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Localization of Urokinase-type Plasminogen Activator in Stromal Cells in Adenocarcinomas of the Colon in Humans

Jan Grøndahl-Hansen,*† Elisabeth Ralfkiær,‡ Lene T. Kirkeby,‖ Peter Kristensen,† Leif R. Lund,† and Keld Danø†

From the Department of Biochemistry and Nutrition, Technical University of Denmark, Lyngby; the Finsen Laboratory† and Department of Pathology,‡ Rigshospitalet, Copenhagen; and the Department of Surgical Gastroenterology, Gentofte Hospital,‖ Copenhagen, Denmark*

Human colon adenocarcinomas and adjacent normal colon tissues were stained immunohistochemically with three different monoclonal antibodies and one preparation of polyclonal antibodies against each of the two plasminogen activators, uPA (urokinase type) and tPA (tissue type). The staining patterns seen with the respective sets of antibodies were identical. In all of 10 cases, staining for uPA in the normal colon tissue was confined to scattered fibroblastlike cells in the lamina propria. Other cells, including epithelial and endothelial cells, were uPA negative. All the tumor infiltrates contained many more uPA-positive cells than the normal tissues, but the staining was confined to fibroblastlike cells and endothelial cells in the tumor stroma, while no staining of the malignant epithelial cells was detected. Analysis for uPA by enzyme-linked immunosorbent assay (ELISA) in four cases showed an average uPA content of 0.15 ng uPA/mg protein in the normal colon tissues and 1.6 ng uPA/mg protein in the tumors. Tissue-type plasminogen activator immunoreactivity was confined to endothelial cells in both the normal colon tissue and in the colon carcinomas. These findings may indicate that colon cancer cells recruit stromal cells to produce uPA involved in degradation of the extracellular matrix during invasive growth. (Am J Pathol 1991, 138:111–117)

The invasive growth of cancer cells, as well as tissue remodeling under normal conditions, involves degradation of proteins of the extracellular matrix. Several proteolytic enzyme systems participate in this process; the most thoroughly studied are the collagenases and the urokinase pathway of plasminogen activation.[1–3]

Urokinase-type plasminogen activator (uPA) is released from cells as a virtually inactive proenzyme (pro-uPA). The single polypeptide chain of this molecule can be proteolytically converted to an active two-chain form of uPA, which in turn specifically activates another proenzyme, plasminogen, to plasmin. Plasmin is a protease with a broad substrate specificity, and can degrade most proteins of the extracellular matrix, such as for example laminin, fibronectin, and proteoglycans.[1] Plasmin can also activate procollagenases[1,3] and latent forms of at least one growth factor.[4]

The uPA pathway of plasminogen activation is regulated in time and space[5] by pro-uPA activation,[6,7] by two well-characterized plasminogen activator inhibitors, PAI-1 and PAI-2,[8] and by a cell-surface receptor specific for uPA (uPAR),[2,9–11] which binds both pro-uPA and active uPA. Pro-uPA can be converted to uPA while receptor bound, and receptor-bound uPA can activate plasminogen.[2] Recent studies indicate that the formation of plasmin is strongly enhanced when both pro-uPA/uPA and plasminogen/plasmin is bound to the cell surface and that cell surfaces may be the physiologic sites of this pathway of plasminogen activation,[12,13] similar to fibrin clots being physiologic sites for the tPA pathway.[14]

The role of uPA in invasion and tissue remodeling has mostly been studied in model systems using cultured or transplanted cells.[15–18] The plasminogen activation system involves a strong cascadelike amplification of the proteolytic activity, and the uPA concentration in tissues is very low. Immunohistochemical studies of the localization of uPA in intact tissues are therefore difficult and few such studies have been reported. In the murine Lewis lung tumor, we previously found that uPA was heterogeneously

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Address reprint requests to Jan Grøndahl-Hansen, Department of Biochemistry and Nutrition, Technical University of Denmark, DK-2800 Lyngby, Denmark.

distributed, but consistently present in and around the tumor cells in areas with invasive growth.[19] We have shown also that uPA is present in some non-neoplastic conditions involving tissue remodeling, such as wound healing,[20] psoriasis,[21] and spermatogenesis.[22]

Other laboratories have studied the localization of uPA in sections of human colon carcinomas, and these studies have suggested that uPA may be present in the neoplastic cells in these malignancies.[23-26] In the present study, we have immunohistochemically examined frozen biopsy samples from 10 human colon carcinomas for reactivity with polyclonal and monoclonal antibodies against uPA and tPA. The results contrast with previously published data and indicate that uPA immunoreactivity is present in stromal elements (fibroblastlike cells and endothelial cells) and not in detectable amounts in the malignant epithelial cells.

Materials and Methods

Materials

All materials, including polyclonal rabbit antibodies, three monoclonal antibodies against human uPA, and three monoclonal antibodies against human tPA, were those described previously.[21,27-29]

Tissue and Tissue Treatment Procedures

Samples of fresh tumor tissue and adjacent normal colon tissue were obtained from 10 patients treated at the Department of Surgery, Gentofte Hospital. The histologic diagnoses were: adenocarcinoma, high to moderately differentiated, Duke stage B (six cases); and adenocarcinoma, moderately to poorly differentiated, Duke stage C (four cases).

The specimens were immediately frozen in isopentane and dry ice and stored at −70°C. Five-micron–thick serial cryostat sections were cut, mounted on chrome-alum-gelatin–coated glass slides, and stored at −70°C until staining.[30] Before staining, sections were thawed in 0.1 mol/l (molar) sodium phosphate (pH 7.4), containing 4% paraformaldehyde, for 1 hour at 4°C.

Immunohistochemical Staining

The staining was performed by the PAP (peroxidase-anti-peroxidase) method of Sternberger,[31] as described in detail previously.[21] Controls included the following: 1) omission of each of the antibody layers used for staining with the polyclonal and monoclonal antibodies; 2) substitution of the primary antibody with either rabbit preimmune and nonimmune immunoglobulin (IgG) or mouse monoclonal antibodies of irrelevant specificity; and 3) substitution of the polyclonal antibodies in the first layer with antibody preparations that had been absorbed by passage through Sepharose columns coupled with purified preparations of either uPA or tPA.[29]

Enzyme-linked Immunosorbent Assay for u-PA

The tissue was homogenized in 0.1 mol/l TRIS-HCl, pH 8.1, 0.5% Triton X-100 (10 μl/mg wet weight), and centrifuged (16,000*g*) at 4°C for 15 minutes. The extracts were stored at −70°C until analyzed. The concentration of protein in the extracts was determined by the Folin-Ciocalteu method, as described by Bonsall and Hunt.[32] The concentration of uPA in the extracts was determined with an enzyme-linked immunosorbent assay (ELISA) with a monoclonal anti-uPA as solid-phase antibody and biotinylated rabbit anti-uPA IgG followed by peroxidase-conjugated avidin, as described.[33] This ELISA detects active u-PA, pro-uPA, and uPA/PAI-1 complexes.

Results

Immunohistologic Staining

All samples were stained with three different mouse monoclonal antibodies against each of the two human plasminogen activators, uPA and tPA. The staining patterns obtained with the use of different monoclonal antibodies to the same activator were identical, although the most intensive staining was seen with anti-tPA clone 3 and with anti-uPA clones 6 and 12, respectively. Three cases were also examined for reactivity with rabbit polyclonal anti-tPA and anti-uPA antibodies. In all these cases, the staining patterns seen with the respective polyclonal and monoclonal antibodies were identical.

In the normal colon tissue, staining for uPA was confined to scattered fibroblastlike cells in the lamina propria. Other cell types, including epithelial cells, goblet cells, and endothelial cells, were uPA negative (Figure 1H). All the tumor infiltrates contained many more uPA-positive cells than the normal tissue, but the labeling was confined to the stromal elements, and staining of the malignant epithelial cells was not detected in any of the specimens. Two types of uPA-positive cells were identified in the tumor stroma, ie, fibroblastlike cells with a similar morphology to those seen in the normal tissue, and endothelial cells in capillaries and venules (Figure 1A, C, E). In individual tumors, the distribution of uPA was similar in different areas

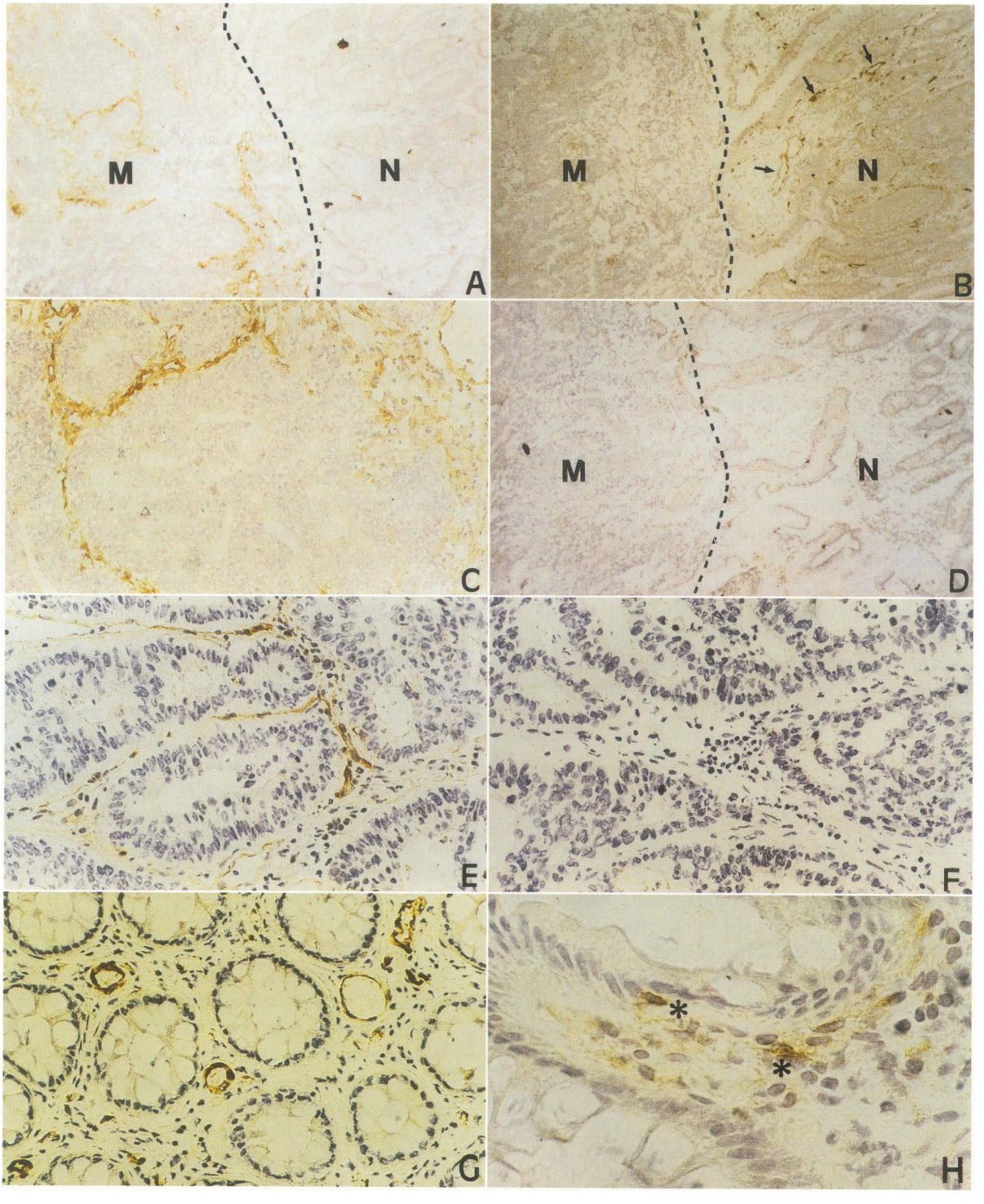

Figure 1. *An adenocarcinoma of the colon with the area of transition (stippled line) between the malignant (M)- and normal (N)-looking tissue. The specimens are stained for uPa (***A** *×200,* **C** *×600), tPA (***B** *×200), and a control antibody of irrelevant specificity (***D** *× 200). Staining for uPA is confined to stromal elements, ie, endothelial cells and fibroblastlike cells (***A** *and* **C**). *uPA labeling of the neoplastic cells is not observed. Staining for tPA is confined to endothelial cells (arrows in* **B**) *and shows a weaker labeling in the tumor area than in the adjacent normal tissue. The staining pattern seen in another tumor stained with anti-uPa (***E** *×600) is similar to the staining pattern seen in* **A** *and* **C**. *The control section stained with an antibody of irrelevant specificity is negative (***F** *×600). In normal colon tissue, staining for tPA is confined to endothelial cells (***G** *×600) and staining for uPA to scattered cells (asterisks) with a fibroblastlike morphology (***H** *×800).*

of the infiltrates. Thus no differences were observed between the central areas as opposed to the areas of transition between normal and malignant-looking tissue.

In all specimens, tPA immunoreactivity was confined to endothelial cells in capillaries, venules, and small and medium-sized veins and arteries (Figure 1B, G). Other stromal elements were tPA negative, and labeling of epithelial cells was not observed. The staining pattern was similar in normal and malignant tissue, although the intensity of the tPA staining of the endothelial cells in the tumor infiltrates varied. Eight tumors showed a rather weak staining of endothelial cells when compared with the endothelial cell labeling seen in the surrounding normal tissue (Figure 1B). In the remaining two cases, the tPA labeling of the endothelial cells in the tumor infiltrates was similar to that seen in the normal vessels.

Control staining experiments in which the specific antibodies were omitted or substituted with nonimmune IgG, preimmune IgG, or mouse monoclonal antibody of irrelevant specificity were negative (Figure 1D, F). Absorption of the polyclonal anti-tPA and anti-uPA antibodies with highly purified preparations of the respective plasminogen activators also completely abolished the staining of the tissues.

ELISA for uPA

In four of the cases, specimens were available that were large enough to allow a determination of uPA by ELISA in extracts from tumor tissue and adjacent normal colon tissue. In these four cases, the tumor tissue contained an average of 1.6 ng uPA/mg protein (standard deviation [SD] = 0.9), while the adjacent normal colon tissue contained an average of 0.15 ng uPA/mg protein (SD = 0.06). On the average, the tumor tissue thus contained over 10 times more uPA than the normal colon tissue. This difference was statistically significant, as evaluated by the *t*-test ($P < 0.05$).

Discussion

The specificity of the staining for uPA and tPA seen in this study is supported by several findings. First our staining and absorption controls clearly indicate that the staining is not due to nonimmunologic binding of the antibodies to tissue components. Second identical results were obtained with polyclonal and three monoclonal antibodies for each of the two activators. At least two of the monoclonal antibodies to uPA are directed against different epitopes, and this is also the case for at least two of the monoclonal antibodies to tPA.[21] Third previous immunochemical and immunohistochemical studies have demonstrated the ability of these antibodies to detect uPA and tPA, respectively, in a variety of tissues and body fluids without any indication of the presence of contaminating antibodies or cross-reaction with other molecules.[20,21,29,34–37] Fourth the results obtained with ELISA for uPA in four of the cases agree well with the immunohistochemical findings. Therefore we believe that it is very likely that the stainings seen in this report represent the presence of genuine uPA and tPA, respectively.

The much higher uPA concentration in the colon tumor tissue than in normal colon tissue determined in this study is in good agreement with previous reports.[38–44] A surprising finding is the localization of the uPA in stromal cells in the colon carcinomas, ie, fibroblastlike cells and endothelial cells, and the absence of uPA staining in the cancer cells. Whether the cancer cells are devoid of uPA, or may contain minute amounts present in concentrations below the detection limit, is not clear. Nevertheless our findings clearly indicate that by far the most uPA found in colon carcinomas is located in the stromal cells.

Urokinase-type plasminogen activator is produced by numerous types of cultured cells of neoplastic origin,[1,45] including cells derived from colon tumors.[46–48] As discussed in detail previously,[1] cultured cells are not necessarily representative of the cells in the intact organism from which they were derived with respect to production of plasminogen activators. Immunohistochemical studies from our own group have, however, previously shown that uPA is consistently present in the cancer cells in invasive areas of the murine Lewis lung tumor.[19,49] Immunohistochemical results from other laboratories have indicated that in colon carcinomas uPA is also located in the cancer cells.[23–26] In two of these studies,[23,25] no uPA staining of stromal cells is described, but, interestingly, Kohga et al[24] reported that while most of the uPA staining was located in the cytoplasm of the cancer cells and at the edge of these cells, a few instances also showed uPA immunoreactivity associated with fibroblasts. Burtin et al[26] reported weak uPA immunofluorescence of the tumor stroma in one third of their cases.

The reason for the apparent discrepancies between the previous studies[23–26] and the present report is not known. It is probable that they are related to the specificity of the antibodies used. All of the previous immunohistochemical studies on colon carcinomas were done with polyclonal antibodies. Our results obtained by ELISA show that uPA constitutes (on a weight basis) only about one-millionth of the protein in the tumor tissue. Even small amounts of contaminating or cross-reacting antibodies may therefore give a relatively strong staining if they are directed against proteins that are present in much higher amounts than uPA, as for example structural proteins. In this context, it should be noted that some of the uPA antibodies reported to stain colon cancer cells[24,25] have

been found in another study[50] to react also with proteins that have a molecular weight different from that of uPA.

We have previously shown[30] that normal colon in the mouse contains uPA-positive cells with a fibroblastlike morphology and that similar uPA-positive connective tissue cells occurred widespread in the lamina propria along the entire gastrointestinal tract. A few such fibroblastlike uPA-positive cells were also found elsewhere in the normal mouse, eg, in the connective tissue of the pancreas and repiratory tract.[30] The present findings of uPA in fibroblastlike cells in the normal human colon are in good agreement with the localization found in the normal mouse. They disagree, however, with data of Markus et al[23] and Kohga et al.[24,25] These authors found uPA immunoreactivity in some, but not all, goblet cells (which in the present study did not stain for uPA). Furthermore they did not describe any uPA staining of connective tissue cells in the normal colon. It is likely that these discrepancies are also due to differences in antibody specificity, as discussed above.

It appears likely that the uPA containing fibroblastlike cells found in normal colon in mouse and man, and being abundant in the human colon tumor tissue, represent the same cell type. This cell type only constitutes a small fraction of all fibroblastlike cells in the normal colon, and we have not been able to characterize it in any detail. A possible explanation for the increased number of these cells in the colon tumor tissue is that they are recruited by the cancer cells to provide uPA needed for invasive growth. The cancer cells may secrete substances that either attract these uPA-containing cells from other parts of the body, stimulate their proliferation, or induce uPA synthesis in fibroblastlike cells. A clarification of this mechanism may demonstrate interactions between cancer cells and their stroma, which are crucial for tumor development. It is interesting that Davies et al[51] have described that a factor secreted by malignant murine cells was capable of inducing plasminogen activator in cultured fibroblasts.

The presence of tPA, but not uPA, in endothelial cells in the normal colon is in good agreement with previous findings in other normal tissues[29,37] and with the assumption that tPA physiologically is a key enzyme in thrombolysis.[14] The occurrence of uPA in endothelial cells, and the weaker intensity of the tPA staining (compared with the normal colon) of endothelial cells observed in some of the colon cancers, have a striking similarity to the staining pattern recently described for these plasminogen activators in acute inflammation of the human appendix.[37] The mechanism and the function of this change in the PA profile of the endothelial cells remain to be clarified.

It should be noted that the uPA-positive cells described in this study do not necessarily produce uPA themselves. A specific cell-surface receptor for uPA has been identified and characterized.[2,9–11] It is possible that uPA is produced and released from one cell type and subsequently bound to the uPA receptor on another cell type.

The presence of uPA immunoreactivity does not necessarily reflect that the conditions for plasminogen activation are present. Urokinase-type plasminogen activator is released from cells as a virtually inactive proenzyme that requires proteolytic activation before it in turn can activate plasminogen.[6,7] The antibodies used in the present study detect both pro-uPA and active uPA[33] (and unpublished results). Furthermore, under physiologic conditions, uPA-catalyzed plasminogen activation seems to require binding of uPA to its receptor,[12,13] and the activator is inhibited by at least two different types of plasminogen activator inhibitors.[8] An evaluation of the possible involvement of plasminogen activation in the pathophysiology of colon cancer therefore requires further study of the occurrence of these other components of the plasminogen activation system.

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