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Localization of Plasminogen Activators in Human Colon Cancer by Immunoperoxidase Staining[1]

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ABSTRACT

The immunoperoxidase technique, using antibodies against human urinary urokinase (M_r 55,000), was used for the localization of this enzyme in histological preparations of human colon tumors and normal colon tissue. The localization of tissue (vascular) activator was also investigated using antibodies against enzyme purified from human malignant melanoma. Both the "indirect method" and the peroxidase-antiperoxidase technique were found to be useful. Urokinase-reactive material was found in all tissues examined (33 primary cancers, 11 metastases, and 8 adenomas). In the normal colon, urokinase was found only in some of the goblet cells of the mucosal epithelium. In colon cancer, diffuse specific staining was observed in the cytoplasm, but the most intense staining was localized at the edge of the cancer cells bordering the lumen of the glands. In some cases, intense supranuclear staining could be observed in a location corresponding to the Golgi apparatus. In a few instances, urokinase could be seen associated with fibroblasts near the advancing front of an invading tumor. Adenoma, a benign tumor but often a precursor of cancer, also showed the presence of urokinase. Most significant were the observations showing that, in regions of the mucosal glands where normal epithelial cells were abruptly replaced by cancer cells, the appearance of cytoplasmic urokinase showed strict and exclusive association with the malignant cells, and the same was the case in transitions from normal epithelium to adenoma.

In contrast to urokinase, tissue plasminogen activator was not associated with cancer cells, but was consistently present in the stroma which separates the cancer glands and was localized in the endothelium of the blood vessels. This visual evidence was supported by results of extraction of plasminogen activators from tumors, and from the separated mucosal and submucosal layers of the normal colon of the same patients, which showed that urokinase is most abundant in the tumor tissue and least abundant in the submucosa, while tissue activator is most prevalent in the well-vascularized mucosa and submucosa and scarce in the usually poorly vascularized adenocarcinomas.

INTRODUCTION

The role of plasminogen activators in the malignant behavior of cancer cells has been the subject of a large number of recent studies. There are now good reasons to believe that these enzymes have more than one function in the neoplastic sequence, and they have been implicated in the process of malignant transformation, as well as in the local and metastatic spread of cancer. Of the 2 main types of plasminogen activator, urokinase and tissue (vascular) activator, the former is more closely associated with neoplasia. Various aspects of this topic have been reviewed in recent years (14, 15, 23, 26). Earlier studies from this laboratory have dealt with the analysis of extracts of human tumors of the lung (18), prostate (4), colon (6), and breast (8), and more recently with the secretion of plasminogen activators by tumor explants in short-term organ culture (16). While these studies yielded valuable information with respect to both the type of activator present in these tumors and the rate of activator release, there remained some uncertainty concerning the actual cell types which gave rise to the various forms of activator.

In order to resolve this uncertainty, we have now carried out a study in which urokinase, and in a smaller number of cases tissue activator, were visualized in tissue sections by an immunoperoxidase technique based on the use of specific antibodies directed against these 2 activators. This study was restricted to colorectal tumors because of the relatively easy availability in this institute of surgical specimens of primary and metastatic origin, of adenomas, and of normal colon tissues from the same patients. In the following, we will discuss the findings based on the examination of 33 colorectal cancers, 12 metastases, and 8 adenomas. In most of the cases, the tissues were also analyzed biochemically for their extractable activator content and for their activator secretion rates in organ culture. In these cases also, the type of plasminogen activator was determined by the use of inhibitory antibodies raised against purified human urinary urokinase.

MATERIALS AND METHODS

Fresh tumor specimens were obtained from the Department of Surgical Oncology of this institute as part of a collaborative effort between the 2 departments. The specimens used included 26 colorectal carcinomas, 2 adenomas, and 8 metastases (liver, lymph node, omentum, ligament) besides normal colon tissues adjacent to the tumor area. Parts of each tumor, having similar macroscopic appearance, were used for short-term organ culture experiments, for plasminogen activator extraction, and for immunohistochemical studies. For the last, tissues were fixed immediately with 10% formalin-0.9% NaCl solution (saline). Additional paraffin-embedded stock samples of 7 colorectal cancers, 6 adenomas, and 4 metastases were kindly made available to us by Dr. Cecilia Lopez of the Department of Pathology of this institute. These were used only for immunoperoxidase studies.

Immunoperoxidase Procedures. Tumor specimens fixed with formalin-saline were routinely embedded in paraffin. The blocks were cut into 4-μm-thick serial sections and mounted on glass slides pretreated by dipping into Elmer's Glue, diluted to 2% with water to prevent detachment of samples during the repeated washings in the subsequent

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procedures. After deparaffinization with xylol and alcohol, the first serial section was stained with hematoxylin-eosin. The sections to be used for immunoperoxidase staining were pretreated with absolute methanol, freshly rendered 0.3% with H_2O_2, for 30 min in order to block the endogenous peroxidase activity of the cells (24). The sections then were rinsed 3 times with distilled water and placed in TBS.[4]

Immunoperoxidase staining was performed either by the "indirect" method or by the PAP method. The former was described recently (16). The PAP method, somewhat modified from the description by Taylor (27), was carried out at room temperature, with all incubations done in a humidifed stainless steel box. The buffer used was TBS. When this buffer was used as a diluent in Steps 2, 3, and 5, it contained 3% nonimmune rabbit serum (for urokinase), or goat serum (for tissue activator). The procedure for the visualization of urokinase was the following: (*a*) pretreatment with nonimmune rabbit serum (previously inactivated by heating at 56 °C for 30 min and diluted to 5% with TBS) for 10 min, followed by shaking off of excess fluid; (*b*) treatment with goat antiserum (usually diluted 1:60 with TBS) or with purified antibody A_{280} of 0.260) against human urinary urokinase for 60 min, followed by thorough jet washing with cold TBS (100 ml) from a squirt-bottle, and additional soaking in the same; (*c*) treatment for 5 min with nonimmune rabbit serum, diluted to 5%, followed by removal of excess fluid, but no washing; (*d*) treatment with rabbit IgG directed against goat IgG, diluted 1:10, and washing with cold TBS. (*e*) treatment with goat PAP, diluted 1:80 with TBS for 30 min, followed by thorough washing, as in Step *b*; (*f*) addition of the substrate solution, 0.5% 3,3′-diaminobenzidine tetrahydrochloride in TBS, containing 0.01% H_2O_2, incubation for 15 min in the dark, followed by jet washing with distilled water and further soaking for 10 min. At this point, in most cases, the slides were lightly counterstained with hematoxylin; and (*g*) dehydration and mounting with Permount. For control slides, in Step *b*, nonimmune goat serum was used in place of the antiurokinase serum, diluted to the same degree.

For the visualization of tissue plasminogen activator, the same procedure was used, but since the source of the primary antibody was rabbit serum, rabbit and goat proteins were interchanged in the rest of the procedure.

Much of the work reported here was carried out using whole goat antiserum against highly purified human urinary urokinase. During the course of this study, we prepared monospecific antibody (see below) and repeated much of the work with this, using the same tumors. The results for the localization of urokinase in tumor tissues were identical. However, use of the monospecific antibody eliminated much, but not all, of the staining of goblet cells in the normal mucosal glands.

Preparation of Monospecific Antiurokinase Antibody and its Specificity. One ml of antiurokinase antibody, purified as described eariler (18), containing 1.3 mg of IgG, was adsorbed on a 2-ml column of Urokinase-Affi-Gel 10 (2 mg of Winkinase coupled to 2 ml). The bound IgG was eluted with 0.1 M acetic acid, containing 0.7 M NaCl, pH 2.5. The IgG which was not held up by the column was repassaged several times through the column (reequilibrated each time) until it was freed of antiurokinase antibody, *i.e.*, it no longer inhibited urokinase activation of plasminogen in an azocaseinolytic assay (18). This fraction, brought to an A_{280} of 0.260, was used as a negative control in the immunoperoxidase procedure. The bound fraction was further purified by absorption with Affi-Gel 10 to which urinary proteins had been coupled. The urinary proteins had been pretreated by repeated passage through a benzamidine-Sepharose column to completely remove urokinase. Passage of the antibody solution through the urinary protein-Affi-Gel column was repeated until no more protein could be removed, as judged by A_{280} measurements. Two kinds of urinary proteins were used in this step; one was from a case of glomerulonephritis, the other from a diabetic tubular proteinuria (the latter kindly supplied by Dr. Pierre Burtin, Villejuif, France). The antibody solution was absorbed with both of these. Finally, this monospecific antibody was concentrated by ammonium sulfate precipitation, dialyzed against TBS containing 10% sucrose, and diluted to A_{280} 0.260, and used without further dilution.

The purified monospecific goat immunoglobulin antibody against urokinase was tested for cross-reactivity by immunodiffusion with the following antigens: tissue plasminogen activator; concentrated urinary proteins; the same after removal of urokinase by benzamidine-agarose; fetal calf serum; and normal human plasma. As shown in Fig. 1, the antibody reacted only with urokinase, with urinary proteins, and with normal plasma. The same figure also shows that the rabbit antibody against tissue activator (see below) reacted only with purified tissue plasminogen activator, and slightly with human plasma; no cross-reactivity with urokinase could be observed.

Plasminogen Activator Assay. The extraction of plasminogen activator and the measurement of its secretion in short-term organ culture was described recently (16). Plasminogen activator activity was assayed by azocaseinolysis in the presence and absence of added human plasminogen, as described earlier (18). The fraction of activator activity in the extracts and culture fluids that was due to urokinase was determined by the measurement of azocaseinolysis in the presence of excess goat antibody directed against human urinary urokinase. The fraction of activity not inhibitable by this antibody was interpreted as being due to tissue activator. Earlier work has shown that such activity is always associated with a M_r 70,000 activator activity in sodium dodecyl sulfate gel electrophoresis, and inhibition by antibody against tissue activator also confirmed this assignment (8).

Reagents. Goat immune serum against highly purified M_r 55,000 human urinary urokinase was described earlier (10). Rabbit antiserum against tissue activator which had been purified from culture fluids of a human melanoma cell line, as well as the activator itself, were the generous gifts of Professor Desiré Collen. Rabbit IgG directed against goat IgG, as well as goat IgG directed against rabbit IgG, were the products of DAKO, Denmark, as supplied by Accurate Chemicals, Westbury, NY. The same company supplied both the goat and the rabbit PAP. 3,3′-Diaminobenzidine was from Sigma Chemical Co. Nonimmune goat and rabbit sera were of local origin.

The black-and-white photographs of immunoperoxidase-stained sections were taken using the high-sensitivity film, Kodak Technical Pan 2415, kindly supplied by Dr. J. Takeuchi of this institute.

RESULTS

Localization of Urokinase. Table 1 contains information on the plasminogen activator content and secretion of the tumors

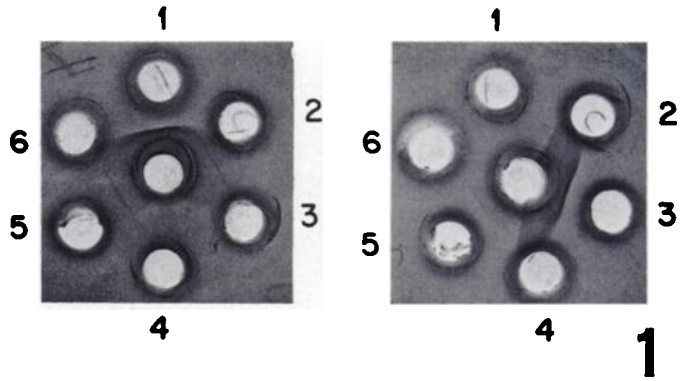

Fig. 1. Double-diffusion patterns of the primary antibodies used in this study. *Left*, specificity of the monospecific antiurokinase immunoglobin. *Center well*, goat antiurokinase IgG, 25.5 mg/ml; *Well 1*, purified human urinary urokinase, M_r 55,000, 1 mg/ml. *Well 2*, concentrated human urinary proteins, 22 mg/ml; *Well 3*, purified tissue plasminogen activator from a human melanoma culture, 0.8 mg/ml; *Well 4*, whole fetal calf serum; *Well 5*, whole normal human plasma; *Well 6*, concentrated human urinary proteins (as in *Well 2*), but after removal of urokinase with benzamidine-agarose. *Right*, specificity of antitissue plasminogen activator immunoglobin. *Center well*, rabbit antitissue plasminogen activator IgG, 3.8 mg/ml. Antigen wells are as in left panel.

[4] The abbreviation used are: TBS, Tris-buffered saline (0.05 M Tris-HCl:0.1 M NaCl, pH 7.6); PAP, peroxidase-antiperoxidase immune complex.

Table 1

Plasminogen activator data and pathology information on the tumors in Figs. 2 to 6

Case	Age (yr)	Sex	Site of origin	Pathology	Stage (Dukes' classification[a])	Extraction		Secretion		Illustrations in article
						CTA units[b] (g tissue)	% of urokinase[c]	Maximum rate CTA units/g/h	% of urokinase[c]	
280	65	M	Right colon	MD[d]	B2	43.9	97	1.86	98	Fig. 2, *B*, *C*, and *D*
277	52	M	Rectal	MD pap	C	16.1	97	0.26	93	Figs. 3*D and 4*, *A* and *B*
285	53	F	Rectosigmoid	INF MD pap	D	17.9	95	1.58	97	Fig. 3*C*
57	77	F	Left colon	WD	D	20.2	85	4.8	98	Fig. 3, *A* and *B*
259	62	M	Cecum	MD muc	C	473.0	97	44.05	95	Fig. 4*C*
64	55	M	Lymph node met.	WD	D	ND		ND		Fig. 5*A*
71	57	M	Omentum met.	MD	D	15.0	95	0.32	100	Fig. 5*B*
269	57	M	Liver met.	WD	D	8.6	98	0.23	97	Fig. 5*C*
259	62	M	Right colon	Adenoma	C	22.6	94	4.78	99	Fig. 2*A*
287	77	F	Sigmoid	INF MD	D	22.04	78	15.24	90	Fig. 6, *A* and *B*
293	48	F	Left colon	INF MD	C1	31.8	81	37.8	97	Fig. 4*D*

[a] See Ref. 3.
[b] See Ref. 16.
[c] Percentage of total activator activity.
[d] MD, moderately differentiated; INF, infiltrating; WD, well differentiated; pap, papillary; met., metastasis; muc, mucinous.

illustrated in the figures, as well as on the pathology of these tumors. Such data on other tumors examined in this study, but not illustrated in the paper, were published earlier (16). Immunoperoxidase staining of colon cancer sections shows consistent localization of the activator in certain areas of the tumor. Thus, we invariably see heavy staining at the luminal edge of the cancer cells which form the glands. This is particularly well visualized in the central gland in Fig. 2*C*, but can also be seen in Figs. 3, *A*, *C*, and *D*; 5*C*; and 6*A*. Normal glands (Fig. 2*C*) do not show this staining pattern. Cytoplasmic staining can be a very impressive feature of abnormal areas, as in the adenoma of Fig. 2*A*, because in general neither normal epithelial cells nor connective tissue cells show such staining. Cytoplasmic staining of varying intensity can be seen in Fig. 4*C* in the cells of a poorly differentiated portion of a mucinous adenocarcinoma removed from the patient who also exhibited the highly stained adenoma shown in Fig. 2*A*. In some cases, it is possible to achieve a closer identification of the intracellular location of the activator, as in the cells of the well-differentiated tubular gland of Fig. 4*D*, where urokinase appears to be concentrated in the supranuclear portion of the cytoplasm, considered to correspond to the Golgi apparatus. In the rare case shown in Fig. 4*A* and *B*, in addition to the ill-developed tubular lamina, the activator can also be seen at the periphery of the cancer nests.

The histological type of the tumor does not seem to influence markedly the localization of urokinase. Thus, the features displayed in the tubular carcinoma of Fig. 2, *B* to *D*, are also present in the papillary carcinomas shown in Fig. 3, *C* and *D*; intense localization of urokinase is seen on the cell surface, which corresponds to the luminal surface in the tubular carcinoma.

Urokinase Staining in Invasion and Metastasis. Urokinase staining follows characteristic patterns in areas of invasive growth, illustrated here in Fig. 3, *A* and *B*. Parts of a cancer gland are shown which appear to have burst open. Intensive urokinase staining is seen in the many desquamated cells in the lumen but, more importantly, in association with the adjacent granulation tissue. This phenomenon is not restricted to a single gland but can be seen along a larger area of the invasive front in the overview shown Fig. 3*A*, *inset*. That the enzyme seen in the stroma originates in the cancer cells is indicated by the observation that the intensity of the color declines rapidly in the direction away from the tumor. The overall orientation of the specific staining in the granulation tissue in Fig. 3*A* is very similar to that of the fibroblasts themselves shown in Fig. 3*B*, suggesting that urokinase may be absorbed on the surface of these cells. This observation will be discussed later.

In the course of this investigation, we observed in some instances the appearance of vesicular structures located just outside the basement membrane of the cancer glands, the walls of which show a positive reaction for urokinase. These structures can be seen in the cancer glands of Fig. 2*C*, and in higher magnification, in Fig. 3*D*. Normal glands do not show such vesicles. These structures may represent products of mesenchymolytic activities of cancer cells, and may serve the purpose of transferring enzymes from the cancer cells to the environment. Electron microscopic studies should reveal more about these structures.

Metastases to 3 different sites, lymph node, omentum, and liver, are shown in Fig. 5. As in the primary tumors, urokinase can be observed both as diffuse cytoplasmic staining and as intensely outlined luminal borders. The remarkable difference in the activator secretion rates between primary and metastatic colon tumors observed earlier (16) is not mirrored in any of the features of immunoperoxidase staining patterns of primary and metastatic tumors. This is not unexpected, since staining is more likely to reflect not secretion rates, but rather the extractable amounts of enzyme, which are not markedly different in the 2 kinds of tumor.

Transitional Areas between Normal and Tumor Epithelium. The most dramatic phenomenon revealed by immunoperoxidase staining for activator is that, in transitional areas, the abrupt change in the histological pattern is accompanied by an equally abrupt change in staining intensity, as can be observed by comparing the hematoxylin-eosin staining of a gland in Fig. 2*B* with the immunoperoxidase-stained image of the same in Fig. 2*C*. In the former, the normal epithelium is easily distinguished by the uniform size and regular arrangement of the nuclei which contrasts with their pleomorphism and disarrangement in the cancerous portion of the gland. This morphological transition is faithfully reflected (Fig. 2*C*) in the appearance of intense staining

in the luminal edge of the cells in the cancer portion, as well as in the diffuse cytoplasmic staining in the same area. The argentaffine staining of the same gland in Fig. 2*D* shows an additional difference between the 2 portions of this gland. The basement membrane of the normal part is uniformly thick, while over the transformed portions it is thin and wavy. It is possible that this phenomenon may indeed be connected with the appearance of urokinase in the same area; there is good experimental evidence to show that plasmin, the product of the enzymatic action of plasminogen activator, can convert latent collagenase to the active enzyme, resulting in proteolytic attack on the basement membrane (22, 30).

A second, equally interesting case of transition can be seen in Fig. 2*A*. Here, the transition is between normal glandular epithelium and the benign tumor, adenoma. Again, the morphological change coincides exactly with the beginning of intense cytoplasmic staining which continues throughout the extent of the tumor. The adenocarcimoma, present in the colon of the same patient, showed equally intense cytoplasmic staining (Fig. 4*C*).

Comparison of the Localization of Tissue Plasminogen Activator with That of Urokinase. The 2 parts of Fig. 6 show a section of colon cancer stained for urokinase and for tissue activator, respectively. In Fig. 6*A*, the activator is localized, as in the other illustrations, mainly at the luminal edge of the cancer cells and in a patchy pattern in the rest of the cytoplasm. In contrast, in Fig. 6*B* staining for tissue activator shows this enzyme to be localized mainly in the stroma which separates the cancer glands, and in particular, in the endothelial lining of the many capillaries and veins that penetrate the stroma. A faint staining of the luminal edge of the cancer glands can also be observed; the desquamated cancer cells also give a positive reaction with this activator as well as with urokinase. In the light of the lack of cross-reactivity between the 2 activators shown in Fig. 1, this observation is not readily interpreted. The cancer cells which form the glands, however, are devoid of this enzyme variant. Fig. 6*B, inset*, magnifies some capillary cross-sections and shows the heavy staining of the endothelial lining.

DISCUSSION

This study presents visual evidence to support the conclusion, drawn by many workers on the basis of biochemical investigations of tumor cell cultures and solid tumors of both animal and human origin, that the production of plasminogen activators is intimately connected with the process of malignant transformation, and indeed, with neoplastic growth in general. The studies of Åstedt and Holmberg (1) and Niklasson *et al.* (21), and those of others (11, 20, 31), including a series on human solid tumors from this laboratory (4, 6, 8, 18), have firmly established that the activator peculiarly associated with neoplasia is urokinase, and not tissue activator. The urokinase-specific immunoperoxidase staining used in the present study fully supports this conclusion. Specific staining for this enzyme in tumor sections is restricted to the cancer cells, and allows an easy distinction between these cells and the fibroblasts and smooth-muscle cells of the surrounding stroma. In contrast, staining for tissue activator (also called vascular activator) localizes this enzyme, not in the cancer cells, but in the endothelium of the blood vessels of the stroma. This finding convincingly supports our previous interpretation (15, 16) of literature data on the distribution of activators in normal and malignant colon tissue. It should be pointed out here that there are several papers in the literature (9, 25, 28) which report that normal colon tissue, or the noncancerous elements of tumor tissue, contain more plasminogen activator than does the tumor itself. In all of these investigations, a fibrinolytic assay was used for the measurement of plasminogen activator activity. It is now well established that the activity of tissue activator is greatly enhanced by the presence of fibrin (4, 5, 19), a property not shared by urokinase. In a fibrinolytic assay of material containing both kinds of activator, the initial clot lysis will mostly reflect the action of the rapidly activating tissue activator; the activation of plasminogen due to urokinase may set in when the clot is already in the process of lysis. The use of this technique, thus, will primarily signal the presence of tissue activator, located mostly in the endothelial lining of the capillaries and small veins of the stroma, but will respond much less strongly to the cancer tissue itself, which contains very little of this type of enzyme, leading to the erroneous conclusion that cancer tissues contain little or no plasminogen activator activity. Quantitative analysis of the extracts of colon tumor tissue and of the separated mucosa and submucosa indeed show that most tumors contain only small amounts of immunologically identifiable tissue activator in comparison to urokinase, while the normal mucosa and submucosa, which contain numerous blood vessels, are relatively rich in this type of enzyme (Table 2). This, however, is not to deny that in rare cases the dominant activator can be of the vascular type, as in some breast cancers (8) and some sarcomas (16) encountered in our studies. The curious case of human melanoma, where cell cultures produce tissue activator while surgically removed solid melanomas contain and secrete mostly urokinase, was presented in a recent paper (17).

Table 2

Plasminogen activator content of colon tumors and of normal mucosa and submucosa

Case	Tissue	Activator (CTA units/g tissue)			Urokinase: tissue activator
		Activator (total)	Urokinase	Tissue activator	
293	T[a]	31.85	25.80	6.05	4.3
	M	6.81	4.63	2.18	2.1
	S	7.42	1.13	6.29	0.2
295	T	32.42	32.10	0.32	100.0
	M	29.86	12.24	17.62	0.7
	S	4.52	0.77	3.75	0.2
301	T	28.94	28.36	0.58	48.9
	M	11.09	8.43	2.66	3.2
	S	3.40	1.33	2.07	0.6
303	T	58.80	56.45	2.35	24.0
	M	11.70	6.55	5.15	1.3
	S	3.91	0	3.91	0
304	T	13.55	12.33	1.22	10.1
	M	23.10	11.3	11.80	1.0
	S	10.16	8.13	2.03	4.0
306	T	23.70	21.57	2.13	10.1
	M	3.49	1.95	1.54	1.3
	S	3.15	1.35	1.80	0.7
Mean	T	31.54 ± 15.0[b]	29.43 ± 14.9	2.11 ± 2.1	32.9 ± 36.6
	M	14.3 ± 10.1	7.52 ± 3.9	6.82 ± 6.5	1.6 ± 0.9
	S	5.43 ± 2.8	2.12 ± 3.0	3.31 ± 1.7	0.95 ± 1.5

[a] T, tumor; M, mucosa; S, submucosa.
[b] Mean ± SD.

The specific staining observed in the fibroblasts in the immediate vicinity of an invasive primary tumor (Fig. 3, *A* and *B*) may be related to some interesting recent observations concerning fibroblast behavior. Baker *et al.* (2) and Low *et al.* (13) found that fibroblasts produce proteins, termed protease-nexins, which can specifically complex with certain enzymes, such as thrombin and urokinase, resulting in the binding of these enzymes to the fibroblast surface. Hoal *et al.* (12) also found a protein of fibroblast origin which bound tissue activator to these cells, preliminary to its internalization and degradation. It is possible that the positive immunoperoxidase reaction shown by the fibroblasts in the invaded area is the result of the absorption of urokinase by such a mechanism. A recent paper by Davies *et al.* (7) may also be relevant which presents data interpreted to mean that several tumor-derived cell lines produce a protein factor which can induce the formation of plasminogen activator when added to cultures of fibroblasts.

The present study has also yielded information on the relative contribution of other types of host cells to the plasminogen activator content of human tumors. Lymphocytes and plasma cells, observable both in primary and in metastatic tumors, do not give a positive reaction for either of the 2 plasminogen activators. The macrophages that were seen in these preparations were mostly negative for urokinase. However, in a few cases they showed very intense staining, attributable either to their own urokinase content (29) or to phagocytosis of RBC which sometimes exhibited nonspecific peroxidase activity. Polymorphonuclear granulocytes also frequently showed strong staining.

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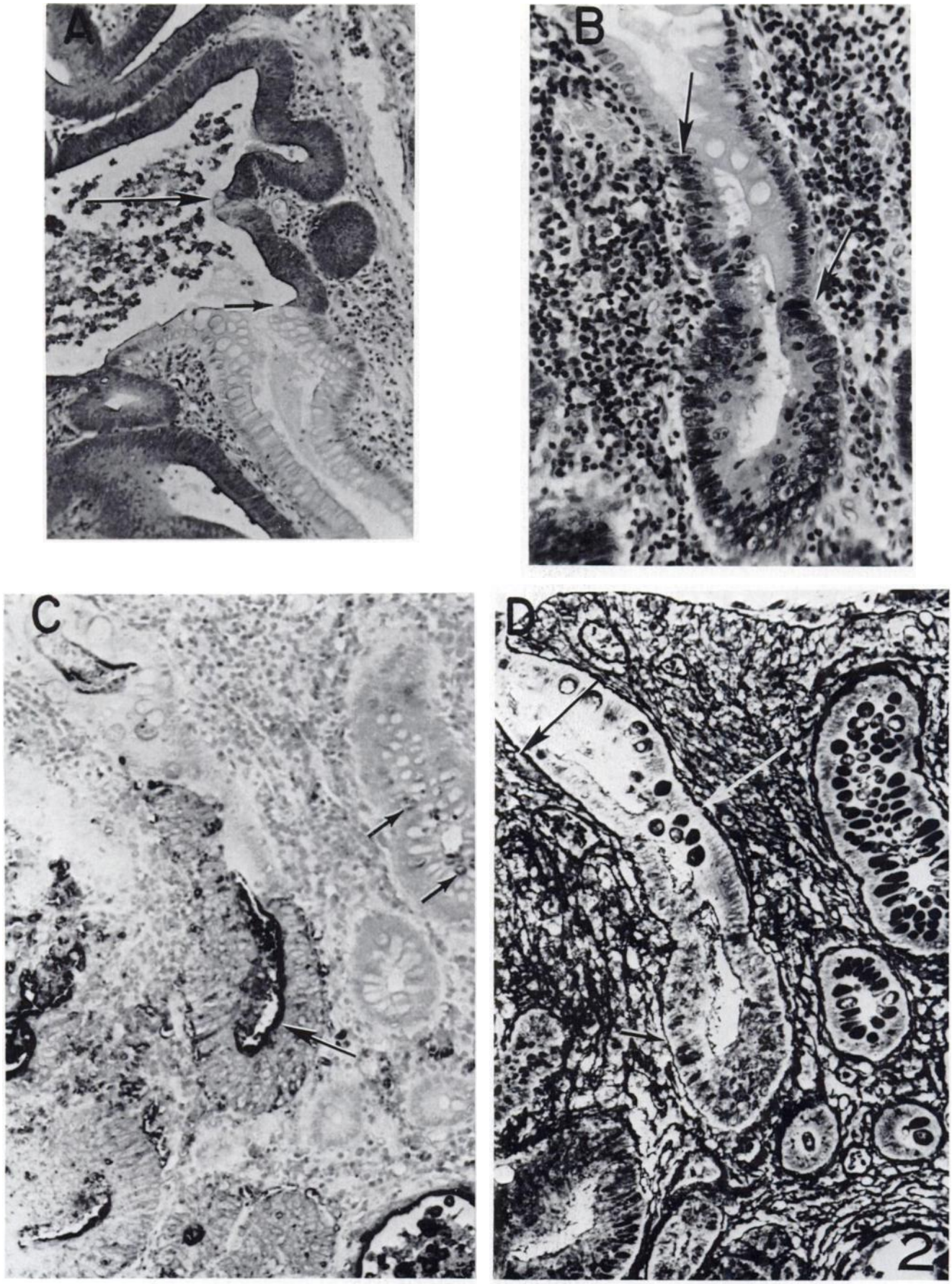

Fig. 2. Immunoperoxidase localization of urokinase in adenoma and colon cancer. *A*, transition between normal colon epithelium and adenoma (counterstained with hematoxylin). Note the abrupt transition to adenoma (*short arrow*) characterized by intense cytoplasmic staining, and by the well-defined luminal edge of the adenoma glands. *Long arrow*, short normal segment within the adenoma; note absence of cytoplasmic staining, and presence of a stained goblet cell. Indirect method. × 240. *B*, hematoxylin-eosin staining of an area of transition from normal to cancerous epithelium in a mucosal gland of the colon. Normal glandular epithelium is changed into cancer as uniform, basally located nuclei are replaced by crowded, irregular ones. *Arrows*, zones of abrupt transition. × 480. *C*, the same gland stained by urokinase-specific immunoperoxidase reaction. Note that only the cancerous portions of the gland are marked by heavy staining of the luminal edge of the cells (*long arrow*). Desquamated cells in the lumina are also heavily stained, and granular staining of the cytoplasm can also be observed. In the normal glands, only an occasional goblet cell is stained (*short arrows*). PAP method. × 430. *D*, the same area stained with Fontana-Masson for reticulin fibers and mucin. Note that the basement membrane of the normal portions of the central gland is uniformly thick (*long arrows*), while that of the cancer area is thin and wavy (*short arrow*). Most of the goblet cells in the normal glands show a positive argentaffin reaction for mucin. × 430.

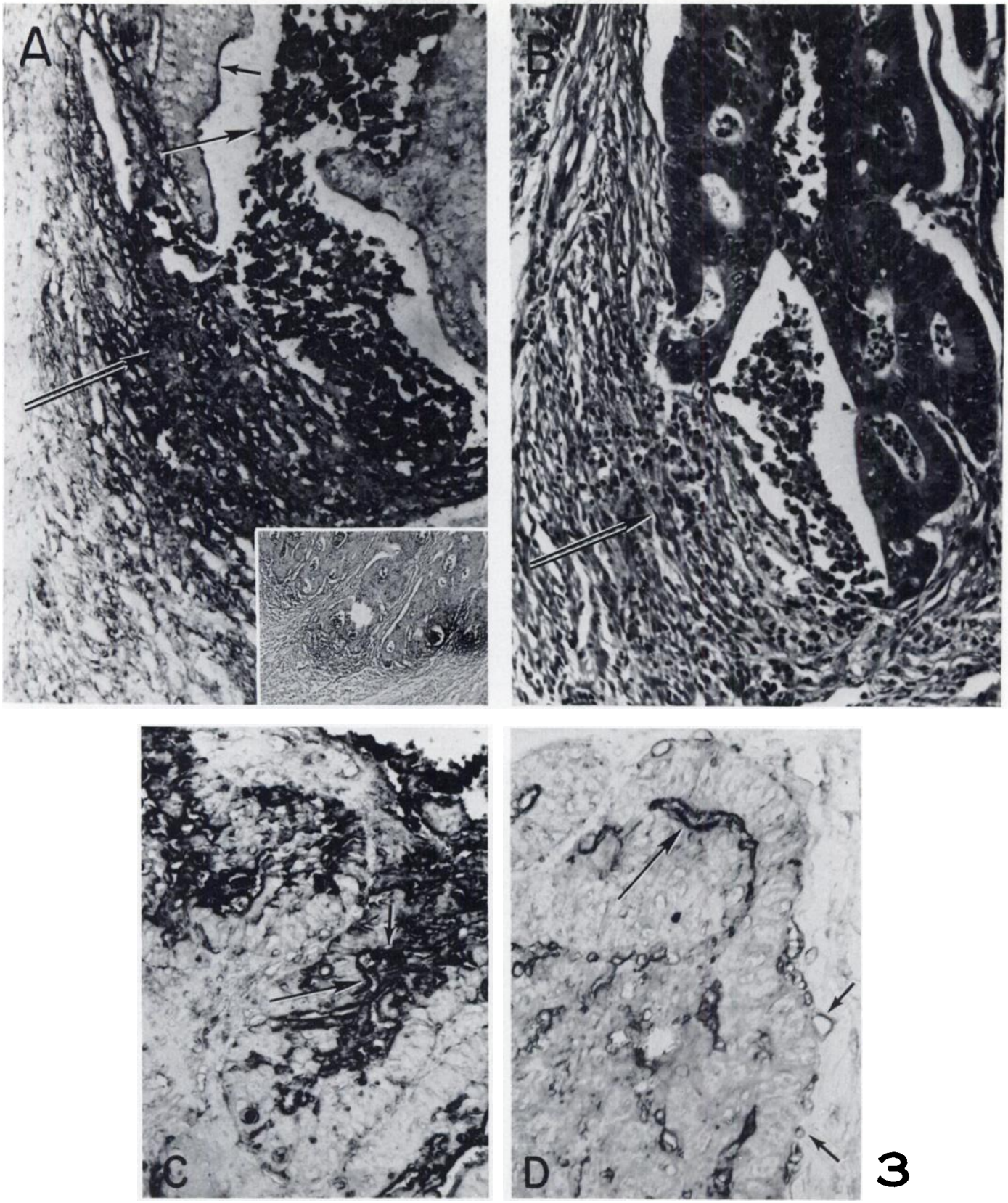

Fig. 3. *A*, apparent diffusion of urokinase at the advancing front of colon cancer. Heaviest staining is found at the luminal edge of cancer cells (*short arrow*) and in the desquamated cells (*long arrow*). The gland appears to have ruptured, and heavy staining is found over the fibroblasts (*double arrow*) just below the ruptured lumen. PAP method. × 430. *Inset*, overview of advancing area into the muscle layer of the colon. Diffusion of urokinase is seen in front of each lobule of the advancing cancer. × 80. *B*, a different area of the same tumor stained by hematoxylin-eosin. Note that the general orientation of the specifically stained regions below the cancer portion in Fig. 2*A* corresponds to the orientation of the fibroblasts in this picture (*double arrow*). × 480. *C*, urokinase-localization in a papillary carcinoma of the colon. Urokinase is observed at the luminal edge of the cancer cells (*long arrow*) and in desquamated cells (*short arrow*). PAP method. × 430. *D*, localization of urokinase in colon cancer with papillotubular growth. Staining is observed at the luminal edge of the glands (*long arrow*) in the cytoplasm, and in vesicular structures just outside the basement membrane (*short arrows*). PAP method. × 480.

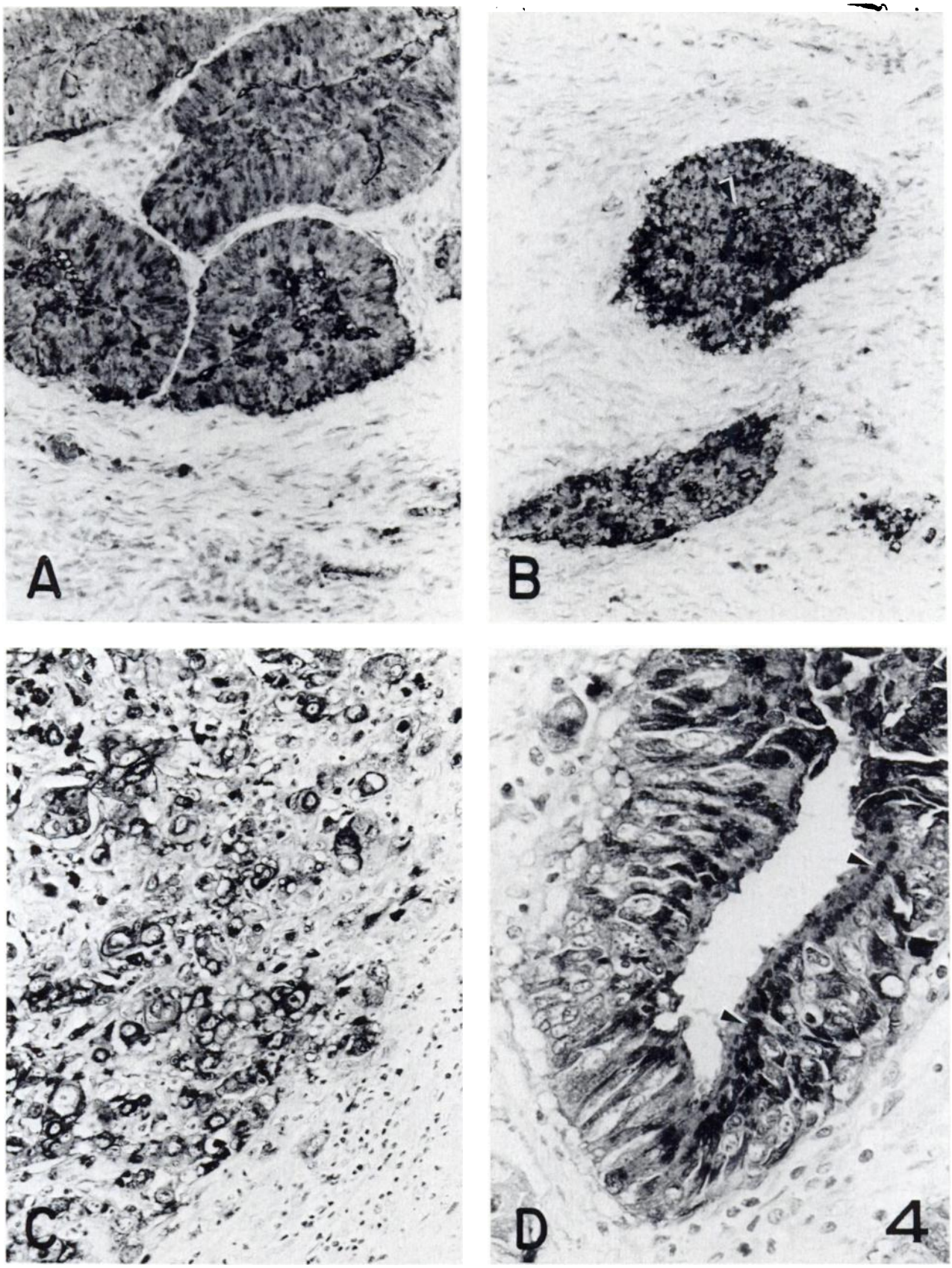

Fig. 4. Localization of urokinase in colon cancer. *A*, staining in this case is intense at the periphery of the cancer nests and is heavier at the advancing front than at the inner surfaces. *B*, same tumor; here, too, staining is at the periphery of the cancer nests, but it can also be seen on the inner surface of the poorly developed tubular lamina (*arrow*). Both *A* and *B* are from the tumor also shown in Fig. 3*D*. PAP, using monospecific antibody against urokinase. × 244. *C*, staining can be seen in the entire cytoplasm of individual cancer cells, but the intensity varies from cell to cell. Poorly differentiated adenocarcinoma of the cecum. Adenoma of the same patient is shown in Fig. 2*A*. PAP method, using monospecific antiurokinase antibody. × 244. *D*, in this well-differentiated tubular-type carcinoma, staining involves the entire cytoplasm of the cancer cells. A characteristic feature is the localization of urokinase at the supranuclear portion (*arrows*), corresponding to the Golgi apparatus. PAP method with monospecific antibody, as above. × 530.

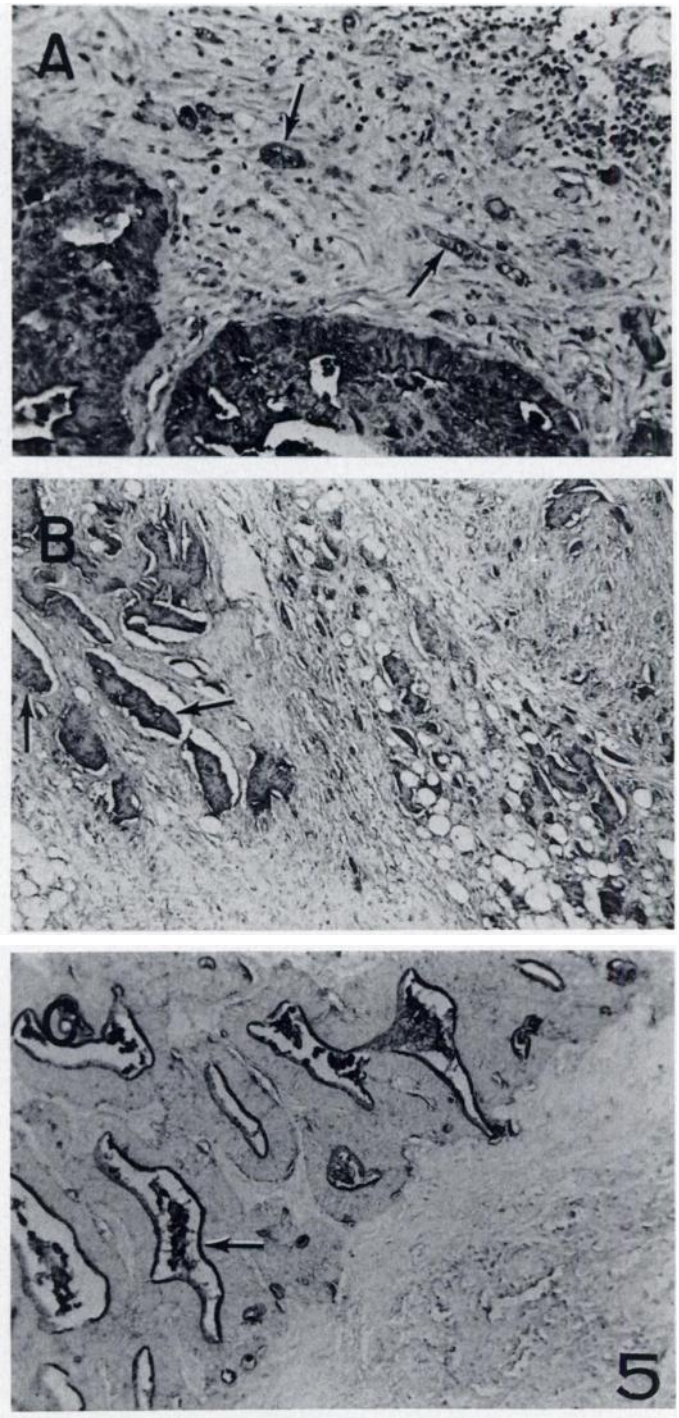

Fig. 5. Urokinase localization in metastatic colon cancer. *A*, lymph node metastasis with glandular growth and with small islands of cancer (*short arrows*). Note that lymphocytes in *upper right corner* show no specific staining (lightly counterstained with hematoxylin). PAP method. × 240. *B*, metastatic colon cancer in the omentum. Islands of well-stained cancer cells (*arrows*) embedded in fibrofatty tissue. PAP method. × 120. *C*, metastatic colon cancer in the liver. The luminal edges of the cancer cells are heavily stained in all the glands shown (*arrow*). PAP method. × 120.

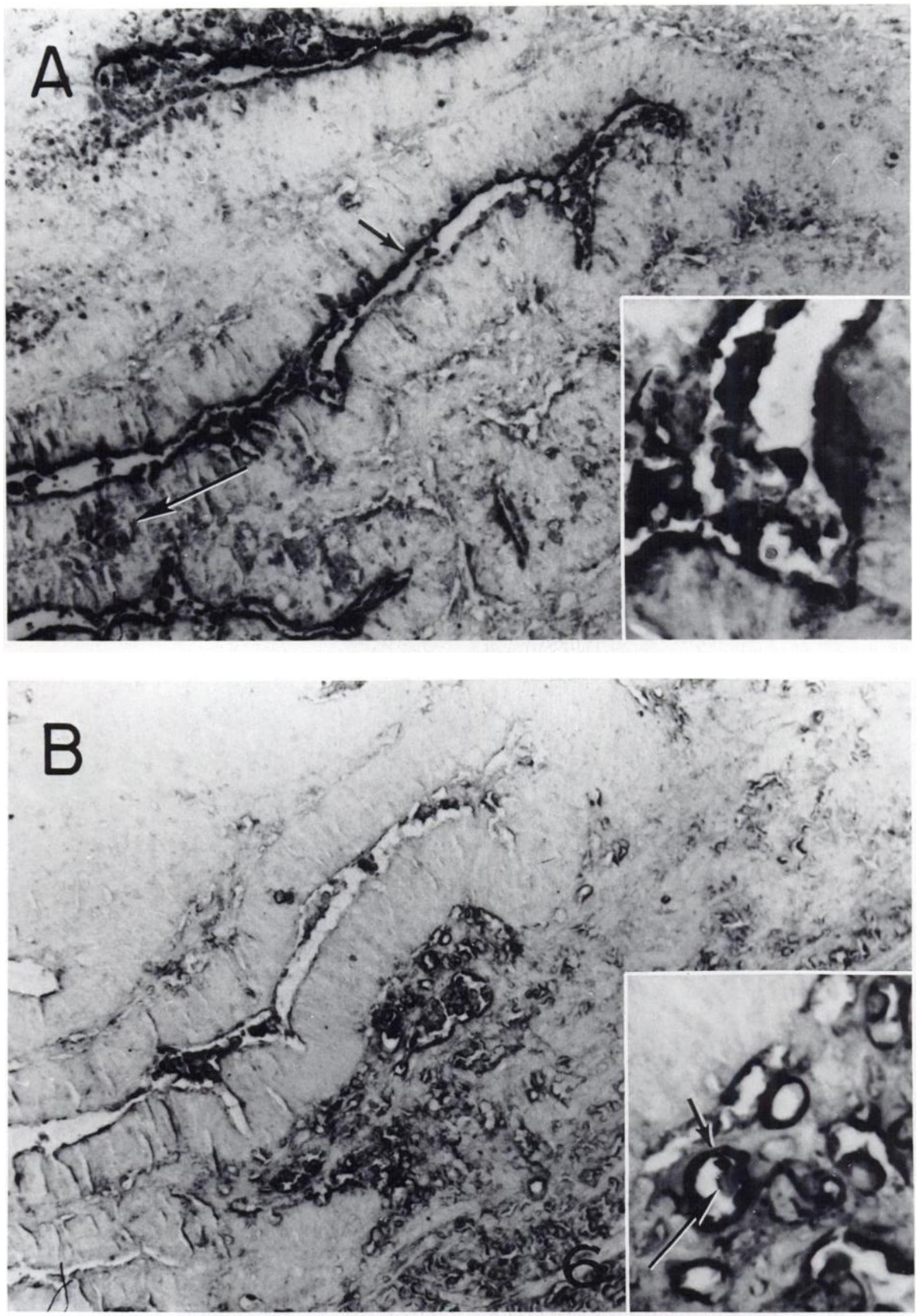

Fig. 6. Contrasting localization of urokinase and tissue activator in colon cancer. *A*, cancer gland stained for urokinase. Staining is observed in the luminal edge of the cancer cells (*short arrow*), and in the cytoplasm (*long arrow*). × 430. *Inset*, detail of the luminal area with strongly reacting desquamated cells in the lumen. PAP method. × 1720. *B*, the same area stained for tissue activator. Heaviest staining is in the strongly vascularized stroma below the cancer gland. Strong staining of desquamated cells can also be seen. × 430. *Inset* shows that most of the staining is in the endothelial layer of the blood vessels (*short arrow*). *Long arrow*, erythrocyte within a vessel. PAP method. × 120.