**Background:** Many antiangiogenic molecules are proteolytically cleaved from larger plasma proteins. For example, plasminogen activators cleave plasminogen into plasmin, and plasmin is converted into angiostatin in the presence of sulfhydryl donors. We thus investigated whether the antiangiogenic activity in plasma could be increased by treatment with recombinant tissue plasminogen activator (rt-PA) and the sulfhydryl donor captopril. **Methods:** Human plasma was treated with rt-PA (10 µg/mL) and/or captopril (1 µM). Angiogenesis was measured *in vitro* by human endothelial cell tube formation and endothelial cell proliferation and *in vivo* in mice with the Matrigel plug assay. Angiostatin was removed from treated plasma by affinity chromatography, immunoprecipitation, or ion-exchange chromatography, and the antiangiogenic activity of the depleted plasma was assessed by tube formation. Three cancer patients were treated with rt-PA and captopril, and their pretreatment and posttreatment plasmas were tested for antiangiogenic activity *in vitro*. **Results:** Angiogenesis *in vitro* was stimulated by untreated plasma and inhibited by plasma that had been treated with rt-PA and captopril but was not affected by treatment with rt-PA and/or captopril alone. **In vivo** angiogenesis in Matrigel plugs was substantially lower in mice treated with rt-PA and captopril than in untreated control mice. Antiangiogenic activity in treated plasma was largely retained after angiostatin was removed: treated plasma inhibited angiogenesis by 64.3% (95% confidence interval [CI] = 46.4% to 82.2%), relative to untreated plasma, and treated plasma depleted of angiostatin by affinity chromatography or immunoprecipitation inhibited angiogenesis by 65.1% (95% CI = 53.8% to 76.4%) or 63.7% (95% CI = 50.9% to 76.5%), respectively. Antiangiogenic activity of plasma from three cancer patients was higher after treatment with rt-PA and captopril than before such treatment. **Conclusion:** Treatment with rt-PA and captopril induced antiangiogenic activity *in vitro* and *in vivo* that appears to be independent of angiostatin. [J Natl Cancer Inst 2003;95:388–99]
In this article, we investigate whether the antiangiogenic activity of human plasma can be increased by treatment with recombinant t-PA (rt-PA) and captopril in vitro and in vivo. We use an in vivo angiogenesis assay in mice to determine whether treatment with rt-PA and captopril decreases angiogenesis in Matrigel plugs, and we measure the antiangiogenic activity in the plasma of cancer patients treated with rt-PA and captopril. Finally, we investigate whether the observed antiangiogenic effects were dependent on angiostatin.

**Materials and Methods**

**Reagents**

rt-PA (Genentech, San Francisco, CA) and captopril (Sigma-Aldrich, St. Louis, MO) were diluted in sterile phosphate-buffered saline (PBS) and used for the bioassays. Heparin (Elkins-Sinn, Cherry Hill, NJ) at 1 U/mL or lepirudin (Aventis Pharmaceuticals, Kansas City, MO) at 5 μg/mL was added to fresh frozen plasma (FFP) or patient’s plasma to prevent clot formation. Matrigel (Collaborative Biomedical Products, Bedford, MA), a basement membrane preparation from the Engelbreth-Holm-Swarm mouse sarcoma, was used at 7 mg/mL for *in vitro* angiogenesis (endothelial cell tube formation) assays and at 10 mg/mL for *in vivo* angiogenesis (Matrigel plug) assays (see below). Basic fibroblast growth factor (bFGF) was purchased from PeproTech, Rocky Hill, NJ. The cell proliferation reagent WST-1 (Roche, Indianapolis, IN) was used for proliferation assays. WST-1 is a tetrazolium salt that is cleaved to formazan by mitochondrial dehydrogenases in viable cells. A murine monoclonal antibody against human angiostatin (Calbiochem, San Diego, CA) was used for western blotting and immunodepletion. A rabbit polyclonal antibody against mouse angiostatin (Affinity BioReagents, Golden, CO) that cross-reacts with human angiostatin was used for western blotting. Human angiostatin (kringles 1–4) was obtained from Calbiochem.

**Concentrations of rt-PA and Captopril Used**

Pharmacokinetic studies have shown that captopril concentrations of 0.1–1 μM are achieved in the plasma after captopril doses of 25–37.5 mg three times a day (30) and that plasma rt-PA concentrations in the range of 0.5–1.8 μg/mL are achieved in healthy volunteers and cardiac patients after rt-PA doses of 0.004 mg/kg/min for up to 90 minutes (31,32). We used captopril and rt-PA concentrations in these dose ranges.

**Case Reports**

Patient 1 was a 46-year-old woman with a history of metastatic malignant fibrous histiocytoma. She had multiple recurrences after surgical resections (pulmonary, hepatic, and subcutaneous nodules), radiation therapy, and thalidomide treatment, and she had refused standard chemotherapy. She was screened for bleeding disorders and for brain metastases and gave written informed consent. She first received 25 mg of captopril by mouth three times a day, and then 1 week later, she received the first of four 12-hour intravenous infusions of rt-PA over a 4-week period with increasing doses each week, from 0.015 mg/kg/h (first dose), to 0.02 mg/kg/h, to 0.03 mg/kg/h, to 0.035 mg/kg/h. Blood was taken for coagulation tests (thrombin, prothrombin, and activated partial thromboplastin times and fibrinogen level) and for bioassays at various times before, during, and after the infusion. Similarly, two additional patients (patients 2 and 3) with advanced melanoma were treated (as part of an Institutional Review Board-approved phase I trial; both provided written informed consent) with captopril at 37.5 mg by mouth three times a day, and 1 week later, they received a 12-hour infusion of rt-PA at a dose of 0.015 mg/kg/h. No patient experienced a substantial adverse reaction during or after the infusions.

**Human Plasma**

Outdated FFP was obtained from Beth Israel Deaconess Medical Center’s blood bank. Blood was collected from patients with cancer from a peripheral vein into citrated tubes. The blood was immediately centrifuged at 3210g for 10 minutes. Both FFP and the patients’ plasma were filter sterilized (0.2-μm [pore size] sterile filters; Millipore, Bedford, MA) and then stored at −20°C for future use. For treated FFP, captopril (1 μM) and rt-PA (10 μg/mL) were added to 1 mL of FFP and incubated for 3 hours at 37°C before use.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells from the lung (HMVEC-Ls) were obtained from Clontech laboratories (Palo Alto, CA) and used between passages 3 and 5. They were maintained in EGM2-MV (full endothelial cell growth) medium (Clontech) that contained endothelial basal medium 2 (EBM-2), supplemented with 5% fetal bovine serum (FBS), gentamicin, amphotericin B, hydrocortisone, ascorbic acid, vascular endothelial growth factor (VEGF), bFGF, human epidermal growth factor, and insulin-like growth factor I. Human renal epithelial (HRE) cells and primary human fibroblasts (IMR-90) were used for specificity assays. HRE cells were maintained in renal epithelial cell growth medium (renal epithelial cell basal medium supplemented with 0.5% FBS, insulin, hydrocortisone, epinephrine, tri-iodothyronine, transferrin, human epidermal growth factor, gentamicin, and amphotericin B; Clontech). IMR-90 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were grown at 37°C in a 100% humidified incubator with an atmosphere of 5% CO2/95% air. When the cell cultures were 80%–90% confluent, cells were harvested with trypsin and resuspended to the cell density required for each assay.

**Matrigel Tube Formation Assay**

Each well of prechilled 48-well cell culture plates was coated with 100 μL of unpolymerized Matrigel (7 mg/mL) and incubated at 37°C for 30–45 minutes. HUVECs were harvested with trypsin, and 4 × 10^4 cells were resuspended in 300 μL of full endothelial cell growth medium and treated with the various agents before plating onto the Matrigel-coated plates. After 12 hours of incubation, endothelial cell tube formation was assessed with an inverted photomicroscope (Nikon, Melville, NY). Mi-
crophotographs of the center of each well at low power (x40) were taken with a SPOT camera (Diagnostic Instruments, Inc., Sterling Heights, MI) with the aid of imaging-capture software (Compix Inc. Imaging Systems, Cranberry Township, PA). Tube formation in the microphotographs was quantitatively analyzed (total tube length) with Simple PCI imaging analysis software (Compix). Tube formation by untreated HUVECs in full endothelial cell growth medium was used as a negative control, and tube formation in cultures treated with actinomycin D (Sigma-Aldrich) at 7.5 μg/mL was used as a positive (inhibitory) control.

**Cell Proliferation Assay**

A total of 4 x 10^3 cells in 100 μL of the appropriate basal medium with 1% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) was placed into each well of a 96-well plate, treated with the test agents, and incubated at 37 °C for 72 hours; control cells were cultured in basal medium, 1% FBS, and antibiotics, as above. Because we observed that plasma was a potent stimulant of endothelial cell proliferation, no additional proliferation stimulus (VEGF or bFGF) was added. After the 72-hour incubation, WST-1 (10 μL) was added to each well, and after a 3-hour incubation at 37 °C, absorbance at 450 nm was determined for each well with a microplate reader (Bio-Rad Laboratories, Hercules, CA). Data presented are the average of triplicate experiments.

**In Vivo Angiogenesis (Matrigel Plug) Assay**

The Matrigel plug assay was performed as previously described (33,34) with the following modifications. Five- to six-week-old male C57/BL6 mice (The Jackson Laboratory, Bar Harbor, ME) were used. Unpolymerized Matrigel (0.5 mL) supplemented with bFGF (500 ng/mL) was injected subcutaneously in the left lower abdominal wall for the stimulated control and treatment groups, and 0.5 mL of Matrigel mixed with a volume of sterile PBS equivalent to that of bFGF was injected for the unstimulated (no bFGF) control group. Each mouse had one plug. Three mice per group were treated for 10 days with

![Fig. 1. Endothelial cell tube formation assay. Approximately 4 x 10^3 human umbilical vein endothelial cells (HUVECs) plus untreated or treated fresh frozen plasma (FFP), as indicated, in full endothelial cell growth medium (EGM2-MV) were plated in each well of a 48-well plate previously coated with Matrigel, incubated at 37 °C, and assessed for tube formation after 12–16 hours of incubation. Heparin (1 U/mL) was added to all FFP samples before plating. When present, the final concentration of recombinant tissue plasminogen activator (rt-PA) was 1 μg/mL, and the final concentration of captopril was 0.1 μM. A) Control tube formation with untreated cells in EGM2-MV. B) Tube formation with 10% untreated FFP in EGM2-MV. C) Tube formation with 20% FFP treated with rt-PA and captopril. D) Tube formation with 10% FFP treated with rt-PA and captopril. E) Tube formation with 1% FFP treated with rt-PA and captopril. F) Tube formation with rt-PA and captopril in phosphate-buffered saline. G) Tube formation with rt-PA alone (compare with panel A). H) Tube formation with captopril alone (compare with panel A). I) Tube formation with heparin alone at 1 U/mL. Bars = 250 μm. J) Quantitative analysis of tube formation. The total length of tubes was measured with the aid of imaging analysis software from the microphotographs of the center of each well taken at x40 magnification. Results are presented as percentage of tube length relative to control (untreated HUVECs).
1) rt-PA (60 \( \mu \)g diluted in 100 \( \mu \)L of 1x sterile PBS per day, subcutaneously) and captopril (150 \( \mu \)g diluted in 100 \( \mu \)L of 1x sterile PBS per day, intraperitoneally); 2) rt-PA alone subcutaneously, with a volume of PBS equivalent to that of captopril intraperitoneally; 3) captopril (150 \( \mu \)g) intraperitoneally, with a volume of PBS equivalent to that of rt-PA subcutaneously; or 4) volumes of PBS equivalent to those of rt-PA and captopril, subcutaneously and intraperitoneally, respectively. Mice were killed on day 10, and the Matrigel plugs were removed, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were examined by light microscopy, and the total number of microvessels containing red blood cells in 10 high-power fields (x400 magnification) was counted in a blinded fashion. Only microvessels that contained red blood cells were counted. Results shown represent the average of counts from three Matrigel plugs per group and 95% confidence intervals (CIs). All animal studies were reviewed and approved by the animal care and use committee of Beth Israel Deaconess Medical Center and were in accordance with the guidelines of the Department of Health and Human Services.

Affinity Chromatography

Lysine–Sepharose (Pharmacia, Peapack, NJ) chromatography was used to separate angiostatin from the other proteins in treated FFP. Briefly, a lysine–Sepharose column (6 mL) was made as described by the manufacturer. The procedure was performed at 4 °C. Treated FFP (10 mL) was loaded onto the column pre-equilibrated with 50 mM sodium phosphate (pH 7.5), and then the column was washed successively with 10 volumes of 50 mM sodium phosphate (pH 7.5), five volumes of PBS, and five volumes of 0.5 M NaCl. Retained proteins were eluted with 200 mM \( \varepsilon \)-aminocaproic acid (Sigma-Aldrich) in water. The eluted protein was dialyzed (dialysis membrane with a molecular weight cutoff of 3000; Pierce, Rockford, IL) against 4 L of PBS for 48 hours, concentrated to the original volume of the plasma, filter sterilized, and stored at −20 °C for future use.

Angiostatin Immunoprecipitation of FFP

Treated FFP (200 \( \mu \)L) was incubated with rocking at 4 °C overnight with a monoclonal antibody against human angiostatin (32 \( \mu \)g/mL). The next day, 50 \( \mu \)L of protein A/G PLUS agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the mixture was rocked for 2 hours at 4 °C and centrifuged (11 750g for 5 minutes). The supernatant (immunoprecipitated plasma) was stored at −20 °C for future use.

Fractionation of Treated FFP

Small-scale anion-exchange chromatographic steps were initially used to optimize the separation of angiostatin from other components in treated FFP with antiangiogenic activity, as follows. Treated FFP (1 mL) was exchanged into buffer A (10 mM Tris–HCl [pH 7.4]/50 mM NaCl by use of a NAP-10 column (Pharmacia), and the sample was applied to a Q-Sepharose column (1-mL HiTrap QXL (Pharmacia) pre-equilibrated with buffer A/50 mM NaCl at a rate of 1 mL/min. The column was washed with buffer A until the absorbance at 280 nm returned to baseline. Proteins were eluted with a step gradient of NaCl in 50-mM increments until 500 mM NaCl was reached. The column was then washed with buffer A/1 M NaCl. All fractions were concentrated and exchanged into 1x PBS before testing for antiangiogenic activity. Components with antiangiogenic activity were eluted between 300 mM NaCl and 400 mM NaCl. Preparative scale separation was performed by applying treated FFP to a 20-ml HiPrep 16/10 QXL column (Pharmacia). The column was washed extensively with buffer A/300 mM NaCl. Absorbed proteins were eluted from the column sequentially with buffer A/400 mM NaCl and buffer A/1 M NaCl. All fractions were concentrated, exchanged into 1x PBS, and stored at −20 °C for further use.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blot Analysis

Protein samples diluted with 1x SDS (Boston BioProducts, Inc., Ashland, MA)/40 mM dithiothreitol were separated by SDS–PAGE in 4%–20% gels (pre-cast gels; Bio-Rad), followed by electroblotting onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 2% bovine serum albumin in Tris-buffered saline/Tween 20 (TTBS: 100 mM Tris, 100 mM NaCl, and 0.1% Tween-20) for 1 hour, the PVDF membrane was incubated overnight with the polyclonal angiostatin antibody.
(2 μg/mL). After washing with TTBS, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL; 1:5000 dilution) for 1 hour. The protein bands were detected by use of SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Statistical Analysis

Means were compared by use of a Student’s t test analysis (data analyzed met all criteria for Student’s t test). The results of the counts for vascular density and cell proliferation are expressed as means with 95% CIs. Differences were considered statistically significant at P<.05. All statistical tests were two-sided.

RESULTS

Inhibition of In Vitro Angiogenesis by Treatment of Human Plasma

In vitro angiogenesis was assessed by endothelial cell tube formation and endothelial cell proliferation. To assess tube formation, 4 × 10⁴ HUVECs in the presence of untreated or treated FFP and full endothelial cell growth medium (EGM2-MV) were cultured on Matrigel-coated plates. HUVECs in full endothelial cell growth medium but without FFP were used as a negative control. After 12–16 hours of incubation, cultures containing treated FFP had less angiogenesis than cultures containing untreated FFP, as demonstrated by inhibition of endothelial cell tube formation (Fig. 1, C–E, and quantitative analysis, J). Angiogenesis of cultures incubated with rt-PA or captopril alone or in combination (Fig. 1, F–H), in the absence of plasma, was equivalent to that in the control cultures. Angiogenesis of cultures increased as the concentration of treated FFP was reduced from 20% to 1% (Fig. 1, C–E). Similar data were obtained when plasma from several patients with cancer was treated in vitro with rt-PA or urokinase and captopril and added to cultures of HUVECs (data not shown).

Additionally, the effects of treated FFP were assessed on cell proliferation by use of HUVECs and HMVEC-Ls. A higher level of cell proliferation was stimulated by untreated FFP than in baseline control cultures without FFP (100%) (for HUVECs, 333% of control [95% CI = 320% to 346%] and, for HMVEC-Ls, 200% of control [95% CI = 180% to 220%]). The results of the counts for vascular density and cell proliferation are expressed as means with 95% CIs. Differences were considered statistically significant at P<.05. All statistical tests were two-sided.

Fig. 3. Matrigel plug assay. Inhibition of in vivo angiogenesis by systemic administration of recombinant tissue plasminogen activator (rt-PA) and captopril was measured by the Matrigel plug. Five- to six-week-old male C57/BL6 mice were injected subcutaneously in the left lower abdominal wall with 0.5 mL of unpolymerized Matrigel supplemented with basic fibroblast growth factor (bFGF; 500 ng/mL) for the stimulated control and treatment groups and with an equivalent volume of sterile phosphate-buffered saline (PBS) for the unstimulated (no bFGF) control group. Three mice per group were treated for 10 days with rt-PA (60 μg/day, subcutaneously) and captopril (150 μg/day, intraperitoneally), subcutaneous rt-PA alone with an equivalent volume of intraperitoneal PBS, intraperitoneal captopril (150 μg) with an equivalent volume of subcutaneous PBS, or equivalent volumes of PBS (subcutaneously or intraperitoneally). On day 10, mice were killed. The Matrigel plugs were removed, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. A) Quantitation of in vivo neovascularization assessed with the Matrigel plug assay. Three plugs per group (one plug per mouse) were examined. The total number of microvessels containing red blood cells from 10 high-power fields was counted and averaged. Error bars = 95% confidence intervals. B–E) Representative light micrographs of sectioned Matrigel plugs. Bars = 50 μm. B) Unstimulated (no bFGF) control, corresponding to bar “no bFGF” in panel A. C) PBS-mock treated group (bar “bFGF” in panel A). D) Group treated with rt-PA and captopril (bar “bFGF + t-PA/captopril” in panel A). E) Group treated with captopril alone (bar “bFGF + captopril” in panel A). Arrowheads = microvessels.
Treated FFP, in contrast, decreased the stimulation induced by untreated FFP (for HUVECs, 213% of control [95% CI = 207% to 219%] and, for HMVEC-Ls, 121% [95% CI = 114% to 127%]; this represents a 36% and 41% inhibition of proliferation, respectively, compared with control untreated plasma; Fig. 2, A and B). When treated or untreated FFP was added to cultures of non-endothelial cells (IMR-90 and HRE), cell proliferation was essentially not affected (data not shown).

**In Vivo Antiangiogenic Effects of Systemic Administration of rt-PA and Captopril**

Antiangiogenic activity of plasma in vivo was assessed in mice with the Matrigel plug assay. Mice were injected with Matrigel and then treated with rt-PA and captopril. The Matrigel plugs of all groups of mice (except the unstimulated control) contained bFGF as a proangiogenic stimulant. None of the groups developed treatment-associated adverse events. Ten days after Matrigel injection mice were killed, and the plugs were analyzed for angiogenesis by measuring the number of endothelial microvessels formed that contained red blood cells. Mice treated with both rt-PA and captopril had substantially lower angiogenesis (i.e., fewer microvessels; 49 microvessels/10 high-power fields, 95% CI = 33 to 65) than those treated with PBS (93 microvessels/10 high-power fields, 95% CI = 83 to 103), rt-PA alone (67 microvessels/10 high-power fields, 95% CI = 58 to 76), or captopril alone (84 microvessels/10 high-power fields, 95% CI = 79 to 89) (Fig. 3, A).

**Fig. 4.** Human umbilical vein endothelial cell tube formation and proliferation assays of plasma from patient 1. Patient 1 was treated with captopril (25 mg by mouth three times a day) for 1 week and then received four cycles, each with a higher dose of recombinant tissue plasminogen activator (rt-PA). Plasma obtained 4 hours into the infusion was used to measure tube formation and cell proliferation; cells were cultured in endothelial cell basal medium containing 1% fetal bovine serum (FBS). When the patient’s plasma was present, cell cultures contained 10% patient’s plasma. A–E) Representative micrographs of the effects of the patient’s plasma on the endothelial cell tube formation assay. Bar = 250 μm. A) Control tube formation induced by the patient’s plasma before the treatment. B) Tube formation after the first rt-PA dose of 0.015 mg/kg/h. C) Tube formation after the second rt-PA dose of 0.02 mg/kg/h. D) Tube formation after the third rt-PA dose of 0.03 mg/kg/h. E) Tube formation after the fourth rt-PA dose of 0.035 mg/kg/h. F) Quantitative analysis of the total tube lengths in panels A–E. G) Inhibition of endothelial cell proliferation with plasma obtained from patient 1 who was treated with rt-PA and captopril. The control culture contained 1% FBS and no plasma; other cultures contained 10% plasma from the patient obtained at the indicated time after the perfusion started. Baseline (bar B) was plasma from patient 1 before the rt-PA infusion began. Plasma was obtained 2 hours into the treatment and then as indicated. Data are the average of triplicate experiments. Error bars = 95% confidence intervals. P < 0.01 (baseline versus 12-hour plasma sample; two-sided Student’s t test).
Induction of Antiangiogenic Activity in the Plasma of Patients Treated With rt-PA and Captopril

Plasma from cancer patient 1 who was treated with captopril and four sequential 12-hour infusions of rt-PA at 0.015, 0.02, 0.03, and 0.035 mg/kg/h was assessed for antiangiogenic activity by its effect on endothelial cell Matrigel tube formation and on cell proliferation. The plasma obtained from patient 1 during infusions of rt-PA at 0.02, 0.03, and 0.035 mg/kg/h had substantial antiangiogenic activity in the HUVEC tube formation assay (Fig. 4, C), and the antiangiogenic activity was slightly higher in plasma obtained during the infusions with higher concentrations of rt-PA (45%, 46%, and 53% inhibition of tube formation from dose levels 2, 3, and 4, respectively, compared with that by pretreatment plasma (control) [Fig. 4, B–E, and F for quantification]). Plasma obtained from patient 1 during the 12-hour infusion also statistically significantly inhibited HUVEC proliferation (67% inhibition; \( P = .01 \), t test) compared with plasma obtained from patient 1 before treatment (Fig. 4, G). This effect lasted for up to 48 hours after the start of the infusion and gradually trended toward baseline over the ensuing 6 days.

Two other cancer patients were treated with rt-PA and captopril as part of an ongoing phase 1 trial for patients with advanced cancer. The Matrigel tube formation assay was performed with plasma obtained during the infusion of patients 2 and 3, and tube lengths were measured. As shown in Fig. 5, A and B, tube formation was moderately inhibited with plasma obtained from both patients during the infusion, one by 25% and the other by 12% (Fig. 5, B). In both patients, angiogenic activity returned to baseline 3 days after the infusion was stopped.

Contribution of Angiostatin to the Antiangiogenic Effects of Treated Plasma

Angiostatin is generated when plasminogen is incubated with plasminogen activators and sulfhydryl donors (17). To determine the contribution of angiostatin to the antiangiogenic effects observed with treated FFP, human angiostatin and treated FFP were tested in Matrigel tube formation assays. Medium containing 10% treated FFP (Fig. 1, D) should contain angiostatin at approximately 10 \( \mu \)g/mL, if full conversion of plasminogen (200 \( \mu \)g/mL in 100% plasma) to angiostatin is assumed. Angiostatin at 10 \( \mu \)g/mL (Fig. 6, A) or at 50 \( \mu \)g/mL (data not shown) essentially did not inhibit tube formation, but 10% treated FFP strongly inhibited tube formation.

Next, affinity chromatography using lysine–Sepharose was performed on treated FFP. Western blot analysis demonstrated that angiostatin had been removed from treated plasma (Fig. 6, E; compare lane 4 versus lane 3). When the tube formation assay was used to assess activity, the antiangiogenic activity retained by angiostatin-depleted FFP (flow-through) (Fig. 6, B) was similar to that of nondepleted treated FFP (angiogenesis was inhibited 64.3% [95% CI = 46.4% to 82.2%] before depletion versus 65.1% [95% CI = 53.8% to 76.4%] after depletion of angiostatin). Untreated plasma was the control for this assay. The lysine-bound fraction (containing angiostatin) had a mild inhibitory effect in the Matrigel tube formation assay (angiogenesis was inhibited 18.8% [95% CI = 15.3% to 22.3%] by the lysine-bound fraction versus 65.1% [95% CI = 53.8% to 76.4%] by flow-through fraction; Fig. 6, C).

To confirm this observation, treated FFP was immunodepleted of angiostatin with monoclonal antibodies against human angiostatin, as shown by western blot analysis (Fig. 6, E, lane 5). The antiangiogenic activity of angiostatin-immuno-depleted treated FFP was retained, as assessed by the Matrigel tube formation assay (angiogenesis was inhibited 63.7% [95% CI = 50.9% to 76.5%] after immunoprecipitation, Fig. 6, D).

Finally, treated FFP was subjected to ion-exchange Q-Sepharose chromatography, and three fractions were obtained (the flow-through after loading at 150 mM NaCl combined with a wash at 300 mM NaCl, the eluate at 400 mM NaCl, and the wash at 1 M NaCl). Each fraction was tested for antiangiogenic activity in the Matrigel HUVEC tube formation assay. Antiangiogenic activity was detected in the fraction eluted at 400 mM NaCl (Fig. 7, B) but not in the flow-through fraction (Fig. 7, A) or the 1 M NaCl wash fraction (Fig. 7, C). Western blot analysis of the three fractions showed that most of the angiostatin was in the flow-through but that some could also be detected in the 400 mM fraction (Fig. 7, D). Thus, angiostatin and antiangiogenic activity clearly could be separated by affinity chromatography, immunodepletion, or ion-exchange chromatography.
Contribution of Angiostatin to the Antiangiogenic Effects of Plasma From the Treated Patient

To determine whether angiostatin was responsible for the antiangiogenic effects seen in vivo with the plasma from patient 1 who was treated with rt-PA and captopril, plasma was obtained 4 hours and 8 hours into the infusion with rt-PA at 0.02 mg/kg/h, and angiostatin was immunoprecipitated with a monoclonal antibody against angiostatin. As shown by western blot analysis, high levels of angiostatin were detected in the plasma before immunoprecipitation, and angiostatin was removed by immunoprecipitation (Fig. 8, F). Angiostatin-depleted plasma retained its antiangiogenic activity, as shown by inhibition of tube formation (Fig. 8, compare B with C and D with E).

DISCUSSION

Our report is the first, to the best of our knowledge, to show that the antiangiogenic activity of human plasma can be increased by treatment with rt-PA and captopril and that the antiangiogenic effects are independent of angiostatin. A goal of this study was to explore whether the antiangiogenic activity of whole human plasma could be increased. Because plasma is rich in coagulation proteins and cytokines—some of which regulate angiogenesis (15,35)—any antiangiogenic effect observed after the treatment would represent a change in the balance between positive and negative regulators of angiogenesis. Soff (36) recently reported that angiostatin could be induced in vivo in a patient who received rt-PA and captopril.

In vitro treatment of human FFP with rt-PA and captopril substantially increased the in vitro antiangiogenic activity of the plasma, as assessed by the Matrigel tube formation assay (Fig. 1) and by endothelial cell proliferation (Fig. 2). We extended these in vitro findings to an in vivo setting by showing decreased neovascularization in the Matrigel plugs of mice treated with rt-PA and captopril (Fig. 3). Moreover, we treated three cancer patients with a combination of captopril and low-dose rt-PA and observed the induction of an antiangiogenic effect in their plasma (Figs. 4 and 5). Patient 1 received captopril and four doses of rt-PA, each at a successively higher concentration, and the antiangiogenic activity of her plasma increased with higher doses of rt-PA. Patients 2 and 3 received captopril and one dose of rt-PA at 0.015 mg/kg/h, which was equal to the lowest dose given to patient 1, and the antiangiogenic activity of their plasma increased slightly. These two patients were part of the first patient cohort in a phase I trial that tested rt-PA and captopril. Because the protocol did not allow intrapatient dose escalation, we could not test higher rt-PA doses in patients 2 and 3, which may explain the lower levels of antiangiogenic activity detected in the plasma of these patients compared with that of patient 1.

The fact that the plasma of patients treated with rt-PA and captopril inhibited endothelial cell proliferation and capillary tube formation suggests that a biologically relevant level of an-
angiostatin activity can be induced at clinically tolerable doses of rt-PA and captopril. Moreover, the finding in the Matrigel plug assay that systemic administration of rt-PA and captopril to mice decreased neovascularization further indicates that the effects induced may be biologically important.

A novel finding that distinguishes our results from those of Gately et al. (17) and Soff (36) is that the antiangiogenic activity in FFP treated with rt-PA and captopril was independent of angiostatin. Pure angiostatin at 10 μg/mL and 50 μg/mL did not substantially inhibit tube formation (Fig. 6, A, and data not shown). Lysine–Sepharose affinity depletion of angiostatin from FFP treated with rt-PA and captopril did not reduce the antiangiogenic activity in the treated FFP (Fig. 6, B). Treated FFP retained antiangiogenic activity after immunoprecipitation of angiostatin (Fig. 6, D). Ion-exchange chromatography of treated FFP demonstrated that most of the antiangiogenic activity could be separated from angiostatin (Fig. 7).

The fact that angiostatin did not play a major role in the antiangiogenic effects of the treated plasma was unexpected. Thus, other antiangiogenic components must be generated by the treatment with rt-PA and captopril that are separable from and may be more potent than angiostatin (Figs. 6 and 7). That angiostatin did not inhibit tube formation in our assay may be related to the conditions of the assays used. In our assays (for ex vivo treatment of plasma), we resuspended the cells in full endothelial cell growth medium that is rich in multiple endothelial cell growth factors. Reports that demonstrated inhibition of tube formation by angiostatin used a less rich medium of VEGF alone, bFGF alone, or low concentrations of serum (37,38).

What are the antiangiogenic compounds whose activity was observed in these studies? In the absence of FFP, rt-PA or captopril, alone or in combination, did not produce substantial antiangiogenic activity in vitro (Fig. 1). Consequently, the activity could arise from proteolytic cleavage of several plasma proteins by rt-PA, either by the plasminogen/plasmin pathway (i.e., angiostatin generation) or by a plasminogen-independent pathway. For example, urokinase and t-PA directly cleave other substrates present in plasma, such as fibrinogen or fibronectin (39,40). In vivo, t-PA might be localized preferentially in the tumor stroma, as are plasminogen and fibrin, and might cleave extracellular matrix proteins to generate antiangiogenic peptides either directly or by activating a proteolytic cascade (e.g., the activation of matrix metalloproteinases by plasmin) (19,41). Alternatively, a (mild) systemic fibrinolytic state induced by rt-PA in plasma (in vitro and in vivo) may increase the antiangiogenic activity of other plasma components, such as proteins of the coagulation cascade.

The increased antiangiogenic activity of the plasma from patient 1 persisted up to 36 hours after the infusion was stopped (Fig. 4, G). The length of this effect was unexpected. Because rt-PA is rapidly cleared from plasma (32), these antiangiogenic effects were probably not mediated directly by rt-PA but must have been mediated by newly generated molecule(s) with a relatively long half-life.

Our studies raise a number of questions. First, is the antiangiogenic effect specific for rt-PA? In the tube formation assay, the antiangiogenic activity of plasma (both FFP and plasma from cancer patients) treated with urokinase and captopril was similar to that of plasma treated with rt-PA and captopril (Merchan JR,
Moreover, we have observed that urokinase-treated plasma, but not rt-PA-treated plasma, induces apoptosis in cow pulmonary arterial endothelial cells and HMVEC-L cells, suggesting that urokinase may have additional substrates in the plasma whose cleavage produces these activities.

Second, what is the role of captopril in the induction of antiangiogenic activity? When we compared the numbers of microvessels in Matrigel plugs from mice treated with rt-PA and captopril with those from mice treated with rt-PA alone, captopril appeared to have made a small but probably real contribution to the antiangiogenic activity observed. In addition to stimulating generation of angiostatin, captopril may have inherent antiangiogenic effects that could be additive to or synergistic with the effects of rt-PA. Antiangiogenic properties of captopril have been reported (41), but the concentrations of captopril used for in vitro inhibition of angiogenesis were in the millimolar range. Because of its inhibitory effect on the angiotensin-converting enzyme (ACE), captopril may alter the balance between endothelial t-PA and PAI-1 (42). ACE generates angiotensin, an important regulator of endothelial PAI-1 (43) production, and degrades bradykinin, one of the most potent stimuli for synthesis and secretion of t-PA (44,45). Captopril decreases the expression of PAI-1 in vitro and in vivo (46,47), which might favor plasminogen activators (t-PA) over inhibitors (PAI-1) in the plasma and in the tumor stroma and, thus, enhance the series of proteolytic events that generate antiangiogenic molecules.

In the tumor microenvironment, rt-PA-generated plasmin (from plasminogen) could trigger a series of proteolytic events leading to degradation of the tumor matrix. During the initial stages of tumor angiogenesis, the formation and deposition of fibrin produce a favorable environment for new vessel formation (46–49). We hypothesize that in vivo rt-PA may activate fibrin-bound plasminogen and enhance degradation of tumor stroma (and fibrin in particular), which would impede new vessel formation. This hypothesis may explain why tumors that overexpress t-PA are associated with fewer metastases in preclinical models (27) and appear to have a better prognosis (improved

**Fig. 8. Immunodepletion of angiostatin from plasma and antiangiogenic activity.** Plasma was obtained from patient 1 treated with recombinant tissue plasminogen activator (rt-PA) and captopril at 4 and 8 hours into the rt-PA infusion (second dose level = 0.02 mg/kg/h) and was immunodepleted with a monoclonal antibody against angiostatin. A) Pretreatment (baseline) plasma. B) Plasma obtained 4 hours into rt-PA infusion. C) Immunodepleted plasma obtained 4 hours into rt-PA infusion. D and E) Plasma obtained 8 hours into rt-PA infusion, before (D) and after (E) immunoprecipitation. Bars = 250 μm. F) Western blot of patient’s plasma before and after immunoprecipitation to demonstrate the immunodepletion of angiostatin. Plasma samples were diluted 1:100, and 5 μL of the diluted samples was applied to each lane. Lane 1 = plasma obtained 4 hours into the rt-PA infusion and before immunoprecipitation; lane 2 = plasma obtained 4 hours into rt-PA infusion after immunoprecipitation; lane 3 = plasma obtained 8 hours after rt-PA infusion before immunoprecipitation; lane 4 = plasma obtained 8 hours after rt-PA infusion after immunoprecipitation; lane 5 = baseline plasma before rt-PA infusion. The 50-kd molecular weight marker is indicated.
metastasis-free survival and overall survival) in patients with breast cancer and melanoma (28, 29).

In summary, human plasma may serve as a rich source of antiangiogenic activity. This activity can be induced at clinically achievable concentrations of rt-PA and captopril, suggesting that rt-PA and captopril should be investigated further as a therapy for cancer and other angiogenesis-dependent disorders. Studies to identify the molecules with antiangiogenic activity generated by this treatment and a clinical trial to evaluate the biologic effects of the treatment in patients with cancer are underway.

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NOTES

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