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Plasminogen Activators and Plasminogen Activator Inhibitors in Human Colorectal Carcinoma Tissues are not Expressed by the Tumour Cells

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Plasminogen activators (PA) have been implicated with the degradation of extracellular matrix during the invasive growth of metastasising tumour cells. The significance of PA expression in tumour cells for the *in vivo* growth of malignant tumours is still a matter of debate. We, therefore, performed immunohistological studies on human colon tumours using monoclonal antibodies against urokinase- (u-PA) and tissue-type plasminogen activator (t-PA) as well as against plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2). Normal colorectal mucosa of seven samples was negative for all four constituents of the PA system. Tumour epithelium of 64 colorectal carcinomas and 10 liver metastases was consistently negative for both, PA and their inhibitors. However, two of four human colon carcinoma cell lines weakly expressed u-PA, PAI-1 and PAI-2. Interstitial dendritic or fibroblast-like cells within the tumour tissue strongly expressed u-PA and, at a lower level, also t-PA, PAI-1 and PAI-2. Vascular endothelial cells were weakly positive for all components of the PA system in colon carcinoma. Our findings indicate that colon carcinoma cells in their natural environment do not express constitutents of the PA system. PA activity, previously found in colon carcinoma tissue, is most likely derived from interstitial cells.
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INTRODUCTION

PLASMINOGEN ACTIVATORS (PA) are thought to play a pivotal role in the invasion of metastasising tumour cells. PA-mediated activation of plasmin leads to the degradation of extracellular matrix components [1] which may facilitate the spread of tumour cells. Studies using *in vitro* cultivated cells seem to confirm this hypothesis. It was shown that many tumour cell lines synthesise and secrete PA in large amounts [2–5]. Furthermore, antibodies specific for u-PA or t-PA had an inhibitory effect on the invasive capacity of cultured tumour cells as determined by *in vitro* assay systems [6, 7]. Measurements of PA activity in primary tumour tissues, however, were disparate and seem to reflect a more complex situation. While most studies reported increased levels of PA in tumour compared to normal tissue [8–13], only some described a positive correlation of PA activity to the stage of malignancy [11, 12, 14]. Oka *et al.* [15] reported a positive correlation of levels of u-PA expression of neoplastic cells in pulmonary adenocarcinoma with tumour size and lymph node involvement at the time of operation. Other studies have, however, presented contradictory results [16]. In colon carcinomas a lower secretion rate of PA was determined in metastatic compared to primary tumours [17]. Some studies were performed to identify the PA-producing cells in tumour tissue. Although studies using polyclonal antisera and monoclonal antibodies (Mab) suggested u-PA might be produced by the neoplastic cells of colon carcinoma [18–21], Grondahl-Hansen *et al.* [22] recently found, by using monoclonal antibodies against urokinase-type PA (u-PA) and tissue-type PA (t-PA), that u-PA-specific staining was confined to fibroblast-like cells within colon carcinoma tissue, whereas malignant cells were apparently negative for both PA types. In a consecutive study mRNA for PA inhibitor 1 (PAI-1) was not detected in colon carcinoma cells [23, 24]. Since PA activity depends on the interaction of PA with their specific inhibitors [1], we extended the study on *in situ* expression of constituents of the PA system by also evaluating the expression of PAI-1 and PAI-2 in colorectal primary tumours and in liver metastases of colon carcinomas.

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MATERIALS AND METHODS

Reagents

The mouse Mab 98/6 was raised against purified low molecular u-PA (33 kDa) and reacts specifically with the 33 and the 54 kD form of active u-PA as well as with pro-u-PA. This Mab did not react with human t-PA and plasminogen as evaluated by enzyme linked immunosorbent assay (ELISA). Monoclonal antibody 98/6 is of IgG1 isotype [25]. The Mab against t-PA and PAI-1 (# 101) were purchased from Monozyme (Charlottenlund, Denmark) and are of IgG1 isotype. The t-PA-specific Mab reacts with the A-chain of two-chain t-PA, binds single-chain t-PA and shows no reaction with u-PA (producer's information). The PAI-1-specific Mab, according to the producer's information, reacts with free PAI-1 and PAI-1/u-PA and PAI-1/t-PA complexes. The Mab against PAI-2 was obtained from American Diagnostics (New York), is of subclass IgG2a and reacts with the high molecular form (60 kD) and the low molecular form (48 kD) of placental PAI-2. This Mab reacts with lower affinity also with PAI-2/u-PA and PAI-2/t-PA complexes. Monoclonal antibody binding was detected with a polyclonal sheep antibody to mouse immunoglobulins (Amersham) and a streptavidin-biotinylated peroxidase complex (Amersham). 3-Amino-9-ethyl-carbazole (AEC) and *N'N*-dimethyl-formamide (DMF) were obtained from Sigma.

Tumour tissue and cell lines

Tissue samples of 64 patients who underwent resection of a colon or rectum carcinoma, were immediately quick frozen in liquid nitrogen. Serial sections of 2–5 μm thickness were cut, air dried and fixed in acetone for 10 min. The tumours were documented, typed, graded and staged according to the UICC classification [26–28]. Seven carcinomas were grade I, 44 were grade II and 13 were grade III. Eleven were of the mucinous type and 53 were non-mucinous. According to the Duke's staging, 17 cases were stage A, 21 cases stage B and 26 cases stage C. Thirty-four carcinomas were located in the rectum, 9 in the sigmoid and 21 in the residual colon. In addition, seven samples of unaffected mucosa and 10 liver metastases of colon carcinomas were investigated.

The colon carcinoma cell line HT29, obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. (ATCC), and three colon carcinoma cell lines (HD-C133, HD-C8, HD-C114), generous gifts of Prof. Dr M. Schwab, German Cancer Research Center, Heidelberg, F.R.G., were cultivated in RPMI 1640 medium (Gibco, Paisley, U.K.) containing 10% fetal calf serum. Cells were detached by short treatment with EDTA, centrifuged and washed. Cytospins were made, air-dried, fixed in acetone for 10 min, and stained. Two human large cell lung carcinoma cell lines, LCLC103H and LCLC97TM1, were used as positive controls. The state of differentiation [29] and synthesis of PA components [4, 25] have been described for these cell lines. The cell line LCLC103H, negative for t-PA production, showed positive staining for u-PA, PAI-1 and PAI-2 whereas cell line LCLC97TM1, negative for PAI-2 production, reacted with Mab against u-PA, t-PA and PAI-1 (Table 1, Fig. 1).

Immunohistochemical staining

All four Mab were diluted 1:100 in phosphate-buffered saline (PBS), pH 7.4; the optimal dilutions were evaluated by staining the two lung carcinoma cell lines with the four Mab. Biotinylated sheep antibody to mouse immunoglobulin was diluted 1:50, and the streptavidin peroxidase complex 1:100 in PBS. Incubation times were 1 h at room temperature for the first antibody and 30 min for the second and third step reagents. Using AEC as the chromogen (0.4 mg/ml in 0.01% H_2O_2 for 30 min), the peroxidase reaction caused an intense red precipitate. The sections were rinsed in tap water, counterstained in Harris' haematoxylin, and mounted with glycerol gelatine. Isotype-matched controls with irrelevant Mab were carried out on a limited number of carcinomas and revealed no isotype-associated side reaction. Each frozen section series contained a negative control without the first reagent; staining was observed in granulocytes whose endogenous peroxidase was not blocked. These reactivities together with the staining observed in areas of tumour necrosis were disregarded. Each frozen-section series contained, as positive controls, cytospins of the lung carcinoma cell lines LCLC103H and LCLC97TM1.

Table 1. *Expressions of u-PA, t-PA, PAI-*1 *and PAI-2 in colon and control lung carcinoma cell lines*

Cell lines	u-PA	t-PA	PAI-1	PAI-2
Colon cell lines				
HT-29	—	—	—	—
HD-C8	—>(+)	—	(+)	—>>+
HD-C114	—>>(+)	—	—	—
HD-C133	—	—	—	—
Control lung cell lines				
LCLC103H	+	—	+	+>>—
LCLC97TM1	+/—	+>—	—>>+	—

+, all cells or structures express the antigen; +>>—, the antigen is expressed in almost all cells; +>—, the antigen is expressed in the majority of cells; +/—, the antigen is expressed in about half of the cells; —>+, the antigen is expressed in a minority of cells; —>>+, the antigen is expressed in few cells; —, the antigen is not expressed; brackets indicate weak antigen expression.

Evaluation of immunohistological staining

For evaluation of intensity of staining, a semiquantitative system was used to determine the antigen expression in the tissue samples and in the tumour cell lines. Antigen expression was scored "+" whenever specific staining was detectable, and "−" when no antigen was detectable. Furthermore, "A ≫ B" indicates that only a minority of cells revealed the staining modality B; "A > B" indicates that cells with staining modality A clearly outnumbered those with modality B; "A/B" indicates that + and − cells were found in equal proportions.

RESULTS

Normal colorectal mucosa and liver parenchyma

Seven tissue samples of normal colorectal mucosa were immunohistochemically investigated for expression of u-PA, t-PA, PAI-1 and PAI-2. Neither in epithelial cells nor in mesenchymal and interstitial cells were PA or inhibitors detected (Fig. 2 a,b). In contrast, in the normal liver parenchyma of 10 colon carcinoma metastases u-PA and t-PA were found expressed in the endothelial compartment of hepatic sinusoids (Fig. 2 c,d), PAI-1 and PAI-2 in few fibroblast-like cells in the interlobular connective tissue.

Colorectal carcinomas and their liver metastases

None of the 64 colorectal carcinomas and 10 liver metastases of colon tumours tested expressed PA or PA inhibitors in their neoplastic epithelium (Table 2, Fig. 3 a–d). In the tumour tissue, endothelium of a few arteries, veins and venules was found to express the PA and inhibitors (Fig. 3 b,c), in addition, t-PA was present in endothelial cells of some capillaries. In contrast, endothelium of lymphatics was negative for all PA components. Although PA proteins were present in the vascular endothelium of colorectal tumours and their liver metastases, their appearance was inconsistent and sparse. No PA or inhibitors were found in structures of the visceral nervous system and in smooth muscle cells of vessels or of gut wall. Lymphocytes in the tumour tissue were negative for PA proteins. The strongest staining for all PA components was observed in interstitial dendritic cells with fibroblast-like morphology (Fig. 3 a,d). In particular, strong expression of u-PA was especially found in those interstitial cells which were in close vicinity of tumour nodules or strains (Fig. 3a). In comparison to u-PA, expression of t-PA was weak in these cells. Only a subset of cells, positive for PA, were also positive for PAI-1 and PAI-2. PAI-2-positive fibroblast-like cells were found in the close vicinity of the tumours (Fig. 3d). As PA-positive fibroblast-like cells were especially found in close vicinity of tumour nodules or strains, there was no difference in PA expression between the tumour invasive front and more centrally located tumour nests.

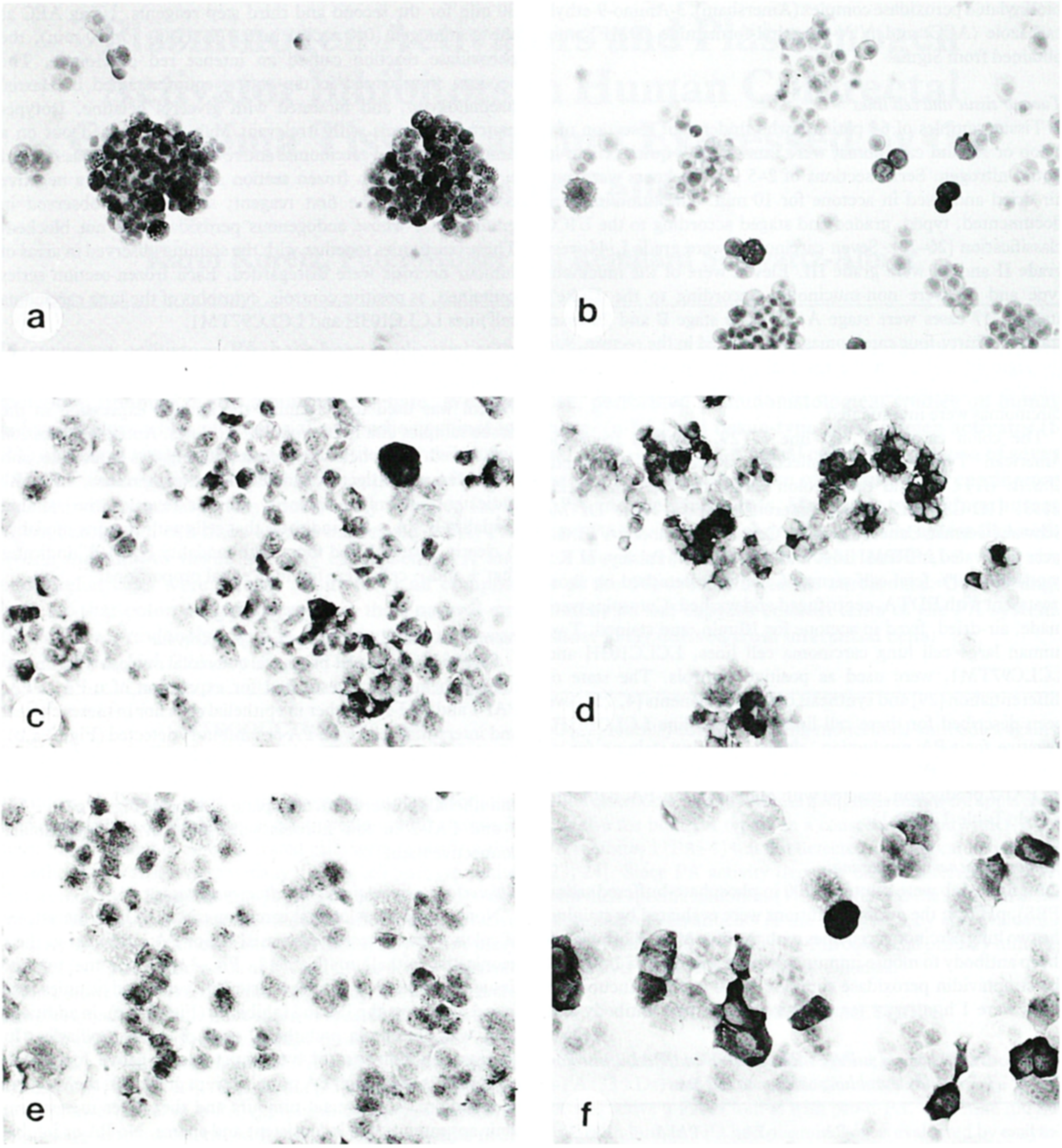

Fig. 1. Expression of PA components in human colon and control lung carcinoma cell lines. (a) u-PA expression in colon carcinoma cell line HD-C8. u-PA is irregularly and inconsistently expressed in the cytoplasm of the tumour cells. (b) PAI-2 expression in colon carcinoma cell line HD-C8. PAI-2 is expressed in the cytoplasm of few tumour cells. (c) u-PA expression in lung cell line LCLC103H. u-PA is expressed in various amounts, faint and spotty or evenly distributed in the cytoplasm. (d) t-PA expression in lung cell line LCLC97TM1. Most cells express high amounts of t-PA in the cytoplasm. (e) PAI-1 expression in lung cell line LCLC103H. PAI-1 is found in minute granules more or less concentrated in a paranuclear cleft within the cytoplasm. (f) PAI-2 expression of lung cell line LCLC103H. PAI-2 is strongly expressed in the cytoplasm of a minor subset of LCLC103H cells. Magnification × 125.

Colon cell lines

Four colon carcinoma cell lines (HT29, HD-C8, HD-C114, HD-C133) were tested for their reactivity with Mab against u-PA, t-PA, PAI-1 and PAI-2 using the immunoperoxidase staining technique. The cell lines displayed heterogeneous expression patterns of PA components. In cell lines HT29 and HD-C133 none of the respective PA-components could be detected (Table 1). The absence of u-PA and t-PA in cell line HT29 has been reported before [2]. A subpopulation of HD-C8 cells weakly expressed u-PA, PAI-1 and PAI-2 (Table 1, Fig. 1 a,b). Few HD-C144 cells expressed u-PA at a low level. The expression of the PA components in the lung control cell lines is shown in Table 1, Fig. 1 c–f.

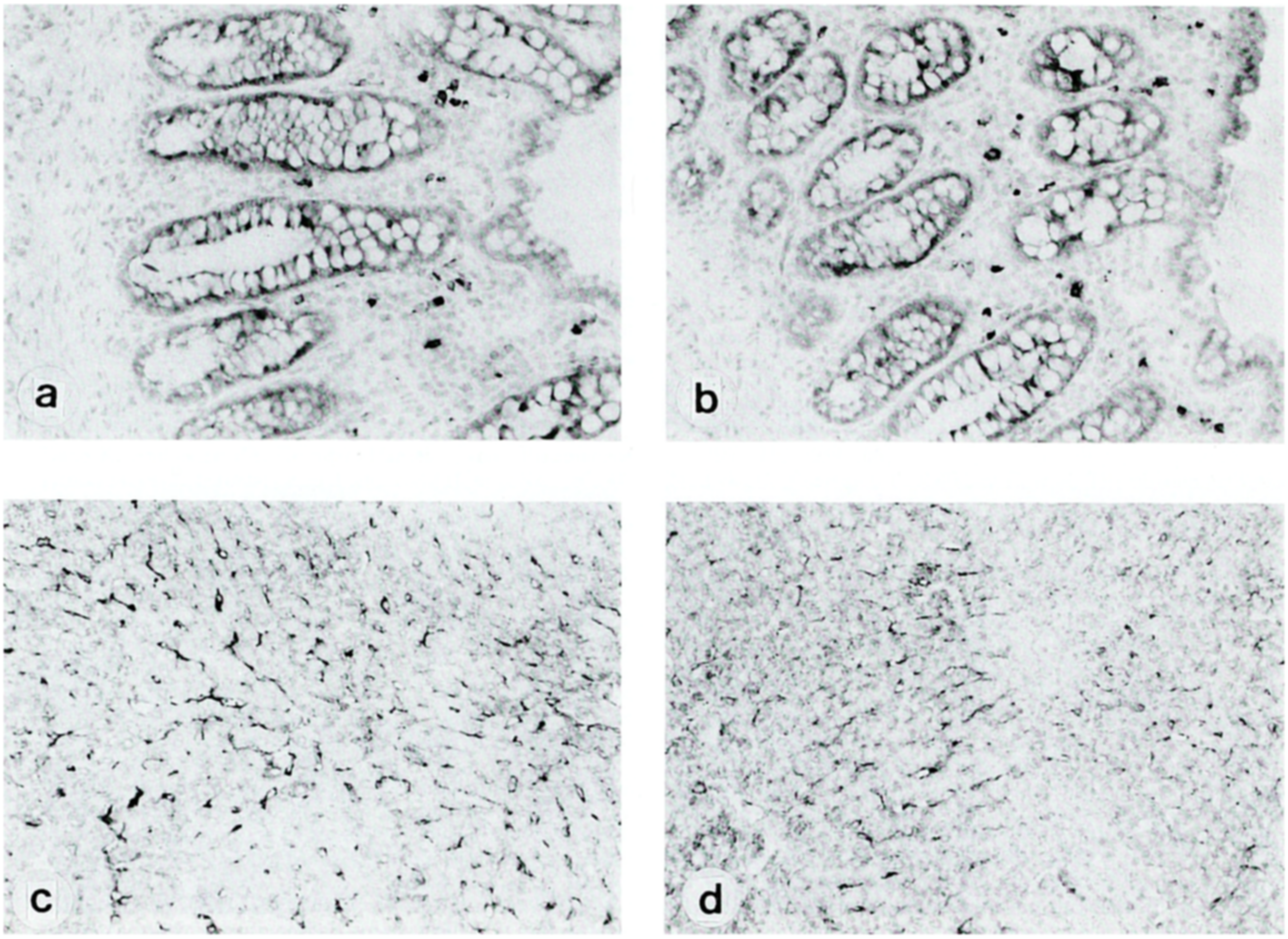

Fig. 2. Expression of PA components in normal colorectal mucosa and in liver parenchyma. Neither in epithelial cells nor in mesenchymal and interstitial cells were u-PA (a) or t-PA (b) detected. Staining was observed in granulocyte of the mucosa whose endogenous peroxidase was not blocked. In the normal liver parenchyma u-PA (c) and t-PA (d) were found expressed in the endothelial compartment of hepatic sinusoids. Magnification × 130.

Table 2. Expression of u-PA, t-PA, PAI-1 and PAI-2 in colorectal carcinomas

Tumour tissue	u-PA	t-PA	PAI-1	PAI-2
Tumour epithelium	—	—	—	—
Smooth muscle				
Vessels	—	—	—	—
Gut wall	—	—	—	—
Endothelium				
Arteries	—>>+	—>>+	—>>+	—>>+
Veins	—>>+	—>>+	—>>+	—>>+
Venules	—>>+	—>>+	—>>+	—>>+
Capillaries	—	—>+	—	—
Lymphatics	—	—	—	—
Nerves				
Gangliocytes	—	—	—	—
Fibres	—	—	—	—
Interstitial dendritic cells	+	(+)	—>+	+>—
Lymphocytes	—	—	—	—
Interstitial matrix	—	—	—	—

For explanation to symbols see Table 1.

DISCUSSION

In this study we have shown that in human colorectal carcinomas, irrespective of histological staging and grade of malignancy, expression of PA and PAI was confined to fibroblast-like stromal cells within the tumour tissue. This expression pattern was consistently found in 64 cases of primary colorectal tumours and 10 metastases of colon tumours. The obvious discrepancy between the result of our study and those which found PA activity in tumour cells may at least in part be explained by different experimental approaches. Most of the earlier studies which described PA activity of tumour cells were performed by using cultured tumour cell lines of different origin. Remarkably, some colon carcinoma-derived cell lines produce little [30] or no PA activity [2]. In a few cell lines, t-PA was detected [2]. In our experiments, u-PA was only measurable in small amounts in two of four colon carcinoma cell lines. Synthesis of PA by colon tumour cell lines may, however, be a feature which is not indicative for the original tumours.

Several publications have described increased levels of u-PA and t-PA in colon tumour tissue extracts compared to normal tissue [9, 10, 12, 31, 32]. Since in these studies the PA producing cells were not defined, it is possible that stromal cells in the tissue are responsible for the observed PA activity. The strong expression of u-PA in interstitial cells, observed by us and others [22], supports this explanation. No correlation was found between PA activity

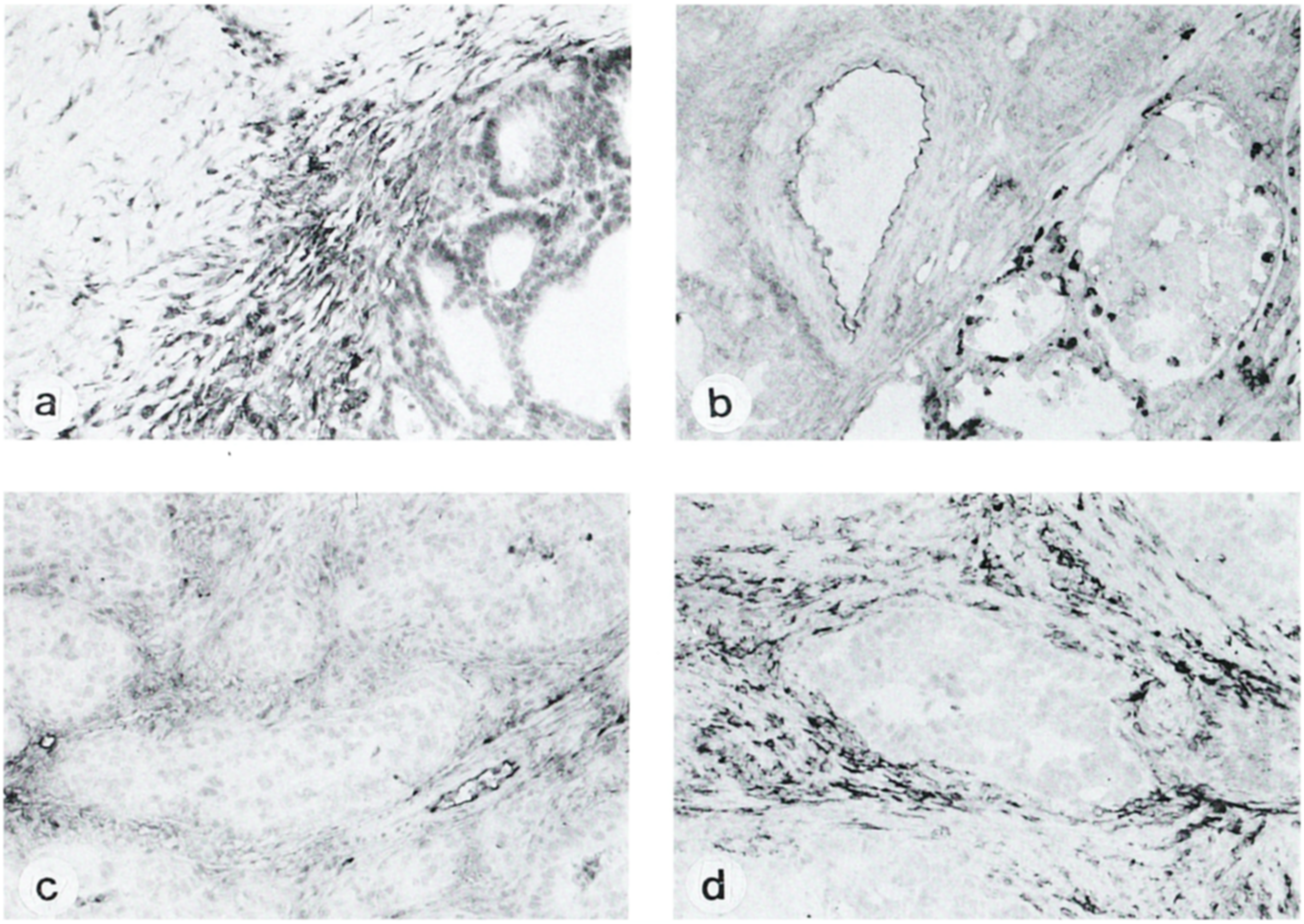

Fig. 3. Expression of PA components in human colon carcinoma tissue. (a) Fibroblast-like cells strongly express u-PA in the close vicinity of a colon carcinoma. The colon carcinoma shows hematoxylin nuclear counterstaining but lacks staining of the cytoplasm with u-PA Mab. (b) Expression of t-PA in the endothelium of an arterial vessel. The smooth muscle layer of the vessel is negative. The tumour mass in a lymphatic vessel is negative, too. The dark stained cells in the interstitium around the lymphatic vessel are granulocytes whose endogenous peroxidase was not blocked and which therefore have reacted with the substrate AEC. (c) PAI-1 is not expressed in the neoplastic epithelium but in the endothelium of a venule. (d) PAI-2 is not expressed in the carcinoma but in fibroblast-like cells surrounding the tumour nodules. Magnification × 125.

and histological type of tumour [11]. Strikingly, one group even reported decreased levels of PA activity in extracts of metastatic compared with primary colon tumours [17].

Earlier studies which examined PA expression of colon carcinomas by immunohistological techniques described association of PA [17, 18, 19, 21] with tumour cells or tumour stroma. These investigations were performed with polyclonal antisera on paraffin sections [33] or with monoclonal antibodies on paraffin sections [15, 34, 35] and in one study with monoclonal antibodies on frozen section [21]. By using monoclonal antibodies specific for components of the PA system and cryostat sections we did not detect PA or PAI expression on neoplastic colon epithelium. This is in contrast to previous findings in gastric carcinomas where u-PA and t-PA have been detected in carcinoma cells using the immunohistochemical technique and monoclonal antibodies on cryostat sections [36]. In another study increased expression and change in localisation of the A chain of u-PA in colorectal carcinomas were found with progressive dedifferentiation whereas the B chain of u-PA and the A and B chain of t-PA did not show similar alterations [21].

Specificity of the Mab applied here in immunoperoxidase staining of frozen tissue samples was confirmed by two findings. Interstitial dendritic or fibroblast-like cells and some endothelium reacted with the antibodies in different intensities. Secondly, the lung cancer cell lines tested for their differential production of PA components [4] yielded corresponding staining pattern, using the same assay system.

We found, in accordance with the observations of Grondahl-Hansen *et al.* [21] and Pyke *et al.* [23], strong expression of u-PA in fibroblast-like interstitial cells. These cells also weakly expressed t-PA and, to a smaller extent, PAI-1 and PAI-2. By contrast, interstitial cells of normal colon tissue were negative for all PA components. Since expression of u-PA was much stronger than of PAI-1 in these cells, it may well be that the PA activity exceeds the inhibitory effects of PAI and may, therefore, account for the increased PA activity measured in colon carcinoma tissue extracts [10–12]. Since we did not find PA expression in normal colon mucosa, one may further speculate that colon carcinoma cells release soluble factors which stimulate these interstitial cells to u-PA production.

Expression of all four PA components was comparatively weak in endothelial cells. The expression of t-PA was found in normal endothelial cells of abdominal and breast skin using polyclonal antisera [37], of u-PA in endothelial cells in inflammatory tissue [38] and in some endothelial cells in colon tumours [22]. PAI-1 expression in cultured endothelial cells [39] is documented and Pyke *et al.* [24] demonstrated PAI-1 mRNA in endothelial cells of tumour stroma in colon carcinoma. We confirm this observation at the protein level. Thus, it seems likely that in tumour tissue, endothelial cells are stimulated for the production of PA components. Tumour necrosis factor and epidermal growth factor have been shown to increase t-PA and PAI-1 production of human microvascular endothelial cells [40].

In view of the strong expression of u-PA restricted to interstitial stroma cells in colorectal carcinomas we conclude that PA activity, which may affect tumour growth, is synthesised, possibly tumour-stimulated, by these cells. Since the same pattern of expression was found in primary tumours as well as in metastases, a functional correlation between increased PA levels and invasive tumour cells seems to be unlikely in colon cancer.

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