In vivo Generation of Angiostatin Isoforms by Administration of a Plasminogen Activator and a Free Sulfhydryl Donor: A Phase I Study of an Angiostatic Cocktail of Tissue Plasminogen Activator and Mesna

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Abstract

Purpose: Angiostatin$_{4.5}$ (AS4.5), the endogenous human angiostatin, is derived from plasminogen in a two-step process. A plasminogen activator converts plasminogen to plasmin, then plasmin undergoes autoproteolysis to AS4.5. A free sulfhydryl donor can mediate plasmin autoproteolysis. To translate this process to human cancer therapy, we conducted a phase I trial of administration of a tissue plasminogen activator (tPA) with a free sulfhydryl donor (mesna).

Patients and Methods: Fifteen patients with advanced solid tumors were treated. The dose of tPA was escalated (cohorts; 1, 2, 3, 5, and 7.5 mg/h for 6 hours). Mesna was administered as a 240 mg/m^2 bolus followed by an infusion of 50 mg/h, concurrent with tPA. Both tPA and mesna were administered 3 consecutive days every 14 days.

Results: No dose-limiting toxicity was observed. Two AS4.5 isoforms were generated, Lys-AS4.5 and Glu-AS4.5. Mean baseline Lys-AS4.5 level was 20.4 nmol/L (SE, 2.9). In the 5 mg/h tPA cohort, Lys-AS4.5 levels increased by an average of 143% or 24 nmol/L (SE, 4.9) above baseline. Glu-AS4.5 (M_r ~ 62,000) was also generated (additional 77 amino acids at amino terminus compared with Lys-AS4.5). Glu-AS4.5 level at baseline was undetectable in four of five patients in the 5 mg/h tPA cohort, but at end of infusion, was ~67 nmol/L (SE, 20). Two patients in the 5 mg/h tPA cohort experienced decreases in tumor markers with treatment, although no clinical objective responses were observed.

Conclusion: This study shows that *in vivo* generation of AS4.5 is safe in humans and may provide a practical approach to achieve antiangiogenic therapy.

Angiogenesis, the process of new blood vessels sprouting from existing vessels, has been intensely studied because Folkman postulated that the development of a neovasculature is required for tumors to grow and metastasize and that inhibition of this process could provide a novel, and theoretically, nontoxic means to suppress cancer growth (1, 2). Because angiogenesis inhibition targets the tumor vascular endothelial cells and not the tumor cells directly, an angiogenesis inhibitor could be active against a broad range of tumor types. Furthermore, because the vascular endothelial cells are genetically normal, it has been suggested that resistance would be less likely to develop after repeated exposure to angiogenesis inhibitors (3). To date, such inhibitors have not been shown to be cytotoxic and thus have been well tolerated. However, despite the great theoretical potential of this treatment modality based on animal models, angiogenesis inhibition has achieved only limited success in the treatment of human cancer.

Angiostatin, a potent angiogenesis inhibitor, is an internal, kringle-containing fragment of plasminogen, which was first identified in 1994 by O'Reilly et al. (4). Angiostatin is found in the urine and serum of tumor-bearing mice, suppresses angiogenesis, and slows the growth of lung metastases of the Lewis lung carcinoma tumor model in mice (4). Human and murine plasminogens contain five kringle domains, defined by their lysine-binding regions, which serve to localize plasminogen or plasmin to their enzymatic substrate (5). Each kringle contains three disulfide bonds that are essential for function. As originally described, murine angiostatin contains three or four of the five kringle domains (4). Several investigators have described different mechanisms by which plasminogen may be proteolytically cleaved to form angiostatin or an angiostatin-like protein (4, 6–15). These different proteolytic processes

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generate proteins with different amino acid contents, different amino or carboxy termini, different tertiary structures, and different antiangiogenic properties (15–17). The mouse angiostatin, originally described by O'Reilly et al. (9), is generated by plasminogen cleavage by matrix metalloproteinase-2. Many of the angiostatin isoforms, although possessing antiangiogenic activity, have not been confirmed to exist *in vivo* in physiologic or pathophysiologic states.

We reported that in a purified, cell-free system, human plasminogen is cleaved to form angiostatin in a two-step process. First, plasminogen is cleaved by a plasminogen activator, urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA), to yield plasmin. Second, plasmin undergoes autoproteolysis to yield angiostatin (10, 11). Similar results were reported by Falcone et al. (12), who noted that plasmin autoproteolysis on the surface of activated murine macrophages yielded angiostatin or angiostatin-like antiangiogenic fragments, and by Stathakis et al. (13, 14), who noted the role of disulfide reduction within kringle 5 to yield angiostatin. The angiostatin isoform resulting from the plasminogen activator/free sulfhydryl donor reaction is called angiostatin$_{4.5}$ (AS4.5) as this isoform includes kringles 1 to 4 and 85% of kringle 5 (amino acids Lys^{78} through Arg^{530} of human plasminogen; refs. 10, 14, 15). In this paper, this isoform is called Lys-AS4.5, specifying the amino terminus. We have recently reported that plasmin autoproteolysis to AS4.5 may occur on the surface of cancer and other cells, mediated by binding to β-actin (18), and previously, we showed that plasmin autoproteolysis may be mediated pharmacologically by a small molecule free sulfhydryl donor, such as captopril, *N*-acetyl-L-cysteine, or others (10). We also have reported that AS4.5 (Lys-AS4.5) was present at low levels in normal human plasma (15).

Therefore, we hypothesized that concurrent administration of a plasminogen activator and a free sulfhydryl donor (called an angiostatic cocktail) would result in conversion of plasminogen to AS4.5. Before human trial with this drug combination, we tested our hypothesis treating human plasma *ex vivo* with uPA or tPA and several free sulfhydryl donors (captopril, mesna, *N*-acetyl-L-cysteine). Treatment of human plasma *ex vivo* with the plasminogen activator/free sulfhydryl donor cocktail did result in conversion of plasminogen to Lys-AS4.5, despite the presence of physiologic inhibitors of plasminogen activators and plasmin in plasma. In addition to Lys-AS4.5, treatment of plasma with the angiostatic cocktail also resulted in generation of Glu-AS4.5, with $M_r \sim 62{,}000$. Based on amino terminal sequencing and immunoblot with the 3641 antibody to the amino-terminal peptide of Glu-plasminogen, Glu-AS4.5 has been identified as an angiostatin isoform with the same amino terminus as Glu^1-plasminogen, corresponding to an additional 77 amino acids at the amino terminus compared with the originally described Lys-AS4.5. The free sulfhydryl donor alone had no effect whereas the plasminogen activator alone resulted in only trace levels of Glu- and Lys-AS4.5.[4] These results supported the hypothesis that a combination of a plasminogen activator and a free sulfhydryl donor could be used to directly generate AS4.5 in human plasma *in vivo*. Several cancer patients received the angiostatic cocktail on a "compassionate use" basis, and were reported in abstract form (15, 19). One experienced a 17 months complete remission from relapsed, refractory Ewing's sarcoma, but her plasma was not available for analysis.

To translate the laboratory experience safely to human cancer therapy, we did a phase I trial of escalating dosages of tPA infusions concurrent with mesna. Whereas tPA has greater fibrin specificity than uPA for the activation of plasminogen to plasmin, tPA was chosen as the plasminogen activator as urokinase had been removed from commercial use due to manufacturing problems. Based on Food and Drug Administration concerns of the hypotensive effects of captopril, mesna was chosen as the free sulfhydryl donor. Patients were monitored to determine the tolerability and dose and schedule of these pharmacologic agents, and patient plasma was evaluated for evidence of *in vivo* generation of AS4.5. In the study, we show a dose-dependent increase in both Lys-AS4.5 and Glu-AS4.5 levels as well as safety and report clinical outcomes.

Materials and Methods

Immunoblot of human plasma. Immunoblots of treated plasma were done as previously described for plasminogen and angiostatin (10, 11). The primary antibody was GMA086 (Green Mountain Antibodies, Burlington, VT) to the plasminogen kringle domains and was directly conjugated to alkaline phosphatase to avoid the artifacts introduced by use of a secondary antibody (i.e., rabbit antimouse IgG-alkaline phosphatase) that would cross-react with the human IgG in the plasma samples. The concentration of the AS4.5 isoforms generated was calculated by comparison of the sample AS4.5 signal with signal from pure Lys-AS4.5 run in parallel by densitometer. VAP antibody to the plasminogen kringle domains was used with comparable results to GMA086 (VAP antibody was kindly provided by V.A. Ploplis and F. Castellino, University of Notre Dame, Notre Dame, IN). Additional immunoblots used the 3641 antibody (American Diagnostica, Inc., Greenwich, CT) specific for the initial 77-amino-acid domain of plasminogen. An ELISA could not be used as there is no antibody available to differentiate human angiostatin from human plasminogen in plasma. Further, the immunoblot allowed for measurement of the relative amounts of the two AS4.5 isoforms generated and confirmation that no other angiostatin isoform was present.

Patient selection. Fifteen patients were enrolled from two sites of the Robert H. Lurie Comprehensive Cancer Center of Northwestern University. All patients gave written informed consent in accordance with federal and institutional review board guidelines. Before study entry, patients with incurable solid tumors were required to have failed at least one standard therapy or have been judged not to be a candidate for such. No therapy for cancer may have been administered in the 4 weeks before entry, with the exception of palliative radiation therapy to a nonmeasurable or nonevaluable lesion. Patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 2. Minimum laboratory requirements for study entry included WBC count >3,000/mm^3, a platelet count >100,000/mm^3, serum creatinine <2.0 g/dL, and liver function tests <5 times the upper limit of normal, a prothrombin time with an International normalized ratio (INR) ≤1.5, and a partial thromboplastin time ≤1.5 times the control. Patients were specifically excluded if they had any history of intracranial metastases, arteriovenous malformations or aneurysms, or any sites of active bleeding or hemorrhage within 4 weeks of study entry, a history of cerebrovascular accident within 3 months, uncontrolled hypertension, or use of anticoagulants.

[4] G. Soff, H. Wang, D. Cundiff et al., Unpublished data.

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Pretreatment evaluation. All patients were required to undergo radiographs of lung and computed tomography of brain, abdomen, and pelvis, and a bone scan within 6 weeks of study entry. A comprehensive history and physical examination, blood tests including a complete blood count and differential, comprehensive chemistry panel, coagulation parameters as noted above, serum pregnancy test (for females still menstruating), urinalysis, and study laboratory parameters were required within 3 weeks of study entry. On-study evaluations were done every other week and included a history and examination, complete blood count and differential, comprehensive chemistry panel, and coagulation tests. Radiographs/scans to follow baseline measurable or evaluable disease were repeated every 8 weeks.

Treatment plan. This study was conducted as a dose-escalation cohort phase I trial, designed to include three to five patients per cohort as per the design proposed by von Hoff et al. (20). As this was a phase I trial, patients were assigned to dose cohorts sequentially without randomization. Patients were treated as outpatients or hospitalized in the General Clinical Research Center in the medical center. The primary objective was to determine the maximum tolerated dosages of a combination of potentially antiangiogenic agents. Secondary objectives were evidence of AS4.5 generation and possible antitumor effect. tPA (activase) was given as a 6-hour continuous infusion, daily for 3 days, repeated every 2 weeks, for 8-week cycles. Successive cohorts received doses of tPA escalated through a predetermined "safe range" of 1, 2, 3, and 5 mg/h delivered for 6 consecutive hours. A single patient was treated at a dose of 7.5 mg/h before tPA drug supply was exhausted and the trial halted before the 7.5 mg cohort could be completed. All patient cohorts received mesna (Mesnex-Bristol Meyers Squibb) as a bolus (240 mg/m^2) immediately before the initiation of the tPA infusion, followed by a 6-hour infusion of the mesna at 50 mg/h concurrent with the tPA. The tPA dose range was felt to be low enough as to not put patients at a high risk of hemorrhagic complications, but still at adequate levels to test the hypothesis of increased generation of endogenous AS4.5. Determination of tolerability for purposes of identification of dose-limiting toxicity was to be made after each 8-week cycle of the mesna/tPA cocktail.

Toxicity and efficacy criteria. Determination of toxicity and dose-limiting toxicities was independently reviewed and verified by clinical trial coordinators of the Robert H. Lurie Comprehensive Cancer Center clinical research office, and the Data Monitoring Subcommittee of the cancer center's protocol review process. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, version 2.0. Patients were evaluable for toxicity assessment after a single dose of study drug was administered. The phase I design for the study was based on the model proposed by Von Hoff et al. (20). Patients were enrolled in cohorts of three. If grade 4 toxicity was observed only in one of these patients, the cohort was expanded by single patients to a maximum of five. Maximum tolerated dose was defined as a dose level at which two of five patients experienced grade 4 toxicity. If >1 patient in three or four patients experienced grade 4 toxicity, the prior lowest dosage would be defined as maximum tolerated dosage.

Patients were evaluable for response after completion of a minimum 8-week exposure to study drug combination. Complete response required disappearance of all evidence of disease considered measurable or evaluable on radiograph or examination for a minimum 4-week period, with resolution of all disease-related symptoms. Partial response required ≥50% decrease in the sum of the products of perpendicular diameters of all measurable lesions identified for outcome at baseline lasting a minimum of 4 weeks with no new lesions identified during this period. Tumor marker changes were supportive of improvement in scans, but were not adequate to determine response alone. Progressive disease required a >25% increase in the sum of the products of perpendicular diameters of all measurable lesions identified for outcome at baseline or from the smallest sum observed after treatment initiation. Time to disease progression was considered the time from the initiation from study treatment to the date of disease progression.

Pharmacokinetic sampling. All patients had plasma obtained to determine levels of AS4.5 on days 1 and 57 (if continuing) of treatment at the following time points: baseline preinfusion, at 2 and 6 hours (end of infusions, time 0) and at 5, 15, 30, 60, 120, and 240 minutes after the end of the infusions, as well as before the infusion start on days 2 and 3.

Statistical methods. A two-factor ANOVA was done with dose and time as the factors. An ANOVA F test was done for each factor. Pairwise comparisons with Bonferroni correction ($P < 0.0167$) were then done to determine which times differed from each other. Data were also analyzed using the Wilcoxon signed rank test with Bonferroni correction for pairwise comparisons of time points.

Results

Patient characteristics. Fifteen patients (8 males and 7 females; 14 Whites and 1 African American) were enrolled in this trial. All patients were eligible and evaluable for toxicity and response. The median age was 54 (range 28-74). Tumor types included renal cell cancer (four), colon cancer (four), melanoma (three), soft tissue sarcoma (two), prostate cancer (one), and urothelial cancer (one). All patients had failed therapy before enrollment. Performance status was Eastern Cooperative Oncology Group 0 or 1 in 10 and 5 patients, respectively.

Toxicity. Overall the therapy was quite acceptable to patients. The median number of cycles of therapy administered per patient was 1 (consisting of four 3-day courses of treatment every other week). Only one patient received <8 weeks of treatment cycle due to grade 4 toxicity. This patient (case 5-2), in the 5 mg/h tPA cohort with advanced hormone refractory prostate cancer, developed a lower extremity deep venous thrombosis and pulmonary embolism requiring anticoagulation and discontinuation of protocol therapy. Thrombosis is not a known toxicity of mesna nor would be expected from the thrombolytic tPA. Duration of therapy ranged from 4 to 16 weeks. Dose-limiting toxicity was determined during the first 4 weeks of protocol therapy for decisions regarding dose escalation. Table 1 shows the spectrum of toxicity experienced by patients while receiving the tPA/mesna combination by dose level.

The therapy was very well tolerated. Dose level cohorts completed and studied included 1, 2, 3, and 5 mg/h (n = 3, 3, 3, 5 per cohort, respectively) of the tPA with fixed mesna dose. The dose of tPA administered was initially limited to 5 mg/h based on institutional review board concerns regarding the potential for life-threatening hemorrhage as a known side effect of tPA, but after completion of the 5 mg/h dose level cohort without dose-limiting toxicity, the study was amended to allow continued escalation. A single patient received tPA at 7.5 mg/h before study discontinuation with no evidence of toxicity. No maximum tolerated dose was established as the 5 mg/h cohort of patients experienced no dose-limiting toxicity that precluded dose escalation.

Grade 3 adverse events were infrequent and often laboratory related and expected, such as prolonged partial thromboplastin time consistent with plasmin generation and fibrinogenolysis. Other grade 3 adverse events were unexpected, such as elevated liver function tests or hypoalbuminemia and were believed to be unrelated to the therapy. Most of the adverse events noted were felt to be unrelated to study drug exposure and more likely secondary to the underlying disease process. Plasminogen

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Table 1. Spectrum of toxicity attributed to the combination of tPA and mesna

Adverse event	1 mg/h			2 mg/h			3 mg/h			5 mg/h				7.5 mg/h		
Grade	1	2	3	1	2	3	1	2	3	1	2	3	4	1	2	3
General																
Asthenia				1	1		2			1	1				1	
Weight loss	1			2												
Pain	1				3		1			1	1	1		1		
Fever				1												
Pulmonary																
Dyspnea					2		2			2						
Cough/hemoptysis				1			1			1						
Neurologic																
Clinical				1						1						
Headache					1		1									
Insomnia							2			1						
Genitourinary																
Hematuria	1			2			1	1		2				1		
Gastrointestinal																
Nausea/vomit				3	2		1	1		2	2					
Anorexia					2		1				2					
Hiccups				1												
Constipation				1							2					
Diarrhea										3					1	
Cardiovascular																
Hypotension				1												
DVT with PE													1			
Infection					1			1	1		2				1	
Skin/mucosa																
Bleeding										2				1		
Rash	1															
Pruritis				1												
Edema										1						1
Laboratory																
Prolonged apt	1		1	1						1						
Elevated LFTs		1				1				1		1				
Anemia	1				3		2			2	2				1	
Thrombocytopenia							1					1				
Hyponatremia				1												
Hypoalbuminemia					1	1	2				1					
Hypocalcemia				1												
Elevated bilirubin					1						1			1		
Elevated creatinine				1										1		

Abbreviations: PTT, partial thromboplastin time; LFT, liver function tests; DVT with PE, deep venous thrombosis with pulmonary embolism.

consumption was <20% compared with baseline in all patients at all dose levels, indicating only partial plasminogen activation to plasmin. No patient experienced clinically significant hemorrhage, the side effect that we were most concerned about before study, although mild skin/mucosal membrane bleeding was common, as was dipstick positive hematuria. One patient had clinically apparent hematuria, which was consistent with known involvement of her urothelium by persistent urothelial cancer and infection.

***Generation of angiostatin 4.5* in vivo.** Plasma samples were drawn at baseline, 2 hours into, and at the conclusion of the 6-hour continuous infusion and at 5, 15, 30, 60, 120, and 240 minutes after the end of the infusions, as well as before the infusion start on days 2 and 3. Using a modified immunoblot assay, Glu-AS4.5 and Lys-AS4.5 levels were measured. Figure 1 illustrates a representative blot from a patient from the 5 mg/h cohort (case 5-4). At 2 hours of infusion, both Lys-AS4.5 and Glu-AS4.5 were detected, as well as the plasmin:α-2-antiplasmin and plasmin:α-2-macroglobulin complexes. At the completion of the infusion, peak Lys-AS4.5 and Glu-AS4.5 levels were observed. Table 2 summarizes the mean increases in the Lys-AS4.5 and Glu-AS4.5 isoforms. In the 1 and 2 mg/h tPA cohorts, there was no significant increases in either AS4.5 isoform. Figure 2A and B illustrates the increases in

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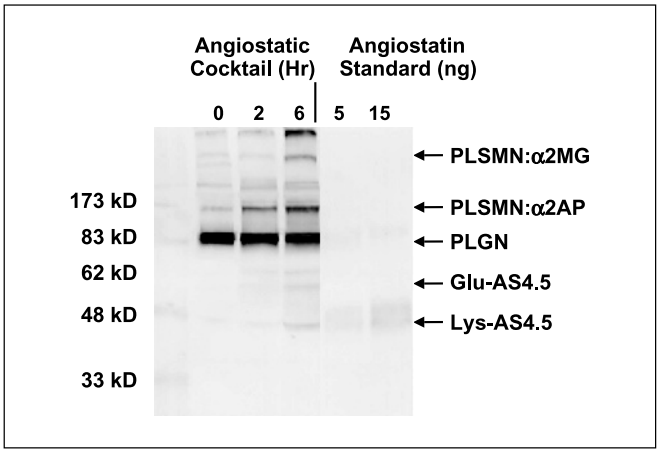

Fig. 1. Immunnoblot of plasma from patient treated in the 5 mg/h tPA cohort (case 5-4). Treatment of this patient resulted in generation of Lys-AS4.5 and Glu-AS4.5, which peaked at completion of the 6-hour infusion. Plasmin (*PLSMN*) complexes with its physiologic inhibitors, α-2-antiplasmin (*α2AP*) and α-2-macroglobulin (*α2MG*), were also observed. The plasminogen band was only slightly reduced indicating that conversion of plasminogen to angiostatin was not maximal. Purified angiostatin (5 and 15 ng) was added in parallel lanes, allowing for densitometric analysis and quantitation of the *in vivo* generated angiostatin.

Lys-AS4.5 and Glu-AS4.5 levels in the individuals from the 3 and 5 mg/h tPA cohorts, and Fig. 2C is the mean levels of those cohorts.

Lys-AS4.5 was detected in pretreatment; baseline samples at ~20.4 nmol/L (SE, 2.9; range, 15-30 nmol/L). The end of infusion Lys-AS4.5 levels ranged from 20 to 41 nmol/L, with a trend for dose dependence. In the 5 mg/h cohort (n = 5), the mean Lys-AS4.5 levels increased by 24.1 nmol/L, from 16.8 nmol/L at baseline to 40.9 nmol/L (SE, 10.4, P = 0.008) at the peak, an increase to ~2.5-fold of baseline levels, and well within the biologically active concentration range (21). The Glu-AS4.5 levels were trace to undetectable at baseline times, and increased to a mean of 5.9, 5.9, and 28.6 nmol/L in the 1, 2, and 3 mg/h tPA cohorts, respectively. In the 5 mg/h tPA cohort (n = 5), there was an increase in the Glu-AS4.5 to a mean of 67.2 nmol/L (SE, 19.1, P = 0.03). Interestingly, the estimated half-lives of the Lys-AS4.5 and the larger Glu-AS4.5 were 2.5 and >12 hours, respectively, indicating that the additional amino acids at the amino terminus of Glu-AS4.5 may result in significantly increased biological half-life and clinical utility.

For both the Lys-AS4.5 and Glu-AS4.5 isoforms, significant increases in peak levels were observed in the 5 mg/h tPA cohort. There also was a trend toward dose-response relationship in the Glu-AS4.5 levels. For Glu-AS4.5, using the ANOVA F-test, there were significant differences across dose (P = 0.049) and across time (P = 0.002). There was significantly more Glu-AS4.5 at the end of infusion versus baseline (P < 0.001). The results from the 1 mg dose differed from the 5 mg dose (P = 0.015) and the 2 mg dose differed from the 5 mg dose (P = 0.025), suggesting a dose response with regard to the tPA.

Although the dose of tPA used was low, plasminogen was confirmed to be converted to plasmin based on the generation of the plasmin:α-2-antiplasmin and plasmin:α-2-macroglobulin complexes. However, the plasminogen levels showed only a modest decrease with a <20% decline in all of the treatment cohorts.

Response. The median survival time for all patients was 11 months. Whereas none of the patients exhibited objective tumor shrinkage, two of the five patients in the 5 mg/h tPA cohort experienced declines in serum tumor markers. In Fig. 3A, the CA 19-9 of a 38-year-old woman with metastatic colon cancer (case 5-4) is shown (see Fig. 1 for the immunoblot of her plasma samples). Treatment administration was associated with a pronounced decline in her serum marker, although her tumor burden measurements did not show a parallel decline by computed tomography imaging. Another patient in the 5 mg/h cohort, with metastatic, hormone refractory prostate cancer (case 5-2), developed a venous thrombosis and pulmonary embolism during the first 8-week cycle and had to be removed from the protocol. Interestingly, he experienced clinical stabilization and then a prolonged gradual decline in the prostate-specific antigen level over 2 years (Fig. 3B). He received no other therapy other than anticoagulation during that period, which could explain his prolonged response.

Discussion

In the past 10 years since angiostatin was first described, much attention has been focused on developing angiogenesis inhibitors for cancer therapy. Whereas efficacy in the treatment of human malignancies has not been proven as quickly as hoped, significant progress has been made in understanding how these factors function and the appropriate methods for use. Bevacizumab (Avastin), a humanized monoclonal antibody to vascular endothelial growth factor, is now approved by the Food and Drug Administration for colorectal cancer therapy and is showing promise in treatment of other malignancies. Angiostatin is one of a series of angiogenesis inhibitors that are internal fragments of other proteins.

Whereas we recently reported that cell surface β-actin can bind plasminogen and plasmin and mediates plasmin

Table 2. Increases in AS4.5 levels in different tPA dose cohorts

Dose of tPA (mg/h)	Increase in Lys-AS4.5 in nmol/L, mean (SE)	Increase in Glu-AS4.5 in nmol/L, mean (SE)
1	3.2 (2.7; NS)	5.9 (4.8; NS)
2	12.2 (11.4; NS)	5.9 (3.5; NS)
3	2.4 (2.3; NS)	28.6 (9.5; P = 0.08)
5	24.0 (10.4; P = 0.008)	67.2 (19.1; P = 0.03)

Abbreviation: NS, not significant.

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autoproteolysis to AS4.5 (18), we also have shown that plasmin autoproteolysis to AS4.5 may be mediated by a small molecule free sulfhydryl donor, such as captopril, mesna, or *N*-acetyl-L-cysteine (10, 15). Therefore, we hypothesized that an angiostatic cocktail, comprising a plasminogen activator and a free sulfhydryl donor, may induce conversion of plasminogen to AS4.5. Before initiating this phase I trial, we showed that treatment of human plasma *ex vivo* with the combination of a plasminogen activator and a free sulfhydryl donor was capable of generating biologically active levels of both Glu-AS4.5 and Lys-AS4.5. Whereas Lys-AS4.5 has been previously described and occurs naturally, Glu-AS4.5 has not been previously described and it is not known if, and under what circumstances, Glu-AS4.5 occurs naturally. The generation of plasmin as an intermediate in this reaction was supported by the generation of complexes of plasmin with α-2-antiplasmin and α-2-macroglobulin (see Fig. 1). Whereas it would have been desirable to test the hypothesis of *in vivo* generation of AS4.5 by the angiostatic cocktail in mice, this was not practical because the commercially available plasminogen activators are human proteins (uPA and tPA) that do not cleave mouse plasminogen.[5] Streptokinase also did not cleave mouse plasminogen. Because plasminogen activators and free sulfhydryl donors are available as Food and Drug Administration–approved drugs, we proceeded to a phase I trial in cancer patients.

Our results confirmed that biologically active levels of both Glu-AS4.5 and Lys-AS4.5 were generated by the combination of

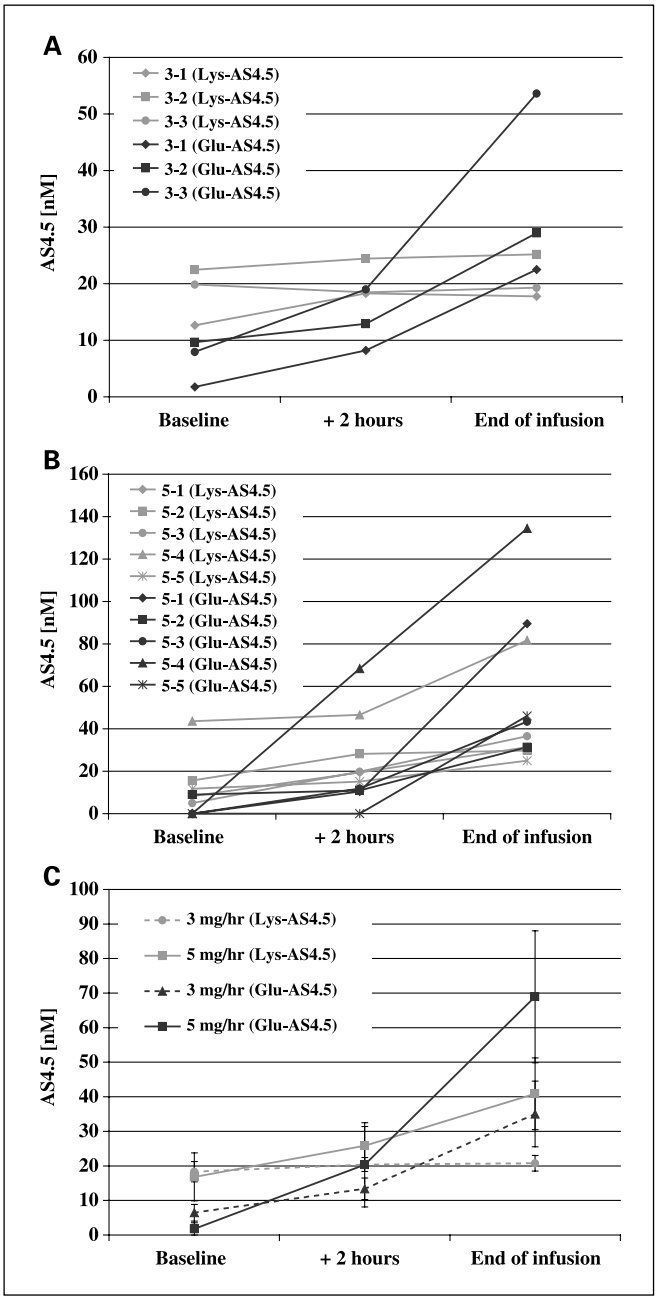

Fig. 2. *In vivo* generation of Lys-AS4.5 and Glu-AS4.5. Lys-AS4.5 and Glu-AS4.5 levels from the three patients in the 3 mg/h tPA cohort (*A*) and the five patients in the 3 mg/h tPA cohort (*B*) are shown. *C, points,* mean; *bars,* SE. The 3 mg/h dose was not sufficient to increase Lys-AS4.5 levels although the mean Glu-AS4.5 levels increased from 6.5 to 35 nmol/L. The 5 mg/h dose resulted in a more pronounced increase in both AS4.5 isoforms. Mean Lys-AS4.5 increased from 16.8 to 40.9 nmol/L and mean Glu-AS4.5 increased from 1.8 to 68.9 nmol/L.

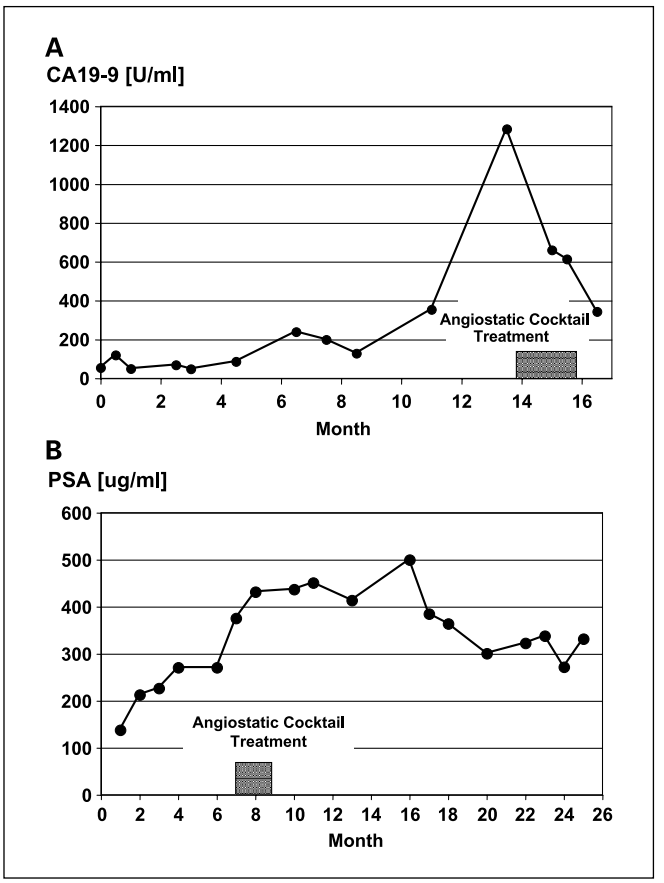

Fig. 3. *A,* tracing of CA19-9 levels in a 38-year-old woman treated in the 5 mg/h cohort (case 5-4). A 38-year-old woman with metastatic colon cancer showed a significant improvement of her CA 19-9 while on study. *B,* prostate-specific antigen tracing of case 5-2, a 64-year-old man with hormone-resistant, metastatic prostate cancer. In the 4 months before the therapy, the prostate-specific antigen increased over 3-fold. Following the treatment with the angiostatic cocktail, the prostate-specific antigen ceased to increase, and gradually declined for over 12 months. He remained clinically stable with no new lesions identified despite no conventional cytotoxic therapy.

[5] J. Henkin, unpublished data.

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tPA and mesna, and that the levels of AS4.5 were a function of the dose of tPA administered. In prior studies, we have reported that AS4.5 potently induces apoptosis of susceptible vascular endothelial cells in culture, with a 50% maximal effect at ~25 nmol/L (22, 23). The levels generated *in vivo* are within the range expected to be biologically active. In this phase I trial, no dose-limiting toxicity was observed. As bleeding was the primary concern, we started with very low doses of tPA and escalated in subsequent cohorts. The study was terminated due to consumption of our available tPA. The minimal decrease in plasminogen levels (<20%) indicates higher doses of tPA are realistic, which would be expected to be associated with further increases in AS4.5.

No objective tumor shrinkage was observed, but two patients of the five in the 5 mg/h tPA cohort exhibited suggestive responses. Case 5-4, a 38-year-old woman with metastatic colon cancer, exhibited a 70% decline of her CA19-9 level while on the angiostatic cocktail. Unfortunately, based on computed tomography scan, her tumor burden did not decrease in the same time period. The most intriguing case was case 5-2, a 64-year-old man with hormone refractory prostate cancer. He developed a proximal deep-vein thrombosis with pulmonary embolism while on the angiostatic cocktail and was removed from the study. His prostate-specific antigen decreased during and gradually after the therapy was discontinued, despite receiving no other chemotherapy or radiation therapy for over 13 months (Fig. 3B). A prolonged clinical response following angiogenesis inhibition was described in mice following repeated cycles of endostatin (24). It is premature to attribute the prolonged disease improvement of this patient to the angiostatic cocktail, yet his response, as well as that of case 5-4, justifies further study.

Our indirect strategy of the *in vivo* generation of AS4.5 by the coadministration of a plasminogen activator and free sulfhydryl donor offers several advantages over the direct approach of production of the recombinant protein. The AS4.5 isoforms are complex proteins consisting of four full kringle domains, plus 85% of kringle 5. Each kringle contains three disulfide bonds and therefore generation of the correctly folded, recombinant protein for human therapy is inefficient and not reliable with current technology. The company EntreMed has produced a smaller angiostatin isoform consisting of kringles 1 to 3, but in clinical trial the angiostatin$_{K1\text{-}3}$ failed to show any partial or complete response (25). However, as previously noted, much of the antiangiogenic activity of the AS4.5 isoforms rests within kringle 5 (15, 17); therefore, it is not appropriate to extrapolate results from the angiostatin$_{K1\text{-}3}$ to effects of AS4.5. The half-life of tPA or uPA is ~5 minutes. In contrast, in a recent clinical trial with recombinant angiostatin$_{K1\text{-}3}$, the half-life of angiostatin$_{K1\text{-}3}$ was 20 minutes (26) shorter than the *in vivo* generated Lys-AS4.5 (~2.5 hours) and Glu-AS4.5 (>12 hours). Another possible advantage of the angiostatic cocktail, compared with administration of recombinant protein, is the possibility that other beneficial activities may be generated *in vivo* by the plasminogen activator and/or free sulfhydryl donor in addition to AS4.5. A recent report independently confirms that there may be antiangiogenic activity with this pharmacologic intervention independent of angiostatin generation (21). Lastly, as we are administering an enzyme and cofactor with an endogenous substrate in plasma (plasminogen) to generate the angiostatin product, it is likely that there is an amplification effect compared with direct administration of the recombinant product.

The mechanism of action(s) of angiostatin-related isoforms remains unclear. Whereas several target or targets have been proposed, it is clear that angiostatin serves to regulate the "angiogenic switch," which serves to maintain the critical balance between proangiogenic and antiangiogenic factors in the tumor microenvironment or the host in general (27). Recent studies have indicated that angiostatin may induce apoptosis of vascular endothelial cells (22, 23, 28). Further, combinations of antiangiogenic agents, including angiostatin, have been shown to have synergistic antitumor effects (29). Until now, the ability to test combinations of investigational agents in humans has been limited by regulatory and practical business hurdles. The recent availability of an approved antiangiogenic, antivascular endothelial growth factor antibody (30, 31), establishes important proof-of-principle for the field in general, but also allows for envisioned combinations of other factors with antivascular endothelial growth factor. The ability to generate *in vivo* the angiostatin isoforms as described herein may also permit novel combinations of antiangiogenic therapy to be tested, e.g., with endostatin, vascular endothelial growth factor antibodies, other small molecules of plasminogen such as kringle 5 alone or angiostatin$_{K1\text{-}3}$, or even radiation/chemotherapy (32, 33), to attempt to validate the laboratory findings (29). Further studies to define the upper limits of plasminogen activator dose that can be safely delivered and exploration of alternative oral free sulfhydryl donors are under way.

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