Protease Activity of Urokinase and Tumor Progression in a Syngeneic Mammary Cancer Model


Background: We and others have previously shown that plasminogen activators generate endogenous angiogenesis inhibitors and induce antiangiogenic activity. Here we assessed the effects of plasminogen activator overexpression on tumor progression in a syngeneic mammary cancer model. Methods: Genes encoding murine tissue plasminogen activator (tPA), urokinase (uPA), and vector controls were stably transfected into 4T1 murine mammary cancer cells, and cell proliferation in vitro was analyzed. Cells were also implanted into female BALB/c mice (n = 12 per group), and tumor growth, lung metastases, and survival were compared. Tumor cell proliferation and microvessel formation were analyzed by immunohistochemistry using antibodies to proliferating cell nuclear antigen and CD31, respectively. 4T1 cells transfected with proteolytically inactive uPA mutants (A and B) were assayed for proliferation in vitro and tumor growth in vivo by using the same syngeneic model (eight to 10 mice per group). All statistical tests were two-sided. Results: In vitro growth of uPA- and tPA-overexpressing and control 4T1 cells was similar. In vivo, however, inhibition of tumor growth and lung metastasis was inhibited in the mice carrying tPA- and uPA-overexpressing tumors, compared with controls (tumor weight at day 34: control, mean = 1760 mg, 95% CI = 1434 to 2087 mg; tPA, mean = 921, 95% CI = 624 to 1217 mg; P < .001; uPA, mean = 395 mg, 95% CI = 161 to 629 mg; P < .001). Number of lung metastases at day 34: control, mean = 117, 95% CI = 74 to 159; tPA, mean = 33, 95% CI = 13 to 52; uPA, mean = 15, 95% CI = 4 to 25; P < .001). Median survival was 42 (95% CI = 36 to 44), 55 (95% CI = 48 to 61), and 73 (95% CI = 51 to 86) days in the control, tPA, and uPA groups, respectively (P < .001). uPA- and tPA-expressing tumors had reduced angiogenesis and cell proliferation compared with controls. Tumors overexpressing uPA mutants grew faster than tumors expressing wild-type uPA (tumor volume at day 30: wild-type uPA, mean = 203, 95% CI = 121 to 285 mm³; control, mean = 534, 95% CI = 460 to 608 mm³; P < .001; mutant A, mean = 600, 95% CI = 520 to 679 mm³; P < .001; and mutant B, mean = 435, 95% CI = 358.9 to 511 mm³; P = .005). Conclusions: In this mouse model, uPA expression delayed tumor progression and had antiangiogenic and antiproliferative effects that may be mediated by uPA’s protease activity. These results challenge the current dogma of proteases being exclusively tumor promoting and provide further rationale for exploring plasminogen activators as antitumor agents. [J Natl Cancer Inst 2006;98:756–64]

The plasminogen activator (PA) system is a family of proteases and inhibitors that regulate various physiologic and pathologic processes, such as coagulation, angiogenesis, and tumor progression (1–3). In addition to acting as proteases, PAs bind to cell surface receptors, leading to cellular signaling events. Urokinase (uPA), for example, binds with high affinity to its cell surface receptor (uPAR), and signaling induces migratory and mitogenic effects (1). It is widely accepted that urokinase and tissue PA (tPA) expression is associated with an aggressive tumor phenotype and an adverse clinical outcome (2, 4–9). However, data are accumulating that suggest that the role of tPA and uPA is far more complex than previously thought. Indeed, several reports have suggested that PAs may negatively regulate angiogenesis and their inhibitors may promote it (10–16).

In addition to promoting tumor invasion by cleaving plasma and stromal proteins, both alone and through interactions with...
matrix metalloproteinases (MMPs), uPA and tPA are also involved in the proteolytic generation of angiogenic peptides, such as angiostatin, endostatin, and tumstatin (15,17–19). We have previously shown that PAs induce angiogenic activity in vitro, in an in vivo angiogenesis model, and in patients with cancer (16). Moreover, we demonstrated that the potent in vitro and in vivo angiogenic effects of PAs are not due solely to the generation of angiostatin. Therefore, we hypothesized that shifting the proteolytic balance in favor of PAs would delay tumor progression. We tested this hypothesis in a syngeneic murine mammary cancer model that resembles human metastatic breast cancer (20) by engineering cancer cells to overexpress PAs and assessing their effects on tumor progression and survival. To investigate the role of uPA’s proteolytic activity on the growth regulatory effects, we engineered proteolytically inactive uPA mutants and compared their in vivo effects with those of proteolytically active uPA in overexpressing cancer cells.

**Materials and Methods**

**Cell culture and reagents**

The spontaneously metastasizing murine mammary carcinoma cell line 4T1 was a gift of Carlos Arteaga (Vanderbilt University, Nashville, TN). The human embryonic kidney epithelial cell line 293T and Lewis lung carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

**Preparation of Expression Constructs**

Total RNA was isolated from BALB/c mouse kidney tissue (Jackson Laboratory, Bar Harbor, ME) (for murine tPA) and Lewis lung carcinoma cells (for murine uPA) by using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Reverse transcription was performed by using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) for reverse transcription–polymerase chain reaction and gene-specific primers 5′-CAAGGCGACACCTTCATCC- 3′ (for tPA) and 5′-AAACCCGAAATGAAACTAAGATGC- 3′ (for uPA) (uPA, NM_008873; tPA, NM_008872). The complete coding regions of murine tPA and uPA were amplified by polymerase chain reaction using tPA primers (forward) 5′-TTGGTGCAG AAAATGGAAGAGAGCTGCTGTTGATC- 3′ and (reverse) 5′-TTAGGATCCCTATTGCTTCGTAATC- 3′ and (reverse) 5′-TTAGGATCCCTATTGCTTCGTAATC- 3′ and uPA primers (forward) 5′-TTGGTGCAG ATGAAATCT GCTGCTGCGCTGCT- 3′ and (reverse) 5′-TTGGATCCCTGAGGGCCGCTGCTGCGCTGCT- 3′. The above cDNAs were cloned into the cytomegalovirus-driven mammalian expression vector pcDNA3.1/Hygro(+) (Invitrogen) between the Nhel and BamHI restriction enzyme sites.

**Generation of Urokinase Mutants**

As a negative control, the proteolytically inactive murine mutant uPA (mutant A) with amino acid serine-to-alanine substitution at position 358 (Ser358→Ala) (21,22) was generated from the pcDNA 3.1–wild-type uPA (see above) by the overlap extension method (23). The oligonucleotides designed to generate the mutations were forward primer wild type (see above) and reverse primer mutant uPA (5′-CGGTCTCTCC AGCATCGCCCTTGCG-3′) and forward primer mutant (5′-TGCAAGGCGCCGCTCTG GAGAGG-3′) and reverse primer wild type (see above). The mutant uPA cDNA was digested at the Bsu36I and BamHI restriction sites, and the fragment containing the mutation was ligated into the wild-type uPA construct that was digested with the same enzymes.

A double-negative (proteolytically inactive and non–uPAR binding) murine uPA (mutant B) was also generated. To abolish uPA’s ability to bind to its receptor, a triple–amino acid mutation in the receptor binding region of murine uPA (arginine-to-asparagine at position 27 [R27N], arginine-to-histidine at position 29 [R29H], and arginine-to-tryptophan at position 30 [R30W]) was introduced (24) using the same method as was used to make mutant A. The oligonucleotides used were forward primer wild type (see above) and reverse primer mutant uPA (5′-CCTTGGGCAAGCTGCACCA GAGATGTTGGAGAAG TACTGTTAGG-3′) and forward primer mutant (5′-CTTAC AAGTACT TCTCCACATTCATGCTGAGCGTAGCCGAGCGAGTCCGAGG-3′) and wild-type reverse (see above). The mutant uPA cDNA was digested at the Nhel and Bsu36I restriction sites, and the fragment containing the triple mutation was ligated into the construct containing the Ser358→Ala mutation after digestion at the Nhel and Bsu36I restriction sites.

All plasmids were transformed into DH5α (Invitrogen), and selected colonies were then cultured. Plasmids were purified from cultures using either QIAprep Miniprep or QIAfilter Plasmid Maxi kit (Qiagen). Full-length sequencing of all constructs was performed and confirmed using the ABI Prism 373 Automated DNA Sequencer (Applied Biosystems, Foster City, CA).

**Preparation of 4T1 Stable Clones**

Constructs containing the genes for wild-type tPA, uPA, and uPA mutants and an empty vector control were transfected into 4T1 cells using Lipofectamine Plus reagent (Invitrogen). Clones stably expressing the genes were selected with 500 μg/mL of hygromycin. Ten to 12 clones each of tPA, uPA wild type, and uPA mutants (and six clones of the empty vector control) were obtained, and expression of the endogenous and transfected genes were assessed by RNA analysis.

**RNA Purification and Northern Blot Analysis**

Total RNA was extracted from stably transfected clones by using the RNeasy Mini Kit (Qiagen). RNA (5 μg) was electrophoresed in a 2 M formaldehyde–1% agarose gel and transferred to a nylon membrane (Roche Diagnostics, Indianapolis, IN) by upward capillary transfer using 10× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate). RNA was cross-linked to the membrane by using a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Blots were prehybridized in Rapid-hyb buffer (Amersham, Arlington Heights, IL) at 65 °C for 30 minutes. 32P-labeled cDNA probes for uPA, tPA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated from full-length murine cDNA obtained by restriction digest from expression vectors described above (uPA and tPA) and from restriction digest of GAPDH cDNA (Accession number NM_002046) by using a Random Primer Labeling kit (Stratagene). Probes were added to the
Western Blot and Urokinase Proteolytic Activity

Constructs for uPA wild type, mutant uPA, and empty vector were transiently transfected into 293T cells as described above. Two days later cells were lysed in RIPA buffer (Boston Bioproducts, Ashland, MA) with complete protease inhibitor cocktail tablets (Roche Diagnostics). Protein concentrations were determined by using the BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins (10 μg) were separated by SDS–polyacrylamide gel electrophoresis on 4%–15% gels (Bio-Rad, Hercules, CA) under reducing conditions, electrophoretically transferred to Immobilon-P polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA), and subjected to standard western blotting procedures, as previously described (16). In brief, membranes were probed with a mouse monoclonal anti-uPA antibody (1 μg/mL, Molecular Innovations, Southfield, MI) in Tris-buffered saline with Tween 20 (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and 2% bovine albumin. Membranes were washed with Tris-buffered saline with Tween 20 four times at room temperature, followed by incubation with ECL anti-mouse immunoglobulin G horseradish peroxidase–conjugated secondary antibody (1:3000 dilution, Amersham Biosciences, UK). Protein–antibody complexes were detected using the SuperSignal West Pico chemiluminescent substrate (Pierce). Western blot analysis of angiostatin and endostatin were performed as above using rabbit polyclonal anti-angiostatin (5 μg/mL) and rabbit polyclonal anti-endostatin (2 μg/mL) (both from Affinity Bioreagents, Golden, CO) and goat anti-rabbit immunoglobulin G horseradish peroxidase–conjugated secondary antibody (1:5000 dilution; Amersham). Western blot analysis of murine uPA was repeated twice. Western blot analysis of angiostatin and endostatin were performed on plasma samples from seven to eight mice per group (see below).

The urokinase proteolytic activity was measured in the conditioned medium (48 hours) of transfected 293T cells by using a colorimetric urokinase activity kit (optical density read at 450 nm), following the manufacturer’s recommendations (Chemicon, Temecula, CA). Optical density experiments were performed in triplicate and repeated twice.

**tPA and uPA Enzyme-linked Immunosorbent Assays**

Murine tPA and murine uPA levels were determined in the conditioned medium (72 hours) and cell lysates of 4T1 clones stably transfected with tPA and uPA constructs by using a murine uPA and tPA enzyme-linked immunosorbent assay (ELISA) kit (Molecular Innovations) by following the manufacturer’s recommendations. Positive control samples (murine uPA and murine tPA) were provided by the manufacturer. Both assays could detect as little as 0.03 ng/mL.

**In Vitro Proliferation Assay**

Growth of 4T1 stably transfected cell lines was assessed as previously reported, with modifications (16). In brief, cells (1000 cells per well) were plated in 96-well plates in complete medium and incubated at 37 °C. Cell proliferation was determined daily for 5 days (vector controls versus tPA and uPA) or 4 days (vector controls versus wild type and uPA mutants) by using the WST-1 proliferation reagent (Roche Diagnostics) (10 μL/well), as previously reported (16). Experiments comparing controls, tPA, and uPA were performed two times in triplicate; those comparing controls and wild type and uPA mutants were performed in quintuplicate.

**In Vivo Studies**

All mouse studies were reviewed and approved by the institution’s (Beth Israel Deaconess Medical Center, Boston, MA) animal care and use committee. Stably transfected 4T1 clones (10^5 cells) were implanted subcutaneously into the fifth left mammary fat pad of 8- to 10-week-old female BALB/c mice (Jackson laboratory) (12 mice per group; independent experiments were repeated at least twice using different clones. Before inoculation, cell viability was assessed using trypan blue (0.4%; Sigma). Primary tumor size was measured every 3–7 days for up to 34 days with Vernier calipers, and tumor volume was calculated using the standard formula (width^2 × length × 0.52). On day 34, mice were killed by CO2 asphyxiation, and primary tumors and lungs were surgically removed. Resected tumors and lungs were rinsed in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4), fixed in 10% neutral-buffered formalin at room temperature overnight, and embedded in paraffin. Paraffin-embedded tissues were stored at room temperature. Immunohistochemistry studies were performed within 3 months of tissue extraction. The experiment was repeated (three to five mice per group); mice were killed at 1–2 weeks to examine tumors at this time. In separate experiments (seven to eight mice/group), tumor-bearing mice were killed (cervical dislocation) on day 31, and blood was obtained by cardiac puncture for plasma extraction. For survival analysis, the experiment was repeated in a separate population of mice (12 per group). Tumors were measured as above, and mice were observed until death or were killed when moribund.

**Histology and Immunohistochemistry**

Tumors and lungs were fixed (10% neutral-buffered formalin), embedded in paraffin, sectioned (5-μm slices, 400 μm apart), and stained with hematoxylin and eosin. Magnified (×20) lung nodules were counted. For immunohistochemistry, tumor sections were deparaffinized and rehydrated before antigen retrieval by heating in a microwave in 10 mM citrate buffer for 1 minute (for proliferating nuclear antigen [PCNA]), or in 0.33 mg/mL protease K for 10 minutes (for CD-31 and urokinase). Sections were incubated overnight at 4 °C with polyclonal rabbit anti-uPA (1:100; Santa Cruz Biotechnologies, Santa Cruz, CA), rat anti-mouse CD-31 (1:50, PharMingen, San Diego, CA), or monoclonal mouse anti-PCNA (1:100, Novocastra Laboratories, Newcastle, UK) antibodies diluted in 3% bovine serum albumin. After washing with PBS three times for 5 minutes each, slides were incubated at room temperature with horseradish peroxidase–conjugated goat anti-rabbit (for uPA, 1:200; DAKO, Carpinteria, CA), biotinylated anti-rat (for CD31), or biotinylated anti-mouse (for PCNA) antibodies (Vector Laboratories, Burlingame, CA). For CD-31 and PCNA staining, the ABC method (Vector Laboratories) was used. After washing in PBS, sections were incubated with 3,3-diaminobenzidine substrate (DAKO, Carpinteria, CA),
counterstained with hematoxylin (3 minutes), washed with water (10 minutes), dehydrated in (70%, 80%, 95%, and 100% ethanol for 3 minutes, twice each), cleared in xylene (10 minutes, twice), and mounted onto slides with Permount (Fisher Scientific, Fair Lawn, NJ). Viable sections of tumors (areas without necrosis) were chosen for evaluation. Five fields per slide per mouse were visualized.

Statistical Analysis

Data are presented as means with 95% confidence intervals (CIs). Differences in means from the in vitro and in vivo experiments were compared by using the Student’s t test and Wilcoxon rank-sum test. Differences were considered statistically significant at \( P < .05 \). Differences of in vivo tumor growth and lung metastases among three or more groups were analyzed by one-way analysis of variance. Pairwise comparisons were performed using the Tukey–Kramer method, in which \( P < .01 \) was considered statistically significant. Overall survival was analyzed by the method of Kaplan–Meier. Differences in survival were analyzed by the log-rank test. All statistical tests were two-sided.

RESULTS

Effects of Early Overexpression of Plasminogen Activator Overexpression on Primary Tumor Growth

We first generated stable 4T1 clones carrying the murine uPA and tPA genes or empty vector (EV) clone 3, tPA clone 9, and uPA clone 11, and these were selected for the first set of experiments, based on maximal expression of the transgene as determined by RNA analysis (Fig. 1, A). uPA and tPA protein overexpression by the selected clones was confirmed by ELISA using conditioned medium (Fig. 1, B and C). Proliferation of PA-overexpressing cells and of the EV control cells were similar (Fig. 1, D). In vivo, however, tumors overexpressing tPA and uPA grew more slowly than the control tumors (82% [95% CI = 56.5% to 106%] reduction in tumor volume in the uPA group and 60% [95% CI = 35% to 85%] reduction in tumor volume in the tPA group at day 34, compared with EV group; \( P < .001 \); Fig. 2, A). The mean tumor weight at day 34 was 1760 mg (95% CI = 1434 mg to 2087 mg) in the EV group, 921 mg (95% CI = 624 mg to 1217 mg) in the tPA group, and 395 mg (95% CI = 161 mg to 629 mg) in the uPA group (uPA versus EV, \( P < .001 \); tPA versus EV, \( P < .001 \); uPA versus tPA, \( P = .017 \); Fig. 2, B).

Effects of Plasminogen Activator Overexpression on Lung Metastases and Survival

Lungs from tumor-bearing mice were removed at day 34, and the number of metastases was assessed. The average number of lung metastases was statistically significantly reduced in the groups (\( n = 12 \) mice per group) bearing tumors derived from cells overexpressing tPA and uPA (in controls, mean = 117, 95% CI = 74 to 159; tPA, mean = 33, 95% CI = 13 to 52; uPA, mean = 15, 95% CI = 4 to 25; \( P < .001 \); Fig. 2, C). Experiments using different clones showed similar results both in tumor growth and lung metastases (data not shown).

To determine whether the effects of tPA and uPA on tumor growth were relevant to survival, the in vivo experiments were repeated. Differences in tumor growth were similar to those in
Fig. 2. In vivo effects of plasminogen activator (PA) overexpression on murine mammary tumor growth. 4T1 cell clones stably expressing PA (tPA), urokinase (uPA), and empty vector (EV) controls were implanted into the mammary fat pad of female BALB/c mice, and tumor growth was measured over time. Mice (12 per group) were killed at day 34 after tumor implantation, and mammary tumors and lungs were surgically removed. A) Tumor growth in tPA- (squares) and uPA-overexpressing (circles) tumors compared with the empty vector controls (triangles). Means and 95% confidence intervals are shown. *, P<.001 tPA versus EV; †, P<.001 uPA versus EV, two-sided Tukey–Kramer test. B) Primary tumor weight at day 34 in the uPA, tPA, and EV groups. *, P<.001, uPA versus EV; †, P<.001, tPA versus EV, two-sided Tukey–Kramer test. C) Number of lung metastases at day 34 in the tPA- and uPA-overexpressing tumors compared with the EV control. *, P<.001, one-way analysis of variance. In panels B and C, bars indicate means and squares show individual data points. Experiments were repeated twice, and one is shown.

the previous experiment (data not shown). Median survival was statistically significantly longer in the uPA group (73 days [95% CI = 51 days to 86 days]) than the tPA (55 days [95% CI = 48 days to 61 days]) and the EV (42 days [95% CI = 36 days to 44 days]) groups (n = 12 mice per group; P<.001; Fig. 3).

Immunohistochemistry Studies

To gain insight into the potential mechanisms of urokinase’s antitumor effects, we assessed microvessel density (using CD31) and proliferation rate (using PCNA) of the primary tumors. No clear differences were observed among the tumors removed at 5 weeks (34 days), probably due to necrosis and high background staining (not shown). Therefore, we removed tumors 1–2 weeks after inoculation (when growth rates in the different groups were similar [data not shown] in an additional group of mice (three to five mice per group) for immunohistochemistry. Microvessel density was decreased, as indicated by reduced CD31 staining, in the uPA and tPA groups compared with the EV control group (Fig. 4, A–C). Proliferation in the uPA and tPA tumors was also decreased, as indicated by the smaller number of PCNA-stained cells compared with that in the controls (Fig. 4, D–F).

Fig. 3. Kaplan–Meier analysis of survival of mice bearing tumors derived from 4T1 cells overexpressing urokinase (uPA), plasminogen kinase (tPA), and empty vector (EV) controls. Mice (12 per group) were monitored until they died or became moribund. Median survival of mice: uPA group, median = 73 days [95% CI = 51 to 86]; tPA group, median = 55 days [95% CI = 48 to 61]); EV group, median = 42 days [95% CI = 36 to 44] (P<.001; two-sided log-rank test).

Fig. 4. Immunohistochemistry studies. A–C) Microvessel density was decreased, as indicated by reduced CD31 staining, in the uPA and tPA groups compared with the EV control group (Fig. 4, A–C). Proliferation in the uPA and tPA tumors was also decreased, as indicated by the smaller number of PCNA-stained cells compared with that in the controls (Fig. 4, D–F).

Role of Urokinase Protease Activity on Tumor Growth Delay

We previously reported that PAs induce antiangiogenic effects in vitro, in vivo, and in cancer patients due to proteolytic cleavage of plasma proteins into antiangiogenic peptides (16). Therefore, we hypothesized that the tumor growth effects of uPA were due to its proteolytic activity. To test this hypothesis, we generated two uPA mutants. Mutant A was a proteolytically inactive uPA mutant with intact receptor binding capabilities, and mutant B was proteolytically inactive and non–receptor binding. Protein expression and proteolytic activity of the wild-type and mutant uPAs were confirmed by western blot analyses of cell lysates and by uPA activity assays of conditioned media of transiently transfected 293T cells (human embryonic renal epithelial cells that do not produce murine uPA) (Fig. 5, A–B).
The constructs were stably transfected into 4T1 cells, and clones (EV, uPA wild-type [WT], uPA mutant A, and uPA mutant B) were selected for in vitro and in vivo experiments on the basis of maximal expression of the transgene, as determined by RNA analysis (Fig. 5, C). Urokinase overexpression in the stable clones was confirmed by ELISA assays (Fig. 5, D). The in vitro growth of maximal expression of the transgene, as determined by RNA analysis (Fig. 5, C). Urokinase overexpression in the stable clones was confirmed by ELISA assays (Fig. 5, D). The in vitro growth

Fig. 4. Proliferation and angiogenesis of tumors derived from 4T1 cells overexpressing tissue plasminogen activator (tPA), urokinase (uPA), or empty vector. Tumors were removed 1–2 weeks after inoculation and stained with rat anti-mouse CD-31 and monoclonal mouse anti–proliferating cell nuclear antigen [PCNA] (A–C). Microvessel density, as determined by CD31 staining, in the uPA (B) and tPA (C) groups and in the empty vector control (A). Arrowheads represent stained microvessels. Bars = 40 μm. (D–F) Tumor cell proliferation as determined by PCNA staining (arrowheads) in uPA- (E) and tPA-overexpressing tumors (F) and empty vector (D) controls. Bars = 80 μm. Pictures shown correspond to representative areas of tumor with viable cells. Five fields were observed per slide per mouse.

Fig. 5. In vitro characterization of 4T1 murine mammary cancer cell clones that stably overexpress urokinase (uPA) mutants that are proteolytically inactive (mutant A) or proteolytically inactive and unable to bind its ligand (mutant B). Expression and proteolytic activity were determined by western blot and uPA activity assays of the cell lysates and conditioned media of transiently transfected 293T cells, respectively. A) Western blot of 293T human kidney epithelial cell lysates using mouse monoclonal anti-uPA antibody. C = mouse uPA protein control; NT = nontransfected 293T cells; WT = wild-type uPA; A = mutant A; B = mutant B; EV = empty vector control. Data from one of two experiments is shown. B) Proteolytic activity (optical density at 405 nm) of WT uPA and mutants A and B was measured in 293T cell–conditioned media (72 hours) by using a colorimetric urokinase activity kit (Chemicon, Temecula, CA). Results are presented as the average and 95% confidence intervals of triplicate experiments repeated twice. C) RNA analysis of transgene expression from 4T1 clones (WT clone 4, mutant A clone 2, and mutant B clone 3). Upper and lower bands represent endogenous and transgene expression, respectively. Glyceraldehyde phosphate-3-dehydrogenase (GAPDH) was used as a control for RNA loading and transfer. D) Urokinase levels in control and uPA-overexpressing 4T1 clones as determined by enzyme-linked immunosorbent assay of the cell lysates. Results are presented as the average and 95% confidence intervals of two experiments performed in duplicate. E) In vitro proliferation of wild-type (triangles) and mutant (mutant A, circles; mutant B, squares) uPA clones (diamonds) and EV controls. Cell proliferation was measured using the WST-1 proliferation reagent, and absorbance was measured at 450 nm. Results are presented as the average and 95% confidence intervals of five experiments.
rate of the different stable transfectants was similar (Fig. 5, E). The above clones were then injected orthotopically into mice. Growth of tumors derived from cells expressing wild-type tPA was statistically significantly reduced compared with that of tumors derived from empty vector control cells and cells transfected with proteolytically inactive uPA mutants. The volume of tumors derived from wild-type uPA at day 30 was 203 mm³ (95% CI = 121 mm³ to 285 mm³), whereas volume of tumors derived from vector control cells was 534 mm³ (95% CI = 460 mm³ to 608 mm³), that in mutant A was 600 mm³ (95% CI = 520 mm³ to 679 mm³), and that in mutant B was 435 mm³ (95% CI = 358.9 mm³ to 511 mm³) (wild-type uPA versus EV, P<.001; wild-type uPA versus mutant A, P<.001; wild-type uPA versus mutant B, P = .005; Tukey–Kramer test) (Fig. 6). Growth of tumors overexpressing uPA mutants was similar to that of empty vector controls. These observations strongly suggest that uPA’s protease activity negatively regulates tumor progression.

**Discussion**

The understanding of the role of the PA system in tumor biology is evolving. Here we have provided evidence that PA overexpression delays tumor progression in a syngeneic mammary cancer model. We generated murine mammary cancer (4T1) cells engineered to overexpress tPA and uPA and assessed in vivo tumor growth and lung metastases after orthotopic implantation of these cells in immunocompetent mice. In addition to reduced primary tumor size, we observed a statistically significant reduction in the number of lung metastases and more importantly, a statistically significant prolongation of survival in PA overexpressing tumor–bearing mice. The tumor growth delaying effects were more marked in the uPA- than tPA-overexpressing tumors and were found to be related to uPA’s protease activity, because in vivo tumors expressing proteolytically inactive uPA mutants grew faster than tumors overexpressing proteolytically active uPA. Immunohistochemical studies suggested that antiangiogenic and antiproliferative mechanisms may mediate uPA’s tumor growth inhibitory effects.

This is the first report, to our knowledge, to assess the effects of PA overexpression in a model (4T1) that closely resembles human metastatic breast cancer (20,25,26). The 4T1 model has several advantages—it is a well-established syngeneic mammary cancer model that is tumorigenic in immunocompetent animals and it is spontaneously metastasizing and highly aggressive (20). Because uPA/uPAR tumor–stromal interactions are species specific (24), we used an all-murine system (murine cancer cells and murine uPA) and an immunocompetent model to resemble human metastatic breast cancer.

The results of this study contrast with those of previous reports, which suggested that uPA overexpression is associated with tumor progression and that inhibition of uPA is associated with antitumor effects (27–30). The models and conditions used in those reports, however, were substantially different from the ones used in this study. Frandsen et al. (30) reported the importance of stromal uPA overexpression in an experimental human breast cancer model in immunodeficient mice. In that study, human cancer xenografts grew more slowly in uPA/− mice than in wild-type mice. They found no differences, however, in tumorigenicity, time to palpable tumors, number of lung metastases, or tumor angiogenesis between uPA-deficient and wild-type mice. Our study is different from theirs in that we investigated the effects of tumor PA (particularly uPA) overexpression in the natural history of a syngeneic, immunocompetent mammary cancer model. In fact, we believe that the studies are not necessarily contradictory. Although Frandsen et al. (30) showed the importance of stromal urokinase on primary tumor growth, which is well established, we show that overexpression of this protease can negatively regulate tumor growth, which emphasizes the importance of a critical balance between proteases and their inhibitors for tumor progression that may be therapeutically exploitable. Also, in contrast to our findings, Choi et al. (29) showed that a nontumorigenic human gastric cancer cell line that expressed human uPA became tumorigenic in immunodeficient mice. However, we cannot directly compare our results with those because of differences in tumor model (murine versus human) and host (immunocompetent versus immunodeficient mice). Furthermore, the objectives of our study were to evaluate the effects of uPA overexpression not on tumor formation but rather on tumor progression, metastasis, and survival.

Several other reports support our findings that proteases in general and PAs in particular may negatively regulate tumor progression and angiogenesis. McCawley et al. (31) reported that MMP-3, another stromal protease, has an antitumor—rather than a tumor-promoting—role in a murine squamous cell carcinoma model. Hayashi et al. (32) have shown that tPA overexpression is associated with reduced liver metastases in a murine colon cancer model. We have previously demonstrated that PAs and c motopril induce antiangiogenic activity in vitro, in vivo, and in cancer patients (16). Bajou et al. (11) and Bergers et al. (33) have demonstrated in uPA- and tPA-knockout mice that single or combined deficiencies of urokinase and tissue PA do not impair tumor growth and angiogenesis, although other reports suggested that the negative regulation of urokinase by antisense constructs or that recombinant PAI-1 reduces tumor growth in xenograft models (27,28). Zacharsky et al. (34) reviewed the literature (spanning more than 50 years) on the
negative regulatory effects of plasminogen activation in the
natural history of experimental animal and human malignancy;
the cumulative data suggested that increased plasmin activity
may be tumor inhibitory. Several studies in the clinical setting
have shown associations between high tPA expression and ac-
tivity and a favorable prognosis in early melanoma and breast
cancer, respectively (13,14). Soff et al. (19) have demonstrated
in vivo generation of angiotatin isoforms in cancer patients to
whom tPA and a sulfhydryl donor were systemically adminis-
tered. Finally, recent clinical trials have shown that broad-
spectrum protease (e.g., MMP) inhibition may not be clinically
beneficial (35,36).

Our findings may provide insight into the well-recognized
clinical finding that PAI-1, the endogenous inhibitor of PAs, par-
adoxically promotes both tumor progression and angiogenesis
and is associated with a poor prognosis in several human cancers
(6,9,10,37,38). In breast and other human cancers, urokinase has
been established as a negative prognostic factor, not alone but in
association with PAI-1 (7,39). Several studies of breast and other
cancers have shown that PAI-1 is an independent prognostic
variable for disease-free survival and overall survival that is as
strong as or stronger than uPA (9,37,38,40–43). Also, Bajou et al.
(11) have shown that PAI-1 promotes angiogenesis by directly
inhibiting proteases, suggesting that excessive plasmin proteoly-
sis may prevent assembly of tumor blood vessels.

The mechanisms of the tumor-inhibitory effects of uPA that
we observed are not completely clear, but our studies with mut-
ant forms of uPA strongly suggest that tumor growth inhibition
depends on uPA proteolytic activity. Our studies suggest that the
in vivo inhibitory effects were not due to direct uPA-induced cy-
totoxicity but may have been due to indirect effects, because in
vitro growth of the uPA-overexpressing tumor cells was similar
to that of controls. Enhanced proteolytic activity of uPA may re-
sult in cleavage of tumor stromal proteins into peptides that in-
hbit angiogenesis and/or proliferation, as suggested by decreased
microvessel density and proliferation. Our finding that plasma
angiostatin or endostatin were not detectable in any of the tumor
groups suggests that local, rather than systemic, antiangiogenic
effects were responsible for the delay in tumor progression. How-
ever, we cannot completely rule out that possibility, because plasma
levels of angiostatin or endostatin may have been below the
detection capabilities of our assays. Another possibility is that
plasmin generated by uPA activity in turn activates other stromal
proteases, such as MMPs (44–46), and may trigger a proteolytic
cascade that leads to tumor matrix disruption and an environment
that is unfavorable for tumor angiogenesis and progression. In
support of this hypothesis, Pepper et al. (47) have shown that
excessive proteolysis is incompatible with normal capillary mor-
phogenesis in vitro, and Liu et al. (48) have demonstrated that a
critical balance between uPA and PAI-1 is necessary for tumor
invasiveness.

The study has several potential limitations. Even though we
have demonstrated that the protease activity of urokinase delays
tumor growth and metastases, we cannot assume that the uPA
overexpression would have the same effects in already-established
tumors or established metastases. Another potential limitation in-
cludes our having used only one tumor model (mammary cancer),
which prevents us from making generalizations about the effects
of uPA overexpression in all solid tumors. The precise cellular
and molecular mechanisms of uPA’s antitumor and antiangiogenic
effects are currently being pursued in our laboratories.

In summary, this study demonstrates for the first time, to our
knowledge, in a syngeneic, orthotopic murine mammary cancer
model that altering the proteolytic balance in favor of PAs—
especially urokinase—delays tumor growth and metastases and
prolongs survival. These effects may be due to uPA’s protease
activity, because tumors overexpressing proteolytically inactive
variants of uPA had a similar growth pattern as the controls.
These results underscore the importance of a tightly regulated
spatial and temporal balance between PAs and inhibitors during
tumor growth and provide evidence that altering this balance in
favor of activators early during tumor development negatively
regulates tumor progression. We propose that enhancing local tu-
mor PA expression may have promise as an antitumor strategy
and are currently investigating whether this strategy may be ap-
icable to other tumor types.

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Notes

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