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Different inhibitors of plasmin differentially affect angiostatin production and angiogenesis

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Abstract

Plasmin is a broad-spectrum serine proteinase, which is presumed to cleave many extracellular proteins and affect angiogenesis. In the present work, we studied the effect of two different inhibitors of plasmin (ε-aminocaproic and α_2-antiplasmin) on angiogenesis in vivo using the chicken embryo chorioallantoic membrane assay, and in vitro using human umbilical vein endothelial cells. ε-Aminocaproic acid inhibited, while α_2-antiplasmin induced, angiogenesis, as well as human umbilical vein endothelial cell proliferation, migration and tube formation on matrigel in a dose-dependent manner. Since plasmin has been implicated in the production of angiostatin, we studied the effect of the two plasmin inhibitors on angiostatin protein amounts in the chicken embryo chorioallantoic membrane. In this tissue, the 38- and 45-kDa isoforms of angiostatin are differentially affected by the two inhibitors: ε-aminocaproic acid increased, while α_2-antiplasmin decreased the amounts of both isoforms. These data suggest that plasmin may have an antiangiogenic role in vivo through generation of angiostatin. Moreover, plasmin inhibitors differentially affect in vivo angiogenesis, depending on the mechanism by which they inhibit plasmin activity.

Keywords: Plasmin; Angiostatin; Angiogenesis; α_2-Antiplasmin; ε-Aminocaproic acid

1. Introduction

Angiogenesis is the growth of new capillary sprouts from preexisting vasculature in response to angiogenic stimuli. Angiogenesis is normally observed during embryonic development, female reproductive cycle, wound healing, organ formation, tissue regeneration and remodeling. However, sustained angiogenesis can be seen in pathological conditions such as inflammation, tumor growth and diabetic retinopathy (Carmeliet and Jain, 2000). Production of proteinases and the subsequent degradation of basement membrane is an essential component of angiogenesis and has consistently been implicated in endothelial cell migration and invasion (Pepper et al., 1996, 2001a).

Urokinase-type (uPA) and tissue-type (tPA) plasminogen activators are serine proteinases that convert the inactive pro-enzyme plasminogen to the active enzyme plasmin, which in turn degrades some components of the extracellular matrix such as fibrin, proteoglycans, laminin and fibronectin (Pepper, 2001a). Furthermore, plasmin activates several matrix metalloproteinases (MMPs) (Pepper, 2001a), resulting in the release of endogenous matrix-bound growth factors (Ribatti et al., 1999). Finally, plasmin and/or plasminogen activators have been implicated in the production of angiostatin and angiostatin-related proteins (Soff, 2000).

Angiostatin is a kringle-containing fragment that can be generated by proteolytic cleavage of plasmin or plasminogen. Different proteinases may yield different isoforms of angiostatin due to different proteolytic cleavage sites (Dong et al., 1997; Gately et al., 1997; Patterson and Sang, 1997; Cornelius et al., 1998; O'Reilly et al., 1999; Morikawa et al., 2000; Soff, 2000). Several MMPs can cleave plasminogen (Patterson and Sang, 1997; Cornelius et al., 1998; O'Reilly et al., 1999) and even plasmin itself undergoes auto-proteolysis to yield angiostatin (Falcone et al., 1994; Soff, 2000). Angiostatin is a potent inhibitor of angiogenesis in vivo and of endothelial cell proliferation and migration in vitro and is considered an endogenous modulator of tumor angiogenesis (Gately et al., 1997; Cornelius

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et al., 1998; Ji et al., 1998; Hari et al., 2000; Soff, 2000). Recent studies have indicated that angiostatin can be produced in non-neoplastic conditions (Soff et al., 1999), reflecting a possible regulatory role also in physiological angiogenesis.

In the present work, we examined the effect of two plasmin inhibitors, ε-aminocaproic acid and α_2-antiplasmin, on physiological angiogenesis. ε-Aminocaproic acid mimics the side chain of lysine and interacts with "lysine binding sites" at the kringle domains of both plasminogen and plasmin (Lerch et al., 1980). Through interactions with these sites, ε-aminocaproic acid disrupts the binding of plasmin to fibrin (Lin et al., 2000) and to the cell surface (Miles and Plow, 1985; Silverstein et al., 1988; Gonias et al., 1989). The serpin α_2-antiplasmin is the primary endogenous inhibitor of soluble plasmin activity (Collen, 1980). We found that ε-aminocaproic acid and α_2-antiplasmin exhibit differential effects on angiogenesis in the in vivo chicken embryo chorioallantoic membrane assay, as well as on the proliferation, migration and differentiation of endothelial cells derived from human umbilical veins. These differences may be due to the different effects of the inhibitors on angiostatin production.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells were isolated from human umbilical cords and cultured as previously described (Pipili-Synetos et al., 1998). The cells were grown as monolayers in medium M199 supplemented with 15% fetal calf serum, 200 μg/ml endothelial cell growth supplement, 4 U/ml heparin sodium, 100 U/ml penicillin–streptomycin and 50 μg/ml gentamycin and were used at passages 1–5. Cultures were maintained at 37 °C, 5% CO_2 and 100% humidity.

2.2. Cell proliferation assay

In order to determine if the plasmin inhibitors affect the proliferation of human umbilical vein endothelial cells, the 3-[4, 5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay was used, as previously described (Papadimitriou et al., 2000). Briefly, cells were seeded at a density of 2×10^4 cells/well in 48-well tissue culture plates in medium supplemented with 5% fetal calf serum. The plasmin inhibitors (Sigma, Athens, Greece) were added to the medium at different concentrations and the number of cells was measured after 48 h. MTT stock (5 mg/ml in phosphate-buffered saline, PBS) at a volume equal to 1/10 of the medium was added and plates were incubated at 37 °C for 2 h. The medium was removed, the cells were washed with PBS pH 7.4 and 100 μl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) was added to each well and agitated thoroughly in order to solubilize the dark blue formazan crystals. The solution was transferred to 96-well plates and immediately read on a microplate reader (Biorad, Hercules, CA, USA) at a wavelength of 490 nm.

2.3. Boyden chamber assay

Migration assays were performed as previously described (Papadimitriou et al., 2001) in a 24-well microchemotaxis chamber (Costar) using untreated polycarbonate membranes with 8-μm pores. Human umbilical vein endothelial cells were harvested and resuspended at a concentration of 10^5 cells/0.1 ml in medium containing 0.25% bovine serum albumin. The bottom chamber was filled with 0.6 ml of medium containing 0.25% bovine serum albumin and plasmin inhibitors. The upper chamber was loaded with 10^5 cells and incubated for 4 h at 37 °C. After completion of the incubation, the filters were fixed with saline-buffered formalin and stained using DiffQuick. The cells that migrated through the filter were quantified by counting the whole area of each filter, using a grid and an Optech microscope at a 20× magnification.

2.4. Matrigel tube formation assay

The tube formation assay was performed as previously described (Papadimitriou et al., 2001). Briefly, matrigel was used to coat the wells of 96-well plates (0.04 ml/well) and was left to polymerize for 1 h at 37 °C. After polymerization, 15,000 cells suspended in 0.15 ml of medium supplemented with 5% fetal calf serum were added to each well. The inhibitors of plasmin were added to the corresponding wells simultaneously with the cells. After 6 h of incubation, the medium was removed, the cells were fixed and the length of the tube network was measured in the total area of the well, as previously described (Pipili-Synetos et al., 1998).

2.5. Chorioallantoic membrane assay

The in vivo chicken embryo chorioallantoic membrane angiogenesis model was used, as previously described (Papadimitriou et al., 2001; Giannopoulou et al., 2001). Leghorn fertilized eggs (Pindos, Ioannina, Greece) were incubated for 4 days at 37 °C, when a window was opened on the egg shell, exposing the chorioallantoic membrane. The window was covered with tape and the eggs were returned to the incubator. Substances tested were diluted in a final volume of 20 μl H_2O and applied at the ninth day of embryo development on an area of 1 cm^2 of the chorioallantoic membrane, restricted by a plastic ring. In order to evaluate the effect of each substance, 48 h after treatment and subsequent incubation at 37 °C, chorioallantoic membranes were fixed in situ, excised from the eggs, placed on slides and left to air-dry. Pictures were taken through a

stereoscope equipped with a digital camera and the total length of the vessels was measured, as previously described (Papadimitriou et al., 2001). Assays for each tested agent were carried out three times and each experiment contained 10–20 eggs per data point.

For the biochemical studies, different doses of each agent were applied on the chorioallantoic membrane and after different time periods of incubation at 37 °C, the chorioallantoic membranes were excised, cut in pieces, washed three times in PBS pH 7.4 and stored at −20 °C until used (Giannopoulou et al., 2001).

2.6. Western blot analysis of angiostatin

Chorioallantoic membrane tissues were homogenized in lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM phenyl–methyl–sulfonyl fluoride, 1 μg/ml aprotinin, 1% Triton X-100) and agitated for 4 h at 4 °C. The lysates were then centrifuged at 20,000*g* for 30 min at 4 °C. The total protein concentration of the supernatants was determined using the Bradford assay and 1 mg of total protein was diluted up to 10 ml with 20 mM HEPES, pH 7.4 and incubated on a rotator with 100 μl lysine–Sepharose beads (PharmaciaBiotech, Sweden) overnight at 4 °C. The beads were washed three times with 20 mM HEPES, pH 7.4, and then boiled in Laemmli loading buffer for 5 min (Stathakis et al., 1997). The extracted proteins were analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis mini gels and transferred to Immobilon P membranes. Blocking was performed by incubating the polyvinylidene difluoride membranes with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS), pH 7.4, overnight at 4 °C under continuous agitation. The membranes were incubated with anti-angiostatin (Alpha Diagnostic International, San Antonio, TX, USA) at a dilution of 1:1250 in 3% (w/v) nonfat dry milk in TBS, 0.05%, Tween-20 (TBS-T) for 1 h at room temperature under continuous agitation and then with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (Sigma) at a dilution 1:2500 in 3% (w/v) nonfat dry milk in TBS-T for 1.5 h at room temperature under continuous agitation. Detection of immunoreactive bands was performed by Super Signal West Pico substrate (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The protein levels that corresponded to the immunoreactive bands were quantified using the image PC analysis software (Scion, Frederick, MD, USA).

2.7. Colorimetric assay for the determination of active plasmin

Leghorn fertilized eggs (Pindos, Greece) were incubated at 37 °C and at different developmental stages, chorioallantoic membrane tissue was excised from the eggs. The tissues were washed in PBS, pH 7.4, cut in pieces and centrifuged at 5000*g* for 5 min at 4 °C. Each pellet was homogenized in lysis buffer (0.1 M Tris–HCl, pH 7.4) and then homogenates were centrifuged at 20,000*g* for 20 min at 4 °C. Total protein concentration was determined in the supernatants and 100 μg of total protein from each sample were diluted in 200 μl lysis buffer, placed in the wells of a 96-well microplate and incubated at 37 °C for 1 h with the plasmin substrate Val-Leu-Lys-*p*-nitroanilide (0.6 mM/well) (Sigma). Substrate cleavage was determined by monitoring the absorbance at 405 nm using a microplate reader (Biorad). The amounts of active plasmin in each sample (mU/mg of total protein) were determined using a standard curve with purified plasmin (Sigma) (Gately et al., 1997; Giannopoulou et al., in press).

2.8. Detection of apoptosis on CAM paraffin sections

CAM tissues were excised from the eggs 24 h after application of plasmin inhibitors, washed in PBS, fixed in saline-buffered formalin, dehydrated and embedded in paraffin. Sections were cut at 4 μm thickness and placed on positively charged glass slides. Apoptosis was studied on CAM paraffin sections using a commercially available apoptosis kit (APOPTOS-I.S., Ylem, Roma, Italy) according to the manufacturer's instructions. Briefly, the slides were incubated with proteinase K for 15 min at room temperature, washed in double-distilled water twice for 5 min each and were covered with peroxidase blocking solution. After 20 min, the slides were washed in double-distilled water twice and sections were preincubated for equilibration in TdT buffer (30 mM Tris–HCl, 140 mM Na cacodylate, 1 mM cobalt chloride, pH 7.2) for 10 min at room temperature. For elongation and labeling of 3′-OH DNA termini, each section of the slide was incubated with 50 μl of reaction mixture, containing TdT enzyme and biotinylated dUTP in TdT buffer for 60 min at 37 °C. The reaction was stopped by transferring the slides in PBS for 5 min at room temperature and the slides were covered with blocking solution for 10 min at room temperature. For detection of poly-dUTP–biotin complexes, the sections were incubated with diluted streptavidin-conjugated peroxidase for 30 min at room temperature. Then, the slides were washed twice for 5 min each in PBS pH 7.4 and covered with freshly prepared DAB solution. After 20 min, the slides were washed with distilled water, counterstained with haematoxylin, mounted in mounting fluid, viewed in Zeiss microscope and photographed using a digital camera (Giannopoulou et al., in press).

2.9. Statistical analysis

The significance of variability between the results from each group and the corresponding control was determined by unpaired *t*-test. Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as means ± S.E.M. from at least three independent experiments.

3. Results

3.1. Effect of plasmin inhibitors on the in vivo formation of new blood vessels

In the present work, we studied if plasmin plays a role in embryonic angiogenesis in vivo in the chicken embryo chorioallantoic membrane.

Initially, we studied if there is plasmin activity in the chicken embryo chorioallantoic membrane. As shown in Fig. 1, plasmin was endogenously produced by chorioallantoic membrane cells and its activity was maximal between Days 6 and 9 of embryo development, after which it progressively declined.

We then studied whether the plasmin inhibitors α_2-antiplasmin and ε-aminocaproic acid affect physiological angiogenesis in the in vivo chicken embryo chorioallantoic membrane. As shown in Fig. 2, α_2-antiplasmin induced angiogenesis in a dose-dependent manner, reaching a stimulation of $35 \pm 3\%$ compared to control at the dose of 4 pmol/cm^2. In contrast, ε-aminocaproic acid inhibited the formation of new blood vessels in a dose-dependent manner, reaching an inhibition of $45.7 \pm 3\%$ compared to control at the dose of 2 nmol/cm^2. The effect of ε-aminocaproic acid was not due to toxicity, as verified on chorioallantoic membrane paraffin sections stained with eosin–haematoxylin (data not shown) or treated with a kit for in situ detection of apoptosis (Fig. 2C).

3.2. Effect of plasmin inhibitors on human umbilical vein endothelial cell proliferation, migration and tube formation

Endothelial cells are the main cellular component of the vessel wall and have important roles in the process of angiogenesis. In order to elucidate whether and how

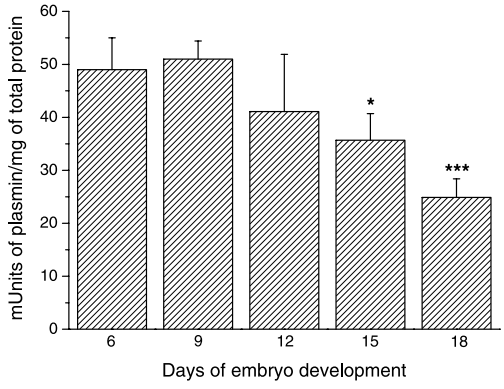

Fig. 1. Chorioallantoic membrane plasmin activity during chicken embryo development. Results are expressed as means ± S.E.M. from three independent experiments. Asterisks denote a statistically significant difference (unpaired *t*-test) from plasmin activity at the ninth day of embryo development. *$P<0.05$, ***$P<0.001$.

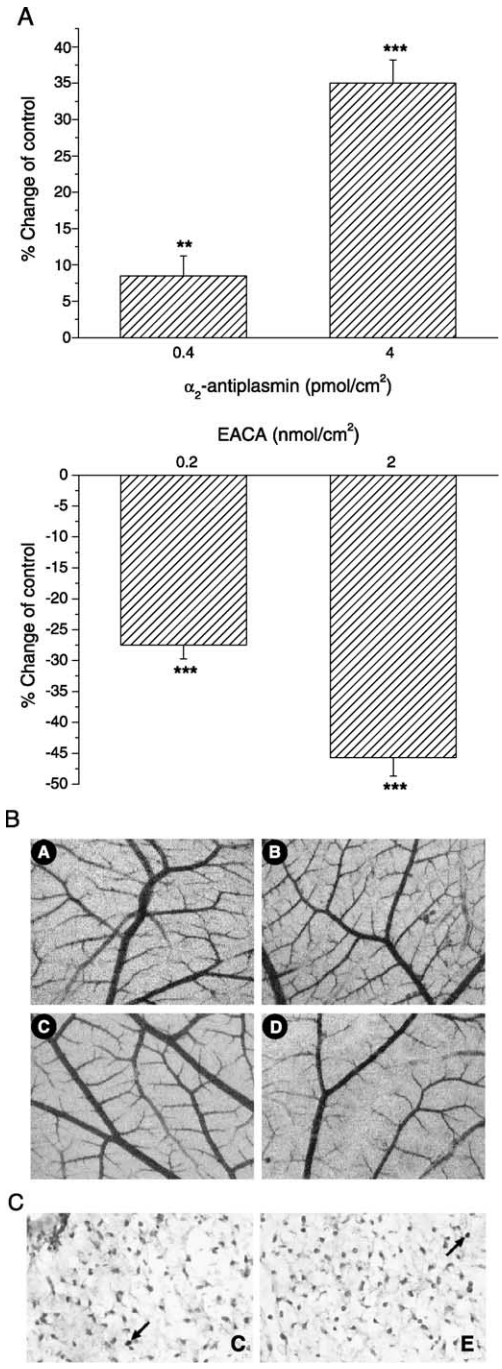

Fig. 2. Effect of plasmin inhibitors on the number of vessels in the chicken embryo chorioallantoic membrane. (A) Different doses of the inhibitors were applied on the chorioallantoic membrane and 48 h later, the number of vessels was estimated using image analysis software. Results are expressed as means ± S.E.M. of the percent change of number of vessels in treated compared to the untreated tissue. Asterisks denote a statistically significant difference (unpaired *t*-test) from the untreated tissue. **$P<0.01$, ***$P<0.001$. (B) Representative pictures showing the vessel network of the chicken embryo chorioallantoic membrane after treatment with plasmin inhibitors. (A) Control, (B) α_2-antiplasmin (4 pmol/cm^2), (C) control and (D) ε-aminocaproic acid (2 nmol/cm^2). (C) CAM paraffin sections treated with an in situ apoptosis detection kit. Sections were counterstained with haematoxylin. C, control; E: tissue treated with ε-aminocaproic acid (2 nmol/cm^2). Arrows indicate apoptotic cells (magnification 40 ×).

ε-aminocaproic acid and α_2-antiplasmin affect different functions of endothelial cells, we studied the effect of the plasmin inhibitors on human umbilical vein endothelial cell proliferation, migration and tube formation on matrigel.

As shown in Fig. 3, α_2-antiplasmin increased, while ε-aminocaproic acid decreased the number of human umbilical vein endothelial cells in a concentration-dependent manner. In the same line, α_2-antiplasmin significantly induced (67 ± 7%, increase compared to control) and ε-aminocaproic acid significantly inhibited (31 ± 7%, decrease compared to control) migration of human umbilical vein endothelial cells (Fig. 4).

As shown in Fig. 5, α_2-antiplasmin significantly induced tube formation on matrigel in a concentration-dependent manner. At the concentration of 20 nM, it caused an 11 ± 0.7, and at the concentration of 200 nM, a 59 ± 10% increase compared to control cells. On the contrary, ε-aminocaproic acid significantly inhibited tube formation (45 ± 8% decrease compared to control cells, at the concentration of 0.1 M).

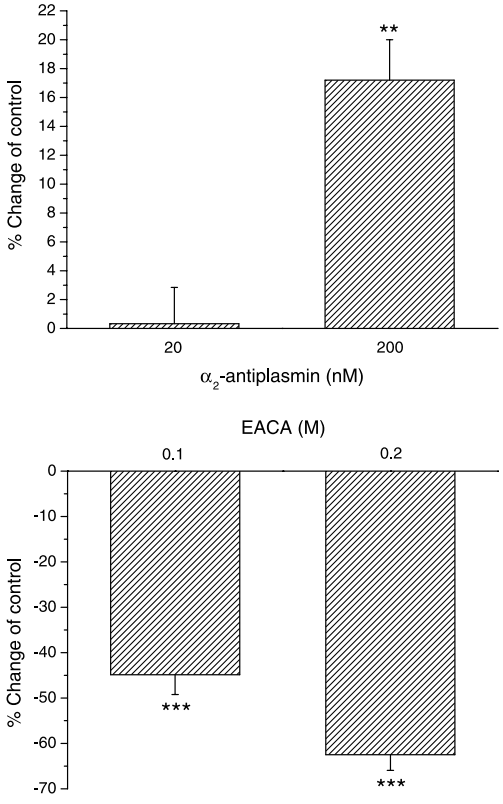

Fig. 3. Effect of plasmin inhibitors on the proliferation of human umbilical vein endothelial cell. Different doses of the inhibitors were applied on human umbilical vein endothelial cell and 48 h later, the number of the cells was estimated with the colorimetric MTT assay as described in Section 2. Results are expressed as means ± S.E.M. of the percent change compared to the untreated cells. Asterisks denote a statistically significant difference (unpaired *t*-test) from the untreated cells. $^{**}P<0.01$, $^{***}P<0.001$.

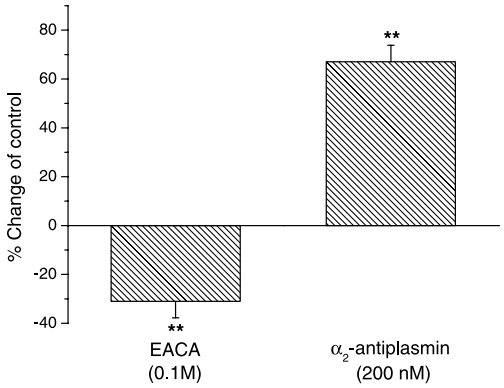

Fig. 4. Effect of plasmin inhibitors on the migration of human umbilical vein endothelial cell. Migration was measured by using the Boyden chamber assay, as described in Section 2. Results are expressed as means ± S.E.M. of the percent change compared to the untreated cells. Asterisks denote a statistically significant difference (unpaired *t*-test) from the untreated cells. $^{**}P<0.01$.

3.3. Effect of plasmin inhibitors on angiostatin protein amounts in the chicken embryo chorioallantoic membrane

Serine proteinases and metalloproteinases have recently been reported to be involved in the generation of angiogenesis inhibitors, such as angiostatin (Soff, 2000), which is an internal fragment of plasminogen or plasmin. Angiostatin has been implicated in the control of pathological angiogenesis (Gately et al., 1997), while it is not clear whether it is also a regulator of physiological angiogenesis. In the present work, we examined whether angiostatin is generated in the chicken embryo chorioallantoic membrane during physiological angiogenesis and if the plasmin inhibitors α_2-antiplasmin and ε-aminocaproic acid affect angiostatin protein levels.

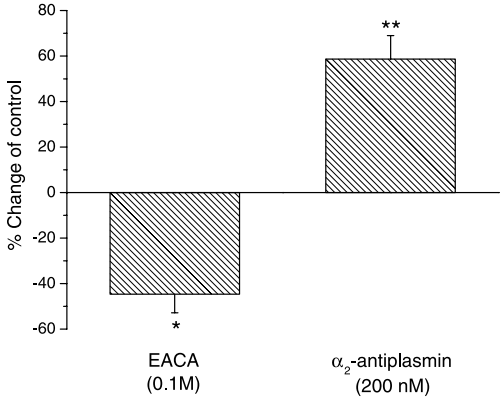

Fig. 5. Effect of plasmin inhibitors on tube formation on matrigel. The cells were fixed and the length of the tube network was estimated using image analysis software. Results are expressed as means ± S.E.M. of the percent change compared to the untreated cells. Asterisks denote a statistically significant difference (unpaired *t*-test) from the untreated cells. $^{*}P<0.05$, $^{**}P<0.01$.

As shown in Fig. 6A, several isoforms of angiostatin were detected in the chicken embryo chorioallantoic membrane (T), as well as in the protein extract of chicken embryo chorioallantoic membrane blood cells (B). The most predominant bands recognized by the anti-angiostatin antibody used, corresponded to peptides with molecular weights 66, 57, 45, 38 and 25 kDa. The bands of 66 and 35 kDa most likely correspond to the heavy and light chains of plasmin (Wiman, 1981). Isoforms of angiostatin of several molecular weights have extensively been described in the literature (Soff, 2000). We chose to estimate the effect of the two plasmin inhibitors on the two best-described isoforms of angiostatin, 45 and 38 kDa.

α_2-Antiplasmin and ε-aminocaproic acid differentially affected angiostatin protein levels in the chicken embryo chorioallantoic membrane. The tested agents were applied on a 1-cm^2 area of the chorioallantoic membrane, restricted by a plastic ring, at the ninth day of embryo development and after 24 h, 45 and 38 kDa angiostatin protein levels were estimated by Western analysis of chorioallantoic membrane protein extracts, as described in Section 2. As shown in Fig. 6B, α_2-antiplasmin reduced the amounts of both isoforms of angiostatin measured. On the other hand, ε-aminocaproic acid significantly increased the amounts of angiostatin. At the lower dose used (0.2 nmol/cm^2), it increased only the 45-kDa isoform by 31 ± 5% compared to the control, while at the dose of 2 nmol/cm^2, it increased both measured isoforms of angiostatin (Fig. 6B).

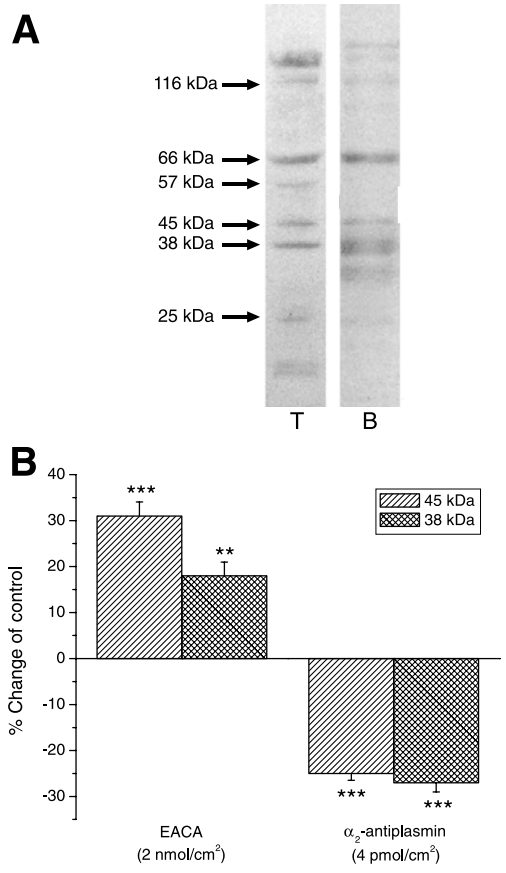

Fig. 6. (A) Representative picture of Western blot analysis of chorioallantoic membrane protein extracts for angiostatin. T: tissue protein extract, B: chicken embryo blood protein extract. (B) Effect of the inhibitors of plasmin on angiostatin protein levels 24 h after substance application on chicken embryo chorioallantoic membrane. The 45- and 38-kDa angiostatin protein amounts were quantified by densitometric analysis of the corresponding bands in each lane. Results are expressed as means ± S.E.M. of the percent change of the amounts of the proteins, compared to the untreated tissues. Asterisks denote a statistically significant difference (unpaired t-test) from the untreated tissues. $^{**}P<0.01$, $^{***}P<0.001$.

4. Discussion

The notion that the plasminogen activator (PA)/plasmin system plays an important role in fibrinolysis, tissue remodeling, tumor invasion, metastasis and angiogenesis has been supported by data published from several groups (Andreasen et al., 1997; Pepper, 2001b). Through proteolytic activity, plasmin can generate several proteins which have either pro- or anti-angiogenic functions (Andreasen et al., 2000) and its involvement in the formation of new blood vessels may depend on the balance between plasmin and other components of the extracellular environment.

In the present work, we studied the involvement of plasmin in physiological angiogenesis in the chicken embryo chorioallantoic membrane. We found that there is a developmentally regulated plasmin activity in this tissue, reaching maximum levels at the ninth day of embryo development, when angiogenesis and endothelial cell proliferation and migration are maximal (Ausprunk et al., 1974). Most of the literature concerning the role of plasmin in angiogenesis considers plasmin as a pro-angiogenic molecule (Pepper, 2001b). However, plasmin may also negatively regulate angiogenesis, through its participation in the production of angiostatin or angiostatin related proteins (this study and Andreasen et al., 2000). A possible antiangiogenic action of the PA/plasmin system is also supported by data showing that in vivo, plasminogen activator inhibitor 1 is proangiogenic at physiological concentrations (Devy et al., 2002) and high levels of plasminogen activator inhibitor 1 are predictive of poor survival prognosis for patients suffering from a variety of different cancers (Pedersen et al., 1994a,b).

As mentioned above, components of the PA/plasmin system are involved in the production of angiostatin in several tumor cells (Gately et al., 1997; Andreasen et al., 2000). Until recently, angiostatin was considered as a molecule being produced by tumors and implicated in inhibition of tumor angiogenesis and metastasis (Soff, 2000). However, the demonstration that angiostatin is also produced by macrophages during inflammation (Falcone et al., 1998) raised possibilities that it is also involved in non neoplastic conditions. Later studies showed that angiostatin is present in normal human plasma at concentrations 6–12 nM (Soff et al., 1999) and is implicated in prevention of

neovascularization in the hypoxic closed-eye environment (Sack et al., 1999). It seems likely that angiostatin plays an important role as a regulator of both physiological and pathological angiogenesis. In the present work, we detected several isoforms of angiostatin during physiological angiogenesis in the chicken embryo chorioallantoic membrane, where it has been shown to inhibit angiogenesis (Cao et al., 1999; Kisker et al., 2001; Scapini et al., 2002).

Angiostatin is well known to inhibit endothelial cell proliferation, migration and tube formation and to induce endothelial cell apoptosis (Ji et al., 1998; Hari et al., 2000). However, besides its effects on endothelial cells, the in vivo effects of angiostatin may be also due to its effects on blood cells. It has been suggested that neutrophils is a key cellular target for angiostatin, which inhibits their migration and neutrophil-mediated angiogenesis (Benelli et al., 2002). Moreover, neutrophils and macrophages generate angiostatin as a by-product of their secreted enzymes (Falcone et al., 1998; Scapini et al., 2002). Inflammatory cells seem to play a significant role in chorioallantoic membrane angiogenesis through inducible nitric oxide synthase production (Pipili-Synetos et al., 2000; Giannopoulou et al., 2002) and protein tyrosine nitration (Giannopoulou et al., 2002). Angiostatin in the chorioallantoic membrane is produced, at least to a significant extent, by chorioallantoic membrane blood cells (Fig. 6A).

In order to clarify the role of plasmin in chicken embryo chorioallantoic membrane angiogenesis, we used the plasmin inhibitors α_2-antiplasmin and ε-aminocaproic acid. Contrary to what was expected, α_2-antiplasmin, which is the primary endogenous inhibitor of plasmin activity (Collen, 1980), induces new vessel formation in vivo and endothelial cell activation in vitro. This induction is possibly due to decreased production of angiostatin from plasmin or/and plasminogen. On the other hand, ε-aminocaproic acid, which mimics the side chain of lysine (Lerch et al., 1980; Lin et al., 2000), inhibits angiogenesis in vivo and in vitro. This inhibition correlates with increased production of angiostatin in the chicken embryo chorioallantoic membrane and decreased production of MMP-2 by endothelial cells (data not shown).

The mechanism(s) through which the two plasmin inhibitors differentially affect angiostatin generation is unclear. ε-Aminocaproic acid does not affect the catalytic activity of plasmin (Lerch et al., 1980) and does not perturb angiostatin formation from (auto)proteolysis of plasmin (Stathakis et al., 1997), but inhibits the binding or dissociates plasmin and plasminogen from the cell surface (Miles and Plow, 1985; Silverstein et al., 1988; Gonias et al., 1989; Humphries et al., 1993). An increase of soluble active plasmin after treatment of chorioallantoic membrane cells with ε-aminocaproic acid may lead to increased production of angiostatin through auto-proteolysis of plasmin or degradation of plasminogen by plasmin. α_2-Antiplasmin, on the other hand, inhibits the activity of soluble plasmin and, thus, leads to decreased production of angiostatin. Aprotinin, which also inhibits plasmin catalytic activity, has similar to α_2-antiplasmin effects on human umbilical vein endothelial cell functions and on chorioallantoic membrane angiogenesis (data not shown).

An alternative mechanism for the inhibitory effects of ε-aminocaproic acid on endothelial cells may concern plasmin binding to the integrin $\alpha_v\beta_3$. It has recently been shown that plasmin-induced migration of endothelial cells requires $\alpha_v\beta_3$ (Tarui et al., 2001). The binding of plasmin to $\alpha_v\beta_3$ is through its kringles (Tarui et al., 2002) and can be inhibited by ε-aminocaproic acid. α_2-Antiplasmin does not significantly affect binding of plasmin on the cell surface (Hall et al., 1991; Humphries et al., 1993).

In summary, the present data suggest that α_2-antiplasmin and ε-aminocaproic acid, which inhibit plasmin via different mechanisms, differentially affect angiostatin production and angiogenesis in vivo and in vitro. These results should be considered during the experimental or clinical use of plasmin inhibitors and, moreover, elaborate a possible antiangiogenic role of plasmin in vivo through generation of angiostatin.

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References

Andreasen, P.A., Kjollerm, L., Christensen, L., Duffy, M.J., 1997. The urokinase-type plasminogen activator system in cancer metastasis. Int. J. Cancer 72, 1–22.

Andreasen, P.A., Egelund, R., Petersen, H.H., 2000. The plasminogen activation system in tumor growth, invasion and metastasis. Cell. Mol. Life Sci. 57, 25–40.

Ausprunk, D.H., Knighton, D.R., Folkman, J., 1974. Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. Dev. Biol. 38, 237–248.

Benelli, R., Morini, M., Carrozzino, F., Ferrari, N., Minghelli, S., Santi, L., Cassatella, M., Noonan, D.M., Albini, A., 2002. Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. FASEB J. 16, 267–269.

Cao, R., Wu, H.-L., Veitonmaki, N., Linden, P., Farnebo, J., Shi, G.-Y., Cao, Y., 1999. Suppression of angiogenesis and tumor growth by the inhibitor K1–5 generated by plasmin-mediated proteolysis. Proc. Natl. Acad. Sci. U. S. A. 96, 5728–5733.

Carmeliet, P., Jain, R.K., 2000. Angiogenesis in cancer and other diseases. Nature 407, 249–257.

Collen, D., 1980. On the regulation and control of fibrinolysis. Thromb. Haemost. 43, 77–89.

Cornelius, L.A., Nehring, L.C., Harding, E., Bolanowski, M., Welgus, H.G., Kobayashi, D.K., Pierce, R.A., Shapiro, S.D., 1998. Matrix metalloproteinases generate angiostatin: effects on neovascularization. J. Immunol. 161, 6845–6852.

Devy, L., Blacher, S., Grignet-Debrus, C., Bajou, K., Masson, V., Gerard, R.D., Gils, A., Carmeliet, G., Carmeliet, P., Declerck, P.J., Noel, A., Foidart, J.M., 2002. The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. FASEB J. 16, 147–154.

Dong, Z., Kumar, R., Yang, X., Fidler, I.J., 1997. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. Cell 88, 801–810.

Falcone, D.J., Borthll, W., McCaffrey, T.A., Mathew, J., McAdam, K., 1994. Regulation of macrophage receptor-bound plasmin by autoproteolysis. J. Biol. Chem. 269, 32660–32666.

Falcone, D.J., Khan, K.M., Layne, T., Fernandes, L., 1998. Macrophage formation of angiostatin during inflammation. J. Biol. Chem. 273, 31480–31485.

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., Volpert, O., Bouck, N., Soff, G.A., 1997. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc. Natl. Acad. Sci. U. S. A. 94, 10868–10872.

Giannopoulou, E., Katsoris, P., Hatziapostolou, M., Kardamakis, D., Kotsaki, E., Polytarchou, C., Parthymou, A., Papaioannou, S., Papadimitriou, E., 2001. X-rays modulate extracellular matrix in vivo. Int. J. Cancer 94, 690–698.

Giannopoulou, E., Katsoris, P., Polytarchou, C., Papadimitriou, E., 2002. Nitration of cytoskeletal proteins in the chicken embryo chorioallantoic membrane. Arch. Biochem. Biophys. 400, 188–198.

Giannopoulou, E., Katsoris, P., Kardamakis, P., Papadimitriou, E., 2003. Amifostine inhibits angiogenesis in vivo. J. Pharmacol. Exp. Ther. 304 (in press).

Gonias, S.L., Braud, L.L., Geary, W.A., Vandenberg, S.R., 1989. Plasminogen binding to rat hepatocytes in primary culture and to thin slices of rat liver. Blood 74, 729–736.

Hall, S.W., Humphries, J.E., Gonias, S.L., 1991. Inhibition of cell surface receptor-bound plasmin by α_2-antiplasmin and α_2-macroglobulin. J. Biol. Chem. 266, 12329–12336.

Hari, D., Beckett, M.A., Sukhatme, V.P., Dhanabal, M., Nodzenski, E., Lu, H., Mauceri, H.J., Kufe, D.W., Weichselbaum, R.R., 2000. Angiostatin induces mitotic cell death of proliferating endothelial cells. Mol. Cell. Biol. Res. Commun. 3, 277–282.

Humphries, J.E., Vasudevan, J., Gonias, S.L., 1993. Fibrinogenolytic and fibrinolytic activity of cell-associated plasmin. Arterioscler. Thromb. 13, 48–55.

Ji, W.R., Castellino, F.J., Chang, Y., Deford, M.E., Gray, H., Villarreal, X., Kondri, M.E., Marti, D.N., Llinas, M., Schaller, J., Kramer, R.A., Trail, P.A., 1998. Characterization of kringle domains of angiostatin as antagonists of endothelial cell migration, an important process in angiogenesis. FASEB J. 12, 1731–1738.

Kisker, O., Onizuka, S., Banyard, J., Komiyama, T., Becker, C.M., Achilles, E.G., Barnes, C.M., O'Reilly, M.S., Folkman, J., Pirie-Shepherd, S.R., 2001. Generation of multiple angiogenesis inhibitors by human pancreatic cancer. Cancer Res. 61, 7298–7304.

Lerch, P.G., Rickli, E.E., Lergier, W., Gillessen, D., 1980. Localization of individual lysine-binding regions in human plasminogen and investigations on their complex-forming properties. Eur. J. Biochem. 107, 7–13.

Lin, L.F., Houng, A., Reed, G.L., 2000. Epsilon amino caproic acid streptokinase–plasminogen activator complex formation and substrate binding through kringle-dependent mechanisms. Biochemistry 39, 4740–4745.

Miles, L.A., Plow, E.F., 1985. Binding and activation of plasminogen on the platelet surface. J. Biol. Chem. 260, 4303–4311.

Morikawa, W., Yamamoto, K., Ishikawa, S., Takemoto, S., Ono, M., Fukushi, J., Naito, S., Nozaki, C., Iwanaga, S., Kuwano, M., 2000. Angiostatin generation by cathepsin D secreted by human prostate carcinoma cells. J. Biol. Chem. 275, 38912–38920.

O'Reilly, M.S., Wiederschain, D., Stetler-Stevenson, W.G., Folkman, J., Moses, M.A., 1999. Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. J. Biol. Chem. 274, 29568–29571.

Papadimitriou, E., Heroult, M., Courty, J., Polykratis, A., Stergiou, C., Katsoris, P., 2000. Endothelial cell proliferation induced by HARP: implication of N or C terminal peptides. Biochem. Biophys. Res. Commun. 274, 242–248.

Papadimitriou, E., Polykratis, A., Courty, J., Koolwijk, P., Heroult, M., Katsoris, P., 2001. HARP induces angiogenesis in vivo and in vitro: implication of N or C terminal peptides. Biochem. Biophys. Res. Commun. 282, 306–313.

Patterson, B.C., Sang, Q.A., 1997. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J. Biol. Chem. 272, 28823–28825.

Pedersen, H., Brunner, N., Francis, D., Osterlind, K., Ronne, E., Hansen, H.H., Dano, K., Grondahl-Hansen, J., 1994a. Prognostic impact of urokinase, urokinase receptor, and type 1 plasminogen activator inhibitor in squamous and large cell lung cancer tissue. Cancer Res. 54, 4671–4675.

Pedersen, H., Grondahl-Hansen, J., Francis, D., Osterlind, K., Hansen, H.H., Dano, K., Brunner, N., 1994b. Urokinase and plasminogen activator inhibitor type 1 in pulmonary adenocarcinoma. Cancer Res. 54, 120–123.

Pepper, M.S., 2001a. Extracellular proteolysis and angiogenesis. Thromb. Haemost. 86, 346–355.

Pepper, M.S., 2001b. Role of the matrix metalloproteinase and plasminogen activator–plasmin systems in angiogenesis. Arterioscler. Thromb. Vasc. Biol. 21, 1104–1107.

Pepper, M.S., Montesano, R., Mandriota, S., Orci, L., Vassali, J.D., 1996. Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. Enzyme Protein 49, 138–162.

Pipili-Synetos, E., Papadimitriou, E., Maragoudakis, M.E., 1998. Evidence that platelets promote tube formation by endothelial cells in vitro. Br. J. Pharmacol. 125, 1252–1257.

Pipili-Synetos, E., Kritikou, S., Papadimitriou, E., Athanassiadou, A., Flordellis, C., Maragoudakis, M.E., 2000. Nitric oxide synthase expression, enzyme activity and NO production during angiogenesis in the chick chorioallantoic membrane. Br. J. Pharmacol. 129, 207–213.

Ribatti, D., Leali, D., Vacca, A., Giuliani, R., Gualandris, A., Roncali, L., Nolli, M.L., Presta, M., 1999. In vivo angiogenic activity of urokinase: role of endogenous fibroblast growth factor-2. J. Cell Sci. 112, 4213–4221.

Sack, R.A., Beaton, A.R., Sathe, S., 1999. Diurnal variations in angiostatin in human tear fluid: a possible role in prevention of corneal neovascularization. Curr. Eye Res. 18, 186–193.

Scapini, P., Nesi, L., Morini, M., Tanghetti, E., Belleri, M., Noonan, D., Presta, M., Albini, A., Cassatella, M.A., 2002. Generation of biologically active angiostatin kringle 1–3 by activated human neutrophils. J. Immunol. 168, 5798–5804.

Silverstein, R.L., Friedlander Jr., R.J., Nicholas, R.L., Nachman, R.L., 1988. Binding of Lys-plasminogen to monocytes/macrophages. J. Clin. Invest. 82, 1948–1955.

Soff, G.A., 2000. Angiostatin and angiostatin-related proteins. Cancer Metastasis Rev. 19, 97–107.

Soff, G.A., Hong, J., Fishman, D., Schultz, R., Cundiff, D., Park, S., Enghild, J., Stack, M.S., Gately, S., 1999. Angiostatin$_{4.5}$: a naturally occurring human angiogenesis inhibitor. Proc. Am. Assoc. Cancer Res. 40, 4088–4096.

Stathakis, P., Fitzgerald, M., Matthias, L.J., Chesterman, C.N., Hogg, P.J., 1997. Generation of angiostatin by reduction and proteolysis of plasmin. J. Biol. Chem. 272, 20641–20645.

Tarui, T., Miles, L.A., Takada, Y., 2001. Specific interaction of angiostatin with integrin alpha(v)beta(3) in endothelial cells. J. Biol. Chem. 276, 39562–39568.

Tarui, T., Majumdar, M., Miles, L.A., Ruf, W., Takada, Y., 2002. Plasmin-induced migration of endothelial cells: a potential target for the anti-angiogenic action of angiostatin. J. Biol. Chem. 277, 22564–33570.

Wiman, B., 1981. Human α_2-antiplasmin. Methods Enzymol. 80, 395–408.