Impact of Apolipoprotein(a) on In Vitro Angiogenesis

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Abstract—Angiostatin, which consists of the kringle I–IV domains of plasminogen and which is secreted into urine, is an efficient inhibitor of angiogenesis and tumor growth. Because N-terminal apolipoprotein(a) [apo(a)] fragments, which also contain several types of kringle IV domains, are found in urine as well, we evaluated the potential angiostatic properties of these urinary apo(a) fragments and of a recombinant form of apo(a) [r-apo(a)]. We used human microvascular endothelial cell (hMVEC)–based in vitro assays of tube formation in 3-dimensional fibrin matrixes. Purified urinary apo(a) fragments or r-apo(a) inhibited the basic fibroblast growth factor/tumor necrosis factor-α–induced formation of capillary-like structures. At concentrations varying from 0.2 to 10 μg/mL, urinary apo(a) fragments inhibited tube formation by as much as 70%, whereas there was complete inhibition by r-apo(a). The highest concentrations of both inhibitors also reduced urokinase plasminogen activator production from basic fibroblast growth factor–induced hMVEC proliferation. The inhibitors had no effect on plasminogen activator inhibitor-1 expression. If our in vitro model for angiogenesis is valid for the in vivo situation as well, our data point toward the possibility that apo(a) may also be physiologically operative in modulating angiogenesis, as the concentration of free apo(a) found in humans exceeds that tested herein. (Arterioscler Thromb Vasc Biol. 2001;21:433-438.)

Key Words: Lp(a) • endothelial cells • kringle • urinary fragments

In adult organs, the turnover of endothelial cells is very slow and accelerates only under a few physiological conditions, such as embryogenesis, ovulation, and wound healing. Under these circumstances, angiogenesis, the formation of new blood vessels from existing ones, lasts for a relatively short period of time.1 Angiogenesis is well regulated through a coordinated balance of angiogenic and angiostatic factors.2 It represents a complex biological process that is initiated by localized activation of endothelial cells and an associated breakdown of the basement membrane.2 Thereafter, endothelial cells undergo an “angiogenic switch” and differentiate in a migrating, tube-forming cell phase that penetrates the surrounding extracellular matrix and forms a capillary “sprout.” During this process, a network of vessels is formed that is accompanied by the formation of new basement membranes.2,3 In contrast, pathological angiogenesis, which becomes uncontrolled due to an imbalance of proangiogenic and antiangiogenic factors, comprises tumor growth and invasion, metastasis, collateral vessel growth in atherosclerosis, ocular neovascularization, rheumatoid arthritis, and psoriasis.4

Angiogenesis in a temporary fibrin matrix, which has been used here as an in vitro assay, focuses on 1 particular pathway, namely, the activation of plasminogen (Plg) by tissue plasminogen activator and urokinase plasminogen activator (u-PA).3,5,6 In addition to the direct effect of plasmin, it also activates metalloproteinases, which initiate breakdown of the basement membrane in the early phases of angiogenesis. Interestingly enough, N-terminal fragments of Plg that are secreted into urine and consist of kringle structures (K-I to K-IV) inhibit angiogenesis,7,8 whereas Plg plays a crucial role in angiogenesis.8 There is also evidence that K-V can inhibit endothelial cell growth.9

Lipoprotein(a) [Lp(a)] is a glycoprotein composed of a core LDL with apolipoprotein(a) [apo(a)] attached by a disulfide bridge. Lp(a) is a highly atherogenic lipoprotein that is correlated with an increased risk for cardiovascular diseases and stroke.10,11 Besides its proposed role in atherogenesis, Lp(a) has been reported to promote the growth of human umbilical vein endothelial cells in synergy with basic fibroblast growth factor (bFGF), during which the latter seems to play a pivotal role.3 Apo(a) is a glycoprotein with repetitive kringle domains exhibiting 75% to 98% structural homology with Plg.12 In particular, apo(a) consists of 10 types of kringle-IV–like domains, which differ from each other by only a few amino acids. Additionally, apo(a) has 1 copy of a kringle-V–like domain and the Plg counterpart of a protease domain. The latter, however, appears to be enzymatically inactive in Lp(a).13–15

With regard to the high homology with Plg, one might expect that apo(a) or apo(a) fragments affect angiogenesis in a similar way as angiostatin, the urinary fragments of Plg. In
this study, we therefore evaluated the effect of recombinant apo(a) [r-apo(a)] and of urinary apo(a) fragments on human microvascular endothelial cell (hMVEC) growth and tube formation in 3-dimensional fibrin matrices. We demonstrate herein that full-length r-apo(a) and the urinary fragments of apo(a) yield a reduction of capillary tube formation and the expression of u-PA but not of Plg activator inhibitor-1 (PAI-1).

Methods

Materials
Penicillin/streptomycin, t-glutamine, and medium 199, with or without phenol red, and supplemented with 20 mmol/L HEPES were obtained from BioWhittaker; newborn calf serum and trypsin were purchased from Life Technologies. Tissue-culture plasticware was obtained from Costar. A crude preparation of endothelial cell growth factor was prepared from bovine hypothyroidal as described by MacG et al.15 Human serum was prepared from freshly obtained blood pooled from 10 to 20 healthy donors from a local blood bank and stored at 4°C. Human serum was obtained from Chiron. BFGF was purchased from Pepro Tech Inc, and human fibrinogen was provided by Lambda. LDL was purchased from Lipoid. Staphylococcal protein A was purchased from Pentapharm Ltd.

Cell Culture
hMVECs, isolated and characterized as previously described,17,18 were cultured on gelatin-coated dishes in medium 199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% human serum, 10% newborn calf serum, 150 μg/mL crude endothelial cell growth factor, 2 mmol/L t-glutamine, 5 U/mL heparin, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C under a 5% CO2/95% air atmosphere. After reaching confluence (~0.7 x 105 cells/cm2), the cells were cultured without growth factor for at least 24 hours before the experiments were started.

Preparation of r-Apo(a) and the Urinary Fragments of Apo(a)

r-Apo(a) was prepared from COS-7 cells transfected with the pSG-5 expression vector, which contained a full-length coding sequence for apo(a) with 18 K-IV repeats, 1 K-V domain, and the protease domain, as described previously.19 The fraction was homogeneous during SDS–polyacrylamide gel electrophoresis and immunologically pure. The yield provided by this procedure was 55% to 60%. Before use, apo(a) was dialyzed against PBS, and then the concentration was determined by dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) as described in detail.20

Urinary fragments of apo(a)22 were prepared in a similar manner. Urine from several probands with elevated plasma Lp(a) levels (>50 mg/dL) was collected, dialyzed against PBS buffer, and chromatographed in a first step over a wheat germ–lectin affinity column. For further purification, the eluted material was dialyzed against PBS and passed over an immunoadsorber, ie, affinity-purified IgG from sheep immunized with Lp(a) and coupled to Australia–Sepharose 6B CL. The coupled antibody was preabsorbed with LDL, Plg, and lipoprotein-free human serum and was specific for apo(a) as tested by Western blotting. Elution of urinary apo(a) fragments was achieved with a 0.2 mol/L glycine solution at pH 2.8.

ELISAs and Antibodies
In-house antisera against apo(a), LDL, and Plg were obtained by immunizing rabbits and sheep according to standard protocols.20 The antisera against Lp(a) was absorbed with Plg and other serum proteins and was monospecific for apo(a). u-PA and PAI-1 antigen determinations were performed by using commercially available immunoassay kits: a u-PA EIA HS (Taurus) and an Imulys PAI-1 (Biopool).

In Vitro Angiogenesis Model

The in vitro tube-forming assay in human fibrin matrixes in 48-tray dishes was performed exactly as described previously.2 The fibrin matrices were soaked in indicator-free medium 199 supplemented with 10% (vol/vol) human serum and 10% (vol/vol) newborn calf serum for 2 hours at 37°C to inactivate the thrombin. hMVECs were seeded in a 1.25:1 split ratio, and highly confluent endothelial cells (0.7 x 105 cells/cm2) were detached from the plates. Endothelial cells were stimulated with 10 ng/mL bFGF and 2.5 ng/mL TNF-α and incubated for the indicated time periods in fresh, indicator-free medium 199 containing 5% human serum.

Invasive cells and the formation of tubulike structures of endothelial cells in the 3-dimensional fibrin matrix were analyzed by phase-contrast microscopy. The total length of tubulike structures of 6 randomly chosen microscopic fields (7.3 mm2/field) was measured by using an Olympus CK2 microscope equipped with a computer and Optimas image analysis software. The extent of tube formation is expressed in mm/cm2, ie, the total length of all tubes found within 1 cm2 of fibrin matrix.

Incorporation of [3H]Thymidine

Endothelial cell proliferation was determined by the incorporation of [3H]thymidine into DNA. Confluent cultures of hMVECs were detached in a trypsin/EDTA solution and allowed to adhere and spread at a density of 105 cells/cm2 on gelatin-coated dishes in medium 199–HEPES solution. After 18 hours, the cells were stimulated with or without growth factors in a preincubation period of 48 hours, a tracer amount of [3H]thymidine (0.5 μCi/2 cm2 well) was added, and the cells were incubated for another 6 hours. Subsequently, the cells were washed and counted in a liquid scintillation counter.

Determination of Specific u-PA Binding

Dissopropylfluorophosphate-treated u-PA was labeled with Na125I by using the Iodo-Gen procedure (Pierce Chemical Co). Binding of 125I–u-PA to hMVECs was determined at 0°C. The cells were plated on melting ice and incubated for 10 minutes with 50 mmol/L glycine-HCl buffer (pH 3.0) to remove receptor-bound, endogenous u-PA. Subsequently, the cells were washed twice with ice-cold medium 199 and incubated with 8 nmol/L 125I–u-PA in endothelial cell–conditioned medium for 3 hours. In parallel incubations, a 50-fold excess of u-PA was included to assess nonspecific binding. Unbound ligand was removed by extensive washing with ice-cold PBS. Cell-bound ligand was solubilized with 0.3 mol/L NaOH and the radioactivity was determined in a gamma counter. Specific binding was calculated by subtraction of nonspecific binding from total binding.

Results

Effect of Apo(a) on Tube Formation

The majority of apo(a) circulating in the blood is bound via apOB to LDL, thereby forming the Lp(a) complex, yet there is a small fraction that is free of apoB.22 Because apo(a) cannot be dissociated from Lp(a) without altering its structure, we used r-apo(a) with 18 K-IV repeats, 1 K-V unit, and the protease-like domain to evaluate the effect on tube formation in fibrin matrixes. The insert in Figure 1 displays the purity of r-apo(a) as assessed by Western blot analysis. bFGF/TNF-α–stimulated hMVECs formed tubulike structures after a stimulation period of 7 days (Figure 1A). Tubes were abundant all over the fibrin matrix, which also penetrated the fibrin matrix–forming microtubule-like structures. Addition of 0.2 or 5 μg/mL r-apo(a) reduced the number and length of the tubes significantly (Figures 1B and 1C). The extent of tube formation was determined by image analysis. Figure 2A shows a representative in vitro tube-forming experiment in which r-apo(a), in amounts of 0.2 to 10 μg/mL, was added to bFGF/TNF-α–stimulated hMVECs. At the
lowest concentration tested [0.2 μg/mL apo(a)], inhibition of 70.0±3.1% compared with control cells was observed. The inhibitory effect of apo(a) on the bFGF/TNF-α–induced tube formation was dose dependent, and at a concentration of 10 μg/mL r-apo(a), tube formation was almost completely abolished.

Effect of Urinary Apo(a) Fragments on Angiogenesis

It has been shown previously that degradation products of apo(a) are secreted into urine. The concentration of apo(a) fragments found in urine amounts to roughly 0.1% of that present in plasma, yet there is large interindividual variation.21 Similar to angiostatin, urinary apo(a) consists of the N-terminal portion of apo(a) containing 2 to 9 K-IV repeats. Purified apo(a) fragments from urine of several donors were also effective in reducing the total tube length but to a lesser extent than did r-apo(a). A small, nonsignificant reduction of tube formation was seen at a concentration of 0.2 μg/mL (Figure 1D), which was dose dependent and reached significance at 1 μg/mL (Figure 2B). At 10 μg/mL a 72.0±3.1% inhibition was observed (Figure 1E). This effect was lower than that of r-apo(a), yet highly significant in comparison with control hMVECs incubated in the absence of apo(a) fragments (Figure 2B).

Apo(a) and Apo(a) Urinary Fragments Inhibit u-PA Accumulation in hMVEC Supernatants

The formation and growth of tubelike structures of hMVECs in our system require u-PA synthesis and secretion into the culture medium,18 binding to the specific cellular receptor, and activation of u-PA.23 We therefore tested the possibility that apo(a) and fragments of apo(a) exert their inhibitory effects on tube formation via the reduction of u-PA production. The u-PA antigen levels in the hMVEC supernatants cultured over a period of 7 days in the absence or presence of r-apo(a) or urinary apo(a) fragments were followed. Adding apo(a) to the medium decreased the amount of u-PA antigen accumulation. Almost total inhibition of the bFGF/TNF-α–stimulated u-Pa accumulation was observed when the hMVECs were incubated with 5 μg/mL apo(a). However, concentrations of 0.2 μg/mL and less did not affect the bFGF/TNF-α–induced u-PA accumulation (Figure 3A). A comparable effect on u-PA accumulation was also observed when the urinary apo(a) fragments were tested (Figure 3B).

Effect of Apo(a) and of Apo(a) Urinary Fragments on u-PA Receptor Expression by hMVECs

The amount of PAI-1, the inhibitor of u-PA, was determined in the supernatants of hMVECs treated with bFGF/TNF-α in the absence or presence of apo(a). Figure 4 shows that none of the apo(a) concentrations used (varying from 0.2 to 10
mg/mL) influenced significantly the bFGF/TNF-α–induced PAI-1 expression.

Does r-Apo(a) or Urinary Apo(a) Fragments Have an Influence on hMVEC Proliferation?
The incorporation of [3H]thymidine into the DNA of human umbilical vein endothelial cells triggered by bFGF and incubated with various concentrations (0.2 to 50 μg/mL) of r-apo(a) or urinary apo(a) fragments was studied in several experiments. Adding 5 μg/mL r-apo(a) blocked both the bFGF-induced human umbilical vein endothelial cell proliferation (up to 70% inhibition, Figure 5A) and the bFGF-induced hMVEC proliferation (up to 100% inhibition, Figure 5B). As already observed in the tube-forming assay, the urinary apo(a) fragments were somewhat less potent. Urinary apo(a) fragments at 5 μg/mL showed a reduction in human umbilical vein endothelial cell proliferation to 84% (Figure 5A), whereas 10 μg/mL of the fragments reduced the hMVEC proliferation to 72% (Figure 5B).

Discussion
The present study was inspired by the findings of Folkman’s group that angiostatin is one of the most efficient inhibitors of angiogenesis and tumor growth. Because N-terminal apo(a) fragments, which also contain several types of kringle IV domain, are found in urine as well as blood, we evaluated the potential angiostatic properties of these fragments and of a recombinant form of apo(a). There are several in vivo and in vitro models for studying angiogenesis and its modulation by various substances, and most of them relate to animal models. To avoid crossing species boundaries, we used an in vitro angiogenesis assay that was based on hMVECs. The urinary apo(a) fragments as well as r-apo(a) inhibited the bFGF/TNF-α–induced formation of capillary-like structures of hMVECs in 3-dimensional fibrin matrixes as well as growth factor–induced human umbilical vein endothelial cell proliferation.

There are some possible mechanisms to explain the inhibitory effect of apo(a) on in vitro tube formation. Our assay depends mainly on cell migration and invasion and requires the binding of u-PA to the u-PA receptor, followed by the activation of Plg and the formation of active plasmin. High concentrations of apo(a) and of the urinary apo(a) fragments inhibited u-PA production, which may explain the inhibition of tube formation under these conditions. However, low concentrations of apo(a) and of urinary apo(a) fragments sufficient to reduce tube formation did not affect the bFGF/TNF-α–induced u-PA accumulation (Figures 2 and 3), suggesting that additional mechanisms are involved. One possibility is that apo(a) competitively inhibits Plg binding to its receptor and the formation of active plasmin.
With respect to that concept, it is also noteworthy that Lp(a) selectively interacts with numerous cells, including monocytes, mesangial cells, liver cells, and endothelial cells, and in some of them, Lp(a) triggers a cell-signaling pathway. Of particular relevance are the studies with endothelial cells: Lp(a) has been reported to enhance dose-dependently the vasoconstrictor response to acetylcholine. Another study has reported that Lp(a) induces the secretion of monocyte chemotactic activity from human umbilical vein endothelial cells. Lp(a) also interferes with Plg binding to the surface Plg receptors on endothelial cells. Angiogenesis or tube formation was not addressed in any of those studies.

Urinary fragments of apo(a) in our in vitro assay were much less effective than was full-size r-apo(a). Because urinary apo(a) consists mainly of K-IV type 2 kringle domains, these results suggest that other structures in apo(a) might be primarily responsible for the described effects. The inhibitory effect of apo(a) and, to a lesser extent, of the urinary apo(a) fragments on endothelial proliferation is not relevant to our in vitro tube-forming model. Proliferation and tube formation are 2 different processes and may occur separately from each other. The presence of TNF-α, a potent inhibitor of growth factor–induced mitogenesis, and the fact that the tyrosine kinase inhibitor tyrphostin A47 had only a moderate inhibitory effect on the outgrowth of tubular structures indicate that stimulation of mitogenesis is not the predominant effect of growth factors during the formation of tubelike structures by hMVECs in fibrin matrices.

So far, there have been discordant reports in the literature concerning the effects of apo(a) on angiogenesis. In a study by Lou et al using the mouse sponge model and apo(a)-transgenic mice, apo(a) had neither a positive nor a negative effect on spontaneous angiogenesis. On the basis of results with the chorio-allantoic membrane assay using chicken embryonic cells, Ribatti et al postulated that Lp(a) induces angiogenesis, whereas Trieu and Uckun, using a model in which Lewis lung carcinoma cells were injected into transgenic mice, reported that apo(a) reduced angiogenesis. It should be emphasized that all of these former assays, including our own, are only models for angiogenesis and cannot be taken as proof that apo(a) in fact acts angiostatically in human species. Such final proof requires results from in vivo studies in humans, which are currently being designed.

In vivo, 3% to 5% of apo(a) is freely available in the circulation, and the rest of the apo(a) is attached via apoB to LDL particles, thus forming the Lp(a) complex. In vivo, 3% to 5% of apo(a) is freely available in the circulation, and the rest of the apo(a) is attached via apoB to LDL particles, thus forming the Lp(a) complex. Assuming that ~15% of the Lp(a) mass consist of apo(a) and a fraction of 5% circulates in free form, an individual with only 15% of the Lp(a) mass consist of apo(a) and a fraction of 5% circulates in free form, an individual with only 26.6 μg/mL (equal to 2.66 mg/dL) Lp(a) may contain some 0.2 μg/mL free apo(a). As the plasma Lp(a) concentration in the human population ranges from 1 to 250 mg/dL, we do believe that our results may well be of physiological relevance.

The physiological role of Lp(a) is unknown. Individuals with low or even undetectable Lp(a) plasma concentrations have no phenotype; nevertheless, it is astonishing that nature has generated this complicated glycoprotein without apparent biological function. Pathophysiologically, however, it is well documented in numerous prospective studies that individuals with plasma Lp(a) levels >25 to 30 mg/dL are at an increased risk for atherosclerotic diseases. The relevant pathomechanism is thought to relate to the structural homology of apo(a) with plasminogen (Plg), an important serine protease involved in the lysis of fibrin clots. The interaction of Lp(a) with plasminogen receptors on endothelial cells has been shown to cause a decrease in cell chemotactic activity from human umbilical vein endothe

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**Figure 4.** Effect of apo(a) on bFGF/TNF-α–induced PAI-1 accumulation. hMVECs were cultured on top of a 3-dimensional fibrin matrix in medium 199 supplemented with 10% human serum and 10% newborn calf serum with bFGF (10 ng/mL) and TNF-α (2.5 ng/mL) in the absence (control) or presence of increasing amounts of apo(a). At days 2, 5, and 7, the supernatants were collected, and PAI-1 antigen in the supernatants was measured by ELISA as described. The data are expressed as mean±SEM of triplicate wells (ng/well) and are representative of 3 independent experiments.

**Figure 5.** Effect of r-apo(a) and urinary apo(a) fragments on bFGF-induced human umbilical vein endothelial cell proliferation. Nonconfluent human umbilical vein endothelial cells (A) or hMVECs (B) were cultured for 48 hours in the absence (control) or presence of 2.5 ng/mL bFGF with or without 5 μg/mL (A), 10 μg/mL (B) apo(a), or 10 μg/mL urinary fragments of apo(a) in medium 199 supplemented with 10% newborn calf serum. After 48 hours, a tracer amount of [3H]thymidine was added to the medium, and incubation was continued in the same medium for another 6 hours; [3H]thymidine incorporation was determined as described in Methods. The data are expressed as mean±SEM of triplicate wells and are representative of 2 independent experiments.
apo(a) to Plg, causing competitive inhibition of fibrinolysis.\textsuperscript{26} Another mechanism by which Lp(a) might contribute to plaque and stenosis formation is the finding that it interferes with transforming growth factor-\(\beta\) activation.\textsuperscript{35}

On the other hand, reports are being accumulated that in the older population, the Lp(a) to apo(a) ratio is significantly increased, pointing toward the possibility that Lp(a)/apo(a) may also have some protective role against life-threatening diseases.\textsuperscript{36,37} Such mechanisms may well be connected to angiogenesis, as this latter process favors tumor infiltration and metastasis formation.\textsuperscript{38,39}

In summary, we have demonstrated herein that human r-apo(a) and the naturally occurring urinary apo(a) fragments affect in vitro tube formation of hMVECs in a fibrin matrix. The mechanism is likely to be related to functional interference with the action of Plg.

Acknowledgments

This work was supported by the SFB-Biomembranes No. F702 and F714, the Austrian National bank ÖNB project No. 7475, and the Dutch Cancer Society (TNOP 97–1511). Technical assistance by A. Ibovnik, H. Grillhofer, and M. Eichholzer is appreciated.

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doi: 10.1161/01.ATV.21.3.433
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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