, die de studies beschreven in dit proefschrift oproepen. De antwoorden kunnen echter alleen door middel van uitgebreid verder onderzoek gegeven worden, waarbij niet alleen een gedegen kennis van het proces van plasminogeen activatie wordt vereist, maar ook het klinische inzicht om de betekenis van die kennis naar de patiënt te vertalen. Dit kan alleen bereikt worden door een nauwe samenwerking tussen klinische en basale onderzoekers.

NAWOORD

Dit proefschrift en de presentatie ervan is tot stand gekomen met de hulp van velen, waarvan ik er een aantal met name wil noemen:

- de collega's van het laboratorium
- de medewerkers van de afdeling endoscopie
- de medewerkers van de operatiekamer
- de medewerkers van de uitsnijkamer van pathologie
- de medewerkers van de audiovisuele dienst
- de medewerkers van de Prof. dr. J. Mulder bibliotheek
- de medewerkers van de huisdrukkerij
- dhr. M. van der Heyden
- dhr. Th. van Rooijen
- mevr. L. Schrama en dhr. F. Brouwer
- dhr. R. de Paus
- dhr. E. Cohen
- dhr. D. de Jongh
- dhr. F. Sier jr.
- de dames L. Niepoth, M. Koster-de Vreese, J. van Spronsen en M. Biewinga-Kok
- mevr. M. Pijnenburg en de heer H. Vloedgraven
- de heren P. de Bruin en S. Ganesh
- de heren C. Fellbaum, M. Schmitt, H. Graeff en H. Höfler (TU, München)
- de heren P. Quax en G. Dooijewaard (IVVO-TNO, Leiden)
- dhr. F. Nagengast (KU, Nijmegen)
- de heren K. Welvaart en C. van de Velde (afdeling Heelkunde)

CURRICULUM VITAE

De auteur van dit proefschrift werd op 24 augustus 1959 te Amsterdam geboren. Hij volgde de middelbare schoolopleiding aan het Lyceum 'Fons Vitae' te Amsterdam en rondde deze in 1979 met het behalen van het Gymnasium-β diploma succesvol af. In hetzelfde jaar startte hij met de studie biologie aan de Vrije Universiteit te Amsterdam, waar in 1983 het kandidaatsexamen werd behaald (B1-wiskunde). Het doctoraalexamen met als hoofdvak Moleculaire Neurobiologie en als bijvakken Medische Microbiologie en Immunologie, aangevuld met de onderwijsaantekening biologie, werd in augustus 1988 met goed gevolg afgelegd. In april 1989 werd een opleiding tot Cobol-programmeur/ systeemanalist (Volmac, Utrecht) met succes afgerond. Vanaf 1 mei 1989 is hij werkzaam op de afdeling Maag-, Darm- en Leverziekten van het Academisch Ziekenhuis Leiden (hoofd: Prof. Dr. C.B.H.W. Lamers). Aldaar en op het Gaubius Laboratorium IVVO-TNO te Leiden werd het in dit proefschrift beschreven onderzoek uitgevoerd.

LIST OF ABBREVIATIONS

APAAP	alkaline phosphatase-anti-alkaline phosphatase
BIA	bio-immunoassay
BM	basement membrane
DFP	diisopropyl fluorophosphate
DSS	disuccinimydyl suberate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FAP	familial adenomatous polyposis coli
GI	gastrointestinal
GPI	glycosyl-phosphatidylinositol
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HEPES	N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid
Ig D/G/M	immunoglobulin class D, G, or M
HMW u-PA	high molecular weight u-PA
LMW u-PA	low molecular weight u-PA
mIU	milli International Unit
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
nd	not done
ns	not significant
OD^{260}	optical density at 260 nm
p	probability
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PMSF	phenylmethylsulfonyl fluoride
R	correlation coefficient
RNA	ribonucleic acid
RPM	rotations per minute
sc-t-PA	single-chain-t-PA
SDS-PAGE	sodiumdodecylsulphate polyacrylamide gelelectrophoresis
SEM	standard error of the mean
TIMP	tissue inhibitor of metalloproteinase
TNM	tumour (lymph)node metastasis classification
t-PA	tissue-type plasminogen activator
Tris	tris(hydroxymethyl)aminomethane
u-PA	urokinase-type plasminogen activator
u-PAR	urokinase-type plasminogen activator receptor
v/v	volume/volume
w/v	weight/volume