Inhibition of Prostate Cancer Neovascularization and Growth by Urokinase-Plasminogen Activator Receptor Blockade

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ABSTRACT

Binding of the serine protease urokinase (u-PA) to its receptor on tumor cell surfaces facilitates proteolysis and tumor invasion. We undertook this study to determine whether the role of u-PA in prostate cancer induced angiogenesis and secondary tumor growth by developing a homologous, immunocompetent in vivo model in which the tumors cells secrete an inhibitor of the murine u-PA receptor. A mutant recombinant murine u-PA that retains receptor binding but not proteolytic activity was made by PCR mutagenesis. Mutant u-PA and a reporter gene prK luciferase were transfected and stably expressed in the highly metastatic rat Dunning MAT-LyLu prostate cancer cell line. Several clones expressing mutant u-PA and luciferase were identified by Western blotting, plasminogen zymography, and reverse transcription-PCR. One of these clones, 5C4, was injected s.c. into Copenhagen rats. Compared to animals injected with clones expressing prK luciferase alone, tumors in animals injected with 5C4 cells were significantly smaller. Moreover, there were fewer lung micrometastases in the 5C4 animals. Primary tumor angiogenesis was measured by microvessel quantification of tissue stained with antibodies against von Willebrand factor. Mean microvessel density in 5C4 tumors was 4.3-fold lower than that in animals with tumors derived from the control tumor cell line (P < 0.0001). Significant inhibition of tumor growth was also observed for two additional MAT-LyLu cell lines expressing mutant u-PA. These findings suggest that cell surface u-PA contributes to prostate cancer growth by enhancing angiogenesis.

INTRODUCTION

Tumor cell invasion and metastasis are multifactorial processes that include adherence to the basement membrane, secretion of proteolytic enzymes, and cell migration into vessels and lymphatics followed by extravasation at distant sites (1). Protease expression by the tumor and/or stromal cells is likely to be required for tumor cell invasion into the surrounding matrix. Protease expression is also likely to contribute to tumor-induced neovascularization that is required for primary tumor growth (2, 3). u-PA has been shown to influence the metastatic behavior of several tumor cell types (4, 5) including prostatic cancer (6–8). Moreover, its expression is stimulated by angiogenic factors that contribute to endothelial cell tubular morphogenesis (9, 10). u-PA cleaves plasminogen to plasmin, a broad-spectrum protease with trypsin-like activity that degrades the extracellular matrix and activates procollagenase. The generation of plasmin is most pronounced when u-PA is bound to its receptor, u-PAR (11). Plasmin bound to tumor cells has been shown to significantly increase the degradation of basement membranes (12).

u-PA is secreted from tumor cells as a single-chain proenzyme that is cleaved to its two-chain active form by serine and cysteine proteases (13). Both the single and two-chain forms can bind to u-PAR. The two-chain form consists of an A (amino acids 1–158) and B (amino acids 159–411) polypeptide chain linked by disulfide bonds. The amino-terminal domain of the A chain mediates binding to u-PAR (14). u-PA proteolytic ability resides in the B chain active site, which contains an essential serine residue (15).

Several interventional strategies have attempted to inhibit tumor invasiveness by blocking u-PA-mediated generation of plasmin on tumor cell surfaces. These include use of antibodies against u-PA (16, 17), competitive inhibition of the receptor with synthetic agents (18, 19), and models using overexpression of the natural u-PA inhibitors PAI-1 and PAI-2 to decrease matrix degradation (11, 20). Tumor cell overexpression of recombinant mutant inactive u-PA or mutant u-PA has been reported to interfere with the generation of cell surface plasmin (21, 22).

Tumor-associated angiogenesis in pathological specimens from patients with prostate cancer has been shown by immunohistochemistry and microvessel quantification to differentiate between localized and metastatic disease (23, 24). The administration of angiogenesis-inhibiting agents in vitro and in vivo models of prostate cancer has been shown to decrease tumor growth (25, 26). The effects of these angiogenesis antagonists on u-PA and plasminogen are unclear and may vary (27). It has been demonstrated that some agonists of angiogenesis modulate u-PA expression by endothelial cell production of bFGF (9, 10). We sought to determine whether the blockade of the urokinase receptor has an inhibitory effect on prostate cancer-induced angiogenesis and the associated tumor growth.

We developed an animal model of prostate cancer inhibition using the highly metastatic and androgen-insensitive Dunning MAT-LyLu cells. In this immunocompetent host, we stably expressed a mutant, recombinant, inactive form of u-PA and the reporter gene luciferase in the tumor cells to determine the role of cell surface u-PA in tumor growth.

MATERIALS AND METHODS

Cell Lines and Urokinase Characterization. Dunning R-3327 rat prostatic adenocarcinoma cell subtype MAT-LyLu was the generous gift of Dr. John Isaacs (The Johns Hopkins University, Baltimore, MD). The MAT-LyLu cell line is anaplastic, androgen-insensitive, tumorigenic, and highly metastatic. The cell culture was maintained in a standard medium consisting of RPMI 1640 with 10% fetal bovine serum (from our institution’s cell culture facility), 25 mM HEPES buffer, penicillin (100 units/ml), streptomycin (100 mg/ml), and dexamethasone (250 nm) in 5% CO2 at 37°C.

u-PA was quantified by an immunocapture assay (12). Secreted u-PA was measured in serum-free CM. When they reached 50% confluence, cell monolayers were washed twice with PBS, and CM was generated by incubating these cells in serum-free standard medium with G418 for 48 h. The CM was concentrated 20-fold with an Amicon concentrator (Beverly, MA) that retained compounds of molecular mass above 10 kDa. Cell surface-bound u-PA was measured after cell surface elution from u-PAR as described previously (12). u-PAR was measured by washing cell monolayers twice with PBS containing 0.2% BSA and then adding 100 nmol/liter mu-PA (a gift from Dr. Stephen
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Rosenberg (The Chiron Corp., Emeryville, CA). After a 1-h incubation at room temperature, unbound mu-PA was removed by two washings with PBS. mu-PA bound to cell surface u-PA was acid-eluted, neutralized as described above, and assayed (12).

Vector Construction, Transfection, and Clone Selection. The expression vector pCEP4 (Invitrogen, San Diego, CA) contains a cytomegalovirus promoter, SV40 polyadenylation tail, and the hygromycin resistance plasmid for mammalian selection. The mu-PA cDNA used for mutant mu-PA construction was obtained from Belin et al. (Ref. 28; University of Geneva, Geneva, Switzerland). The amino acid serine at position 358 in the active site of the 1.3-kb u-PA coding domain was changed to alanine by PCR mutagenesis as described previously (12).

The resulting pCEP plasmid containing mutant mouse u-PA/Ala-358 (pCEPMmu/A358) was linearized with the restriction endonuclease ClaI and transfected into MAT-LyLu cells by electroporation. Transfected cells were grown in medium containing G418 (500 µg/ml), and resistant clones were selected and grown in G418 for an additional 20 days. Clones were screened for mutant mu-PA expression by a competitive immunocapture assay. The assay was performed as described previously (12), except that CM collected from monoclonal populations was equalized for protein concentration, combined with 20 µg/ml wild-type mu-PA, and then added to each well. Clones expressing mutant mu-PA competitively inhibited binding of the anti-mu-PA antibody to wild-type u-PA. Therefore, medium from the highest expression of mutant u-PA yielded the weakest chromogenic signal. Clones expressing the most mutant u-PA by the competitive immunocapture assay were further tested by Western blotting and plasminogen zymography.

Immunoblotting was performed to evaluate soluble u-PA expression. After protein equalization, prestained molecular weight markers and CM samples were electrophoresed in SDS-polyacrylamide gels as described previously (29). Electrophoresis was performed under conditions of constant amperage, and samples were then transferred to nitrocellulose membranes as described previously (30). Membranes were blocked and then washed and exposed to rabbit anti-mu-PA IgG (American Diagnostica, Greenwich, CT). Horseradish peroxidase-conjugated goat antiserum IgG (Pierce) was added, followed by ECL chemiluminescence (Amersham, Arlington Heights IL) and exposure to Hyperfilm-ECL film (Amersham).

Cell-bound u-PA was assessed for functional activity by plasminogen zymography. Total protein concentrations of glycine eluates were determined for MAT-LyLu cell cultures (43 µg/ml), clone 5C4 (104 µg/ml), and clone 6A1 (273 µg/ml); 20 µg of each were added to Laemmli SDS sample buffer and electrophoresed in a 10% SDS-polyacrylamide gel containing 50 µg/ml Lys-plasminogen (American Diagnostica) and 1 mg/ml casein. After SDS removal by a Triton wash, gels were incubated in Tris-HCl buffer (pH 7.8) for 40 h at 37°C. Gels were stained with Coomassie Blue (0.5% Coomassie Blue, 10% acetic acid, and 30% isopropanol alcohol) and then destained. Substrate degradation was observed by the loss of staining in the region occupied by the electrophoresed u-PA. Gels were fixed and dried, and the signals were imaged in reverse optical polarity with PrismView optics and processed using a Macintosh computer.

Controls consisted of the pCEP4 expression vector transfected alone into MAT-LyLu cells. Two groups of 27 and 36 clones expressing hygromyacin only (hygro-only 1 and 2, respectively) were collected and pooled together. The luciferase vector pPKluciferase was the generous gift of Dr. Robert Cohen (Genentech Corp., South San Francisco, CA). pPKluciferase and the neomycin resistance plasmid pPKneo were cotransfected into the selected mutant mu-PA-expressing clone 5C4 and the two hygromyacin-only controls by electroporation. After selection in 400 µg/ml neomycin-containing medium, clones were assayed for luciferase expression (Promega, Madison, WI).

mRNA Purification and RT-PCR of Mutant u-PA. mRNA was obtained from one flask (75 cm²) at 50% confluence of the hygro-only 1 and clone 5C4 cell lines. The cells were trypsinized, and the cell pellet was collected by centrifugation (300 × g). With the QiAshredder and Oligotex mRNA kit (Qiagen, Chatsworth, CA), cells were lysed and homogenized, and purified mRNA was extracted from the cell pellet according to the vendor’s suggested protocol. mRNA was quantified by measuring the absorbance at 260 and 280 nm. RT-PCR was performed with the first-strand cDNA synthesis kit according to the manufacturer’s protocol (Boehringer Mannheim, Indianapolis, IN). The resulting single-stranded cDNA was amplified in a final volume of 100 µl. Each reaction containing 1× reaction buffer, 2.5 mM MgCl₂, 0.2 mM deoxynucleotide mix, 0.01 µg/ml gelatin, 50 pmol of each mutant mu-PA primer (mutant mu-PA-F, 5'-GATTCCCTGAAGGGCCATG; mutant mu-PA-R, 5'-ATTGAAATCCATCAGGAAGAATG), 2.5 units of Taq polymerase, and 20 µl of template cDNA underwent 28 PCR cycles. Aliquots (10 µl) of the RT-PCR product were electrophoresed in 2% NuSieve and 1% agarose gel in 1× TAE (40 mM Tris acetate/2 mM sodium EDTA/glacial acetic acid, pH 8.8) containing 0.5 µg/ml ethidium bromide.

Immunohistochemistry. Fresh primary tumors were placed in Carnoy’s fixative for 1 h, transferred to 100% ETOH overnight, and then embedded in paraffin. Sections (5 µm) were air-dried overnight, warmed at 65°C for 30 min, and then sequentially placed in xylene, 95% ETOH, 70% ETOH, and 0.2 M HCl and water. After three washes in PBS and 0.1% Tween 20 and 20 min in 0.3% hydrogen peroxide, slides were blocked with goat serum, washed three times in PBS and Tween 20, and incubated with rabbit anti-human von Willebrand factor antisera (DAKO). Slides were washed three times and goat anti-rabbit biotinylated antibody was added for 1 h. The 3,3′-diaminobenzidine substrate kit and Vectastain Elite ABC kit (Vector Laboratories) were used according to the manufacturer’s suggested protocol. Slides were dehydrated and air-dried overnight, and vessels were counted at ×25. Fifty fields from 10 slides (3 animals) were counted for each tumor group, and the means were compared.

Cell-doubling Times and Luciferase Standard Curves. To determine the cell-doubling times of clones SC4 and the hygromyacin-only cell lines, 20,000 cells were plated in multiple 30-mm dishes. Duplicate wells were trypsinized every 2 days, and the cells were quantified on a Coulter counter. The doubling times were calculated by a regression analysis similar to the method described by Issacs et al. (31).

Before in vivo experiments, standard curves for luciferase expression in the SC4 and hygromyacin-only clones were established. Copenhagen rats were sacrificed, and the lungs were harvested. Serial dilutions of trypsinized cells from the SC4 and hygromyacin-only clones were added to lungs that had been quick-frozen in liquid nitrogen and pulverized. These lungs were assayed with the luciferase assay system, and standard curves were established for each cell line.

Animal Experiments. Primary tumor growth and metastasis were studied in imbed adult male Copenhagen rats (Harlan Sprague Dawley, Indianapolis, IN). Animals were anesthetized with pentobarbital, weighed, and injected s.c. in the flank with 50,000 harvested cells. Rats were followed for 14 days and then sacrificed. Primary tumors and lungs were excised and weighed. Specimens were quick-frozen in liquid nitrogen, pulverized, incubated with lysis buffer (Promega), and prepared for fluorescence on a luminometer according to the vendor’s protocol. The number of cells in the primary and metastatic sites was determined according to the standard curves generated for each cell line. A second in vivo study was performed with 4 × 10⁶ cells/200 µl; animals were followed for 18 days, and then primary tumors and lungs were harvested.

Four mutant u-PA-expressing clones (SC4, 4D4, 6A1, and 5C1) and the mock transfected hygromyacin-only clones were screened for in vivo primary tumor growth. A total of 400,000 cells of each was injected s.c. in the flank and harvested at 14 days and weighed. There were four to six rats in each group.

Statistical Analysis. Results shown are the mean ± SE. Groups were compared for significance using Wilcoxon’s rank-sum test. The mean angiogenic activity for the two groups was compared by a heteroscedastic t test.

RESULTS

Characterization of Secreted and Cell Surface u-PA on Parental and Transfected Cell Lines. MAT-LyLu cells secreted 1.2 ng/ml u-PA and expressed a range of 10,000–30,000 surface urokinase receptors/cell.

After transfection with the pCEPMmu/A358 plasmid and growth in hygromyacin-containing medium, 150 resistant clones were selected for an additional 20-day growth in hygromycin. The 90 most viable-appearing monoclonal cell populations were assayed in the competitive immunocapture assay. Nineteen clones that inhibited wild-type u-PA binding to rabbit anti-mu-PA IgG to the greatest extent in this assay were selected for screening by immunological means.

Secreted u-PA production by parental and transfected cell lines was
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lytic activity as demonstrated by plasminogen zymography. This confirms that the overexpressed u-PA is indeed the mutant u-PA.

In Vitro Growth of 5C4 Cells. The in vitro cell-doubling times of the control pooled group of clones expressing hygro-only 1 and of the mutant u-PA-expressing clone 5C4 were measured. During exponential cell growth, the doubling times for hygro-only 1 cells and for clone 5C4 were calculated at 23.00 and 23.01 h, respectively. Thus, the effects of mutant u-PA on tumor growth do not seem to be due to an effect on intrinsic cell proliferation.

Micrometastasis. Standard curves plotting the number of cells versus luciferase counts for each cell line were generated (data not shown). Regression analysis of the predicted versus actual luciferase counts was performed, and a regression coefficient of 0.99 was achieved for each curve, indicating the strong relationship between increasing cell number and luciferase expression. For in vivo experiments, the luciferase activity was measured in primary tumors or lungs and plotted on the corresponding standard curve to calculate cell number.

Animal Experiments. Primary weights of tumor clones with variable expression of mutant urokinase are shown in Fig. 4. The difference in primary tumor weight derived from three clones was statistically smaller than that of hygromycin-only rats (P = 0.01). In the first experiment, animals demonstrated mild morbidity from the primary tumor burden at sacrifice at 14 days. Table 1 shows primary tumor weights and the calculated number of cells in the primary lesions and lungs of rats injected with hygro-only 1 and clone 5C4 cells. Animal weight did not change significantly over the 2-week observation period. No lung metastases were detectable in the clone 5C4 group. Of 12 rats injected with hygromycin-only cells, 5 had a luciferase measure assessed by chemiluminescent immunoblotting, as shown in Fig. 1. Although u-PA secretion by MAT-LyLu cells was detectable in the immunocapture assay, it was not detectable by Western blotting. The 4D4 clone expresses relatively low amounts of u-PA compared to the high u-PA-expressing clones 6A1 and 5C4. mu-PA is shown as a positive control.

That the cell-bound u-PA from the mutant transfectants is the proteolytically inactive mutant mu-PA was demonstrated by plasminogen zymography. Fig. 2 compares cell surface-eluted receptor-bound mu-PA from control lines hygro-only 1 and 2 and clones 5C1, 5C4, and 6A1 were added to Laemmli SDS sample buffer and electrophoresed in a 10% SDS-PAGE gel containing 50 mg/ml Lys-plasminogen and 1 mg/ml casein. mu-PA in the samples caused the conversion of plasminogen in the gel to plasmin with enzymatic degradation of casein in the gel at the 43-kDa position. Clone 5C4 has the most u-PAR occupied with mutant mu-PA and thus has the least degradation. Quantification of signal volume by densitometry shows the following values: hygro-only 1, 228; hygro-only 2, 173; clone 5C1, 159; clone 6A1, 344; and clone 5C4, 33. This experiment was performed in triplicate.

Fig. 1. A chemiluminescent Western blot for detection of soluble u-PA secreted by MAT-LyLu cells and high mutant mu-PA-expressing clones 6A1 and 5C4 and the low mu-PA-expressing clone 4D4. Equal protein concentrations of serum-free CM are loaded in each lane and electrophoresed in a 10% SDS-polyacrylamide gel. mu-PA is used as a positive control.

Fig. 2. Plasminogen zymography of cell surface eluates. Glycine-eluted receptor-bound mu-PA from control lines hygro-only 1 and 2 and clones 5C1, 5C4, and 6A1 were added to Laemmli SDS sample buffer and electrophoresed in a 10% SDS-PAGE gel containing 50 mg/ml Lys-plasminogen and 1 mg/ml casein. mu-PA in the samples caused the conversion of plasminogen in the gel to plasmin with enzymatic degradation of casein in the gel at the 43-kDa position. Clone 5C4 has the most u-PAR occupied with mutant mu-PA and thus has the least degradation. Quantification of signal volume by densitometry shows the following values: hygro-only 1, 228; hygro-only 2, 173; clone 5C1, 159; clone 6A1, 344; and clone 5C4, 33. This experiment was performed in triplicate.

Fig. 3. RT-PCR of hygro-only 1 and clone 5C4 cell lines. mRNA was purified as described in "Materials and Methods," and cDNA was generated. Forward and reverse primers for mutant u-PA were used for PCR amplification. A 200-bp PCR product is seen in the clone 5C4 cell line but not in the hygro-only 1 or control (no DNA) lanes.
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5C4, 4D4, and 6A1 had significantly smaller tumors (P = 0.01).

To confirm the trend toward fewer lung metastases in rats injected with the 5C4 cell line, a second experiment was performed. An 8-fold increase in cell number (4 × 10^5 cells) was injected, and the animals were observed for a longer period (18 days). At the time of sacrifice, the primary tumor burden was responsible for sufficient morbidity to obviate a longer observation period. Primary tumors were again significantly larger in the hygro-only 1 group versus the 5C4 group (19.1 ± 5.5 versus 8.5 ± 3.2 g, respectively, P = 0.001). Tumor size and peritumor vascularity can be seen in Fig. 5. Lung involvement by tumor cells was again found to be greater in the hygro-only 1 group than in the 5C4 group (342.9 ± 245 versus 36.5 ± 26 cells, P < 0.05).

Angiogenic Activity. One possible explanation for the effect of the mutant u-PA on tumor growth is that tumor-induced angiogenesis is inhibited. When tumors were analyzed for neovascularization, clone 5C4 primary tumors had a significantly lower mean microvesSEL count than did primary tumors from the hygromycin-only animals (6.8 ± 4.9 versus 28.9 ± 9.4, P < 0.001).

DISCUSSION

u-PA has been shown to be an important component of the invasive process in various models of prostate cancer. Binding of u-PA to its receptor on tumor cells facilitates degradation of the surrounding matrix. This step is an essential part of tumor invasion, which leads to metastatic spread. Hoosein et al. (32) found that the metastatic and androgen-insensitive human PC3 and DU145 prostate cells expressed both u-PA and u-PAR but that the nonmetastatic androgen-sensitive LNCaP cells did not; the PC3 and DU145 cells invaded the basement membrane in a Matrigel model, whereas LNCaP cells did not. Gaylis et al. (6) showed that an aggressive variant of PC3 cells secreted 3.5-fold more soluble u-PA than did parental PC3 cells, but cell-bound u-PA was not measured. In our studies, we demonstrated that u-PA plays a role not only in the metastatic spread of prostate cancer cells but also in primary tumor growth.

The Dunning rat prostatic carcinoma animal model has been well characterized (31). Primary tumor growth of MAT-LyLu cells is rapid and results in significant animal morbidity. Many investigators inject tumor cells into the hind limb and perform a hind limb amputation at day 14, once the primary lesion has metastasized (33). Microscopic metastases can then progress, with the number of gross lung metastatic foci used as an end point. In another study, MAT-LyLu cells that overexpress wild-type rat u-PA were injected intravenicularly to enhance skeletal metastases (8); spinal cord paralysis and organ histology were the endpoints in this model. The stable expression of luciferase in our experiments allowed for the detection of fewer than 10 metastatic cells in an organ and the quantification of viable cancer cells in a tumor that may contain hemorrhagic, cystic, or necrotic areas. This level of sensitivity obviates lengthy studies or removal of the primary tumor, thus decreasing experimental morbidity. In addition, unlike the experiments reported by Crowley et al. (22), we used a host with a competent immune system, a situation that more closely resembles spontaneous tumorigenesis in an otherwise normal host.

Attempts to block the u-PA cascade have included a variety of strategies. Ossowski et al. (16) used anticytotoxic antibodies that recognize u-PA to decrease squamous cell carcinoma invasion in a nude mouse. Other potential antagonists include the u-PA inhibitor amiloride (18) and the synthetic bicyclic heteroaromatic amides, (19) which competitively inhibit u-PA. Other investigators have used expression of PAI-1 to decrease matrix solubilization (20) and to show that the binding of PAI-1 to matrix vitronectin inhibits cell surface plasminogen activation (11). Approaches that use cell transfection and overexpression of either mutant u-PA or mutant u-PAR to interfere with generation of cell surface plasmin have been reported.

Masucci et al. (21) developed a truncated version of u-PA that does not anchor to mouse LB6 cell membranes. The secreted mutant

| Table 1 Results of in vivo experimentation in Copenhagen rats |
|-----------------|-----------------|-----------------|-----------------|
| Primary tumor weight | 5.9 ± 1.8 cells | 1.3 ± 0.5 cells | P = 0.001 |
| Mean no. of cells in primary tumor | 5.38 × 10^6 ± 9.6 × 10^5 | 1.4 × 10^5 ± 6.1 ± 10^3 | P = 0.001 |
| Mean no. of cells in lungs | 1307 ± 1600 cells | 54 ± 26 cells | P = 0.06 |
| No. of animals with lung metastases | 5 | 0 |

* A total of 50,000 hygro-only 1 cells or clone 5C4 cells were injected into each animal's flank, and the rats were sacrificed 14 days later.

Fig. 4. Mean primary tumor weights from rats injected with mock transfectant (hygro-only 1) cells and clones expressing various levels of mut-PA by in vitro assays. Mean tumor weights are shown above each bar. Compared to hygro-only 1 cells, clones 5C4, 4D4, and 6A1 had significantly smaller tumors (P = 0.01).

Fig. 5. Primary tumors from hygro-only 1 (A) and clone 5C4 (B) cell lines 14 days after s.c. injection of 50,000 cells. The control hygro-only 1 tumors are larger with greater peritumor vascularity in the s.c. tissue surrounding the tumor.
receptor acts as a scavenger for soluble u-PA, thus preventing its binding to wild-type u-PAR.

A hybrid protein consisting of the amino-terminal fragment and albumin was reported to inhibit u-PA binding to u-PAR competitively in a reconstituted basement membrane model (34). Min et al. (35) constructed a u-PAR antagonist consisting of the epidermal growth receptor acts as a scavenger for soluble u-PA, thus preventing its binding to wild-type u-PAR.

The antagonist inhibited formation of capillary networks by murine brain endothelial cells in vitro fibrin gels. Mice injected s.c. with Matrigel/BFGF plugs had greater neovascularization compared to plugs containing Matrigel/BFGF and the u-PA antagonist, although vessels were not quantitated. These investigators also injected plugs containing B16 melanoma cells and Matrigel with or without the fusion protein s.c. into mice. Tumor size measured with calipers revealed smaller tumor growth in animals receiving plugs containing the fusion protein. Smaller tumors were measured in animals injected with fusion protein, but microvessel density was not assessed. In the experiments reported here, we used microvessel quantitation in tumor specimens to show a close correlation between inhibition of neovascularization and tumor growth.

The significantly smaller primary tumor growth in the mutant u-PA-expressing clones that we observed may be secondary to alterations in tumor angiogenesis. Also, clonal variation of in vitro mutant mu-PA expression correlated inversely with in vivo primary tumor volumes, suggesting a relationship between increasing expression of mutant mu-PA and inhibition of tumor growth. We compared the motility of the hygromycin-only and clone 5C4 cells in a Boyden chamber migration assay and found no difference. The mechanism by which inhibition of u-PA is likely to inhibit tumor-induced neovascularization is unclear. u-PA expression and binding to u-PA is up-regulated in migrating endothelial cells (36). Growth of primary tumors requires angiogenesis including increased focal vascular protease activity for new vessel formation. Expression and binding of u-PA to u-PA on endothelial cells are likely to contribute to tumor angiogenesis, a process our data suggest may be inhibited by the presence of mutant u-PA. A variety of angiogenic growth factors including bFGF and vascular endothelial growth factor also stimulate expression of u-PA and u-PA on capillary endothelial cells (37, 38). Focal expression of u-PA and other proteases by endothelial cells promotes invasion of the surrounding matrix and new capillary formation (27). One possible mechanism for the decrease in primary tumor growth that we observed may be that secreted mutant mu-PA by the tumor cells has host-modulating effects including binding to host stromal macrophages, fibroblasts, and vascular endothelium in the tumor environment. As noted above, Crowley et al. (22) studied mutant human u-PA-expressing PC3 cells in athymic mice and found a marked decrease in metastatic spread but found no difference in primary tumor growth compared with that of controls. The failure to observe an effect of mutant human u-PA on the primary growth of PC3 cells in athymic mice may be related to the inability of human u-PA to bind to mouse u-PAR (22).

These data indicate that synthetic ligands that target u-PA may be of therapeutic value in patients with prostatic cancer at risk for progression. This model also suggests that the u-PA amino-terminal fragment could be used as a potential delivery system for drugs or toxins in the form of fusion proteins.

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