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Prevention of metastasis by inhibition of the urokinase receptor

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ABSTRACT **The plasminogen activator urokinase (u-PA) mediates proteolysis by a variety of human tumor cells. Competitive displacement of u-PA from cellular binding sites results in decreased proteolysis *in vitro*, suggesting that the cell surface is the preferred site for u-PA-mediated protein degradation. We studied the effect of u-PA receptor blockade on the metastatic capacity of human PC3 prostate carcinoma cells, using transfectants which expressed chloramphenicol acetyltransferase (CAT). Eight weeks after subcutaneous inoculation of these cells into nude mice, CAT activity was detected in regional lymph nodes, femurs, lungs, and brain, thereby mimicking the organ tropism observed for naturally occurring metastases of prostate cancer. In a second transfection, CAT-expressing PC3 cells received cDNA encoding a mutant u-PA (Ser^{356} → Ala) which lacks enzymatic activity but which retains full receptor binding affinity. Three mutant u-PA expressors, each with <5% of wild-type cell-associated u-PA activity, were compared *in vivo* with independently derived controls. Primary tumor growth was similar in each group of animals and all tumors expressed comparable CAT activity. In contrast, metastasis (as assessed by CAT activity) was markedly inhibited when cell surface u-PA activity was blocked. Levels of CAT activity were reduced by a factor of >300 in regional lymph nodes, 40–100 in brain tissue, and 10–20 in lung tissue. Metastatic capacity was inhibited similarly when animals were given intermittent intraperitoneal injections of a u-PA/IgG fusion protein capable of displacing u-PA activity from the tumor cell surface. Our results indicate that cell surface u-PA activity is essential to the metastatic process. In addition, the assay system employed in these experiments may be generally useful in testing other therapeutic modalities to limit the spread of primary tumors.**

Metastasis results from a series of interdependent processes including tumor cell proliferation, invasion into normal host tissue, angiogenesis, dissemination via blood or lymph, and ultimately, outgrowth of new tumor masses at distant sites. Many human tumors and cancer cell lines exhibit a high level of expression of the plasminogen activator urokinase (u-PA) (1). *In vitro* studies have implicated u-PA in tumor cell invasion into substrates such as human amniotic membranes and extracts of basement membrane-like material (2, 3). A cDNA which encodes a receptor for u-PA has been cloned (4). Binding of the protease to this receptor is dependent on the noncatalytic N-terminal domain of u-PA (5). Competitive displacement of u-PA from cellular binding sites decreases plasminogen-dependent degradation of basement membrane proteins by tumor cells, suggesting that the cell surface is the preferred site for u-PA-mediated proteolysis (6).

Although the implication from *in vitro* studies is that cell surface-associated u-PA may be important in naturally occurring tumor metastasis, only modest effects of anti-u-PA antibodies have been observed in nude mice inoculated with human tumor cells. The reported effects of anti-u-PA antibodies have included a qualitative decrease in tumor cell invasiveness at the site of the primary tumor without a difference in pulmonary metastases (7). In addition, the number of pulmonary nodules following tail vein injection of B16 melanoma cells was decreased by preincubation of the tumor cells with anti-u-PA antibodies (8).

We developed a sensitive and quantitative nude mouse model system to study the role of u-PA in spontaneous metastasis. Our initial studies focused on the human prostate carcinoma cell line PC3 because histologically proven metastases had been documented in nude mice (9). We expressed the bacterial enzyme chloramphenicol acetyltransferase (CAT) in these cells to provide an easily detectable marker for tumor cells in host tissue (10). In this report, we use the CAT assay to demonstrate that competitive displacement of u-PA from its cellular binding site by catalytically inactive proteins abolishes the metastatic capacity of a human prostate carcinoma cell line. These studies establish that the metastatic process *in vivo* is sensitive to u-PA receptor blockade. The metastasis assay described is generally useful and can also be used to examine other potential antimetastatic therapeutic approaches.

MATERIALS AND METHODS

Vector Construction. All expression vectors used in this study were derived from pRK (11), which contains the human cytomegalovirus promoter and enhancer and the simian virus 40 polyadenylylation signal. The neomycin-resistance plasmid pRKneo was constructed by cloning the neomycin phosphotransferase gene (12) into the *Hin*dIII and *Sma* I sites of pRK. The hygromycin-resistance plasmid pRKhyg contained a cDNA encoding hygromycin B phosphotransferase between the *Hin*dIII and *Bam*HI sites of pRK. The CAT expression vector pRKcat was constructed by inserting the CAT gene (10) into the same position in pRK. The wild-type u-PA expression vector pRKupa contained the entire human u-PA coding domain within a 1.3-kb *Hin*dIII–*Stu* I cDNA fragment. The active-site serine at amino acid 356 was mutated to alanine by the polymerase chain reaction using a mismatched downstream primer as described (6). The resulting plasmid, pRKupa/Ala^{356}, was used to derive mutant u-PA-expressing cell lines.

Cells and Cell Culture. PC3 cells were obtained from the American Type Culture Collection and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium H-16 and Ham's nutrient mixture F12, supplemented with 10% fetal bovine serum. Transfections were performed by the calcium phosphate method (13). Transfectants were selected with

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Abbreviations: CAT, chloramphenicol acetyltransferase; u-PA, plasminogen activator urokinase.

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either the neomycin analogue G418 (400 μg/ml; GIBCO/BRL) or hygromycin B (400 μg/ml; Boehringer Mannheim). CAT1 and CAT2 cells were obtained by cotransfection of PC3 cells with pRKcat and pRKneo, selection in G418, and screening for CAT expression. Subsequently, cotransfection of pRKupa/Ala^{356} with the hygromycin-resistance vector pRKhyg allowed selection of transfected clones which overexpressed mutant u-PA.

Production of Recombinant u-PA/IgG Fusion Protein. The 5′ region of the u-PA cDNA encoding amino acids 1–137 (containing the receptor-binding and kringle domains) was ligated in frame to a cDNA fragment that encoded 227 residues of IgG, including the hinge and Fc domains (14). The chimeric DNA was inserted into a pRK vector and stably transfected into Chinese hamster ovary (CHO) cells for protein expression (15). The fusion protein was purified from conditioned medium by affinity chromatography using protein A-Sepharose (Pharmacia). Bound protein was eluted with acid citrate buffer [50 mM sodium citrate, pH 3.0/20% (wt/vol) glycerol]. Eluates were neutralized promptly with 1/20th volume of Tris·HCl (pH 9.0) and dialyzed against phosphate-buffered saline prior to injection.

CAT Assays. CAT assays were performed by a phase-separation method (16). Briefly, extracts of cells or tissues containing CAT activity were incubated with sodium [^{3}H]acetate (DuPont/NEN) and chloramphenicol in the presence of ATP, coenzyme A, and acetyl-coenzyme A synthetase (EC 6.2.1.1). Acetylated products were isolated by benzene extraction and quantitated with a scintillation counter. Extracts of cells were prepared by three freeze–thaw cycles in 1 ml of 0.25 M Tris·HCl (pH 7.8). Extracts of whole organs or tissues were prepared similarly except that the material was mechanically homogenized prior to freezing. After centrifugation to remove insoluble material, extracts were assayed immediately or stored at −70°C until use.

Cell Surface u-PA Activity Measurement. PC3 transfectants were grown to confluency in 24-well dishes. Confluent cell monolayers were washed, cell surface-associated proteins were eluted, and u-PA activity was measured as described (6, 17). Briefly, elution was performed with acid glycine (pH 3.0). Eluates were then neutralized with 1 M Tris·HCl (pH 8.0). u-PA was collected by immunocapture in 96-well plates coated with a polyclonal goat anti-u-PA antiserum (American Diagnostica, Greenwich, CT). Immobilized u-PA was then incubated with plasminogen. Plasmin activity generated during this incubation was measured with a chromogenic substrate. Purified recombinant u-PA (a gift of Jack Henkin, Abbott) was used as a standard.

***In Vitro* Invasion Assay.** The ability of PC3 transfectants to traverse a basement membrane-like substrate was assayed by using Matrigel (Collaborative Research) essentially as described (3, 18). Briefly, Transwell chambers (Costar) with a filter diameter of 6.5 mm and pore size 8 μm were coated with 60 μg of Matrigel. Cells (5×10^4) were then introduced into the chamber and cultured in serum-containing media for 3 days. Invading cells were then enumerated by using the vital stain 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) as described (3). Some experiments were performed in the presence of aprotinin (200 kallikrein inhibitory units/ml; American Diagnostica). The results were expressed as a percentage of the total cell number present following the 3-day incubation period. Determinations were performed in quadruplicate.

Metastasis Assay. Six-week-old female BALB/c homozygous nude (*nu/nu*) mice were purchased from Life Sciences (St. Petersburg, FL) and were maintained in a specific pathogen-free environment throughout the experiment. Animals were injected with 10^6 CAT$^+$ tumor cells in each thigh and were sacrificed 8 weeks after inoculation. Primary tumors were excised and weighed. Necropsies were performed and various organs and tissues were harvested. These tissues were then homogenized in 1 ml of CAT assay buffer (0.25 M Tris·HCl at pH 7.8). CAT assays were performed on 1/10th volume of the homogenates.

RESULTS

Two clones of CAT$^+$ PC3 cells, CAT1 and CAT2, were obtained by stable transfection. Assays of CAT activity by a phase-separation technique showed that cultured CAT1 and CAT2 cells yielded ≈0.7 cpm per cell. Similar results were obtained when the assay was performed in the presence of a lysate of mouse lung. As few as 250 cells could be detected in organ lysates containing 10^8–10^9 cells, and CAT activity varied linearly with tumor cell number up to at least 10^5 cells (Fig. 1). Cells were then injected subcutaneously into nude mice. Eight weeks after injection, animals were sacrificed and primary tumors, organs, and tissues were recovered for assay of CAT activity. Abundant CAT activity was detected in inguinal lymph nodes, lungs, brains, and femurs (Table 1). The observed pattern of CAT activity mimics the organ tropism observed for naturally occurring metastases of human prostate cancer.

PC3 cells secrete a large amount of u-PA and exhibit saturating amounts of u-PA activity bound to the cell surface (19). Acid elution of cell surface-associated proteins from monolayers of PC3-derived clones CAT1 and CAT2 confirmed the presence of cell surface u-PA activity (Fig. 2). Northern blot analysis using a cDNA probe for the u-PA receptor (6) revealed the presence of the expected 1.5-kb mRNA (data not shown), indicating the likely involvement of the u-PA receptor in the display of cell surface u-PA activity. To examine the role of cell-associated u-PA activity in spontaneous metastasis, we competitively displaced the active protease from the tumor cell surface by stable expression of a u-PA variant capable of u-PA receptor binding, but lacking protease function. The proteolytically inactive protein we expressed (u-PA[Ala^{356}]) was identical to wild-type u-PA except that its active-site serine, Ser^{356}, was changed to alanine.

Subsequent transfections yielded two clones expressing [Ala^{356}]u-PA derived from CAT1 (CAT1-Ala^{356}/1 and CAT1-

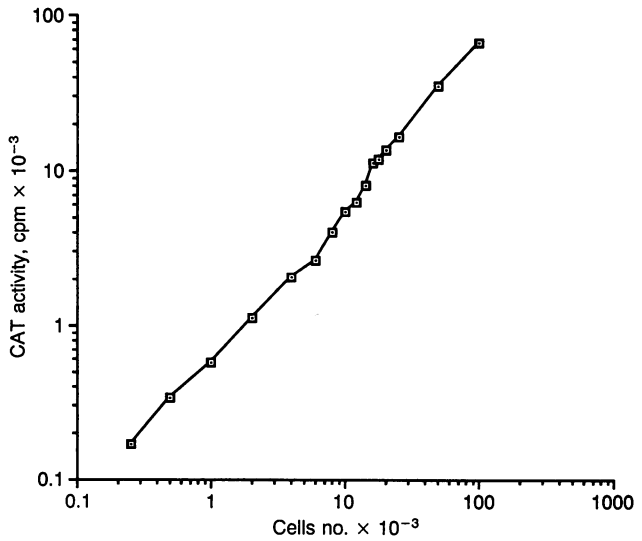

FIG. 1. Detection of CAT-expressing PC3 cells in lung lysates. Cultured CAT1 cells were lysed in CAT assay buffer, and aliquots of the lysate corresponding to between 2.5×10^2 and 1×10^5 cells were mixed with 100 μl of lung extract prepared from a control nude mouse. CAT assays were performed as described in *Materials and Methods*. The data are displayed as a log–log plot.

Table 1. Metastasis of CAT-expressing PC3 cells after subcutaneous inoculation into nude mice

Tissue	CAT activity, cpm	
	CAT1	CAT2
Lung	24,980 ± 8,840	67,800 ± 5,030
Inguinal node	60,240 ± 29,280	46,240 ± 19,440
Brain	36,220 ± 14,060	73,600 ± 46,620
Femur	12,900 ± 5,760	3,900 ± 3,060
Spleen	1,480 ± 320	840 ± 180
Kidney	0 ± 20	80 ± 40
Pancreas	560 ± 60	660 ± 380

Mice were injected with 10^6 CAT$^+$ tumor cells in each thigh. Six animals were injected with CAT1 cells and six with CAT2 cells. Animals were sacrificed 8 weeks after inoculation and extracts were prepared from whole organs or tissues. CAT assays were performed on 1/20th volume of these extracts. Data shown reflect the total activity within a given organ or tissue and are displayed as mean ± SEM. Background values were derived from measurements using extracts prepared from uninjected control animals and were subtracted from the values shown. Under these assay conditions, cultured CAT1 and CAT2 cells generate ≈0.7 cpm/cell.

Ala^{356}/2) and a single clone derived from CAT2 (CAT2-Ala^{356}). Controls derived from CAT1 included two hygromycin-resistant clones (CAT1-Hyg/1 and CAT1-Hyg/2), as well as a pool representing ≈100 individual clones (CAT1-HygPool). With an acid elution technique, all controls showed similar expression of cell surface u-PA activity corresponding to ≈3000 molecules of active protease per cell. The [Ala^{356}]u-PA clones all showed markedly decreased cell surface u-PA activity (<5% residual activity in each case) compared with either the parent cells or the hygromycin-resistant controls (Fig. 2). As expected, competitive displacement of active u-PA from the cell surface resulted in increased u-PA in conditioned medium (29–87% increased compared with either the parent cells or control transfectants).

The effect of competitive displacement of active u-PA on invasive capacity was studied *in vitro* by measuring the ability of tumor cells to traverse a barrier composed of Matrigel, a mixture of proteins having a composition similar to that of basement membrane. Under these conditions we found that invasiveness of [Ala^{356}]u-PA transfectants was reduced by 50–60% compared with the parent cell lines (data not shown). A similar degree of inhibition was observed when CAT1 parent cells were cultured in the presence of the plasmin inhibitor aprotinin. Taken together, these results suggest that invasiveness of PC3 cells *in vitro* depends at least partially on plasmin activity generated by cell surface u-PA.

To test the effect of reducing cell surface-associated u-PA activity on tumor growth and metastasis *in vivo*, CAT$^+$ control cells and transfectants expressing u-PA competitors were injected into nude mice as described above. Primary tumor growth was remarkably consistent. Of 50 animals given bilateral tumor inoculations, 49 developed tumors after 8 weeks. Tumors ranged in size from 0.42 to 1.00 g and did not differ significantly among groups. To test for metastasis from these primary tumors, inguinal lymph nodes, brains, and lungs were harvested and assayed for CAT activity (Fig. 3). All of the lymph nodes harvested from animals that had been injected with the five control cell lines had abundant and relatively consistent CAT activity, varying only about 4-fold from animal to animal. In contrast, all three [Ala^{356}]u-PA-expressing clones exhibited background levels of lymph node CAT activity in each animal tested, indicating that these clones were unable to establish metastatic tumor foci within the regional lymph nodes. Similarly, CAT activity ranged from 40- to 100-fold higher in brains and from 10- to 20-fold higher in lungs from control animals than in organs from animals expressing mutant u-PA.

Clones expressing u-PA competitors could differ from the control cell lines in some unanticipated fashion (e.g., by consistent decreases in u-PA receptor or by increased production of plasminogen-activator inhibitors). To show that competition for the cell surface u-PA binding site was sufficient to inhibit the formation of metastases, we expressed and purified a u-PA competitor protein and tested its effects directly in nude mice. The competitor protein consisted of the u-PA cell-binding domain (amino acids 1–137) at the N terminus and a C-terminal portion derived from the hinge and Fc regions of human IgG (14). The protein competed efficiently with endogenous u-PA for the cell surface receptor on

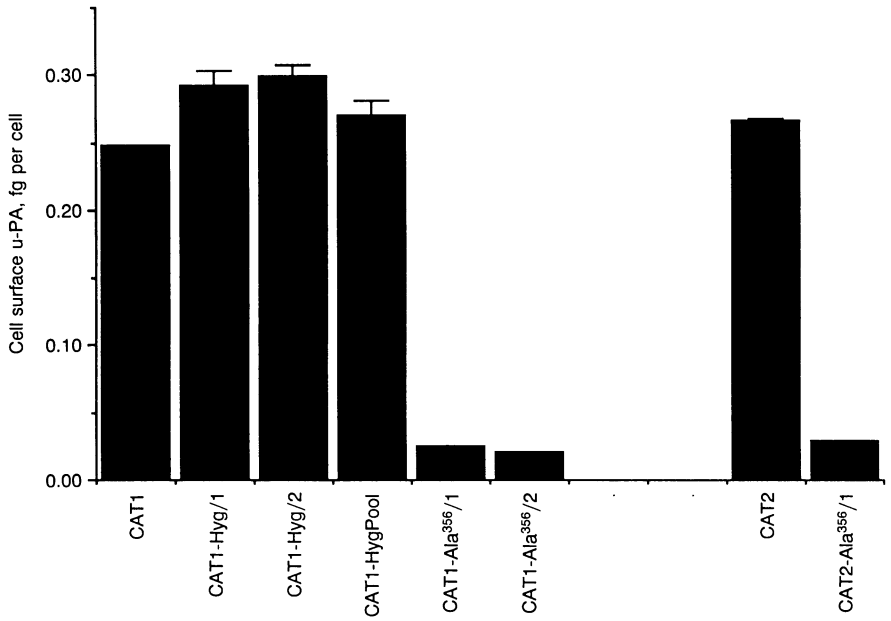

FIG. 2. Cell surface-associated u-PA activity of CAT$^+$ PC3 transfectants expressing inactive mutant u-PA and controls. Samples (from left to right) included CAT1 parent, CAT1-Hyg/1, CAT1-Hyg/2, CAT1-HygPool, CAT1-Ala^{356}/1, CAT1-Ala^{356}/2, CAT2 parent, and CAT2-Ala^{356}. A complete description of these cell lines is given in the text. Purified recombinant u-PA was used as a standard. Values shown represent means ± SD of determinations done in triplicate.

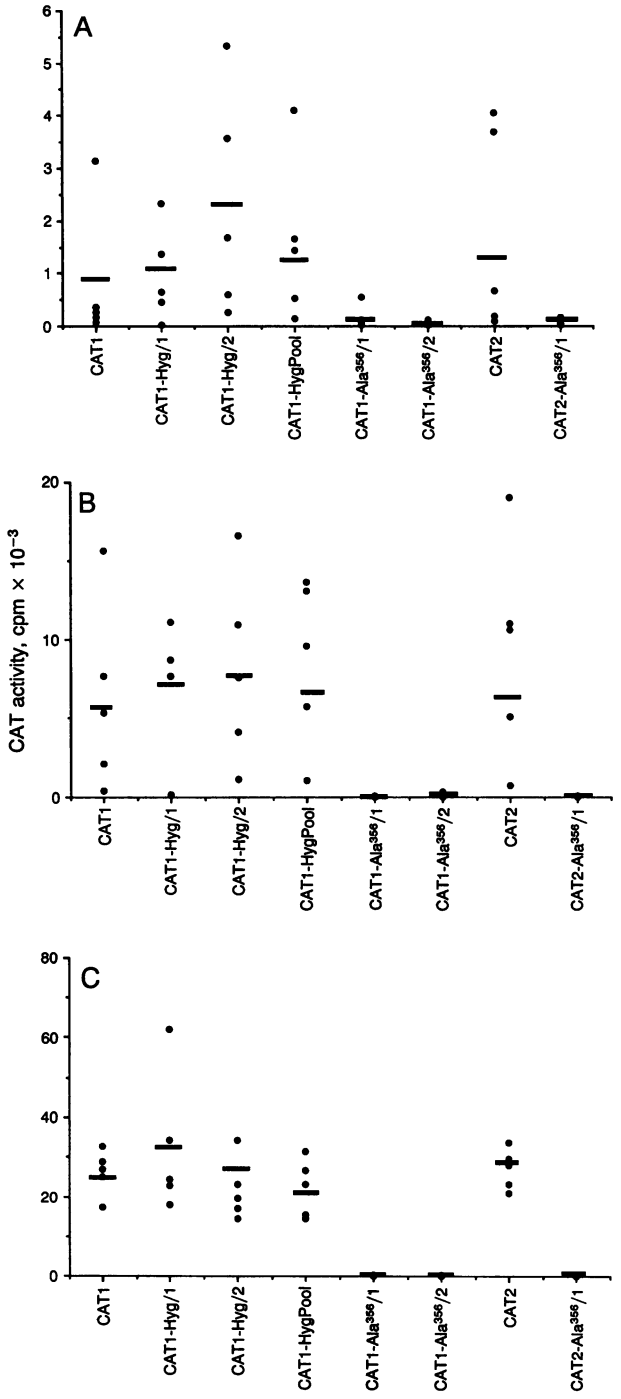

FIG. 3. CAT activity in lungs (*A*), brains (*B*), and lymph nodes (*C*) from nude mice inoculated with PC3 cells expressing inactive mutant u-PA and controls. For each transfectant, a group of five to seven mice was injected in each thigh. Mice were sacrificed and tissues harvested 8 weeks after tumor inoculation. One-tenth volume of each tissue extract was used to measure CAT activity. The construction of control cells (CAT1, CAT1-Hyg/1, CAT1-Hyg/2, CAT1-HygPool, and CAT2) and cells expressing the [Ala^{356}]u-PA competitor (CAT1-Ala^{356}/1, CAT1-Ala^{356}/2, and CAT2-Ala^{356}) is described in the text. Values for individual animals are shown by the data points. The mean for each group is indicated by a horizontal bar. Background values were derived from measurements using organ lysates prepared from uninjected control animals and were subtracted from the values shown.

PC3 cells, with 95% inhibition achieved at a concentration of 200 ng/ml. Pharmacokinetic experiments indicated that plasma levels of 10 μg/ml could be maintained with biweekly

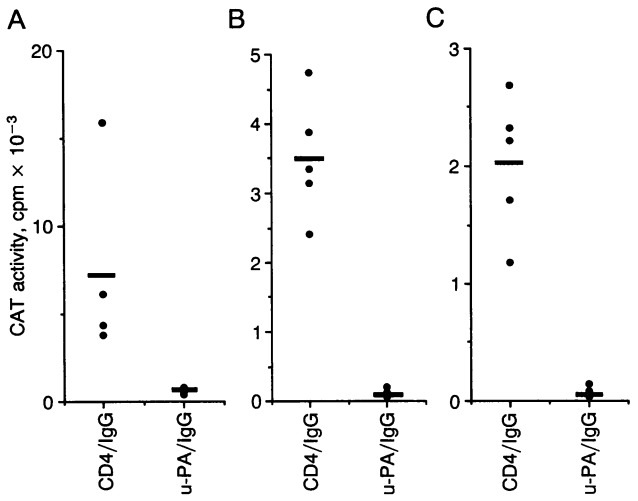

FIG. 4. CAT activity in lungs (*A*), brains (*B*), and lymph nodes (*C*) from nude mice treated with intermittent intraperitoneal doses of u-PA/IgG or control following inoculation of PC3 cells. CAT-expressing cells were injected subcutaneously into each thigh of recipient mice. The tumor inoculum consisted of 10^6 cells and was composed of an equal mixture of CAT1 and CAT2 cells. Animals were given 800 μg of either u-PA/IgG or the CD4/IgG control intraperitoneally at the time of tumor inoculation. Subsequent intraperitoneal injections of 400 μg twice weekly maintained serum levels of the fusion proteins at >10 μg/ml. Eight weeks following tumor cell injection the animals were sacrificed and CAT assays were performed on 1/10th volume of each tissue extract. Values for individual animals are shown. The mean for each group is indicated by a horizontal bar.

injections of 400 μg. Animals were injected subcutaneously with CAT1 cells and received either the u-PA/IgG fusion protein or a control preparation consisting of a CD4/IgG fusion protein (14). The results were similar to those obtained with stable transfectants. Primary tumor growth was consistent and did not vary significantly among groups (data not shown). However, metastatic capacity as assessed by CAT activity present in lymph nodes, brains, and lungs was markedly inhibited in animals injected with the u-PA/IgG fusion protein (Fig. 4).

DISCUSSION

These experiments indicate that cell surface u-PA enzymatic activity is essential for spontaneous metastasis of a human prostate carcinoma cell line in nude mice. Secreted u-PA activity alone is not sufficient, since nonmetastatic clones expressing the inactive u-PA variant secrete greater amounts of active u-PA than metastatic controls or parent cell lines. Thus, our results implicate the cell surface u-PA receptor as a critical component of the metastatic machinery utilized by these human tumor cells *in vivo*. The finding that inhibition of invasion through a basement membrane-like substrate correlates with decreased metastatic capacity suggests that binding of active u-PA to the cell surface receptor may be important *in vivo* for tumor cell invasiveness and/or locomotion. Additional possible effects of tumor cell surface u-PA on cooperative cellular processes such as angiogenesis remain to be explored.

The effects on metastatic capacity seen in the present study are qualitatively similar to those observed by Hearing *et al.* (8). These authors assayed lung colonization following intravenous inoculation of B16 melanoma cells into syngeneic animals. They found that the number of tumor nodules was decreased 40–80% by preincubation of tumor cells with anticatalytic antibodies directed against murine u-PA. It is

interesting that Hearing *et al.* were not able to achieve more than 60–70% inhibition of cell surface u-PA. In the current study, the more dramatic effects observed may be related to more complete inhibition of cell surface proteolytic activity (see Fig. 2). Ossowski *et al.* (7) noted decreased local invasiveness of primary tumors when anti-u-PA antibodies were administered to nude mice injected subcutaneously with HEp3 human epidermoid carcinoma cells, but no difference in the incidence of pulmonary and nodal metastases. Possible explanations for the lack of an observed effect on metastasis in this study include differences in the cell types used as well as differences in the methods by which metastatic capacity was assessed.

The spontaneous metastasis model described in this study provides advantages over approaches which depend on histologic demonstration of metastatic tumor foci. Histologic analysis of regional lymph nodes confirmed the presence of micro-metastases in animals injected with CAT-expressing parent cells, whereas no tumor cells were found in the nodes of animals injected with cells expressing [Ala^{356}]u-PA (data not shown). However, the CAT assay may be a more sensitive means of identifying metastases than histology, since relatively few detectable tumor cells were found in nodes in which the level of CAT activity averaged more than 100× background. Lower levels of tumor infiltration in substantially larger target organs such as the brain or lungs might well be missed by histologic evaluation. In addition to its sensitivity, measurement of metastatic burden by CAT assay is rapid, inexpensive, and quantitative. The major limitation of the procedure is the need to establish stable transfectants of the cell lines to be evaluated. Development of efficient retroviral vectors which yield consistent, high-level expression of the marker protein may simplify the process and expand the range of human tumor cell lines and short-term cultured explants which are amenable to this approach. Use of other marker proteins such as firefly luciferase may result in further improvements in sensitivity.

The experiments described here assessed total metastatic burden and therefore did not permit dissection of the precise steps in the metastatic process which depend on cell surface u-PA. The earlier studies cited above suggest effects both on the primary tumor, resulting in a decreased likelihood of vascular entry (7), and at the ultimate site of metastatic seeding (8). Use of the CAT expression strategy and the u-PA/IgG fusion protein will allow additional experiments to address these issues directly. For example, the hypothesis that cell surface u-PA is important for tumor cell entry into the vasculature can be evaluated by using the CAT assay to measure the number of tumor cells present in the blood of animals bearing primary tumors in the presence or absence of the competitor protein. Similarly, the role of cell surface-associated u-PA at sites of metastases can be addressed by measuring the kinetics of tumor cell accumulation in various organs following intravenous administration.

Metastasis of human tumor cells in nude mice has been observed only rarely (20). The CAT expression assay will permit reevaluation of this issue. We anticipate that a variety of human tumor cell lines will be found to have measurable metastatic capability by this analysis. Preparation of a panel of metastasizing human cell lines would expand research into the basic mechanisms underlying the metastatic process and provide models to test the efficacy of potential therapeutic agents *in vivo*. Since currently available therapies are largely ineffective for the treatment of metastatic disease, new approaches are clearly needed. On the basis of our initial results we suggest that blockade of the u-PA receptor may hold therapeutic promise. A more complete description of the tumor types in which metastatic capacity depends on cell surface u-PA activity, together with a precise delineation of the u-PA-dependent events in the metastatic process, will be important in identifying the subgroups of patients that might benefit from inhibitor-based therapies.

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