Plasminogen activators (PA) 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 lead 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 Kdaltons (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 antipeptidase-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 electrophotography was removed.

**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 rinsing Fc22-22, soaked at 4°C for 30 min at 4°C, briefly rinsed in TBS-Triton and exposed to 1% glutaraldehyde. The site of antigen-antibody reaction was visualized by exposure to methanol:H2O (42,48). Peroxidase activity was demonstrated by the diaminobenzidine-H2O2 reaction (42,47), in which spots of the activator preparations were applied to Whatman paper. The glutaraldehyde-polymer depleted of plasminogen activator by three passagesthrough a 4-aminobenzamide-vasculitis-transformed plasminogen activator-producing 3T3 cells (3T3/MSV-10), soybean trypsin inhibitor (M, 20.1Kdaltons) and a-lactalbumin (M, 14.4Kdaltons).

RESULTS

Extracts of nine Lewis lung tumors (primary tumors as well as lung metases) from different passages during a period of more than one year, were all found to contain PA activity, as measured by the 125I-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 antimmune 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, ~48 Kdaltons) was detected (13). Occasionally, a small amount of PA with an electrophoretic mobility corresponding to murine t-PA (M, ~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 metases. 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
Distribution of u-PA immunoreactivity in Lewis lung tumor metastasis invading the chest wall. Antiperoxidase-peroxidase staining with 5 μg/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). Endogeneous 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:H2O2 before immunocytochemical staining. × 35 (a); × 140 (b); × 350 (c).
as the inactive proenzyme pro-u-PA.

is present in the one-chain form and, therefore, presumably the murine urokinase-type activator in Lewis lung carcinoma. These results demonstrate that the major part (>80%) of the activator is present in the Lewis lung carcinoma extracts, separated under nonreducing conditions, showed only one stained band with an apparent Mₐ 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 Mₐ 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 carcino 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 preparations 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.

**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 Mₐ of 48 Kdaltons that was identical to that of purified murine pro-u-PA. 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 Mₐ 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.

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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 Camilo 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 ofu-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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