Demonstration of Urokinase Expression in Cancer Cells of Colon Adenocarcinomas by Immunohistochemistry and *in Situ* Hybridization

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The question whether urokinase is expressed in human colon cancer by the cancer cells themselves or by surrounding stromal elements such as fibroblasts, macrophages, and leukocytes, which transfer the activator to the receptors of the cancer cells, has been a controversial one. In the present study 12 cases of colorectal cancer were investigated by immunohistochemical methods using three monoclonal antibodies of different specificity against urokinase. Cytoplasmic staining of strongly varying intensity was observed in all cases, with the antigen expressed most strongly in the apical and the basal regions of the cancer cells. In some cases, staining was also found in stromal elements surrounding the cancer glands. That the activator was indeed the product of the cancer cells was demonstrated by *in situ* hybridization using a uPA-cDNA probe, which detected the presence of uPA-mRNA in both the basal and the apical regions of the cancer cells. A monoclonal antibody against the receptor for uPA showed similar localization. These findings indicate that the activator is expressed by the cancer cells and is not recruited by them from stromal elements. (Am J Pathol 1999, 155:1115–1120)

The essential role proteolysis plays in the local invasion and metastatic dissemination of cancer is well recognized and has been extensively reviewed in recent years.^{1–6} Work from this laboratory demonstrated the greatly enhanced plasminogen activator activity in extracts of cancer tissues from the colon, lungs, breast, prostate, ovaries, and malignant melanoma (reviewed in Ref. 7). Secretion of activators from short term organ culture of these tumors^{8,9} was also shown, and the activator was identified as urokinase. Since these studies were carried out on specimens that contained both tumor tissue and interstitial matrix, the exact location of the activator could not be established unequivocally. To clarify this issue, an immunohistochemical study was undertaken using a polyclonal goat anti-urokinase antibody that had been rendered monospecific. Using this antibody, strong staining was obtained in specimens of colo-

rectal carcinoma localized in the cancer cells, with strong accumulation at the apical end of the cells and also at the basal region, while normal glands showed staining only in some goblet cells.^{10,11} Strong staining was also observed occasionally in polymorphonuclear leukocytes, macrophages, and fibroblasts.

Grøndahl-Hansen et al¹² in a study on the immunohistochemical localization of urokinase in colon adenocarcinomas using monoclonal antibodies showed a positive reaction only in fibroblast-like and endothelial cells in the stroma, but none in the cancer cells. *In situ* hybridization studies by the same group also failed to detect specific mRNA in the cancer cells.¹³ These results formed the basis of the theory that in colon cancer urokinase is expressed by stromal cells from which the activator is recruited to the receptors of the cancer cells in a paracrine fashion. While subsequently several studies were published documenting the presence of uPA in the colon cancer cells, none of them presented evidence as to the site of origin of the activator.

We have now extended our investigations using three monoclonal antibodies directed against different domains of uPA, and with one directed against the uPA receptor. Our results again show abundant presence of urokinase in the cancer cells. *In situ* hybridization studies establish that these cells are indeed the site of synthesis of the enzyme. The present results cast doubt on the general applicability of the paracrine theory mentioned above.

Materials and Methods

Tissue Samples

Of the 12 specimens analyzed 8 were from the rectosigmoid segment of the colon, 2 from the sigmoid colon, 1 from the rectum, and 1 from the transverse colon. All tumors were invasive well- to moderately differentiated adenocarcinomas.

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Antibodies

Monoclonal antibodies (MAb) to uPA B chain 3689 (Ab1) and 394 (Ab2) and antibody to the A chain of uPA 3921 (Ab3), and antibody to domain II of the uPA receptor 3932 (Abr) were obtained from American Diagnostica (Greenwich, CT). The specificities of these antibodies were reported earlier by others^{14–16}: for Ab1,¹⁷ for Ab2,¹⁶ for Ab3, and¹⁸ for Abr. The Ab1 is strictly active site-specific, whereas Ab2 encompasses a larger domain than just the active site region.

Other Reagents

A DAB detection kit which includes the biotinylated universal secondary antibody was obtained from Ventana Medical Systems (Tucson, AZ). Super Block blocking buffer in Tris-buffered saline was purchased from Pierce (Rockford, IL). Trypsin was from GIBCO (Grand Island, NY), Triton X-100 from Sigma (St. Louis, MO), pretreatment reagent kit for paraffin-embedded sections used for *in situ* hybridization and fluorescein isothiocyanate (FITC)-labeled MAb was from Vysis (Downers Grove, IL). Digoxigenin-11-dUTP was purchased from Boehringer Mannheim (Indianapolis, IN). The plasmid containing the uPA construct (pHUK-8, ATCC 57328) was purchased from American Tissue Culture Type Collection (Rockville, MD). Wizard plus SV mini preps DNA purification system was from Promega (Madison, WI), QIAquick gel extraction kit was from QIAGEN (Valencia, CA).

Immunohistochemistry

A Ventana ES automated immunohistochemical stainer (Ventana Medical Systems) programmed for a three step immunoperoxidase technique (ABC) was used for the detection of uPA and its receptor. The instrument was programmed for a 20-minute treatment with 0.05% trypsin containing 0.01% Triton X-100 at 37°C and a blocking step for 10 minutes at 37°C with Super Block. The primary antibody was allowed to react at 37°C for 32 minutes, followed by incubation with biotinylated secondary antibody for 8 minutes. Subsequent to this the slides were incubated with avidin-horseradish peroxidase and the DAB substrate, followed by a 4 minutes copper enhancement. The slides were then stained with hematoxylin and photographed using an Olympus model BX 40 microscope equipped with a Hitachi model HV-C 20 video camera and an Image Pro 3.0 software (Media Cybernetics LP, Spectrum Services Inc., Webster, NY). The primary antibodies were used at the following concentrations of the IgG: MAb1, MAb2, and irrelevant mouse IgG at 40 µg/ml; MAb3 and MAb-uPA_r at 25 µg/ml.

Purification of uPA cDNA

The plasmid containing the uPA cDNA was purified using Promega's Wizard Plus Minipreps DNA purification system. The purified DNA was then digested with *Bam*HI and

*Bgl*II restriction enzymes and the insert was gel purified using a QIAquick gel extraction kit.

Fluorescence in Situ Hybridization (FISH)

Paraffin-embedded tissue sections 4 µm thick were pretreated for FISH using a paraffin pretreatment kit (Vysis) according to the manufacturer's instructions. The probe, uPA-cDNA, was labeled with digoxigenin-11-dUTP by random priming¹⁹ and detected with MAb to digoxigenin labeled with FITC.²⁰ Sections were counterstained with the nuclear stain DAPI (4'-6-diamidino-2-phenylindole) for better visualization. Signals were analyzed using appropriate filters on a Nikon Optiphot fluorescence microscope. Images were captured and analyzed using a monochrome CCD camera (Cohu Inc., San Diego, CA) and image analysis software (MacProbe, PSI, Inc., League City, TX).

Results

Altogether 12 colorectal adenocarcinomas were examined for the presence of uPA using MAb1 directed against an epitope on the B chain. Of these 12 specimens four were also stained with MAb2 and MAb3 and MAb-uPA_r. In a positive control experiment, the MAb1 gave a strong staining of the basement membrane of tubules of normal kidney, which is known to be the site of synthesis of urokinase (data not shown). Normal colon tissue known not to express urokinase was used as a negative control and showed no staining with MAb1 (see below). In 3 cases *in situ* hybridization was also performed using a cDNA for the detection of the presence of uPA mRNA in cancer cells and in stromal elements. Every one of the cancers showed some degree of positive reaction in the immunohistochemical studies. The intensity of staining, however, varied from case to case, and it also depended strongly on the antibody used. Figure 1, A and B, shows the results obtained by using antibodies 1 and 2, which are directed toward different epitopes on the B chain of uPA, ie, on the one carrying the active center, and are inhibitory to enzyme activity. Staining is strongest at the apical end of the cells, but it is also pronounced at the basal pole. Figure 1D, at a higher magnification, shows uniform granular staining throughout the entire cytoplasm of the cancer cells. The specificity of the reaction is shown by the absence of staining in Figure 1E, where the antibody (MAb1) had been absorbed with immobilized uPA before application to the section. The same tumor is shown in Figure 1C, this time treated with MAb3, directed toward an epitope on the A chain of uPA. The staining is much weaker in this case and does not accentuate the polar regions of the glands. Staining is also observed in the lumina. According to the manufacturer, reaction with this antibody compromises the ability of uPA to react with its receptor. Since much of the uPA present is believed to be bound to receptor,^{21,22} the weak binding may be due to masking of the epitope by the receptor. Figure 2B shows that the normal glands of the same tumor do not react with antibody 1.

Figure 1. A–E: Immunoperoxidase staining of tumor 1346, a moderately well differentiated invasive adenocarcinoma of the colon for uPA with domain-specific monoclonal antibodies. Antibodies used were: MAb1 (A), MAb2 (B), MAb3 (C). Magnification, $\times 200$. D: MAb1 at a magnification of $800\times$. E: MAb1 after prior absorption with immobilized uPA, $\times 200$. Sections were counterstained with hematoxylin. F: *In situ* hybridization using uPA-cDNA for the detection of uPA-mRNA. Arrow points to green fluorescence given by the mRNA at apical regions of the cancer cells. Blue fluorescence visualizes nuclei. Magnification, $\times 800$. All sections were taken from the same tissue block. In this tumor, staining is confined to both the apical and basal regions of the cancer cells. Specificity of the antibody is shown in E, where no staining is observed after absorption. The bar in A represents $50\ \mu\text{m}$.

That the uPA present in the cancer cells is synthesized there and is not recruited from matrix elements is shown by the intense reaction given by uPA mRNA following treatment with uPA cDNA in *in situ* hybridization experiments. The green fluorescence in Figures 1F and 2F signals the presence of mRNA specific for uPA, and its localization matches that of uPA itself, as shown by immunohistochemistry, ie, it is most pronounced at the apical end, but it is also expressed at the basal portion of the cells (compare Figures 1D and 1F). As a control, the plasmid devoid of the uPA cDNA insert used as an irrelevant DNA showed only background signal (data not shown).

Figure 2A shows that uPA receptor is localized in the cancer cells, as has been reported by others,^{21,25,26} and

it shows a predominantly cytoplasmic presence, with no apparent concentration on the cell membranes.

The localization of uPA is similar in another colorectal tumor shown in Figure 2C. In both tumors shown here, 1346 and 6765 (Figures 1A, 1D, and 2C), there is only an occasional presence of uPA-containing stromal elements. This is not the case, however, with tumor 290 (Figure 2, D and E) where uPA is evident in the cytoplasm of various stromal elements, including fibroblasts, macrophages, and polymorphonuclear neutrophils. However, even in these cases the strongest staining is still observed in the cancer cells. In tumor 1617 (Figure 2F) *in situ* hybridization shows the presence of uPA mRNA in a number of stromal elements, but also at both poles of the cancer cells.

Figure 2. A: Immunoperoxidase staining of tumor 1346 (see Figure 1) showing diffuse cytoplasmic staining with mAb1 for uPA receptor. Magnification, $\times 800$. B: The same tumor, adjacent normal glands, showing no staining with MAb1. Magnification, $\times 400$. C: Tumor 6765, another invasive moderately differentiated rectosigmoidal adenocarcinoma, treated with MAb2, shows patchy granular cytoplasmic staining, with the apical regions showing the most intense staining. Magnification, $\times 800$. D and E: Immunoperoxidase staining of tumor 290, a moderately differentiated adenocarcinoma of the colon, with MAb1 showing both tumor and stromal areas with intense uniform cytoplasmic staining of the tumor glands. Stromal elements, ie, fibroblasts, macrophages and polymorphs are also stained. Magnification, $\times 200$. F: *In situ* hybridization for the detection of uPA-mRNA in tumor 1617, a well- to moderately differentiated invasive adenocarcinoma of the colon. Strongest green fluorescence is seen at the basal region of the tumor cells. Some of the adjacent stromal elements also show green fluorescence. In addition, the lumen of the gland is full of cellular elements, some of which show green fluorescence. These cells are both desquamated and necrotic cancer cells, as well as inflammatory cells such as polymorphs and macrophages. Magnification, $\times 800$.

Discussion

The results reported here, obtained with the use of three monoclonal antibodies directed against different domains of the uPA molecule, as well as the *in situ* hybridization studies using cDNA to localize the mRNA for uPA, clearly point to the cancer cells as the major site of synthesis of uPA in human colon cancer, and also validate the results published earlier from our laboratory.^{10,11}

As mentioned above, Grøndahl-Hansen et al published in 1991 an immunohistochemical study in which they used a monoclonal antibody to demonstrate the presence of uPA in human colon cancer.¹² They found no staining in the cancer cells but did observe reactions in

stromal elements, such as fibroblasts, endothelial and inflammatory cells in the vicinity of the cancer tissue, while staining for tPA was restricted to endothelial cells. In a subsequent report from the same laboratory, Pyke et al¹³ published the results of *in situ* hybridization using an ³⁵S-labeled antisense RNA probe for the detection of uPA mRNA in colon cancer tissue. Sporadic spots confined to isolated cells were observed in the stroma, but not in the cancer cells. Endothelial cells, found to be positive in the immunohistochemical study, however, were negative in this study. On the basis of these findings they theorized that in colon cancer uPA is produced by stromal elements and may subsequently be recruited to the recep-

tors at the invasive areas of the cancer cells. uPA receptors were indeed identified by this group in the invasive regions of the cancer cells.²² These authors' view of the general significance of this type of stroma-to-tumor transfer was extended to other proteases, and was summarized in a recent review article.²³ To resolve the discrepancy between their results and those from our laboratory^{10,11} which showed very strong staining for uPA in the colon cancer cells, Grøndahl-Hansen et al¹² proposed that the polyclonal antibody used in the studies quoted contained a contaminant antibody directed toward some substance present in the cancer cells, which was not urokinase.

In subsequent years several reports appeared documenting the presence of uPA in the cancer cells of colon tumor sections, as detected by immunohistochemical means. Sier et al in 1991¹⁴ using a monoclonal antibody directed to the B-chain of uPA (same as our mAb1), found strong diffuse staining in the columnar epithelial cells of colon cancer and of adenomas as well. They also observed strong staining in the lamina propria of adenomas which they interpreted to be due to eosinophilic granulocytes. Buo et al^{24,25} found strong staining at the invasive regions of the carcinomas which involved the cytoplasm of the cancer cells as well as some cells of the adjoining matrix. Tan et al,²⁶ using a monoclonal antibody obtained from Grøndahl-Hansen, found intense staining at the invasive front of carcinomas, which also involved the cancer cells, as well as connective tissue elements. While all these studies indeed documented the presence of uPA in the cancer cells, they did not prove that they were the site of origin of the activator. The present results establish the presence of uPA, as well as the site of its expression in the cancer cells.

The argument put forward by Grøndahl-Hansen et al¹³ that the polyclonal antibody used in our previous studies^{10,11} lacked uPA specificity appears to be invalid, since 1) the polyclonal antibody was rendered monospecific by absorption with urinary proteins from various sources from which uPA had been completely removed by benzamidine affinity chromatography; and 2) when the polyclonal antibody was completely absorbed with uPA, all staining was abolished. It has also been repeatedly demonstrated by others that colon cancer cells in culture do secrete urokinase.^{27,28} The present results obtained with the monoclonal antibodies support the same conclusion as that of Kohga et al.^{10,11}

It is important to emphasize, however, that the expression of urokinase by the cancer cells is, in itself, insufficient for the promotion of invasive behavior: the presence of the receptor for this enzyme is equally important, as has been shown in several studies.^{29,30} The coincident localization of enzyme and receptor in the present study suggests that most of uPA may be bound to its receptor. Its intracellular localization (Figure 2A) suggests that the uPA-receptor complex was internalized.

The reason for the inability of Grøndahl-Hansen et al to demonstrate uPA in the cancer cells is not clear. The three monoclonal antibodies that have been used in their work were characterized earlier, were strongly inhibitory, and gave positive results with sections of psoriatic skin.³¹ The fact that they found positive reactions only in some

stromal elements suggests that treatment of the sections with the antibodies may not have been sufficiently intense. We have found in our earlier work that colon cancer cells did not give a strong signal with the polyclonal antibody unless the sections had been pretreated either with 0.1% trypsin or 0.1% Triton X-100.¹¹ It should also be noted that if the paracrine theory were right, and the interstitial cells indeed transferred uPA to the receptors of the cancer cells, the authors should have observed staining in the cancer cells, which they did not. The positive results shown with endothelial cells in Figure 1, A and C in the paper by Grøndahl-Hansen et al¹² is also unexpected. In none of the published studies on colon cancer, with the exception of that by Tan et al,²⁶ who used Grøndahl-Hansen's antibody, was such staining observed. In fact, the same group in an earlier paper³² clearly demonstrated in veins and small vessels of human skin, that the endothelium reacted only with an antibody against tPA, and not with one against uPA, a result identical to the one obtained earlier by Kohga et al¹⁰ in examining stromal vessels of colorectal carcinoma.

The negative results of the *in situ* hybridization study of Pyke et al¹³ are also difficult to interpret in view of the evidence for strong transcriptional activity shown in the cancer cells in the present study. It is now known that the results obtained using the fluorescence *in situ* hybridization technique is more sensitive than the autoradiographic technique.²⁰

While there is no longer any reason to assume that the localization of uPA in the cancer cells as described by Kohga et al was artifactual, the positive reaction obtained with some goblet cells of the normal glands, reported in that study, was not observed in the present one, or in any of the other studies quoted. It may have been due to cross-reaction by the polyclonal antibody with a mucinous component present in the goblet cells. Finally, the predominant localization of uPA in the cancer cells, as well as the abundance of mRNA for the same in these cells, coupled with the relative paucity of the same in the surrounding stroma, speaks against the general applicability of the theory which proposes that uPA is generated in the stromal cells, and is then transferred by them to the receptors located on the surface of the cancer cells.²³ In fact, it is well known that carcinomas elicit a desmoplastic reaction in the stroma. Thus it is more likely that uPA present in the stromal elements in the vicinity of the invasive front had been released from the cancer cells, and subsequently taken up by inflammatory cells, ie, macrophages, polymorphonuclear neutrophils, and other stromal elements. Tumor cells have also been known to release urokinase into their environment.^{8,9,27,30}

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