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Rapid Communication

Urokinase-type Plasminogen Activator Is Expressed in Stromal Cells and Its Receptor in Cancer Cells at Invasive Foci in Human Colon Adenocarcinomas

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In this study* in situ *hybridization methods were used to examine biopsy samples from 13 adenocarcinomas of the colon for the presence of mRNA for the urokinase-type plasminogen activator (u-PA) and its specific cell-surface receptor (u-PAR). In all cases, u-PA mRNA was present in fibroblastlike cells in the stroma adjacent to the invasive tumor nodules. Urokinase-type plasminogen activator mRNA was not detected in the malignant cells. All specimens also contained u-PAR mRNA in cells located at the tumoral–stromal interface of invasive foci, but in contrast at least some of these cells were in all but one case identified as being of malignant origin. Stromal cells, probably tumor-infiltrating macrophages and neutrophils, also were positive in these areas. These results support the view that components of the plasminogen activation system may act to influence proteolytic events occurring at the interface between stroma and malignant cells in adenocarcinomas of the colon in humans. (Am J Pathol 1991, 138:1059–1067)

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Several proteolytic enzyme systems participate in the degradation of extracellular matrix proteins during cancer invasion. One of the most extensively studied is the urokinase pathway of plasminogen activation. Urokinase-type plasminogen activator (u-PA) is released from cells as a virtually inactive proenzyme (pro–u-PA) that can be proteolytically converted to an active two-chain molecule. Active u-PA specifically activates the ubiquitous proenzyme plasminogen into plasmin, a protease with a broad substrate specificity that is capable of degrading most proteins of the extracellular matrix, either directly or through activation of latent collagenases.[1–3]

The u-PA catalyzed plasminogen activation is regulated in time and space by several mechanisms, such as factors affecting the degree of pro–u-PA activation, specific plasminogen activator inhibitors, of which two different types have been characterized, PAI-1 and PAI-2 (for a review, see Andreasen[4]) and a specific cell-surface receptor for u-PA, u-PAR. This receptor has been identified on human monocytes and on a variety of cultured cell lines of neoplastic origin.[5–7] Recently it was purified[8] and its complete cDNA isolated.[9] u-PAR is a 55 to 60-kd highly glycosylated protein that is attached to the cell membrane by a phosphoinositol glycolipid anchor.[10] It binds both pro–u-PA and active u-PA with high affinity (0.01 to 10 nmol/l [nanomolar] depending on cell type.[5,7,11] Pro–u-PA can be converted to active u-PA while receptor bound, and receptor-bound active u-PA can activate plasminogen.[12] Indeed receptor binding of pro–u-PA is associated with a strong enhancement of plasmin formation at the cell surfaces.[13,14] Ultrastructural studies have shown that u-PAR on the surface of some cultured cells, eg, fibrosarcoma cells, localize u-PA to cell–cell and focal cell–substratum contact sites.[15,16]

Previous immunohistochemical studies have supported the hypothesis that the u-PA pathway of plasminogen activation is involved in tissue remodelling processes in normal and pathologic conditions. These in-

clude prostate involution,[17] wound healing,[18] psoriasis,[19] inflammation,[20] and cancer invasion.[21–23] In one of the latter studies,[23] we found that the u-PA immunoreactivity in human colon adenocarcinomas was more abundant in the tumor tissue than in the adjacent noninvolved colon. Surprisingly, however, all detectable u-PA immunoreactivity was seen in fibroblastlike cells and endothelial cells. Staining of the malignant epithelial cells was not observed. No data are available showing the distribution of the u-PA receptor *in vivo*. In this study we used *in situ* hybridization methods to examine the distribution of mRNA for u-PA and the u-PA receptor in adenocarcinomas of the colon in humans.

Materials and Methods

Materials

The following materials were obtained from the indicated sources: T7 and T3 polymerase, pBluescriptKS(+) plasmid vector (Stratagene; La Jolla, CA); RNasin and DNase I (Promega, Madison, WI); [35]S-UTP (1300 Ci/mmol/l [millimolar]) (Amersham DK, Birkeröd, Denmark); Dithiothretiol and restriction endonucleases (Boehringer Mannheim, Mannheim, FRG); K5 autoradiographic emulsion (Ilford, Cheshire, UK); Formamide (Fluka, Buchs, Schwitzerland); Salmon Sperm DNA (Type III, Sigma, St. Louis, MO). All other materials were as described previously.[22]

Tissue preparation

Routinely processed, formalin-fixed, and paraffin-embedded specimens from 13 adenocarcinomas of the colon, operated on during 1989, were drawn from the files of the Department of Pathology at the Rigshospital. The specimens were assessed in accordance with standard criteria and included three cases of Duke's B, moderately differentiated; eight Duke's B, highly differentiated; one Duke's C, moderately differentiated; and one Duke's C, highly differentiated. Desmoplasmic reactions were not observed in any of the specimens. In another five cases of colon adenocarcinomas, fresh frozen specimens were available. These had been frozen in liquid nitrogen immediately after surgery and were stored at −80°C until use.

Preparation of RNA probes

Fragments of the human u-PA and u-PAR cDNA were subcloned using standard techniques,[24] and the following subclones were prepared in pBluescriptKS(+): pHUPA10 (EcoRI(32)-EcoRI(627)), pHUPA13 (AccI(791)-AccI(1303)), pHUR04 (PstI(184)-PstI(451)), and pHUR06 (BamHI(497)-BamHI(1081)). Basepair numbers correspond to sequences as listed in EMBO database (AQC. numbers: K03226) (u-PA) and (u-PAR).[9] In pHUPA10, the first EcoRI site (at basepair number 32) was created by extension from blunt end. Pure plasmid preparations were prepared by banding in CsCl gradients. For pHUPA10 and pHUR06, the correct identity of inserted fragments was assured by sequencing by the dideoxy method of Sanger.[25] The plasmids were linearized for transcription using restriction endonucleases and 5 μg of the linearized plasmids was extracted with phenol and with chloroform/isoamylalcohol (25:1), precipitated with ethanol, and redissolved in water. Each transcription reaction contained 1 μg linearized DNA template and transcriptions were performed essentially as recommended by the manufacturer of the polymerases. The RNA was hydrolyzed in 0.1 mol/l (molar) sodium carbonate buffer, pH 10.2, containing 10 mmol/l dithiotreitol (DTT) to an average size of 100 bases. Probe preparations always contained more than 2×10^6 cpm/μl, and the amount of trichloroacetic acid precipitable material was usually more than 90%. For pHUPA10 and pHUR06, RNA probes transcribed from opposite strands of the same plasmid template, yielding sense and antisense transcripts, were adjusted to the same radioactivity concentration. Probes were stored at −20°C until they were used.

In Situ *Hybridization*

In situ hybridization was performed using a method adapted from a number of published procedures.[26] Briefly, paraffin sections were cut, placed on gelatinized slides, heated to 60°C for 30 minutes, deparaffinized in xylene, and rehydrated through graded alcohols to phosphate-buffered saline (PBS) (0.01 mol/l sodium phosphate buffer pH 7.4, containing 0.14 mol/l NaCl). The slides then were washed twice in PBS, acid treated in 0.2 mol/l HCl for 20 minutes, and washed for 5 minutes in PBS. This was followed by incubation in 5 μg/ml Proteinase K in 50 mmol/l TRIS-HCl, pH 8.0, with 5 mmol/l EDTA for 7.5 minutes, washing twice in PBS (2 minutes), and fixation in 4% (wt/vol) paraformaldehyde in PBS for 20 minutes. Fixative was removed by washing with PBS and slides were immersed in 0.2% (vol/vol) acetic acid anhydrid in 100 mmol/l triethanolamine in a beaker on a magnetic stirrer for 5 minutes. Finally the slides were washed in PBS (5 minutes), dehydrated in graded etha-

nols, and airdried before RNA probe was applied (80 pg/μl), in a solution of deionized formamide (50%), dextran sulphate (10%), t-RNA (1 μg/μl), Ficoll 400 (0.02% (wt/vol), polyvinylpyrrolidone (0.02% [wt/vol]), bovine serum albumin (BSA) Fraction V (0.02% (wt/vol)), 10 mmol/l DTT, 0.3 mol/l NaCl, 0.5 mmol/l EDTA, 10 mmol/l TRIS-HCl, and 10 mmol/l $NaPO_4$ (pH 6.8). Sections were covered by alcohol-washed, autoclaved coverslips and hybridized at 47°C overnight (16 to 18 hours) in a chamber humidified with 10 ml of a mixture similar to the hybridization solution, except for probe, dextran sulphate, DTT, and t-RNA (washing mix). After hybridization, slides were washed twice in washing mix for 1 hour at 50°C. Sections then were washed in NTE (0.5 mol/l NaCl, 1 mmol/l EDTA, 10 mmol/l TRIS-HCl [pH 7.2]), with 10 mmol/l DTT at 37°C for 15 minutes, and treated with RNase A (20 μg/ml) in NTE at 37°C for 30 minutes. This was followed by washing in NTE at 37°C (2 × 30 minutes) and in 2 l of 15 mmol/l sodium chloride, 1.5 mmol/l sodium citrate, pH 7.0 with 1 mmol/l DTT for 30 minutes at room temperature with stirring. Sections then were dehydrated and air dried. Finally autoradiographic emulsion was applied following the manufacturer's recommendations and sections were stored in black, air-tight boxes at 4°C until they were developed after 1 to 2 weeks of exposure.

Immunohistochemistry

Tissue macrophages were identified by PAP-immunostaining (a modification of the method of Sternberger[27]) using a monoclonal anti-macrophage antibody (F8H) reactive with macrophages in routine sections (provided by Dr. Bruno Falini, Perugia, Italy).

Results

u-PA mRNA

In all 13 cases investigated, u-PA mRNA was confined to fibroblastlike cells in the tumor stroma. These cells were numerous in the stroma surrounding the invasive tumor nodules, and the hybridization signal was especially abundant in areas in which tissue degradation and/or inflammatory reactions were present (Figure 1a). Hybridization to the malignant cells was not seen in any of the cases. Endothelial cells also were negative.

In mucosal tissue uninvolved by the tumor, scattered fibroblastlike cells located just below the free surface epithelium showed a weak hybridization signal in six cases.

In all cases, the hybridization signal for u-PA mRNA was much stronger in the tumor tissue than in the uninvolved colon tissue.

u-PAR mRNA

Cells at the leading edge of invasively growing tumor glands showed hybridization signal to u-PAR mRNA in all 13 specimens. In 12 cases, u-PAR mRNA was clearly present in some malignant epithelial cells at these sites. At a predominant number of invasive foci in the 12 cases, both some cancer cells and some of the tumor-infiltrating stromal cells contained u-PAR mRNA (Figure 1c). These cells could not, by the present method, be identified with certainty but they might represent a subset of tissue macrophages and neutrophils because these cell types at other localizations were found to contain u-PAR mRNA (see below). However, at some invasive foci, a few cancer cells located at the edge of the invading tumor were positive, whereas the surrounding stromal tissue was negative (Figure 1b). In the remaining one case (classified as moderately differentiated Duke's B), relatively few cells located at the tumoral–stromal interface contained u-PAR mRNA, and it was not possible to establish with certainty the identity of these cells due to a weak and diffuse pattern of silver grain deposition.

The dysplastic epithelium adjacent to areas with invasion showed a moderately high hybridization signal for u-PAR mRNA in four cases. In these cases hybridization was confined to short stretches of epithelium.

A general feature of the hybridizations to u-PAR mRNA was the absence of signal in both cancer cells and stromal cells in parts of cancerous tissue distant from sites of invasion. However we found two exceptions to this pattern. First large, rounded cells, present in necrotic intratumoral areas, were positive for u-PAR mRNA in 6 of the 13 cases (Figure 1d). These cells were macrophages as shown by immunostaining of adjacent sections with a monoclonal anti-macrophage antibody (not shown) (see Materials and Methods). Second granulation tissue of ulcerated mucosa, where present, consistently showed a weak signal. Although this signal was very diffuse it probably represented neutrophilic granulocytes because the pattern of silver grain deposition completely matched the distribution of these cells in the tissue.

In normal-appearing mucosa, the free surface of the luminal epithelium showed a weak signal for u-PAR mRNA in five cases. In these cases the hybridization signal for u-PAR mRNA was much stronger in the tumor tissue than in the uninvolved colon tissue. The normal-

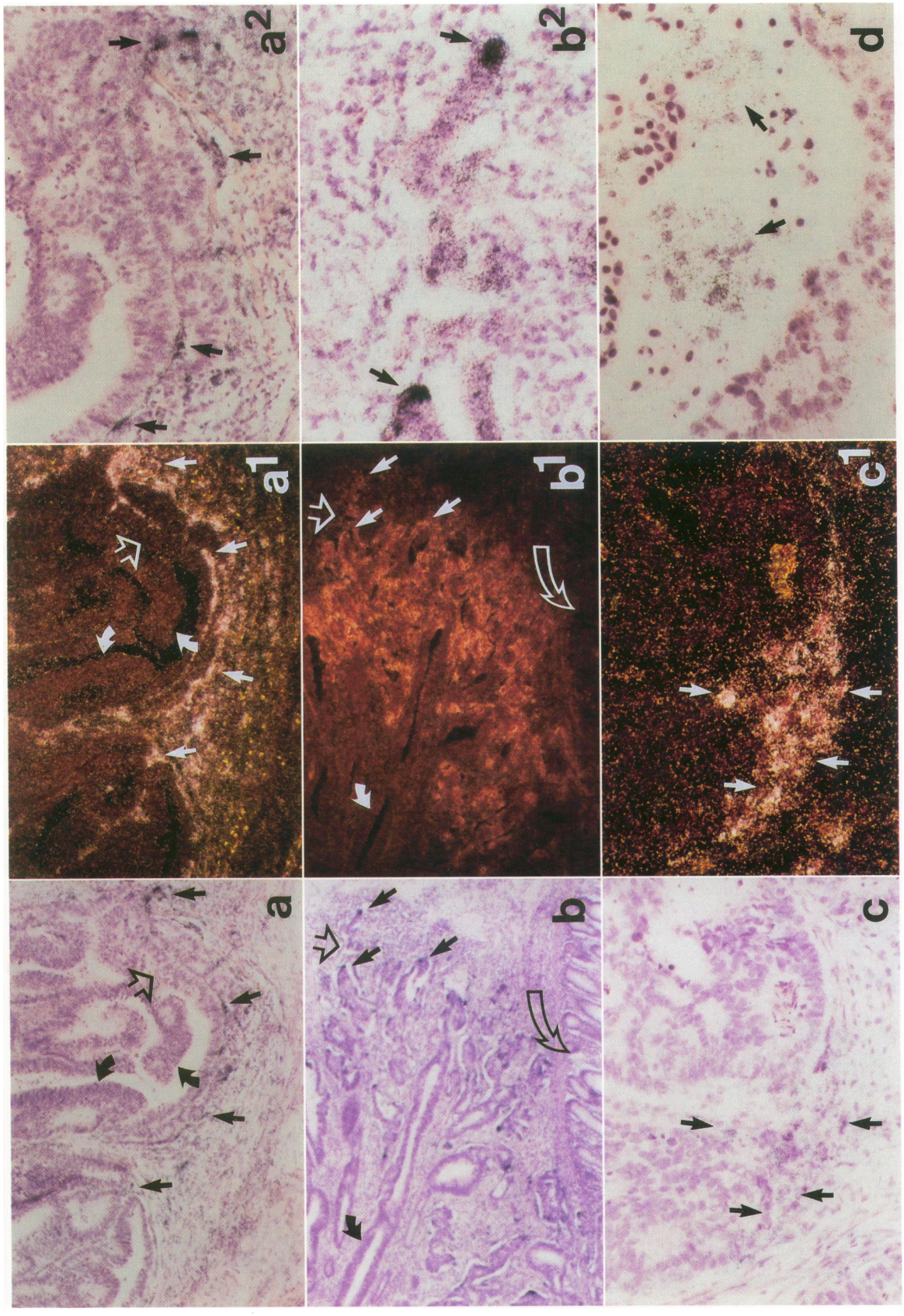
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Figure 1. In situ *hybridization of colon cancer with ^{35}S-labeled anti-sense RNA probes for u-PA* (**a**) *and u-PAR* (**b, c, d**). *u-PA mRNA is located in fibroblastlike stromal cells* (**a**, *straight filled arrows), while no signal is observed in malignant epithelium (curved arrows). In contrast, hybridization to u-PAR mRNA is seen in malignant cells at the leading edge of an invasively growing tumor* (**b**, *straight filled arrows), while there is no signal in cancerous tissue distal to the site of invasion (curved filled arrow). Also note the lack of signal in the adjacent mucosa uninvolved by the tumor (curved open arrow). In another case* (**c**), *u-PAR mRNA containing cells at the contour of a tumor nodule seem to be of both malignant and nonmalignant nature (arrows). In an intratumoral necrotic area* (**d**), *u-PAR mRNA containing macrophages can be seen (arrows).* **a**^{1}, **b**^{1}, *and* **c**^{1} *are darkfield images of* **a**, **b**, *and* **c**, *respectively.* **a**^{2} *and* **b**^{2} *are high magnifications of areas (straight open arrows) in* **a** *and* **b**, *respectively (magnifications: ×100,* **a**, **a**^{1}, **b**, **b**^{1}; *×640,* **a**^{2}, **c**, **c**^{1}, **d**; *×1000,* **b**^{2}).

looking surface epithelium in the remaining cases were negative, as were all other tissue elements in the uninvolved mucosa of all specimens.

In all tumors investigated, the u-PAR mRNA containing cells appeared to constitute a cell population distinct from those showing hybridization signal for u-PA mRNA. This differential expression was seen particularly clearly at the contours of the tumor nodules (Figures 2a and b).

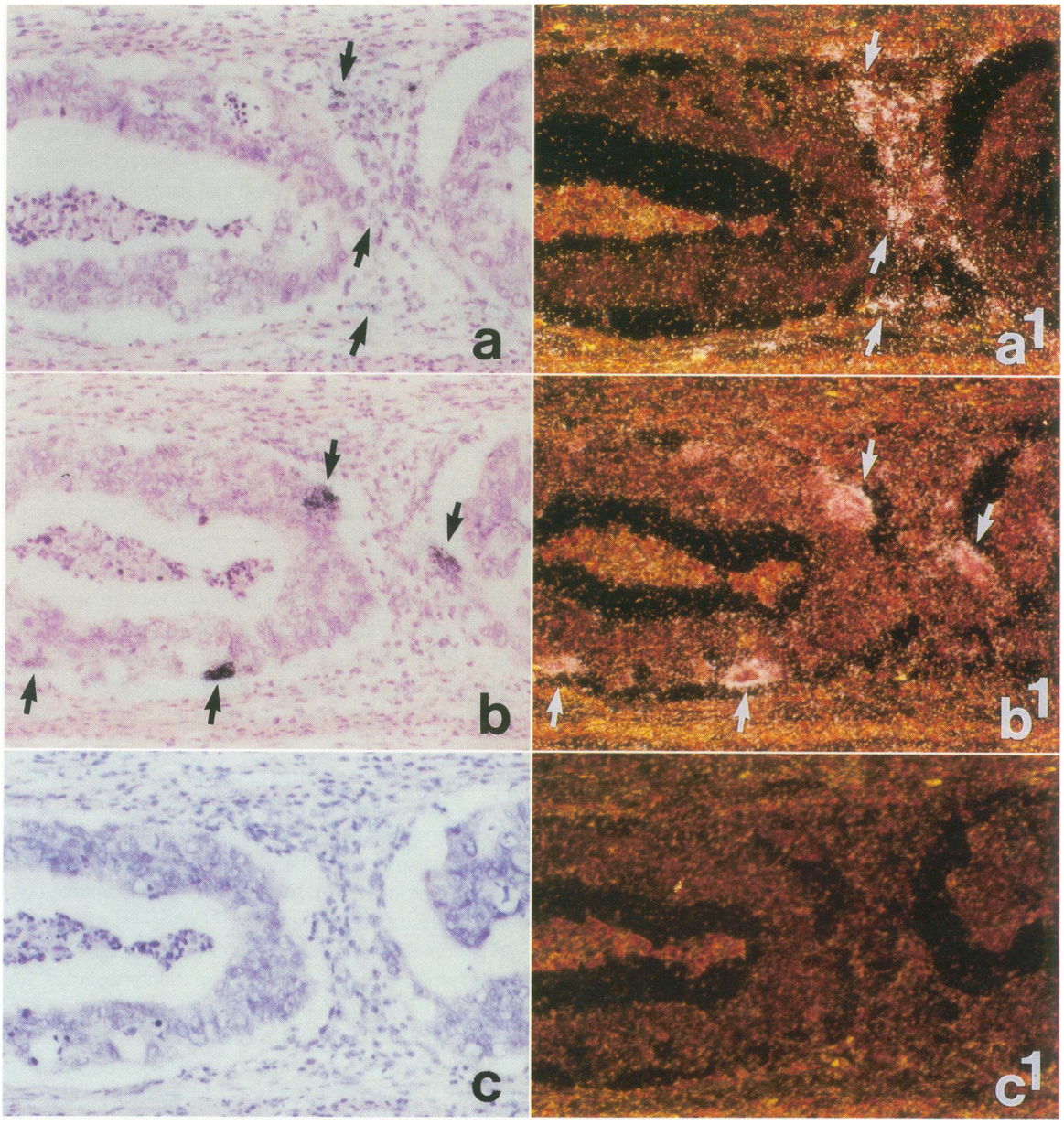

Figure 2. *Comparison of u-PA* (**a**) *and u-PAR* (**b**) *mRNA containing cells at the contours of malignant epithelium.* In situ *hybridization for the two mRNA species were performed on adjacent sections. Fibroblastlike cells containing u-PA mRNA are clearly located in the peritumoral stroma* (**a**, *arrows), whereas u-PAR hybridization signal can be seen in cells that are integrated in the malignant epithelium* (**b**, *arrows). As a negative control hybridization to an adjacent section with a sense probe for u-PAR mRNA show no signal* (**c**). **a**^{1}, **b**^{1}, *and* **c**^{1} *are darkfield images of* **a**, **b**, *and* **c**, *respectively (magnifications, ×640).*

Control Experiments

Positive control experiments were performed by application of two different anti-sense probes representing two non-overlapping parts of each of the two cDNAs (see Materials and Methods). These probes were adjusted to the same specific radioactivity and applied to adjacent sections of five of the tumors. In all cases, the two probes showed virtually identical hybridization patterns.

To test the reproducibility of the method, sections from all tumors were hybridized on two separate occasions with one probe for each of the two mRNA species (pHUPA10 and pHUR06; see Materials and Methods). Again similar results were obtained.

As a negative control, sense RNA probes transcribed from CDNA for one of each of the two mRNA species (pHUPA10 and pHUR06) were applied to adjacent sections of all 13 tumors. In these sections no signals were seen (Figure 2c).

To investigate the influence of fixation and paraffin-embedding procedures on the hybridization signal, we hybridized frozen sections from five colon tumors (see Materials and Methods) with one of the u-PA RNA probes (pHUPA10). In these specimens, the hybridization pattern was similar to that seen when using routinely processed paraffin-embedded tissue.

Discussion

In this study we used *in situ* hybridization methods to examine the distribution of mRNA for u-PA and u-PAR in adenocarcinomas of the colon in humans. The results indicate that mRNA for both u-PA and u-PAR are present at the interface between tumor and stroma at invasive foci. The specificity of these results is supported by the fact that identical hybridization patterns were obtained with the use of two different anti-sense RNA probes transcribed from non-overlapping parts of each of the respective cDNAs, and that no signal was obtained with the corresponding sense RNA probes. In the case of the u-PA mRNA-containing cells, a similar cell population previously was found to contain u-PA immunoreactivity (see below). Therefore we conclude that the hybridization patterns demonstrated in this study are due to the presence of authentic u-PA mRNA and u-PAR mRNA in the tissue sections.

In the tumor tissue, the hybridization signal for u-PA mRNA was found to be much stronger than in the uninvolved colon mucosal tissue. This correlates well with previous reports of increased amounts of u-PA protein in extracts of colon cancer tissue as compared to normal colon tissue.[23,28–33] The localization of u-PA mRNA found in this study also correlates with our previous immunohistochemical findings in colon cancer; using a polyclonal and three different monoclonal antibodies, u-PA protein was found in fibroblastlike cells in the tumor stroma but was not detected in the malignant cells.[23]

In the immunohistochemical study of human colon cancer[23] we found that also some endothelial cells in capillaries and venules in the tumor stroma contained u-PA. In the present study no u-PA mRNA was detected in any endothelial cells. This suggests that the u-PA protein seen in these cells is produced elsewhere and subsequently is bound to or internalized by the endothelial cells. We recently found that many endothelial cells in the stroma of colon carcinomas contain mRNA for plasminogen activator inhibitor type-1 (Pyke C, unpublished observation). PAI-1 has a high affinity for u-PA (see Andreasen et al[4]) and it is possible that the u-PA protein detected in the endothelial cells in our previous report is produced by and released from the fibroblastlike cells and then bound to PAI-1 present in the endothelium. In this context it should be noted that some cells internalize u-PA/PAI-1 complexes.[34]

The lack of detectable u-PA protein[23] and u-PA mRNA in colon cancer cells is in apparent disagreement with several reports on the production of u-PA by cultured colon cancer cells.[35,36] There are, however, several examples that cultured cells are not representative of the cells in the intact organism from which they are derived with respect to production of components of the plasminogen activation system (see Danø et al[1]). The present findings apparently add another example of this phenomenon that can be explained by the different occurrence of factors (eg, hormones, growth factors, cytokines) modulating the synthesis of these components in the microenvironment of the cells under *in vivo* and *in vitro* conditions.

Cultured colon cancer cells previously were reported to contain u-PAR as detected by cross-linking[35] while there are no previous reports on immunocytochemistry or *in situ* hybridization for u-PAR. Studies with a recently isolated cDNA for mouse u-PAR have, however, shown that its mRNA is present in Lewis lung carcinoma cells *in vivo* (Kristensen P, unpublished results).

The presence of u-PA mRNA in a small number of fibroblastlike cells just below the free surface of the luminal epithelium in uninvolved colon tissue correlates with immunohistochemical findings.[23] Furthermore these findings correlate with studies of the gastrointestinal tract of the normal mouse in which u-PA immunoreactivity[38] and u-PA mRNA[26] was found in a similar cell type. Also the observations in the present study that normal-appearing mucosal tissue in a number of cases showed weak signals for u-PAR mRNA in the luminal epithelium are comparable to findings in the normal mouse of u-PAR mRNA in some epithelial cells (Kristensen P, unpublished data). The physiologic relevance of the differential expression of

u-PA and u-PAR in the normal gut mucosa could be speculated to be in regulating the shedding of epithelial cells at this site. Interestingly the pattern of u-PA and u-PAR expression found in cancerous areas is highly analogous to that seen in the normal colon mucosa. It appears likely that u-PA is produced and released from the fibroblastlike stromal cells, and subsequently is bound to the u-PA receptor on the surface of cancer cells at the leading edge of the invasively growing tumor.

The lack of u-PA immunostaining of cancer cells in colon adenocarcinomas[23] appears to contradict the mechanism proposed above. These results do not preclude, however, that receptor-bound u-PA is present on the cancer cells in amounts less than the detection limit. Indeed receptor binding may in itself render the u-PA less detectable by immunohistochemical methods.*

The production of u-PA by the many peritumoral fibroblastlike cells in colon cancer may be a result of a cancer cell-mediated induction. This could be achieved in two ways, either by a recruitment of host cells that already are producing u-PA, or the cancer cells could, in a paracrine manner, induce the resident fibroblastlike cells to produce u-PA. Many cytokines and growth factors (eg, TGF-β, TNF, EGF, and bFGF) induce the synthesis of u-PA in certain cell types, and such cytokines and growth factors often are produced by cancer cells (see Laiho[39] for review). Whether any of these factors are involved in a paracrine u-PA induction in colon cancer remains to be explored, eg, by studies on their occurrence in colon cancer tissue. Recent reports that plasmin is capable of interfering with the activity of growth factors either by activating latent proforms[40] or by releasing latent matrix-bound factors[41] may indicate that the interaction between the cancer cells and the stromal cells is even more complex. It will be important to determine whether the expression by different cell populations of u-PA and u-PAR is a widespread phenomenon in cancer and whether other normal epithelial–stromal interfaces show similar patterns of u-PA and u-PAR expression.

The presence of u-PAR mRNA in a subpopulation of neutrophilic granulocytes and tissue macrophages support evidence for a role of u-PAR in providing these cells the proteolytic potential necessary for migrating into areas of inflammation.[5,37]

The findings in the present study has potential clinical implications. Immunostaining and *in situ* hybridization for u-PA and u-PAR may contribute to the histologic diagnosis and, as indicated by reports on u-PA levels in human breast cancers,[42,43] quantitation of the u-PA and u-PAR content in tumor extracts may have prognostic value. Interference with the interaction between u-PA and u-PAR inhibits cell-surface plasminogen activation[13,14,44] and may be of therapeutic value. It is probable that the production of the ligand and the receptor by different cell types in colon cancer will facilitate this interference.

*The lack of u-PA immunostaining of the colon cancer cells has been confirmed during the present study (unpublished results). Several of the monoclonal and polyclonal antibodies used for the immunostaining[23] have been found to react with receptor-bound u-PA on cultured cells.[14,15,44,45] It is possible, however, that receptor-bound u-PA is extracted selectively during tissue preparation or that binding of inhibitors to active receptor-bound u-PA[44,46] may decrease its detectability.

Acknowledgments

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