Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation

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Angiostatin, a kringle-containing fragment of plasminogen, is a potent inhibitor of angiogenesis. The mechanism(s) responsible for the anti-angiogenic properties of angiostatin are unknown. We now report that human angiostatin blocks plasmin(ogen)-enhanced *in vitro* invasion of tissue plasminogen activator (t-PA)-producing endothelial and melanoma cells. Kinetic analyses demonstrated that angiostatin functions as a non-competitive inhibitor of extracellular-matrix (ECM)-enhanced, t-PA-catalysed plasminogen activation, with a K_i of $0.9 \pm 0.03 \mu\text{M}$. This mechanism suggests that t-PA has a binding site for the inhibitor angiostatin, as well as for its substrate plasminogen that, when occupied, prevents ternary complex formation between t-PA,

plasminogen and matrix protein. Direct binding experiments confirmed that angiostatin bound to t-PA with an apparent K_d [$K_d(\text{app})$] of $6.7 \pm 0.7 \text{ nM}$, but did not bind with high affinity to ECM proteins. Together, these data suggest that angiostatin in the cellular micro-environment can inhibit matrix-enhanced plasminogen activation, resulting in reduced invasive activity, and suggest a biochemical mechanism whereby angiostatin-mediated regulation of plasmin formation could influence cellular migration and invasion.

Key words: angiogenesis, $\alpha 2$ -antiplasmin, endothelium, extracellular matrix, tissue-type plasminogen activator.

INTRODUCTION

Regulation of angiogenesis is a fundamental mechanism for control of tumour progression [1–4]. Angiostatin, a kringle-containing polypeptide fragment of plasminogen, is a potent inhibitor of angiogenesis, tumour growth and metastasis [5–9]. Recent studies have demonstrated that plasminogen activation and subsequent plasmin activity are required intermediary processes for direct angiostatin formation by cancer cells [10,11]. Angiostatin can also be generated *in vitro* via limited proteolysis of plasminogen using pancreatic elastase, metalloelastase or matrix metalloproteinase-3, -7 or -9 [12–15]. On the cellular level, angiostatin has been shown to block both endothelial cell migration and proliferation. It has been suggested that the apparent anti-proliferative effect might result from induction of endothelial cell apoptosis [6] or inhibition of cell cycle progression [16]. Although the kringle domains are important in mediating the angiosuppressive properties of angiostatin [17,18], the mechanism by which angiostatin inhibits cellular migration is unknown.

Vascular endothelial cells secrete plasminogen activators (PAs), the function of which is to activate the plasma zymogen plasminogen into the active proteinase plasmin. Plasmin is active against a number of macromolecules present in the basement membrane or provisional tumour stroma, including laminin, fibrin(ogen) and fibronectin [19,20]. Whereas cultured endothelial cells secrete both tissue-type and urinary-type PAs [t-PA and u-PA (or urokinase) respectively], t-PA is the predominant serine proteinase expressed by endothelial cells *in situ* [21]. Furthermore, t-PA activity is required for endothelial cell organization and growth-factor-dependent tube formation *in vitro* [22,23]. In addition, a subset of highly invasive cells, such as melanoma and neuroblastoma, produce t-PA, indicating that t-PA can also initiate pathological connective tissue proteolysis [24–27]. In the

solution phase, t-PA is an inefficient activator of plasminogen; however, the catalytic efficiency of t-PA is substantially enhanced by interaction with numerous macromolecules that function as non-essential cofactors, including fibrin(ogen) [28], intact extracellular matrix (ECM) [29,30] or individual ECM proteins, such as type IV collagen or laminin-1, and thrombospondin [31–33]. The increased pericellular plasmin resulting from this matrix-enhanced plasminogen activation can then facilitate cell migration and invasion [19].

In the present study we have assessed the mechanism by which angiostatin alters the invasive activity of endothelial and melanoma cells. Plasmin(ogen)-enhanced cellular invasion was blocked by both angiostatin and anti-catalytic t-PA antibodies, suggesting that angiostatin might exert its function via interaction with t-PA. Direct binding experiments confirmed the presence of an angiostatin-binding site on t-PA. Kinetic analyses demonstrated that angiostatin functions as a non-competitive inhibitor of matrix-enhanced plasminogen activation by preventing ternary complex formation between t-PA, plasminogen and matrix protein. The data in the present study provide a biochemical mechanism by which angiostatin might regulate plasmin formation in the extracellular milieu, and thereby influence cellular migration and invasion.

EXPERIMENTAL

Proteins

Plasminogen was purified from human plasma by affinity chromatography on L-lysine-Sepharose as described previously [32]. Plasmin was generated by incubating plasminogen (100 μg) with 100 μl of u-PA-Sepharose (Calbiochem, La Jolla, CA, U.S.A.) at 25 °C for 4 h, followed by centrifugation for 3 min at 3000 rev./min in an Eppendorf model 5415C benchtop centrifuge to remove the Sepharose resin. Protein concentrations were

Abbreviations used: $\alpha 2$ -AP, $\alpha 2$ -antiplasmin; BCIP/NBT, 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium; ECM, extracellular matrix; (t/u)-PA, (tissue/urinary)-plasminogen activator; VLK-pNA, D-Val-Leu-Lys-p-nitroanilide.

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determined spectrophotometrically at 280 nm using an $A_{1\text{ cm}}^{1\%}$ value of 16.9 and molecular masses of 92000 and 81000 Da for plasminogen and plasmin respectively. Affinity-purified human angiostatin (Angiogenesis Research Industries, Chicago, IL, U.S.A.) was generated by incubating plasminogen with u-PA in the presence of *N*-acetyl-L-cysteine, followed by repurification on L-lysine-Sepharose as described previously [10]. α 2-Antiplasmin (α 2-AP) was purified by affinity chromatography on plasminogen-Sepharose [34,35], and protein concentration was determined spectrophotometrically using an $A_{1\text{ cm}, 280}^{1\%}$ of 13.0 and a molecular mass of 65000 Da [35]. Two-chain-recombinant t-PA was generously given by Dr. Henry Berger (Glaxo-Wellcome, Research Triangle Park, NC, U.S.A.). Biotinylation of t-PA was performed by incubating with *N*-hydroxysuccinimide-biotin (Pierce, Rockford, IL, U.S.A.) at a final concentration of 0.1 mg/ml for 1 h at 25 °C, followed by extensive dialysis against 20 mM Hepes, pH 7.4. The presence of label was confirmed by Western blotting with alkaline-phosphatase-conjugated streptavidin (Sigma Chemical Co., St. Louis, MO, U.S.A.). Human type IV collagen and murine laminin-1 were purchased from Sigma. The synthetic basement-membrane extract Matrigel, composed predominantly of type IV collagen and laminin-1, was purchased from Becton Dickinson (Bedford, MA, U.S.A.). Goat anti-(human melanoma t-PA), anti-catalytic t-PA antibody (#374B) and u-PA were products of American Diagnostica (Greenwich, CT, U.S.A.). A monoclonal antibody recognizing the kringle 1–3 fragment of plasminogen was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN, U.S.A.). Plasminogen kringle 4, anti-(goat IgG) alkaline phosphatase conjugate and anti-(mouse IgG) alkaline phosphatase conjugate were obtained from Sigma. The alkaline phosphatase substrates *p*-nitrophenyl phosphate and 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) were also products of Sigma.

Cell culture and *in vitro* invasion

Bovine aortic endothelial cells were isolated from calf aorta and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum as described previously [36,37]. B16F10 murine melanoma cells were obtained from Dr. I. J. Fidler (MD Anderson Cancer Center, Houston, TX, U.S.A.) and were maintained in Eagle's minimal essential medium, as described previously [38]. OVCA 429 ovarian carcinoma cells were obtained from Dr. Robert Bast, Jr. (MD Anderson Cancer Center, Houston, TX, U.S.A.), and were cultured as previously described [39]. Invasive activity *in vitro* was assessed by determining the ability of cells to invade Matrigel [40]. Briefly, polycarbonate filters (8- μ m pore size; Becton Dickinson, Franklin Lakes, NJ, U.S.A.) were coated with Matrigel (11 μ g/filter) and placed in a modified Boyden chamber. Cells (1×10^5) were added to the top chamber in serum-free medium, or medium containing angiostatin, plasminogen, anti-tPA antibody or control IgG, and incubated for 6 h (B16F10) or 48 h (bovine aortic endothelial cells). Following incubation, non-invading cells were removed from the top surface of the membrane filter with a cotton swab, and filters were excised and stained with Diff-Quik (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Cells on the lower surface of the filter were enumerated using an ocular micrometer and counting a minimum of six high-powered fields. Each experiment was performed twice with triplicate samples.

Analysis of binding

Qualitative analysis of t-PA binding to angiostatin and plasminogen was obtained by ligand blotting, as previously described

[33]. Briefly, plasminogen (5 μ g) or angiostatin (5 μ g) was electrophoresed on 5–15% gradient gels and electroblotted to Immobilon. Following electroblotting, one membrane was stained with Coomassie Blue for protein identification. Before ligand binding, blots were incubated in 10 mM Tris/HCl, 0.15 M NaCl, 0.05% (v/v) Nonidet P-40, 3% (w/v) BSA for 30 min at room temperature to block non-specific sites. Blots were then incubated with t-PA (3 μ g/ml) overnight at 4 °C. After washing to remove unbound ligand, bound t-PA was detected using goat anti-(human melanoma t-PA) followed by rabbit anti-(goat IgG) alkaline phosphatase conjugate, and the phosphatase substrate BCIP/NBT. In control experiments, binding of u-PA (3 μ g/ml) to immobilized angiostatin was assessed as described above, and bound u-PA was detected using murine anti-(u-PA) followed by an alkaline-phosphatase-conjugated anti-mouse secondary antibody and BCIP/NBT. Non-specific binding was determined by evaluating the interaction of either t-PA or u-PA with immobilized low-molecular-mass markers (Sigma).

Direct binding of t-PA to angiostatin-coated microtitre wells was determined as described previously [33]. Briefly, t-PA (0–100 nM) was added to angiostatin-coated microtitre wells in 10 mM Tris, 0.15 M NaCl, 0.05% (w/v) Tween 20, 3% (w/v) BSA at 37 °C for 2 h. After washing three times, bound ligand was detected using the appropriate primary antibody and alkaline-phosphatase-conjugated secondary antibody with *p*-nitrophenyl phosphate (1 mg/ml in 0.1 M glycine, pH 10.4, 1 mM MgCl_2 , 1 mM ZnCl_2); A_{405} was monitored. Data were corrected for background binding to albumin-coated wells and apparent dissociation constants [$K_d(\text{app})$] were determined by non-linear regression analysis using Sigmaplot (SPSS Inc., Chicago, IL, U.S.A.). Similar experiments were performed to assess the binding of u-PA (0–250 nM) to angiostatin-coated wells using the appropriate primary and secondary antibodies, as described above. Competition binding experiments were also performed by adding biotinylated t-PA (20 nM) to angiostatin-coated microtitre wells in the presence of a 0–100-fold molar excess of unlabelled t-PA. After washing, as described above, bound biotin-labelled t-PA was detected using alkaline-phosphatase-conjugated streptavidin (at a dilution of 1:200), followed by addition of *p*-nitrophenyl phosphate and monitoring A_{405} .

Binding of angiostatin or t-PA to microtitre wells coated with type IV collagen or laminin-1 was also determined, as described previously [33]. Briefly, angiostatin (0–5000 nM) or t-PA (0–100 nM) was added to collagen- or laminin-coated wells in 10 mM Tris, 0.15 M NaCl, 0.05% (w/v) Tween, 3% (w/v) BSA at 37 °C for 2 h. After washing, bound ligand was detected as described above. Data were corrected for background binding to albumin-coated wells and values of $K_d(\text{app})$ were determined by non-linear regression analysis using Sigmaplot. In competition binding experiments, t-PA (20 nM) was incubated with increasing molar ratios of angiostatin (0–25-fold molar excess, 0–500 nM angiostatin), and binding of t-PA was assessed as described above. Experiments were repeated in triplicate.

Plasminogen activation kinetics

The initial rate kinetics of t-PA- and u-PA-catalysed plasminogen activation were determined at 37 °C using a coupled assay [41] to monitor the amidolytic activity of generated plasmin with the synthetic plasmin substrate D-Val-Leu-Lys-*p*-nitroanilide (VLK-pNA; Sigma). Plasminogen (0–200 nM) was incubated in 20 mM Hepes, pH 7.4, in a total volume of 175 μ l with VLK-pNA (0.3 mM). Plasminogen activation was initiated by the addition of t-PA (0.55 nM) or u-PA (0.48 nM), and the resulting plasmin

hydrolysis of VLK-pNA was monitored by measuring the change in absorbance at 405 nm with time using a Molecular Devices Thermomax kinetic microtitre plate reader. Initial velocities (v_i) were calculated from plots of A_{405} against t^2 , as described previously [32]. Reactions were carried out in microtitre wells coated with type IV collagen or laminin-1 in the presence or absence of angiostatin (0–1000 nM). Control reactions were performed in albumin-coated wells. Data were analysed by using the Dixon plot to characterize the kinetic mechanism of inhibition [42,43]. The amidolytic activity of t-PA (5.5 nM) was determined by incubating proteinase with 0.3 mM D-Ile-Pro-Arg-p-nitroanilide (IPR-pNA; Sigma) at 37 °C, and monitoring substrate cleavage by absorbance at 405 nm. Plasmin amidolytic activity was analysed by incubating plasmin (50 nM) with VLK-pNA (0.3 mM) and monitoring absorbance at 405 nm. The effect of angiostatin on t-PA or plasmin amidolytic activity was assessed by incubating t-PA or plasmin with increasing amounts of angiostatin (added in a mol:mol ratio) in the presence of the appropriate proteinase substrate. Kinetic analyses were performed in triplicate, and repeated a minimum of three times.

α 2-AP inhibition studies

Inhibition of plasmin by α 2-AP was analysed by titrating plasmin (50 nM) with α 2-AP (0–100 nM, i.e. up to 2-fold molar excess) by incubating enzyme and inhibitor for 10 min at 37 °C, followed by addition of VLK-pNA and monitoring of the residual plasmin activity. The effect of angiostatin on the inhibitory reaction was assessed by including a 10-fold molar excess of angiostatin (500 nM) and determining the inhibition of activity, as described above. In additional experiments, a constant amount of plasmin (50 nM) and α 2-AP (25 nM) was incubated in the presence of increasing angiostatin (0–2500 nM, i.e. up to a 50-fold molar excess over plasmin), and the remaining plasmin activity was determined by the addition of VLK-pNA, and monitoring A_{405} , as described above. Inhibition experiments were performed in triplicate.

RESULTS

Angiostatin inhibits plasmin(ogen)-enhanced cellular invasion

Angiostatin has been reported to inhibit endothelial cell migration, a component of the angiogenic process [44–46]. To determine whether angiostatin regulates invasion, the ability of endothelial cells to penetrate a synthetic basement membrane (Matrigel) was assessed. In the absence of plasminogen, angiostatin had no effect on endothelial cell penetration of the type IV collagen and laminin-1-rich matrix (Table 1). However, addition of plasminogen and subsequent plasminogen activation by cellular t-PA resulted in a 2.5–3.0-fold increase in invasive activity. Angiostatin effectively blocked the plasmin(ogen)-enhanced cellular invasion, reducing invasiveness to baseline levels (Table 1). Similar results were observed following the addition of anti-catalytic t-PA antibodies (Table 2). In the absence of plasminogen, endothelial cell invasive activity was unaffected, whereas plasmin(ogen)-enhanced invasion was completely inhibited by anti-(t-PA) function-blocking antibodies. These data suggest that the anti-invasive effect of angiostatin might result from an interaction between angiostatin and t-PA. This is supported by the observation that plasmin(ogen)-enhanced invasion of t-PA-producing melanoma cells is also blocked by angiostatin (Table 1), whereas u-PA-producing ovarian carcinoma cells are unaffected (Table 1).

Table 1 Effect of angiostatin on plasmin(ogen)-enhanced cellular invasion

Bovine aortic endothelial cells (BAEC), murine melanoma cells (B16F10) or human ovarian carcinoma cells (OVCA 429) (at a cell density of 1×10^5) were added to a modified Boyden chamber containing an 8- μ m pore filter coated with Matrigel (11 μ g/filter) in the presence or absence of plasminogen (Pg) (50 nM) and/or angiostatin (AS) (5-fold molar excess, i.e. 250 nM), as described in the Experimental section. After incubation at 37 °C, filters were excised, non-invading cells were removed from the top surface of the membrane, stained with Diff-Quik, and invading cells were enumerated by using an ocular micrometer and counting a minimum of ten high-powered fields. Data shown represent the means $\pm$ S.D. from experiments performed in triplicate. Incubation times were 48, 6 and 72 h for BAEC, B16F10 and OVCA respectively.

Cell type	[Pg] (nM)	[AS] (nM)	Relative invasion (no. of cells/field)
BAEC	0	0	65.8 $\pm$ 8.7
	0	250	56.8 $\pm$ 4.0
	50	0	171.0 $\pm$ 13.1
	50	250	88.0 $\pm$ 9.7
B16F10	0	0	9.1 $\pm$ 3.1
	0	250	11.5 $\pm$ 2.5
	50	0	29.5 $\pm$ 5.7
	50	250	10.6 $\pm$ 4.5
OVCA 429	0	0	41.6 $\pm$ 14.3
	0	250	55.2 $\pm$ 8.7
	50	0	98.0 $\pm$ 11.7
	50	250	92.2 $\pm$ 11.8

Table 2 Effect of anti-catalytic t-PA antibodies on endothelial cell invasion

Bovine aortic endothelial cells (1×10^5) were added to a modified Boyden chamber containing an 8- μ m pore filter coated with Matrigel (11 μ g/filter) in the presence or absence of plasminogen (Pg) (50 nM) and/or anti-catalytic t-PA antibody (anti-tPA) at the indicated concentration. After incubation at 37 °C for 48 h, filters were excised, non-invading cells were removed from the top surface of the membrane, stained with Diff-Quik, and invading cells were enumerated by using an ocular micrometer and counting a minimum of ten high-powered fields. Data shown represent the means $\pm$ S.D. for experiments performed in triplicate.

[Pg] (nM)	[anti-tPA] (μ g)	Relative invasion (no. of cells/field)
0	0	51.5 $\pm$ 15.0
0	5	62.9 $\pm$ 14.0
0	10	61.6 $\pm$ 13.0
50	0	234.5 $\pm$ 43.0
50	5	56.0 $\pm$ 20.0
50	10	36.0 $\pm$ 12.0

Analysis of angiostatin binding to t-PA

To assess qualitatively the interaction between angiostatin and t-PA, initial experiments utilized ligand blotting. Both immobilized angiostatin (Figure 1A, lane 2) and a plasminogen control (Figure 1A, lane 1) bound to soluble t-PA. Binding of t-PA to molecular-mass markers was not observed (with the exception of soya-bean trypsin inhibitor, which binds serine proteinases; Figure 1A, lane 3), demonstrating that the interaction of t-PA with angiostatin does not result from non-specific binding to denatured proteins. In addition, binding of u-PA to angiostatin was not observed (Figure 1A, lane 5), suggesting that the interaction with angiostatin is specific for t-PA. Direct binding of t-PA to angiostatin was confirmed by analysing t-PA interaction with angiostatin-coated microtitre wells. Binding of t-PA to

Figure 1 Binding of t-PA to angiotensin

(A) Blot of t-PA ligand binding to angiotensin and plasminogen. Plasminogen (5 μ g; lanes 1, 4 and 7), angiotensin (5 μ g; lanes 2, 5 and 8) and molecular-mass markers (lanes 3 and 6) were electrophoresed on SDS/5–15% polyacrylamide gels, and electroblotted on to Immobilon. Ligand blots (lanes 1–3) of Immobilon incubated with 3 μ g/ml t-PA and probed with an anti-t-PA antibody, as described in the Experimental section, are shown. Lanes 4–6: a ligand blot of immobilized angiotensin incubated with 3 μ g/ml u-PA and probed with an anti-u-PA antibody. Immobilon membrane stained with Coomassie Blue is shown in lanes 7 and 8. The migration positions of angiotensin and plasminogen are indicated by arrowheads. (B) Binding of t-PA to angiotensin. Increasing concentrations of t-PA (0–40 nM) were added to microtitre wells coated with angiotensin. Binding was analysed as described in the Experimental section, and corrected for non-specific binding to albumin-coated wells. Saturation binding data were analysed by non-linear regression to determine K_d (app) values. Data represent the means for triplicate samples. (C) Competition binding of biotinylated t-PA to angiotensin. Biotinylated t-PA (20 nM) was added to angiotensin-coated microtitre wells in the presence of increasing concentrations of unlabelled t-PA (0–100-fold molar excess) and bound t-PA was quantified as described above. Results are expressed as the percentages of biotinylated t-PA bound to angiotensin in the absence of unlabelled t-PA (designated as 100%). Data represent the means for triplicate samples. (D) Angiotensin inhibits t-PA binding to type IV collagen and laminin-1. t-PA (20 nM) was incubated with angiotensin in increasing molar ratios (up to 25-fold molar excess; 0–5000 nM angiotensin) as indicated, and binding to type IV collagen (●) or laminin-1 (○) immobilized in microtitre wells was analysed as described above. Results are expressed as the percentages of t-PA bound in the absence of angiotensin (designated as 100%). Data represent the means for triplicate samples.

angiotensin in the presence of 3% BSA was both concentration-dependent and saturable (Figure 1B). In control experiments, however, concentration-dependent, saturable binding of u-PA to angiotensin was not observed at u-PA concentrations up to 100 nM. Competition binding experiments using biotinylated t-PA (20 nM) demonstrated that $\approx 60\%$ inhibition of binding in the presence of a 100-fold excess of unlabelled t-PA occurs (Figure 1C), indicating the specificity of binding. The value of K_d (app) for binding of t-PA to angiotensin was 6.7 ± 0.7 nM.

As these data demonstrated that angiotensin forms a complex with t-PA, the effect of complex formation on t-PA binding to matrix proteins was determined. Binding of t-PA to type IV collagen and laminin-1 is of high affinity, with K_d (app) values of 2.7 ± 0.34 and 4.5 ± 1.2 nM respectively. Incubation of t-PA with increasing molar ratios of angiotensin inhibited binding to type

IV collagen and laminin-1 (Figure 1D) with a maximum inhibition of 50%, as predicted on the basis of similar K_d (app) values for t-PA binding to collagen IV, laminin-1 and angiotensin. Maximal inhibition was obtained at a 3–5-fold molar excess of angiotensin over t-PA. As the plasma concentration of t-PA is 0.75–3 pM [21,47], these data indicate that as little as 3.75–15 pM angiotensin would significantly suppress the matrix association of t-PA. Interestingly, angiotensin did not bind directly to the matrix proteins type IV collagen or laminin-1 in a concentration-dependent, saturable manner, as indicated by K_d (app) values > 500 nM. This is in contrast with plasminogen, which binds in a kringle-dependent manner to type IV collagen and laminin-1 with K_d (app) values of 12 and 18 nM respectively [33,48], thereby suggesting that the conformation of the kringle domains in angiotensin is distinct from that of plasminogen.

Figure 2 Angiostatin inhibits matrix-enhanced plasminogen activation

Plasminogen (20 nM) was incubated with increasing molar ratios of angiostatin (0–100-fold molar excess), followed by the addition of t-PA (0.55 nM) and VLK-pNA (0.3 mM). Plasminogen activation was detected by monitoring A_{405} . Activation velocity was analysed on wells coated with type IV collagen (●), laminin-1 (▽) or albumin (■). In control experiments, 20 nM plasminogen (◆) was incubated in albumin-coated wells with increasing molar ratios of angiostatin (up to 100-fold molar excess), followed by the addition of u-PA (0.48 nM) and VLK-pNA (3 nM). Plasminogen activation was detected by monitoring A_{405} . Kinetic data are the means for triplicate data, and are representative of three individual experiments.

Table 3 Effect of angiostatin on amidolytic activity of t-PA and plasmin

The effect of angiostatin (AS) on t-PA and plasmin amidolytic activity was determined by incubating t-PA (5.5 nM) or plasmin (50 nM) in the presence or absence of angiostatin in a 50-fold molar excess relative to each enzyme. The reaction was initiated by addition of the indicated substrate (0.3 mM) and substrate cleavage was determined by monitoring A_{405} , as described in the Experimental section. IPR-pNA, D-Ile-Pro-Arg-p-nitroanilide.

Enzyme	Substrate	Velocity (pmol of pNA released/min)	
		– AS	+ AS
t-PA	IPR-pNA	5.7 ± 0.53	6.5 ± 0.83
Plasmin	VLK-pNA	187.2 ± 7.5	192.4 ± 9.9

Angiostatin inhibits matrix-enhanced plasminogen activation

Intact endothelial cell ECM or individual matrix proteins, such as type IV collagen and laminin-1 function kinetically as non-essential cofactors by binding to both plasminogen and t-PA, and significantly enhancing the efficiency of t-PA-catalysed plasminogen activation [29,32,33,42,43]. In contrast, u-PA-catalysed activation is not regulated by matrix proteins [49]. Because angiostatin binds t-PA and blocks matrix association, the effect of angiostatin on matrix-enhanced plasminogen activation was analysed. The initial velocity of plasminogen activation by t-PA was stimulated 13–14-fold in microtitre wells coated with type IV collagen or laminin, relative to albumin-coated wells (Figure 2; also see [32]). Addition of angiostatin blocked this matrix-enhanced plasmin formation in a dose-dependent manner, reducing activation velocity to levels observed in the absence of matrix stimulation (Figure 2). No inhibition was observed in the presence of up to a 250-fold molar excess of plasminogen kringle 4. In control experiments on albumin-coated wells, angiostatin had no effect on solution-phase activation of plasminogen by t-PA (Figure 2). At a 100-fold excess

Figure 3 Effect of angiostatin on the initial-rate kinetics of type IV collagen-enhanced plasminogen activation

Upper panel: Dixon plot showing $1/\text{velocity}$ against $[\text{angiostatin}]$ at various concentrations of the substrate plasminogen. The concentrations of plasminogen used were $0.01 \mu\text{M}$ (●), $0.02 \mu\text{M}$ (○) and $0.05 \mu\text{M}$ (▼). Data were analysed by linear regression, and the lines shown represent the theoretical fit to the data [correlation coefficient (r) > 0.97]. Lower panel: Michaelis–Menten plots showing the effect of increasing angiostatin on the initial-rate kinetics of plasminogen activation by t-PA. Increasing plasminogen concentrations (0–0.2 μM) were incubated with t-PA (0.55 nM) in microtitre wells coated with type IV collagen, and plasminogen activation was determined by monitoring plasmin cleavage of VLK-pNA (0.3 mM), as described in the Experimental section. Angiostatin concentrations used were 0 μM (●), 0.2 μM (▽), 0.5 μM (■) and 1.0 μM (◇). Kinetic data were analysed by non-linear regression analysis, and the lines shown represent the theoretical fit to the data (r > 0.97). The plots shown are the means for data measured in triplicate, and are representative of three individual experiments.

of angiostatin, the velocity of activation on type IV collagen and laminin-1 surfaces decreased to 11 % and 23 %, respectively, of control reactions in the absence of angiostatin. In contrast, activation of plasminogen by u-PA was not significantly affected by angiostatin (Figure 2). In the presence of a 100-fold excess of angiostatin, the velocity of u-PA-catalysed activation remained at 83 % of the control rates obtained in the absence of angiostatin. Because plasminogen activation is measured using a coupled reaction, the angiostatin-induced decrease in activation velocity could result from a reduction in the enzymic activity of t-PA, plasmin, or both. Analysis of the individual amidolytic activities of t-PA and plasmin showed no significant difference in the presence of a 50-fold molar excess of angiostatin (Table 3).

Figure 4 Effect of angiotensin on plasmin inhibition by $\alpha 2$ -AP

(A) Inhibition of plasmin in the presence of excess angiotensin. Plasmin (50 nM) was incubated with $\alpha 2$ -AP (25 nM) in the presence of increasing molar ratios of angiotensin (up to 50-fold molar excess over plasmin). Residual plasmin activity was determined by the addition of VLK-pNA (0.3 mM), with subsequent monitoring of substrate cleavage at A_{405} . Results are expressed as the percentage of plasmin activity relative to control reactions containing 50 nM plasmin, but lacking $\alpha 2$ -AP (designated as 100%). Data represent the means for triplicate experiments. (B) Effect of angiotensin on plasmin: $\alpha 2$ -AP titration. Plasmin (50 nM) was incubated on type IV collagen (∇), laminin-1 ($\square$) or albumin ($\circ$)-coated wells with increasing molar ratios of $\alpha 2$ -AP (up to 2-fold molar excess of $\alpha 2$ -AP; 0–100 nM) in the presence of a 10-fold molar excess of angiotensin (500 nM). Residual plasmin activity was determined as described above. Results are expressed as the percentage of plasmin activity relative to control reactions containing 50 nM plasmin and 500 nM angiotensin, but lacking $\alpha 2$ -AP (designated as 100%). Data represent the means of triplicate experiments.

Taken together, the binding and initial-velocity data suggest that t-PA might contain binding sites for its substrate plasminogen, the inhibitor angiotensin, and a cofactor (such as type IV collagen). To confirm the mechanism by which angiotensin inhibits plasminogen activation on matrix surfaces, the initial-rate kinetics of plasminogen activation by t-PA on type IV collagen were analysed in the presence of increasing concentrations of angiotensin. Michaelis–Menten plots of activation velocity against [plasminogen] on type IV collagen-coated microtitre wells confirmed the dose-dependent inhibitory effect of angiotensin (Figure 3, lower panel). To determine the kinetic mechanism of angiotensin inhibition, data were analysed by Dixon plot analysis (1/activation velocity against [angiotensin] at various concentrations of the substrate plasminogen). The individual lines on the Dixon plot have a common x-axis intercept

(Figure 3, upper panel), characteristic of non-competitive inhibition [41,42]. Replotting the slopes of the Dixon plot against 1/[substrate] (results not shown) indicates an apparent K_i of approximately $0.91 \pm 0.025 \mu\text{M}$. Similar results were obtained on laminin-1-coated wells, whereas activation on albumin-coated wells was unaffected. A non-competitive mechanism of inhibition is consistent with the data reported above, since it suggests that angiotensin binds directly to t-PA at a site distinct from the active site, thereby preventing its interaction with the non-essential cofactor type IV collagen. This is supported by the observation that angiotensin has no effect on the activation reaction in the absence of the cofactor (Figure 2), indicating that angiotensin effectively blocks the ternary complex formation between t-PA, plasminogen and matrix, which is essential for efficient plasmin formation.

Angiotensin does not alter $\alpha 2$ -AP inhibitory activity

Plasmin activity is controlled *in vivo* by complex formation with proteinase inhibitors, such as the serpin $\alpha 2$ -AP. The initial association between plasmin and $\alpha 2$ -AP involves a kringle-mediated recognition event, followed by interaction of the plasmin active site with the reactive site loop of $\alpha 2$ -AP [34]. To determine whether the kringle structure of angiotensin might modify the ability of $\alpha 2$ -AP to inhibit plasmin, $\alpha 2$ -AP (25 nM) was preincubated with increasing concentrations of angiotensin, followed by the addition of plasmin (50 nM) and analysis of the remaining plasmin activity. Angiotensin did not alter the inhibitory potency of $\alpha 2$ -AP, even when present in a 50-fold molar excess over plasmin (Figure 4A). This was confirmed by titration of plasmin with $\alpha 2$ -AP in the presence of a 10-fold molar excess of angiotensin (Figure 4B). Plasmin was inhibited by $\alpha 2$ -AP at a 1:1 molar ratio, regardless of the presence or absence of angiotensin or matrix proteins. Together, these data suggest that $\alpha 2$ -AP modulation of plasmin activity is not regulated by angiotensin.

DISCUSSION

Angiotensin is a potent inhibitor of both growth-factor-induced corneal angiogenesis *in vivo* and endothelial cell migration, proliferation and tube formation *in vitro* [5,44]. Because primary and metastatic tumour growth requires angiogenesis, angiotensin therefore also inhibits the growth of a variety of murine and human tumours [6–9,13,44]. The mechanism(s) responsible for the angiostatic activity of angiotensin is/are unknown. However, the data reported here suggest that inhibition of matrix-enhanced t-PA-catalysed plasmin formation might contribute to the anti-angiogenic activity of angiotensin. These data are consistent with reported pharmacological inhibitors of angiogenesis that also modify the plasminogen/plasmin system [50,51]. It should be noted, however, that the anti-proliferative effects of angiotensin require direct endothelial cell binding [52], suggesting multiple distinct mechanisms whereby angiotensin might regulate endothelial cell behaviour.

Invasion of endothelial cells or tumour cells into the basement membrane requires proteolytic modification of ECM proteins. Plasmin activity is not an absolute requirement for physiological angiogenesis, as mice deficient in plasminogen (Plg^{-/-}), t-PA (t-PA^{-/-}) or u-PA (u-PA^{-/-}) develop relatively normally [53,54]. However, delayed dissemination of Lewis lung carcinoma to lymph nodes and a corresponding decrease in morbidity and mortality are also observed in Plg^{-/-} animals [55], suggesting that plasmin activity contributes to tumour growth and dissemination. This is supported by the data in the present study, which

Scheme 1 Model for angiostatin inhibition of invasion by blocking matrix-enhanced plasminogen activation

(A) t-PA (tPA) is secreted by endothelial or melanoma cells and binds ECM cofactors, such as type IV collagen or laminin-1. Activation of matrix-bound plasminogen (Pg) to plasmin (Pm) is rapid (B), resulting in the formation of pericellular plasmin (C). Limited plasmin proteolysis of matrix macromolecules facilitates cellular invasion (C). In the presence of angiostatin (AS), an angiostatin AS-tPA complex is formed (D), which prevents matrix association of tPA. The AS-tPA complex can still bind to and activate Pg (E), albeit at a significantly reduced rate relative to the matrix-bound complex. The resulting decrease in pericellular plasmin is manifested functionally as a loss of invasive activity.

demonstrate that, in the presence of plasminogen, endothelial cell invasion is enhanced significantly as a result of cell-mediated plasmin formation. Angiostatin effectively blocked the plasmin(ogen)-enhanced invasive activity in a manner similar to that of anti-catalytic t-PA antibodies, suggesting that angiostatin might regulate t-PA activity. In contrast, angiostatin had no effect on plasmin catalytic activity. Direct binding experiments confirmed the interaction between angiostatin and t-PA; however, binding of angiostatin to u-PA was not observed. t-PA and u-PA share a highly homologous B chain containing the serine proteinase domain, but there is a significant variation in the structure of their regulatory A chains: the t-PA A chain consists of a pair of kringle domains and a fibronectin-like finger domain, whereas that of u-PA is composed of a growth-factor-like domain and a single kringle [56]. Although the exact sequence of the angiostatin-binding site on t-PA has not been elucidated, a plasminogen-binding site distinct from the B chain active site was previously reported on the t-PA A chain [57]. Binding of angiostatin to the t-PA A chain is supported by the observation that t-PA amidolytic activity was not inhibited by angiostatin, indicating that angiostatin does not directly interfere with either substrate binding or catalytic activity.

Intact endothelial cell ECM and individual matrix proteins function as non-essential cofactors for the t-PA-catalysed plasminogen activation reaction by binding both t-PA and plasminogen, subsequently leading to significantly enhanced plasmin production [29–33,49]. Angiostatin significantly inhibited the cofactor-enhanced reaction, functioning kinetically as a non-competitive inhibitor, supporting the observation that angiostatin does not directly compete with plasminogen for binding to the t-PA active site. Together, these data support a model in which the inhibitory effect of angiostatin results from a blocking of the interaction between t-PA and matrix cofactor (see Scheme 1). Interestingly, although the interaction of plasminogen with matrix proteins is kringle-dependent [33,48], no direct binding of

angiostatin to matrix proteins was observed. The angiostatin utilized in these experiments was re-purified on lysine–Sephacrose, indicating that the lysine-binding sites are intact and functional [10]. These data indicate that the affinity of the lysine-binding sites is significantly altered in the angiostatin polypeptide relative to intact plasminogen, and suggest that kringle conformation might play a substantial role in matrix-cofactor recognition.

Plasmin activity is tightly controlled *in vivo* by covalent complex formation between plasmin and the serpin α 2-AP. The initial interaction of plasmin with α 2-AP involves a kringle-mediated recognition event prior to plasmin cleavage of the α 2-AP reactive site peptide bond and covalent complex formation [34]. Angiostatin had no effect on the inhibitory potency of α 2-AP, even when present in a 50-fold molar excess over plasmin, or when preincubated with the inhibitor, indicating that kringle conformation might also mediate inhibitor recognition. Inhibition of plasmin activity by α 2-AP in collagen IV- or laminin-1-coated wells was also unaffected by angiostatin. These data suggest that while angiostatin regulates the rate of plasmin generation, it does not effect an essential physiological control mechanism for limiting plasmin activity.

On the basis of these data, the following model depicts a potential mechanism whereby angiostatin might block endothelial cell invasion (Scheme 1). Endothelial cells produce t-PA (A in the Scheme), which can bind directly to the matrix cofactor in close proximity to matrix-bound plasminogen (B). This results in a substantial increase in the rate of plasmin formation, contributing to matrix proteolysis, which facilitates endothelial cell invasion (C). In the presence of angiostatin, formation of an angiostatin–t-PA complex inhibits t-PA interaction with the matrix cofactor (D). Plasminogen in the angiostatin–t-PA–plasminogen ternary complex is still converted into plasmin, albeit at a much slower rate (E), resulting in reduced endothelial cell invasion. Together, these data suggest that angiostatin in the endothelial cell micro-environment can inhibit matrix-enhanced

plasminogen activation, resulting in a reduced invasive activity, and suggest a biochemical mechanism whereby angiostatin-mediated regulation of plasmin formation might influence cellular migration and invasion.

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REFERENCES

- Folkman, J. and Shing, Y. (1992) *J. Biol. Chem.* **267**, 10931–10934
- Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. and Ferrara, N. (1993) *Nature (London)* **362**, 841–844
- Hori, A., Sasada, R., Matsutani, E., Naito, K., Sakura, Y., Fujita, T. and Kozai, Y. (1991) *Cancer Res.* **51**, 6180–6184
- Millauer, B., Shawver, L. K., Plate, K. H., Risau, W. and Ulrich, A. (1994) *Nature (London)* **367**, 576–579
- O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H. and Folkman, J. (1994) *Cell* **79**, 315–328
- O'Reilly, M. S., Holmgren, L., Chen, C. and Folkman, J. (1996) *Nature Med.* **2**, 689–692
- Lannutti, B. J., Gately, S. T., Quevedo, E., Soff, G. A. and Paller, A. S. (1997) *Cancer Res.* **57**, 5277–5280
- Cao, Y., O'Reilly, M. S., Marshall, B., Flynn, E., Ji, R. W. and Folkman, J. (1998) *J. Clin. Invest.* **101**, 1055–1063
- Tanaka, T., Cao, Y., Folkman, J. and Fine, H. A. (1998) *Cancer Res.* **58**, 3362–3369
- Gately, S., Twardowski, P., Stack, M. S., Cundiff, D. L., Grella, D., Castellino, F. J., Enghild, J., Kwaan, H. C., Lee, F., Kramer, R. A. et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10868–10872
- Stathakis, P., Fitzgerald, M., Matthias, L. J., Chesterman, C. N. and Hogg, P. J. (1997) *J. Biol. Chem.* **272**, 20641–20645
- Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E. and Magnusson, S. (1978) in *Progress in Chemical Fibrinolysis and Thrombolysis* (Davidson, J. F., Rowan, R. M., Samama, M. M. and Desnoyers, P. C., eds.), pp. 191–209, Raven Press, New York
- Dong, Z., Kumar, R., Yang, X. and Fidler, I. J. (1997) *Cell* **88**, 801–810
- Lijnen, H. R., Ugwu, F., Bini, A. and Collen, D. (1998) *Biochemistry* **37**, 4699–4702
- Patterson, B. C. and Sang, Q. A. (1997) *J. Biol. Chem.* **272**, 28823–28825
- Griscelli, F., Li, H., Bennaceur-Griscelli, A., Soria, J., Opolon, P., Soria, C., Perricaudet, M., Yeh, P. and Lu, H. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6367–6372
- Cao, Y., Ji, R. W., Davidson, D., Schaller, J., Marti, D., Sohndel, S., McCance, S. G., O'Reilly, M. S., Llinas, M. and Folkman, J. (1996) *J. Biol. Chem.* **271**, 29461–29467
- Sim, B. K. L., O'Reilly, M. S., Liang, H., Fortier, A. H., He, W., Madsen, J. W., Lpachovich, R. and Nacy, C. A. (1997) *Cancer Res.* **57**, 1329–1334
- Mignatti, P. and Rifkin, D. B. (1993) *Physiol. Rev.* **73**, 161–195
- Tsirka, S. E., Bugge, T. H., Degen, J. L. and Strickland, S. (1997) *Proc. Nat. Acad. Sci. U.S.A.* **94**, 9779–9781
- Menashi, S., Lu, H., Soria, C. and Legrand, Y. (1993) *Bailliere's Clin. Haematol.* **6**, 559–576
- Sato, Y., Okamura, K., Morimoto, A., Hamanaka, R., Hamaguchi, K., Shimada, T., Ono, M., Kohno, K., Sakata, T. and Kuwano, M. (1994) *Exp. Cell Res.* **204**, 223–229
- Schnaper, H. W., Barnathan, E. S., Mazar, A., Maheshwari, S., Ellis, S., Cortez, S. L., Baricos, W. L. and Kleinman, H. K. (1995) *J. Cell Physiol.* **165**, 107–118
- Rijken, D. C. and Collen, D. (1981) *J. Biol. Chem.* **256**, 7035–7041
- Neuman, T., Stephens, R. W., Salonen, E. M., Timmus, T. and Vaheri, A. (1989) *J. Neurosci. Res.* **23**, 274–281
- Bizik, J., Lizonova, A., Stephens, R. W., Grofova, M. and Vaheri, A. (1990) *Cell Regul.* **1**, 895–905
- Bizik, J., Stephens, R. W., Grofova, M. and Vaheri, A. (1993) *J. Cell Biochem.* **51**, 326–335
- Hoylaerts, M., Rijken, D. C., Lijnen, H. R. and Collen, D. (1982) *J. Biol. Chem.* **257**, 2912–2919
- Knudsen, B. S., Silverstein, R. L., Leung, L. L. K., Harpel, P. C. and Nachman, R. L. (1986) *J. Biol. Chem.* **261**, 10765–10771
- Stack, M. S., Rinehart, A. R. and Pizzo, S. V. (1995) *Eur. J. Biochem.* **226**, 937–943
- Silverstein, R. L., Harpel, P. C. and Nachman, R. L. (1986) *J. Biol. Chem.* **261**, 9959–9965
- Stack, M. S., Gonzalez-Gronow, M. and Pizzo, S. V. (1990) *Biochemistry* **29**, 4966–4970
- Moser, T. L., Enghild, J. J., Pizzo, S. V. and Stack, M. S. (1993) *J. Biol. Chem.* **268**, 18917–18923
- Wiman, B., Lijnen, H. R. and Collen, D. (1979) *Biochim. Biophys. Acta* **579**, 142–154
- Enghild, J. J., Valnickova, Z., Thogersen, I. B., Pizzo, S. V. and Salvesen, G. (1993) *Biochem. J.* **291**, 933–938
- Folkman, J., Haudenschield, C. C. and Zetter, B. R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5217–5221
- Soff, G. A., Jackman, R. W. and Rosenberg, R. D. (1991) *Blood* **77**, 515–518
- Stack, M. S., Gray, R. D. and Pizzo, S. V. (1993) *Cancer Res.* **53**, 1998–2004
- Moser, T. L., Young, T. N., Rodriguez, G. C., Pizzo, S. V., Bast, R. C. and Stack, M. S. (1994) *Int. J. Cancer* **56**, 552–559
- Bafetti, L. M., Young, T. N., Itoh, Y. and Stack, M. S. (1998) *J. Biol. Chem.* **273**, 143–149
- Wohl, R. C., Summari, L. and Robbins, K. C. (1980) *J. Biol. Chem.* **255**, 2005–2013
- Dixon, M. and Webb, E. C. (1964) *Enzymes*, pp. 322–330, Academic Press, New York
- Segel, I. H. (1975) *Enzyme Kinetics*, pp. 125–135, John Wiley and Sons, New York
- Gately, S., Twardowski, P., Stack, M. S., Patrick, M., Boggio, L., Cundiff, D. L., Schnaper, H. W., Madison, L., Volpert, O., Bouch, N. et al. (1996) *Cancer Res.* **56**, 4887–4890
- Claesson-Welsh, L., Welsh, M., Ito, N., Anand-Apte, B., Soker, S., Zetter, B., O'Reilly, M. and Folkman, J. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5579–5584
- Ji, W. R., Barrientos, L. G., Llinas, M., Gray, H., Villarreal, X., DeFord, M. E., Castellino, F. J., Kramer, R. A. and Trail, P. A. (1998) *Biochem. Biophys. Res. Commun.* **247**, 414–419
- Mullertz, S. (1984) *Semin. Thromb. Hemostasis* **10**, 1–5
- Stack, M. S., Moser, T. L. and Pizzo, S. V. (1992) *Biochem. J.* **284**, 103–108
- Moser, T. L., Enghild, J. J., Pizzo, S. V. and Stack, M. S. (1995) *Biochem. J.* **307**, 867–873
- Takano, S., Gately, S., Neville, M. E., Herblin, W. F., Gross, J. L., Engelhard, H., Perricone, M., Eidsvoog, K. and Brem, S. (1994) *Cancer Res.* **54**, 2654–2660
- Pepper, M. S., Vassalli, J. D., Wilks, J. W., Schweigerer, L., Orci, L. and Montesano, R. (1994) *J. Cell Biochem.* **55**, 419–434
- Moser, T. L., Stack, M. S., Asplin, I., Enghild, J. J., Hojrup, P., Everitt, L., Hubchak, S., Schnaper, H. W. and Pizzo, S. V. (1999) *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 2811–2816
- Carmeliet, P., Schoonjans, L., Kleckens, L., Ream, B., Degan, J., Bronson, R., deVos, R., van den Oord, J. J., Collen, D. and Mulligan, R. C. (1994) *Nature (London)* **368**, 419–424
- Bugge, T. H., Flick, M. J., Daugherty, C. C. and Degen, J. L. (1995) *Genes Dev.* **9**, 794–807
- Bugge, T. H., Kombrinck, K. W., Xiao, Q., Holmback, K., Daugherty, C. C., Witte, D. P. and Degen, J. L. (1997) *Blood* **90**, 4522–4531
- Lijnen, H. R., Bachmann, F., Collen, D., Ellis, V., Pannekoek, H., Rijken, D. C. and Thorsen, S. (1994) *J. Intern. Med.* **236**, 415–424
- Geppert, A. G. and Binder, B. R. (1992) *Arch. Biochem. Biophys.* **297**, 205–212

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