Angiotensinogen and Its Cleaved Derivatives Inhibit Angiogenesis

Jérôme Célérier, Amauri Cruz, Noël Lamandé, Jean-Marie Gasc, Pierre Corvol

Abstract—The members of the serine protease inhibitor (serpin) family, which share a common tertiary structure and a role as serine protease inhibitors, are involved in a variety of newly discovered functions. For example, antithrombin III exerts a strong antiangiogenic activity. Angiotensinogen, the renin substrate, has a folded structure and is a member of the noninhibitory serpin subfamily. Two other noninhibitory serpins, maspin and pigment epithelium-derived factor, have antiangiogenic properties. We investigated the antiangiogenic effect of angiotensinogen and 2 related compounds: (1) des(angiotensin I)angiotensinogen, the product of angiotensinogen cleavage by renin, and (2) the reactive center loop-cleaved angiotensinogen, which is produced after selective and limited proteolysis by the protease V8. We used well-established in vitro (endothelial cell proliferation and migration, and capillary-like tube formation on Matrigel) and in vivo (the chick chorioallantoic membrane assay) models of angiogenesis to evaluate the antiangiogenic activities of these 3 related molecules. Our data demonstrated that these compounds exerted a clear and equipotent antiangiogenic effect, thus attributing a novel function to angiotensinogen and des(angiotensin I)angiotensinogen, for which no function was previously known. (Hypertension. 2002;39:224-228.)

Key Words: angiotensinogen ■ angiotensin I ■ endothelial growth factors

Angiotensinogen (AGT) is the precursor of angiotensin I (Ang I), an inactive decapeptide that is converted into Ang II, the main effector of the renin-angiotensin system (RAS). Its only role known is as a substrate for renin, a highly specific aspartyl protease. Renin cleaves the N-terminal end of AGT to generate Ang I. This leaves a much larger fragment intact (97.8% of the whole amino acid sequence), called des(angiotensin I)angiotensinogen (des[Ang I]AGT), which until now did not have any known function (Figure 1 for a schematic representation of AGT and its derivatives).

The biochemical, enzymological, and structural characteristics of AGT have been thoroughly investigated as it is the rate-limiting step in the first reaction of the RAS cascade: its concentration in human plasma (1 μmol/L) is close to its affinity (Km) for renin. The liver is the main site of AGT synthesis, but other sites include the glial cells, adipocytes, kidney, and the walls of large vessels. The concentration of AGT in the plasma is regulated by several endocrine factors and also depends on the genotype of the AGT gene. An AGT gene variant at position 235 (235T) is associated with a 10% to 20% increase in plasma AGT concentration and high blood pressure.

Des(Ang I)AGT was long considered to be a degradation product and thus has not been studied extensively. What has been suggested is that des(Ang I)AGT may inhibit the renin AGT reaction. AGT shares amino acid sequence and structural homologies with the serine protease inhibitor (serpin) family of proteins, but it has no inhibitor activity. Indeed, like 3 other noninhibitory serpins (ovalbumin, pigment epithelial-derived factor [PEDF], and maspin), AGT does not undergo the classical stressed-relaxed pathway of the inhibitory serpins such as antithrombin III or α1-antitrypsin.

Surprisingly, serpins appear to be involved in angiogenesis. The antiangiogenic properties of 2 serpins were discovered when antiangiogenic factors were screened for their ability to block endothelial cell growth in culture: the first is the reactive center loop (RCL)-cleaved form of antithrombin III, a protein with known anticlotting activity, and the second is PEDF, a protein with neurotrophic activity at its N-terminus. In addition, it was shown that maspin, a serpin with antitumor activity, may exert its antitumoral effect by inhibiting angiogenesis.

These studies suggest a common inhibitory mechanism of endothelial cell proliferation by serpins during angiogenesis. The mechanism is probably not related to an inhibitory serpin function because PEDF and maspin are noninhibitory serpins, and the RCL-cleaved antithrombin III has lost the ability to bind to its specific target. The tertiary structure of the cleaved form of antithrombin III was among the first serpin structures to be determined, and Fitzpatrick et al predicted that maspin contains a serpin fold. Therefore, the antiangiogenic effect of serpins is related to a common structural feature.
Sodium dodecyl sulfate (SDS) separation of the agarose by centrifugation, and its cleaved form was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

A, AGT comprises 452 amino acids (aa). B, AGT is cleaved by renin to produce the decapeptide Ang I and the derivatives. A new specific des(Ang I)AGT antibody D-854. This molecule was also recognized by the N-terminal antibody (N-1345) but was immunoprecipitated by the C-terminal antibody (C-1350). Des(Ang I)AGT antibody D-854. This molecule was also recognized by antibody (D-854) was raised against the first 8 amino acids residues of the renin product (NH2-Val11-Ile-His-Asn-Glu-Ser-Thr-Cys18-COOH) to specifically recognize des(Ang I)AGT.

Preparation of Des(Ang I)AGT and Proteinase V8-Cleaved AGT (RCL-cleaved AGT)

Human recombinant AGT was extensively hydrolyzed with human renin in 100 mMol/L citrate/NaH2PO4 buffer (pH 5.7). The renin reaction was stopped by the addition of 1 μmol/L pepstatin, and Ang I was removed by extensive dialysis (20 mmol/L Tris-HCl, pH 8.0, and 150 mmol/L NaCl). Immunohistochemical studies demonstrated further that the renin product had lost its Ang I moiety. The des(Ang I)AGT produced was not recognized by antibody N-1345 (directed against Ang I) and the C-terminal antibody (C-1350). A new specific des(Ang I)AGT antibody (D-854) was raised against the first 8 amino acids residues of the renin product (NH2-Val11-Ile-His-Asn-Glu-Ser-Thr-Cys18-COOH) to specifically recognize des(Ang I)AGT.

Proteins, Enzymes, and Antibodies

Human antithrombin III and porcine pancreatic elastase were obtained from Calbiochem (Bachem). The RCL-cleaved antithrombin III was prepared, using porcine pancreatic elastase, as described by O’Reilly et al. Purified human recombinant renin (Hoffmann La Roche) was used to generate des(Ang I)AGT from AGT. The AGT antipeptide antibodies were the Ang I antibody (N-1345) and (Hoffmann La Roche) was used to generate des(Ang I)AGT from AGT.

The optimal stimulatory concentrations of 3 ng/mL for vascular endothelial growth factor (VEGF), a gift from Dr J. Plouet, and 2.5 ng/mL for basic fibroblast growth factor (bFGF) (Clonetics) were used for all proliferation experiments. AGT or des(Ang I)AGT between 0.1 and 500 mmol/L plus 1 growth factor were incubated for 24 hours with 3H-thymidine (1 μCi/mL, Amersham). The remaining material after 10% trichloroacetic acid treatment was solubilized (200 mmol/L NaOH), and the incorporated radioactivity was measured by liquid scintillation counting.

Statistics

Data were compared statistically by use of a nonparametric t test (StatView). Significant values were taken as P≤0.05.

Endothelial Cell Migration

Endothelial cell migration assays were performed in modified Boyden chambers (Costar) with filters of 0.008-mm pore size. HUVECs were seeded at a density of 50 000 cells per well (in 200 μL of migration medium, EBM-2/0.1% BSA). Migration was induced by adding the migration buffer supplemented with 30 ng/mL VEGF to the lower part. Lower concentrations of VEGF did not stimulate migration during the study period. AGT and des(Ang I)AGT were added to the migration medium (1 μmol/L, 100 mmol/L, and 1 mmol/L) at the same time as VEGF. HUVECs were allowed to migrate for 6 hours at 37°C. After migration, the cells remaining on the upper side of the filter were removed mechanically. The filters were fixed with 4% paraformaldehyde, and the cells were stained with crystal violet (0.1% in PBS). The filters were then mounted on glass slides to count the cells that had migrated to the lower surface in 3 randomly selected areas per filter. Each assay was performed in triplicate.

Capillary-like Tube Formation on Matrigel

HUVECs in EBM-2 (2%FCS) were plated (10 000 cells per well) in 24-well plates (Costar) that had been precoated with 400 μL of Matrigel (Becton Dickinson) in the absence or presence of 100 mmol/L and 1 μmol/L AGT, des(Ang I)AGT, or RCL-cleaved AGT. After 24 hours at 37°C, the medium was aspirated, and cells were fixed with 4% paraformaldehyde. Capillary-like tubes were photographed under a dissecting microscope (Leica).

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Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells from lung (HMVECs-L; Clonetics) were cultured in EBM-2 medium (Clonetics) as recommended by the manufacturer. Only cells from passages 3 to 7 were used.

For the proliferation experiments, collagen-coated wells (24-well culture plate) were seeded with 5000 HUVECs or 10 000 HMVECs-L. After 24 hours, endothelial cells were starved in EBM-2 supplemented with 0.2% fetal calf serum (v/v) for 30 hours. The optimal stimulatory concentrations of 3 ng/mL for vascular endothelial growth factor (VEGF), a gift from Dr J. Plouet, and 2.5 ng/mL for basic fibroblast growth factor (bFGF) (Clonetics) were used for all proliferation experiments. AGT or des(Ang I)AGT between 0.1 and 500 mmol/L plus 1 growth factor were incubated for 24 hours with 3H-thymidine (1 μCi/mL, Amersham). The remaining material after 10% trichloroacetic acid treatment was solubilized (200 mmol/L NaOH), and the incorporated radioactivity was measured by liquid scintillation counting.

Nonendothelial Cell Culture

Aortic smooth muscle cells and normal human keratinocytes were obtained from Clonetics and cultured in the medium provided by the manufacturer, as recommended. For proliferation experiments, the same protocol was used as described above for the endothelial cells.

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Chorioallantoic Membrane Angiogenic Assay

Three-day-old chicken embryos were removed from their shells and placed in plastic Petri dishes. After 7 days of incubation, we laid a silicone ring (10-mm ID) randomly on the chorioallantoic membrane (CAM) for the deposition of testing solutions. All protein samples were extemporaneously diluted (20 mmol/L Tris-HCl, pH 8, and 150 mmol/L NaCl). AGT and des(Ang I)AGT (1 and 10 μg) and RCL-cleaved AGT (10 μg) dissolved in 20 μL of buffer were applied inside the ring twice at an interval of 24 hours. Photographs were taken just before application (day 0) and 48 hours after the first treatment.

First- and second-order centripetal blood vessels were quantified in a randomly selected zone representing one-third of the treated zone as previously shown. The control nontreated areas consisted of ~50 first-order vessels and ~20 second-order vessels and were always compared with the treated area of the same embryo. All observations were made in a rigorous double-blind manner.

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Statistics

Data were compared statistically by use of a nonparametric t test (StatView). Significant values were taken as P≤0.05.
Results

Full-Length AGT, Des(Ang I)AGT, and RCL-cleaved AGT are Antiangiogenic in the CAM

Observation at low magnification showed that when human AGT or its cleaved derivatives were placed on the CAM at day 7 of incubation, the density of small blood vessels was reduced 2 days later (Figure 2A through 2D). This antiangiogenic effect of AGT, des(Ang I)AGT, and RCL-cleaved AGT was caused by a reduction in the number of first- and second-order vessels compared with control in the untreated zone of the CAM in the same embryo. Preexisting larger vessels were not affected by the treatment. Quantification of microvascular density showed that 1 μg AGT or des(Ang I)AGT significantly reduced (20% to 35%) the density of small vessels (Figure 3), and a dose of 10 μg further reduced the vascular density to 50% of controls (P<0.01). At the same dose of 10 μg, RCL-cleaved AGT reduced angiogenesis by 65%. In the same assay, cleaved antithrombin III, but not intact antithrombin III, exhibited a marked antiangiogenic effect (data not shown), consistent with the results of O’Reilly et al. 10

Antiproliferative Effect of AGT and Its Cleaved Derivatives on Endothelial Cells In Vitro

Two types of endothelial cells, from micro- or macrovascular beds (HMVECs-L or HUVECs, respectively) were used for assessing the effect of AGT and its derivatives. VEGF- or bFGF-stimulated HUVEC proliferation was inhibited by AGT, des(Ang I)AGT, and RCL-cleaved AGT in a dose-dependent and similar fashion. The half maximal inhibition (EC50) was ≈50 to 100 nmol/L after VEGF stimulation (Figure 4A) and ≈100 nmol/L after bFGF stimulation (Figure 4B). AGT and/or des(Ang I)AGT had similar inhibitory effects on HMVECs(Figure 4C and 4D). All these results were significant by ANOVA (P<0.01).

In the same culture conditions, human aortic smooth muscle and human keratinocytes were not responsive to the inhibitory effect of AGT and des(Ang I)AGT, even when the inhibitor concentration was increased 10-fold (data not shown). No inhibitory effect of AGT and des(Ang I)AGT on

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Figure 2. Antiangiogenic effect of AGT and its cleaved forms des(Ang I)AGT and RCL-cleaved AGT on the CAM. A, Normal vascularization of the CAM at day 9 of chick development. B, Forty-eight hours after beginning the treatment of AGT, a marked inhibition of new small blood vessels in the CAM was observed (asterisks). Note that preexisting medium- and large-sized vessels are not affected (arrows). Similar antiangiogenic effects were also observed with similar doses of des(Ang I)AGT (C) and RCL-cleaved AGT (D). Bar, 2.5 mm.

Figure 3. Inhibition of angiogenesis by AGT and its derivatives on the CAM. Percentage of inhibition was evaluated by a comparison between the treated area and the nontreated area (absence of inhibition) of the same CAM. The graph shows the quantification of the CAM assay by counting the number of small vessels (first- and second-order as described in Methods) 48 hours after beginning the treatment with inhibitors. Values are mean±SD. *P<0.05.

Figure 4. Inhibition of endothelial cell proliferation. HUVECs (A and B) or HMVECs-L (C and D) stimulated by the optimal concentration of VEGF (3ng/mL, A and C) or bFGF (2.5ng/mL, B and D) were incubated with various concentrations of AGT (●), des(Ang I)AGT (♦), and RCL-cleaved AGT (◇). The effect of the inhibitors was compared with the basal control condition in the absence of inhibitor, arbitrarily evaluated as 0% of inhibition. Each point represents the mean±SD of triplicate samples, and each curve is representative of 2 or 3 independent experiment. Statistical significance by ANOVA, P<0.01.
cell proliferation was also observed when these cells were cultured in their cell culture proliferative medium.

Inhibition of Endothelial Cell Migration

To determine whether AGT and its derivatives affect endothelial cell migration, HUVECs were allowed to migrate in modified Boyden chambers in response to VEGF. At a dose of 30 ng/mL, VEGF promoted endothelial cell migration through the chamber membrane. When AGT, des(Ang I)AGT, and RCL-cleaved AGT (0.1, 1, and 10 nmol/L) were added, migration diminished by 50% to 75% in a dose-dependent manner (Figure 5).

Inhibition of Capillary-like Structure on Matrigel by AGT and Des(Ang I)AGT

The ability to form capillary-like structures in Matrigel is another specific property of endothelial cells and an important step in angiogenesis. When seeded on Matrigel, HUVECs formed a network of capillary-like structures with numerous intercellular contacts within 6 hours (Figure 6A). The addition of 1 μmol/L AGT, des(Ang I)AGT, or RCL-cleaved AGT to the Matrigel markedly decreased the length of these capillary-like structures and the number of junctions (Figure 6B through 6D).

Discussion

We have demonstrated that AGT and its related molecules, des(Ang I)AGT and RCL-cleaved AGT, exhibited similar effects in well-established models of angiogenesis: the CAM angiogenic assay, endothelial cell proliferation and migration, and the formation of capillary-like structures on Matrigel. The EC_{50} dose that inhibits endothelial cell migration (1 nmol/L) is close to that of PEDF (EC_{50}, 0.4 nmol/L) and lower than that of maspin (EC_{50}, 200 to 300 nmol/L). Endothelial cell proliferation was inhibited at higher AGT and des(Ang I)AGT concentrations (EC_{50}, 50 to 100 nmol/L) than those required for endothelial cell migration.

AGT, like maspin and PEDF, is antiangiogenic. In our study, neither the absence of Ang I nor the cleavage of the RCL affected the antiangiogenic property of AGT. This result is also consistent with the fact that maspin still inhibits endothelial cell migration, even though its RCL is truncated or disrupted by a point mutation. Thus, an intact RCL, which is a characteristic of the serpin fold, is not required for the antiangiogenic activity. Therefore, a common molecular mechanism probably accounts for the inhibition of angiogenesis by the noninhibitory serpins.

Our data raise a series of important questions concerning this new property of AGT and des(Ang I)AGT and its putative physiological relevance. First, it should be noted that the in vitro antiangiogenic effect of AGT and des(Ang I)AGT is observed at concentrations close to that of plasma AGT, ie, in the micromolar range. The amount of des(Ang I)AGT circulating in the plasma is similar to the amount of AGT circulating in the plasma. AGT is synthesized in different tissues and is rapidly secreted by a nonregulated pathway. Although several experiments have shown that Ang I is generated from local AGT, the amount of AGT and des(Ang I)AGT in tissues has not been reported.

Second, if AGT and des(Ang I)AGT have a physiological antiangiogenic activity, these proteins should be present near or within vascular structures and may be associated with extracellular matrix proteins. In this respect, it is interesting that AGT is produced not only in the liver AGT but also in the aorta and adipose tissue, which are major sites of angiogenesis. The glial cells also synthesize AGT, and to find a function to AGT produced by these cells, Kakinuma et al studied the effect of cold injury on the brain of AGT-deficient mice. They observed that AGT-deficient mice, exposed to cold for several days, exhibited an abnormal vascular brain barrier permeability that was associated with defective glial cells. Recently, the same group showed that renin-deficient mice do not exhibit this phenotype, which suggests that the...
vascular brain barrier abnormality in AGT-deficient mice was owing to a lack of AGT per se and not to a local production of Ang II. In the light of the antiangiogenic effect of AGT, we speculate that AGT produced by the glial cells surrounding the brain capillaries contributes to the stability of the blood brain barrier. The absence of AGT, as in AGT-deficient mice, would lead to a destabilization of the blood barrier, an increase in the vascular permeability, and ultimately plasma extravasation during a stresslike cold injury.

Third, Ang II has been reported to have angiogenic properties. This is controversial because quinapril, an ACE inhibitor, increased rather than decreased vascular density in a model of acute limb ischemia in the rabbit. The proangiogenic activity of Ang II was first demonstrated on the chicken CAM by Le Noble et al. and later in a sponge model by Machado et al. This angiogenic effect of the renin system on microvessels has been documented in a model of electrical stimulation–induced angiogenesis. It may seem paradoxical that the same molecule, AGT, exhibits an antiangiogenic effect as a serpin and an angiogenic effect as the precursor of Ang II. However, local conditions may determine whether the direct antiangiogenic effect of AGT or the proangiogenic effect of Ang II prevails. These local conditions are the consequence of multiple factors. For example, the clearance rate of Ang II is higher than that of AGT, which leads to a relatively stable concentration of AGT and des(Ang I)AGT as opposed to that of Ang II. AGT could also exert its antiangiogenic effect in a site devoid of renin and, therefore, of Ang II production; des(Ang I)AGT could exert an antiangiogenic effect when Ang II has been cleaved from AGT. It should be noted that in our experiments, Ang I was not produced when AGT was applied to the CAM as measured by radiomunoassay (J. Célérié, A. Cruz, N. Lamandé, J-M. Gasc, and P. Corvol, unpublished data, 2000). This was expected because of the strong renin species specificity.

The data reported in this study suggest that AGT possesses an antiangiogenic property, as well as being the precursor of Ang I. The putative physiological role of des(Ang I)AGT may be to participate to the angiogenesis/antiangiogenesis balance via its serpin fold.

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References

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