

## Immunocytochemical Localization of Urokinase-type Plasminogen Activator in Lewis Lung Carcinoma

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**ABSTRACT** The invasively growing and metastasizing Lewis lung carcinoma consistently contained urokinase-type plasminogen activator (u-PA) enzyme activity. When investigated immunocytochemically with antibodies against u-PA, different parts of individual tumors showed a pronounced heterogeneity in staining intensity. Strong staining was found in areas with invasive growth and degradation of surrounding normal tissue, while other areas were completely devoid of staining. Immunoreactivity occurred both with a perinuclear cytoplasmic localization in tumor cells and associated with apparently extracellular material. SDS PAGE of tumor extracts, under both reducing and nonreducing conditions, followed by immunoblotting, showed only one immunocytochemically stainable band with an electrophoretic mobility corresponding to that of purified proenzyme to u-PA, while no two-chain u-PA was detected. This indicates that the major part of the activator in Lewis lung carcinoma is present as one-chain pro-u-PA.

Plasminogen activators (PA)<sup>1</sup> are serine proteases that can convert the zymogen plasminogen into the active proteolytic enzyme plasmin. Plasmin, which is also a serine protease, can in turn degrade most proteins. Plasmin plays a role in a variety of normal and pathological processes, in which tissue degradation and thrombolysis have been the most extensively studied, and mobilization of plasmin by cellular release of PA may be a general mechanism for producing localized extracellular proteolysis (1). Recent findings have demonstrated that PA are secreted from cells in culture as inactive proenzymes (2–5). The overall process leading to the formation of plasmin is thus a cascade reaction that can be influenced by regulatory factors and leads to a large amplification of the proteolytic activity.

Two types of PA can be distinguished, based on molecular weight ( $M_r$ ) and immunological reactivity (6–17), immunohistochemical distribution (18, 19), and the amino acid sequences of the proteins and the nucleotide sequences of the corresponding cDNA (20–22). One type, with  $M_r$  of approximately 70 Kdaltons (tissue-type PA, t-PA), is assumed to play a role in plasminogen activation leading to thrombolysis (18, 23–26). Another type, with  $M_r$  of ~50 Kdalton (urokinase-type PA, u-PA), is believed, among other functions, to play a role in certain normal and pathological processes that

involve tissue degradation, such as implantation of the fertilized egg into the uterus (27, 28), postlactational involution of mammary glands (29), inflammation (1, 30), and cancer (1, 10, 31–40). We have reported the purification of murine u-PA (41), the development of rabbit antibodies against the purified enzyme (13), and the use of these antibodies for an immunocytochemical mapping of the distribution of u-PA in the normal mouse (19). We now report their use for the immunocytochemical detection of u-PA in the invasively-growing, transplanted Lewis lung carcinoma.

### MATERIALS AND METHODS

The following materials were obtained from the indicated sources: Lewis lung carcinoma (as a gift from Mammalian Genetics and Animal Production Section, National Cancer Institute, Bethesda, MD, Urokinase (Leo Pharmaceuticals, Ballerup, Denmark); cyanogen-bromide-activated Sepharose (Pharmacia, Uppsala, Sweden); swine IgG anti-rabbit immunoglobulins and rabbit antipeoxidase-peroxidase (Dakopatts, Copenhagen, Denmark); Millipore nitrocellulose paper GSWP 000 10 (Millipore, Molsheim, France). All other materials were those described previously (2, 3, 10, 13, 16, 19, 41, 42), or of the best commercially-available grade.

**Tumors:** Lewis lung carcinoma (43) was stored frozen in liquid nitrogen or propagated in female C57/B1 mice at the age of 8–10 wk by subcutaneous inoculation of ~0.5-mm fragments (44). After ~2 wk, tumors were either retransplanted or used for experiments. In the latter case, mice were anesthetized with diethylether and perfusion-fixed with cold (4°C) 0.01 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl (PBS), followed by a cold (4°C) 4% (wt/vol) paraformaldehyde solution in 0.1 M sodium phosphate buffer, pH 7.4, as described (19). Tissue used for enzyme assay and electroblotting was removed

<sup>1</sup> Abbreviations used in this paper: PA, plasminogen activator; u-PA, urokinase-type PA; t-PA, tissue-type PA.

from animals perfused with PBS alone. The tumors used in this study were in their 11–36th transplantation generation.

**Tissue Treatments:** Specimens from animals perfused with paraformaldehyde were, as described (19), cut into 1–2-mm cubes and postfixed overnight (typically, 16 h) at 4°C. This was followed by a 24 h rinsing in 0.1 M sodium phosphate buffer, pH 7.3, containing 20% sucrose. The tissue cubes were then frozen in melting Freon-22, sectioned at 4–8  $\mu\text{m}$  on a cryostat at  $-20^\circ\text{C}$ , and mounted on chromealungelatin-coated slides.

Extracts from frozen samples of freshly PBS-perfused tumor tissue were prepared, as described (35), with 0.1 M Tris/HCl, pH 8.1, containing 0.5% (wt/vol) Triton X-100, 10  $\mu\text{l}/\text{mg}$  wet tissue weight. The samples were homogenized and centrifuged (4,000 g) at 4°C for 10 min.

**Antibodies:** Antibodies against highly-purified murine u-PA were produced by immunization of rabbits, as described (13). The IgG fraction was purified by sodium sulphate precipitation and DEAE-Sephadex ion exchange chromatography (45). The IgG was, as described (19), absorbed against a glutaraldehyde polymer of murine proteins (46). The glutaraldehyde polymer was prepared from a mixture of (a) conditioned medium from murine sarcoma virus-transformed plasminogen activator-producing 3T3 cells (3T3/MSV-10), depleted of plasminogen activator by three passages through a 4-aminobenzamide aminododecyl cellulose column (41), (b) conditioned medium from murine embryonal fibroblasts (10), and (c) normal mouse serum. All three parts were further depleted of u-PA by being passed through a column of rabbit antimurine u-PA IgG coupled to Sepharose 4B. The glutaraldehyde polymer was prepared from 30-ml protein solution in 0.2 M sodium acetate buffer, pH 5.0, containing BSA (25 mg/ml), by slowly adding 6 ml of 2.5% glutaraldehyde (wt/vol). The IgG preparations were applied to and eluted from the polymer in PBS at 4°C (46).

Preimmune and nonimmune IgG were purified and absorbed in the same way. The purified IgG preparations were screened for their ability to stain spots of murine pro-u-PA and u-PA (3) with an immunocytochemical model system (47), in which spots of the activator preparations were applied to Whatman No. 1 paper.

Affinity purified antimurine u-PA IgG was prepared by chromatography on a column containing purified murine u-PA coupled to Sepharose, as described previously (19).

**Immunocytochemistry:** Cryostat sections or paper models were soaked, as previously described (19), in 0.05 M Tris HCl, pH 7.4, with 0.15 M NaCl and 1% (wt/vol) Triton X-100 (TBS-Triton) for 15 min, exposed to 1% (wt/vol) human serum albumin or 10% normal goat serum in TBS-Triton for 30 min at room temperature, briefly rinsed in TBS-Triton and exposed to varying concentrations of antibodies purified as described above. Optimal IgG concentrations for staining were found to be 5  $\mu\text{g}/\text{ml}$  (dilution performed in TBS with 0.25% BSA) using 20 h of incubation at 4°C followed by 2 h of re-equilibration at room temperature. The site of antigen-antibody reaction was revealed by the antiperoxidase-peroxidase method of Sternberger, as described (42, 48). Peroxidase activity was demonstrated by the diaminobenzidine- $\text{H}_2\text{O}_2$  method (48) and sections were counterstained lightly with haematoxylin.

In some experiments, endogenous peroxidase activity of mouse tumor tissue was quenched by exposure to methanol: $\text{H}_2\text{O}_2$  prior to immunocytochemical staining (42). This treatment somewhat compromised, but did not totally inhibit, immunocytochemical staining, and was therefore not used routinely. Whenever employed, adjacent sections not exposed to the methanol: $\text{H}_2\text{O}_2$  reagent were analyzed in parallel.

Controls were those recommended by Sternberger (48) and included (a) omission of either the first, second, or third layer of antiserum; (b) substitution of the primary antibodies by preimmune or nonimmune IgG (at concentrations of 5–50  $\mu\text{g}/\text{ml}$ ), or by control hyperimmune sera (antigastrin serum 4652, antisomatostatin serum R213/3); (c) absorption of the primary antibodies against varying concentrations (4.2–63 KU/ml), of highly-purified murine pro-u-PA (3, 41). The pro-u-PA used for antibody absorption showed one Coomassie Blue stainable band corresponding to  $M_r$  48 Kdaltons after SDS PAGE under reducing as well as nonreducing conditions (3) (detection limit for possible contaminating proteins  $\sim 5\%$ ).

**Plasminogen Activator Assay:** The PA content in tissue extracts was determined by a modification of the  $^{125}\text{I}$ -labeled fibrin plate assay, in which catalytic amounts of plasmin were added to allow for the simultaneous determination of pro-u-PA and u-PA (3). Each assay well contained 0.5 ml of 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100, 0.25% gelatin, 1  $\mu\text{g}$  human glu-plasminogen and 7 ng human plasmin. In parallel assays, 0.25  $\mu\text{g}$  affinity purified antimurine u-PA IgG (13) was added. This completely inhibited the enzymatic activity of the purified pro-u-PA and u-PA, while it did not inhibit murine t-PA (13). Pro-u-PA/u-PA in the samples was calculated as that part of the activity that was inhibited by the antimurine u-PA IgG. In the assay, a highly-purified murine pro-u-PA standard preparation (3) (0.5–2 U/ml; see reference 10 for definition of 1 U murine u-PA) was used for calibration. The

same preparation was used as an internal standard to check for the possible inhibition of the assay by inhibitors of plasmin and/or PA (3). In some of the extracts, a strong inhibition of the internal standard was observed under these conditions. In these cases, the assay was modified, as described (19), so that the incubation period was 20 h. Under these conditions the inhibition of the internal pro-u-PA standard was  $<15\%$  in all cases.

**Electrophoresis and Immunoblotting:** SDS PAGE was performed in a stacking system using slab gels with a gradient of 6–16% (wt/vol) polyacrylamide (10) and a potential difference of 60 V, for 16–18 h. In some cases, samples were reduced immediately before application to the gel, by being boiled for 2 min in a buffer containing 5% 2-mercaptoethanol. Following SDS PAGE, proteins were transferred electrophoretically (10 V, 250 mM, 20 h) from the polyacrylamide gel onto nitrocellulose paper using a modification (19) of the procedure of Burnette (49) and stained either with 0.5% amido black in 45% methanol, 10% acetic acid or by the peroxidase-antiperoxidase method, as described (19).

In each gel, the following mixture of marker proteins was electrophoresed and transferred by electroblotting: rabbit phosphorylase ( $M_r$  94 Kdaltons), BSA ( $M_r$  67 Kdaltons), ovalbumin ( $M_r$  43 Kdaltons), carbonic anhydrase ( $M_r$  30 Kdaltons), soybean trypsin inhibitor ( $M_r$  20.1 Kdaltons) and  $\alpha$ -lactalbumin ( $M_r$  14.4 Kdaltons).

## RESULTS

Extracts of nine Lewis lung tumors (primary tumors as well as lung metastases), from different passages during a period of more than one year, were all found to contain PA activity, as measured by the  $^{125}\text{I}$ -fibrin plate method using plasmin-enriched plasminogen (this allows for the simultaneous determination of u-PA and its proenzyme pro-u-PA, see reference 3). Extracts from a variety of murine tissues contain both u-PA and t-PA (19, 41, and unpublished observations). Rabbit antibodies directed against murine u-PA completely inhibited its enzyme activity, without inhibiting t-PA (13). Addition of rabbit antimurine u-PA in the assay, therefore, enabled us to determine the proportion of enzyme activity due to u-PA. This was consistently  $>90\%$  in all tumor extracts assayed, and the pro-u-PA/u-PA content in the extracts was found to vary between 2 and 64 U/mg wet weight of tissue (mean 25 U/mg, SD 21 U/mg). The finding that u-PA consistently constituted the majority of the plasminogen activator activity in the Lewis lung carcinoma extracts was further substantiated by results of zymography with overlaying of SDS polyacrylamide gels on plasminogen-containing agarose gels (50). In most cases, only PA with an electrophoretic mobility corresponding to murine u-PA ( $M_r$   $\sim 48$  Kdaltons) was detected (13). Occasionally, a small amount of PA with an electrophoretic mobility corresponding to murine t-PA ( $M_r$   $\sim 75$  Kdalton, see reference 13) also was found (results not shown).

### *Immunohistochemical Localization of u-PA*

Lewis lung carcinomas consistently contained u-PA immunoreactivity, as detected with the peroxidase antiperoxidase technique (48). There was a pronounced heterogeneity in the staining of different parts of individual tumors: some parts stained strongly and other parts were devoid of immunoreactivity (Fig. 1 a). This was the case for both primary tumors and lung metastases. Typically, the most intense immunoreactivity was observed in areas of the tumors that were adjacent to the surrounding tissue, and where, from histological criteria, the growth appeared to be invasive with an active degradation of normal tissue, e.g., of muscle tissue, as shown in Fig. 1 b. Some of the staining apparently represented extracellular material, while other parts seemed to represent intracellular material, often with a perinuclear localization (Fig. 1 c). Without the aid of electron microscopy, it was not

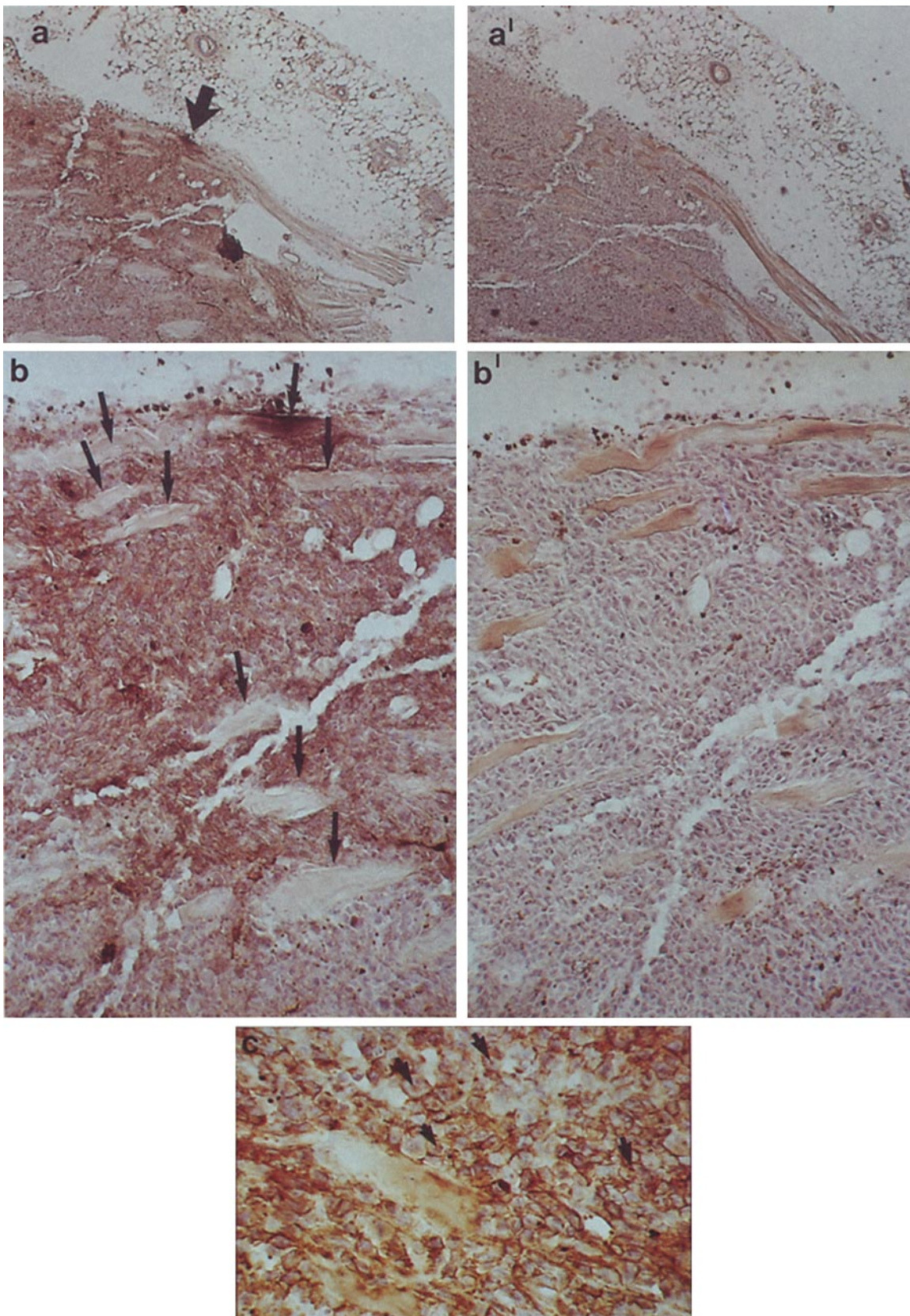


FIGURE 1 Distribution of u-PA immunoreactivity in Lewis lung tumor metastasis invading the chest wall. Antiperoxidase-peroxidase staining with 5  $\mu\text{g}/\text{ml}$  of rabbit anti-murine u-PA IgG (a). Adjacent section stained with the same concentration of the antibody preabsorbed with 21 KU/ml of a purified u-PA preparation (a'). b, b' as a, a', respectively; c as a. Note in a the pronounced staining heterogeneity: most staining occurring in the part of the tumor invading the chest wall (arrow). b is a close up view of this area. Muscle fiber remnants are seen (arrows). c is a higher magnification of tumor cells surrounding a muscle remnant. Most of the stain is located extracellularly or associated with the cell membranes (see text) but in scattered cells perinuclear staining also occurs (arrows). Endogenous peroxidase activity of granulocytes and pseudoperoxidase activity of erythrocytes is seen in a, a' and b, b'. This activity was quenched if sections were treated with methanol:H<sub>2</sub>O<sub>2</sub> before immunocytochemical staining.  $\times 35$  (a);  $\times 140$  (b);  $\times 350$  (c).



possible to determine exactly how much of the immunoreactivity was located extracellularly, or whether some of the apparently extracellular material was attached to the cell membrane.

Staining controls, including the use of up to 50  $\mu\text{g}/\text{ml}$  nonimmune or preimmune IgG and unrelated hyperimmune sera, were negative, as were experiments in which the various antibody layers were deleted. Absorption of the anti-u-PA antibodies with highly-purified pro-u-PA or u-PA completely abolished the staining reported above (Fig. 1 *a'*, *b'*). In all specimens, erythrocytes and granulocytes were the only cell types displaying significant endogenous peroxidase activity.

### Immunoblotting Analysis

To further investigate the character of the stained material, extracts of Lewis lung carcinoma tissue, purified murine pro-u-PA, and purified murine u-PA were separated by SDS PAGE under reducing as well as nonreducing conditions, and the proteins electrophoretically transferred to nitrocellulose sheets. Immunocytochemical staining of the nitrocellulose replicas of the Lewis lung carcinoma extracts, separated under nonreducing conditions, showed only one stained band with an apparent  $M_r$  of 48 Kdaltons that was identical to that of purified murine pro-u-PA (Fig. 2). We have previously demonstrated (3) that the murine pro-u-PA consists of a single polypeptide chain, while the active form, u-PA, consists of two polypeptide chains with apparent  $M_r$  of 29 Kdaltons and 19 Kdaltons, respectively, held together by one or more disulphide bridges. As shown in Fig. 3, purified pro-u-PA and u-PA could still be detected immunocytochemically on nitrocellulose replicas after electrophoresis under reducing conditions. Pro-u-PA showed one 48-Kdalton band (lane *b*) and active u-PA showed a 29-Kdalton band (lane *c*), but the 19-Kdalton band was not detected under these conditions. When the Lewis lung carcinoma extracts were separated by SDS PAGE under reducing conditions, immunocytochemical staining of the nitrocellulose replicas showed only one band with an electrophoretic mobility that was indistinguishable from that of murine pro-u-PA electrophoresed in parallel (Fig. 3). These results demonstrate that the major part (>80%) of the murine urokinase-type activator in Lewis lung carcinoma is present in the one-chain form and, therefore, presumably as the inactive proenzyme pro-u-PA.

### DISCUSSION

Antibodies developed against a highly purified preparation of murine u-PA were used for the immunocytochemical staining. The staining and absorption controls clearly demonstrate that nonimmunological binding of the purified IgG preparation to tissue components can be excluded. The absorption controls were performed with pro-u-PA and u-PA preparations that were pure, as evaluated by SDS PAGE. This makes it unlikely that the staining is due to contaminating antibodies. As for the possibility of the staining being due to cross reaction with an antigen different from pro-u-PA/u-PA present in the Lewis lung tumor, this appears less likely because of the results obtained in the immunoblotting and zymography experiments. In these experiments, only one band with electrophoretic mobility identical to that of pro-u-PA could be stained on nitrocellulose replicas from SDS polyacrylamide gels, and zymography with corresponding nonreduced gels demonstrates enzymatic activity with the same electrophoretic mobility.

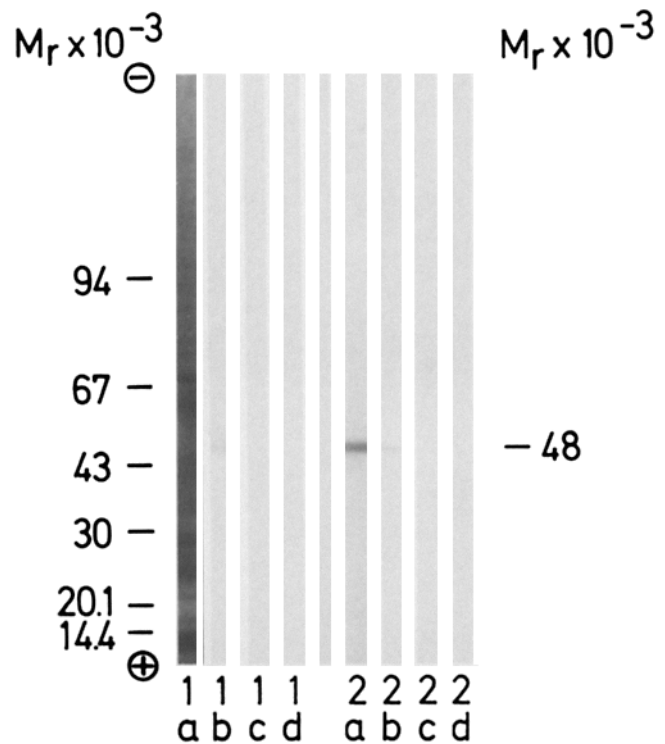


FIGURE 2 Immunoblotting analysis of murine u-PA after SDS polyacrylamide gel electrophoresis under nonreducing conditions of Triton X-100 extracts of Lewis lung tumor (1) and purified preparations of murine pro-u-PA (2). Tumor tissue was extracted with 0.1 M Tris HCl, pH 8.1, containing 0.5% Triton X-100, and 40  $\mu\text{l}$  of extract containing 110 U u-PA activity was applied to each of four lanes (*a-d*). Highly purified murine pro-u-PA was applied to each of four other lanes (42 KU to lane *a* and 105 U to lanes *b-d*). Following SDS PAGE, the proteins were electrophoretically transferred onto nitrocellulose paper. The paper was cut longitudinally and the lanes were either stained with amido black (*a*) or by the PAP method using antimurine u-PA IgG (5  $\mu\text{g}/\text{m}$ ) (*b*), antimurine u-PA IgG preabsorbed with murine pro-u-PA (21 KU/ml) (*c*), or nonimmune IgG (5  $\mu\text{g}/\text{ml}$ ) (*d*).

The specificity of the staining for u-PA is also supported by other findings. First, all of the Lewis lung tumor studied contained less u-PA enzyme activity and fewer immunoreactive cells than superficial mucosa of stomach, while both parameters in the Lewis lung carcinomas varied within the range previously observed in vas deferens, kidney, lung, and placenta in the normal mouse (19). Secondly, the ability of these antibodies to detect u-PA specifically is supported by a previous study of the distribution of u-PA immunoreactivity in the normal mouse, in which we found that, besides u-PA, none of the protein bands transferred to nitrocellulose replicas, were stained, although blotting experiments were performed on extracts of a variety of tissues. In summary, we find that these results provide convincing evidence that the immunoreactivity detected in the Lewis lung carcinoma represents authentic pro-u-PA/u-PA. It should be noted, however, that the results only determine the presence of a certain amount of pro-u-PA or u-PA in cells or extracellular space at the time of fixation, and do not necessarily reflect the amount of production of the activator.

As judged from Fig. 1, a considerable part of the activator seems to be located extracellularly. The localization might be an artifact resulting from the processing of the tissue, but this explanation seems unlikely, however, because the intact anti-

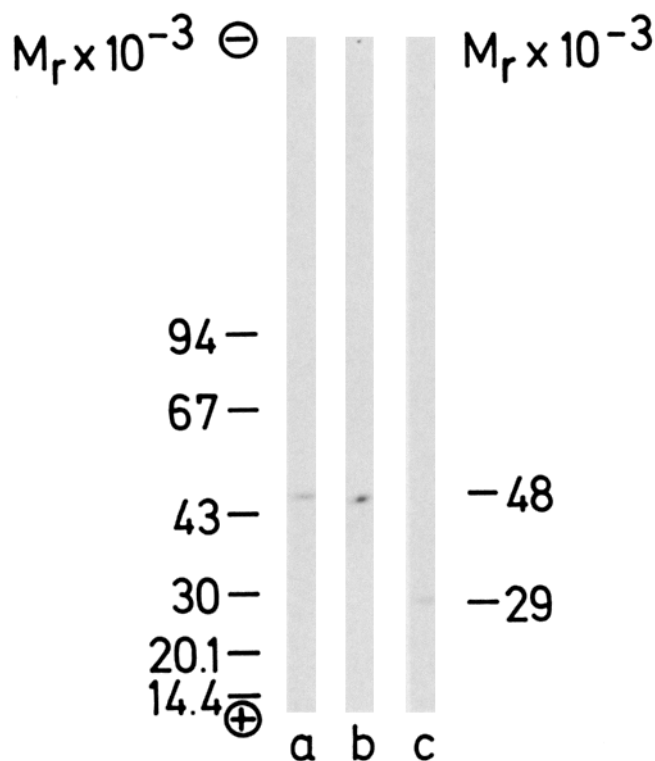


FIGURE 3 Immunoblotting analysis of murine u-PA after SDS PAGE under reducing conditions of Triton X-100 extracts (80  $\mu$ l, 220 U u-PA) of Lewis lung tumor (a) and purified preparations (220 U per lane) of murine pro-u-PA (b) and u-PA (c). Experimental conditions were as described for lanes b in Fig. 2, except that each sample was boiled with 5% 2-mercaptoethanol for 2 min immediately before electrophoresis.

mals were fixed by perfusion with paraformaldehyde before the specimens were excised and postfixed. The apparently extracellular location of a part of the activator is similar to findings in some organs in the normal mouse, e.g., u-PA immunoreactivity apparently was located extracellularly along the basement membrane and the lamina propria in the small and large intestine (19).

The heterogeneity in the cellular content of u-PA within the same tumor, as documented by this study, agrees well with the heterogeneity with respect to a variety of other properties, typical for most malignant neoplasms (51, 52) including Lewis lung carcinoma (53). The heterogeneity found in the Lewis lung carcinoma cells is also in agreement with a similar cellular heterogeneity in u-PA content found in an immunofluorescence study of cultured human glioblastoma cells with monoclonal antibodies against the activator (54).

PA activity has previously been found both in cultured Lewis lung carcinoma cells (55) and in extracts of Lewis lung carcinoma (56). Numerous reports have dealt with the presence of PA in cultured cells derived from malignant neoplasms of both human and animal origin. A large variation in PA activity has been observed among different cultures derived from the same animal tumor strain, e.g., the B16 melanoma (57, 58), and among cell cultures derived from tumors of the same histological type from different patients (59). The present finding of u-PA heterogeneity within individual tumors indicates that selection of different tumor cell populations during establishment of cell cultures may contribute to these apparent discrepancies.

u-PA was found in and around the tumor cells but not in connection with non-neoplastic cells in the tumor, such as inflammatory cells. This finding agrees with recent immunohistochemical studies by Markus et al. (39) and Camiolo et al. (60), who demonstrated u-PA in human colon and prostate carcinoma cells, respectively. In view of the fact that u-PA immunoreactivity in the normal mouse previously was found to be confined to a certain number of well-defined cell types (19), the findings in the present study and those reported for human carcinomas (39, 60) suggest that immunocytochemical demonstration of u-PA may be valuable for the histopathological diagnosis of cancer.

The preferential localization of u-PA in areas of the tumors with invasive growth and tissue degradation agrees with the hypothesis that u-PA in this tumor is involved in these processes. Invasive growth may be a necessary part of the metastatic process, and the present results therefore agree with a recent report by Ossowski and Reich (40), who found that antibodies that can inhibit the enzyme activity of human u-PA also inhibited metastases, but not local growth, of a human tumor transplanted onto the chorion-allantoic membrane of chicken embryos.

As evaluated by immunoblotting, >80% of the activator in the Lewis lung carcinoma extracts was present in the one-chain proenzyme form. Taken together with the apparent extracellular localization of a considerable part of the u-PA immunoreactivity, this points to the activator being released from the tumor cells in the pro-enzyme form. This was previously found to be the case for PA released from virus-transformed murine cells in culture (3) as well as from cultured human cells of neoplastic origin (2, 4, 5). Pro-u-PA can be activated by catalytic amounts of plasmin. However, it appears likely that as yet unknown factors are involved in initiating the process leading to plasminogen activation, by converting extracellular pro-PA to the active form, and that such factors may be decisive for plasmin-mediated extracellular proteolysis.

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