TAFI and Pancreatic Carboxypeptidase B Modulate In Vitro Capillary Tube Formation by Human Microvascular Endothelial Cells

Ana H.C. Guimarães, Nancy Laurens, Ester M. Weijers, Pieter Koolwijk, Victor W.M. van Hinsbergh, Dingeman C. Rijken

Objective—Besides having a key role in fibrinolysis, the plasminogen system has been implicated in cell migration and angiogenesis. A common mechanism is the binding of plasminogen to carboxy-terminal lysine residues in partially degraded fibrin or on cellular surfaces. Here we examined the involvement of thrombin activatable fibrinolysis inhibitor (TAFI) and pancreatic carboxypeptidase B (CPB) in an in vitro capillary tube formation system, which is largely plasminogen-dependent.

Methods and Results—Human microvascular endothelial cells (hMVECs) were seeded on a 3D plasma clot matrix and subsequently stimulated with bFGF/tumor necrosis factor (TNF)-α. Tube formation was analyzed and fibrin degradation products (FbDP) were determined in the medium. Supplementation of the matrix with additional TAFI or CPB produced a reduction in tube formation. Pretreatment of hMVECs with CPB before seeding resulted in a similar effect. FbDP-levels indicated a concomitant reduction in matrix proteolysis. A TAFI inhibitor increased tube formation and FbDP release into the medium. In separate assays, CPB impaired the migration of hMVECs in a dose-dependent manner, whereas proliferation and adhesion remained unaffected.

Conclusions—Overall, these results demonstrate that TAFI and CPB in these systems modulate the plasminogen system both in the matrix and on the cell surface, thus leading to the inhibition of endothelial cell movement and tube formation.

(Arterioscler Thromb Vasc Biol. 2007;27:2157-2162.)

Key Words: TAFI ■ carboxypeptidase B ■ angiogenesis ■ plasma clot matrix ■ plasminogen

Activated thrombin activatable fibrinolysis inhibitor (TAFIa) is a basic carboxypeptidase that inhibits fibrinolysis by preventing the positive feedback in plasmin generation (reviewed in1-2). The proenzyme TAFI is synthesized in the liver and present in plasma and can be activated by trypsin, plasmin, and thrombin by a single cleavage at Arg-92. TAFIa, which is intrinsically unstable (half-life of about 8 minutes at 37°C), cleaves carboxy-terminal basic residues from proteins with a preference for carboxy-terminal arginine residues over carboxy-terminal lysine residues.3 Pancreatic carboxypeptidase B (CPB), a digestive basic carboxypeptidase, displays high homology with TAFIa but in contrast to TAFIa, CPB is a stable protease.

The conversion of plasminogen (Plg) into active plasmin is initiated either by the tissue-type plasminogen activator (tPA) or the urokinase-type plasminogen activator (uPA). tPA is mainly involved in the dissolution of fibrin in the circulation and uPA in the induction of pericellular proteolysis.3 The interaction between plasminogen and fibrin is dependent on lysine binding sites on plasminogen. Plasmin is able to generate new carboxy-terminal lysine and arginine residues in fibrin enhancing its own binding as well as the binding of plasminogen.6 This enhanced binding results in an increased catalytic efficiency of plasmin formation7 and can be blocked by TAFIa.8 TAFIa was shown to inhibit tPA and uPA in vitro plasma clot lysis,9,10 whereas in vivo inhibition of TAFIa was shown to enhance tPA-induced thrombolysis.11-14

Besides the key role that these carboxy-terminal lysine residues play in fibrinolysis they have also been implicated in cell migration, wound healing and angiogenesis where they function as binding sites for plasminogen.15 Lysine analogs, such as ε-amino-caproic acid (ε-ACA) and tranexamic acid (Cyclobalan) efficiently prevent plasmin formation16 and inhibit tumor cell metastasis and primary tumor growth.17,18 Moreover, binding of plasminogen to the cell surface can be abrogated by treatment with pancreatic CPB as well as with TAFIa.19

It is therefore possible that TAFI functions as a broad modulator of the plasminogen system in its various functions.

Original received May 24, 2006; final version accepted July 4, 2007.
From the Laboratory for Physiology (A.H.C.G., N.L., E.M.W., P.K., V.W.M.v.H.), Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, the Department of Hematology (A.H.C.G., D.C.R.), Erasmus University Medical Center, Rotterdam, and the Department of Biomedical Research (P.K.), TNO Quality of Life, Leiden, The Netherlands.
A.H.C.G. and N.L. contributed equally to this study.
Correspondence to Dr D.C. Rijken, Erasmus University Medical Center, Department of Hematology, Room Ee1393, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. E-mail D.Rijken@erasmusmc.nl
© 2007 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at http://atvb.ahajournals.org

DO: 10.1161/ATVBAHA.107.150144

2157
Although an TAFI−/− mice did not present an overt phenotype, recently an in vivo role for TAFI as a modulator of the plasminogen system has been demonstrated during fibrinolysis and during cell migration. Furthermore, TAFI-deficient mice were shown to display an impaired healing of cutaneous wounds and of colonic anastomoses. However, little is known about the possible effects of TAFI on endothelial cell migration and neovascularization.

Here, we investigated the participation of TAFI in the formation of capillary-like tubular structures in vitro using a model for tube formation that relies mainly on the Plg/plasmin system. The addition of antibodies against uPA, aprotinin, or antibodies against the uPA receptor (uPAR) completely inhibited bFGF/TNF-α–stimulated tube formation, whereas the addition of anti-uPA antibody or of a general matrix metalloproteinase (MMP)-inhibitor resulted only in a moderate inhibition. Frequently, neovascularization occurs in adults under conditions, in which a fibrinous exudate is formed, and this can facilitate the angiogenesis process. To our knowledge, this is the first time that TAFI has been demonstrated to have an effect on in vitro capillary-like tube formation. Moreover, this effect could not be solely ascribed to the cleavage of carboxy-terminal lysine residues from partially degraded fibrin, which composes the known substrate for TAFI and rather points to the existence of additional physiological substrates. We propose that TAFI might be involved in neovascularization processes during thrombus resolution, wound healing, and atherosclerosis.

**Materials and Methods**

For details on methods, please see the supplemental material (available online at http://atvb.ahajournals.org).

**Materials**

CPB was purchased from Sigma-Aldrich, and TAFI was isolated as described previously. TAFI-depleted plasma was prepared using an anti-TAFI IgG Sepharose column.

**Cell Culture**

Foreskin hMVECs were isolated, cultured, and characterized as previously described.

**In Vitro Tube Formation Assay**

The formation of capillary-like tubes in plasma clot matrices was performed and evaluated essentially as previously described in fibrin matrices.

**Results**

For Supplemental Figure I and II, please see the supplemental material.

**Involvement of TAFI in Tube Formation in a 3D-Plasma Clot Matrix**

To study the involvement of TAFI during tube formation, we used a 3D in vitro model where a plasma clot is used to mimic the provisional wound matrix. hMVECs were seeded on top of the plasma clot matrix and stimulated with bFGF/TNF-α to form capillary-like structures (Figure 1A). Figure 1B demonstrates that addition of the TAFIa-specific inhibitor PCI stimulated the formation of tubular structures by 57% (P<0.01), whereas increasing the TAFI concentration (by 50 nmol/L) in the plasma clot matrix inhibited tube formation (42%, P<0.05). In agreement with previous findings, the plasmin inhibitor aprotinin caused a strong inhibition of tube formation (80%, P<0.01) confirming the dependency of the model on the activity of plasmin. Similarly, inhibition of uPA by anti-uPA and of uPAR by anti-uPAR (MoAb H2) also reduced tube formation corroborating the involvement of cell-bound u-PA on tube formation (both over 80%, P<0.01; not shown). The inhibitory effect of TAFI during tube formation was also examined in experiments where a direct thrombin inhibitor (hirudin, 40 U/mL) was added to the stimulation medium. Addition of PCI under these conditions resulted in a similar stimulation of capillary tube formation (1.6-fold, P<0.001). This implies that not thrombin but an alternative pathway is responsible for TAFI activation in our model. Addition of hirudin increased capillary tube formation with stimulation with bFGF/TNF-α by 139% (P<0.001).

Because TAFIa is an unstable enzyme, pancreatic CPB was incorporated into the plasma clot matrix. Addition of CPB (1 to 10 U/mL) to the matrix induced a significant and dose-dependent inhibition of tube formation, which was significant at 5 and 10 U/mL CPB (both P<0.01; Figure 1C). The inhibition of tube formation observed with the addition of 50 nmol/L TAFI to the matrix was comparable to the inhibition observed with 1 to 5 U/mL CPB (compare Figure 1B and 1C).

The inhibitory effect of TAFI during tube formation might emerge from its known downregulation of fibrin matrix degradation or from an effect on hMVECs themselves. To further explore this, the hMVECs were pretreated with the stable active enzyme CPB. Subsequently, the hMVECs were washed and seeded on top of the plasma clot matrix. After 24 hours they were stimulated with bFGF/TNF-α alone (CPB−) or combined with the addition of CPB to the medium (25 U/mL, CPB+). This pretreatment (CPB+) resulted in an inhibition of tube formation (58%, P<0.01) pointing to a direct effect on the hMVECs (Figure 1C). Furthermore, when CPB was also added to the stimulation medium (CPB+), a supplementary inhibition in tube formation was observed (73%, P<0.01). It is interesting to notice that either supplementation of the matrix with 10 U/mL CPB or treatment of hMVECs with CPB in combination with CPB in the medium (CPB+++) resulted in an inhibition of tube formation comparable to the addition of aprotinin. This suggests that the observed effects reflected complete interference with the uPA/plasmin system.

**Fibrin Degradation Products and uPA Accumulation During Tube Formation**

The amount of fibrin degradation products (FbDPs) accumulated in the conditioned medium markedly increased during tube formation, as evaluated during 2 48-hour periods after initial stimulation by bFGF/TNF-α (Figure 2A). This accumulation was largely inhibited by aprotinin (81%, P<0.01). Addition of PCI in the matrix enhanced FbDP accumulation in the medium (87%, P<0.01) whereas addition of TAFI (Figure 2A) inhibited the release of FbDPs compared with bFGF/TNF-α stimulation only (57%, P<0.01). Moreover, when the matrix was supplemented with CPB (Figure 2B), FbDP levels decreased...
(71% CPB₁, 86% CPB₅, and 89% CPB₁₀, all P<0.01) pointing to a downregulation of fibrinolysis. Pretreatment of hMVECs with pancreatic CPB also inhibited FbDPs release (87% CPB₁, and 93% CPB₁₀, both P<0.01). These results point once more to a downregulation of the Plg/uPA system.

The accumulation of uPA in the stimulation medium during tube formation was significantly decreased when...
hMVECs were pretreated with CPB (46% CPB and 48% CPB compared with bFGF/TNF-α, both P < 0.01; not shown). The amount of uPA found in the medium under bFGF/TNF-α stimulation corresponded to about 16 ng/mL. The addition of CPB, PCI, or TAFI to the matrix did not alter the uPA accumulation compared with bFGF/TNF-α.

TAFI Concentration in the Plasma Clot Matrix and Consequences for Tube Formation
We examined the effect of reducing TAFI concentration in the plasma clot matrix by testing serial dilutions of normal pooled plasma in TAFI-depleted plasma. Reduction of the TAFI content of the plasma clot matrix caused an acceleration of the tube formation (Figure 3A) and an increase in FbDP accumulation (Figure 3B). Depletion of TAFI in the plasma clot matrix or addition of PCI to normal plasma clot matrix had similar results (Figure 3A and 3B).

The reduction of TAFI content not only caused an increase in capillary tubes but also modified their structure. Cross-sections were made from these plasma clot matrices perpendicularly to the matrix surface and the morphology of the tubes formed in the matrices was examined. The tubes formed in matrices containing reduced TAFI levels displayed a more extensive network and were accompanied by wider tubes/lumen-like structures, often in the upper area of the plasma clot matrix, suggesting an increased fibrin degradation (Figure 4A through 4C) and also possibly an altered migration.

Effect of CPB on hMVEC Proliferation, Adhesion, and Migration
The results above suggest that the basic carboxypeptidases CPB and TAFI are able to modulate hMVECs functions, probably via the regulation of the uPA/plasmin system. We therefore used CPB to study the effect of basic carboxypeptidase activity on the proliferation, adhesion, and migration of hMVECs. Proliferation, in the presence of bFGF was not affected by addition of increasing amounts of CPB (1, 10, and 100 U/mL; not shown). In addition, the adhesion of hMVECs was not affected in the presence of 10 or 100 U/mL CPB in the culture medium, as estimated by cell counting (not shown). A wound assay was used to investigate the effect of carboxypeptidase B activity on hMVEC migration under similar stimulation conditions as used for the tube formation assay, namely stimulation by bFGF/TNF-α. In this assay the...
migration of hMVECs was impaired in a dose-response manner by increasing CPB concentrations in a 24-hour period (supplemental Figure I). The uPA/plasminogen system was also involved during the migration of hMVECs, under bFGF/TNF-α stimulation, as the addition of anti-uPAR, anti-uPA, or aprotinin delayed the migration of hMVECs.

**Localization of TAFI in an Atherosclerotic Plaque**

To investigate the presence and localization of TAFI in new vascular structures formed in a fibrinous environment, immunohistochemical analysis of TAFI was performed in tissue sections of atherosclerotic plaques with organized thrombi. The neointima with incorporated thrombus contained new capillaries. The endothelial cells of the newly formed microvessels were visible after staining for CD31 (PECAM-1) (supplemental Figure II A). Staining with an antibody against TAFI (supplemental Figure II B and II C) revealed that TAFI was, as expected, present in the fibrinous exudate, and accumulated in many of the vascular structures in this thrombus. This occurred possibly by the colocalization of TAFI with the endothelial cells lining the vessels.

**Discussion**

Currently, TAFI is primarily seen as an inhibitor of the plasminogen system during fibrinolysis. Yet, there is increasing evidence that TAFI function may not be restricted to fibrinolysis but that TAFI may also act as a regulator of inflammation and as a modulator of the plasminogen system during tissue remodeling and cell migration. In this report we have provided evidence for the involvement of TAFI in the formation of capillary-like tubular structures by hMVECs in a 3D-plasma clot matrix. To our knowledge, these results represent the first attempt to investigate the role of TAFI in tissue remodeling processes in vitro.

The absence of a dramatic phenotype for TAFI−/− mice20–22 is shared with deficiencies of other components of the fibrinolytic system32–34 and does not necessarily mean that TAFI does not fulfill a physiological role. Supporting this notion, TAFI−/− mice have impaired wound healing and abnormal keratinocyte migration25 and using TAFI−/−, Plg−/− mice it was demonstrated that TAFI regulates the functions of the plasminogen system both in fibrinolysis and in cell migration in vivo.21 Moreover, it should be noted that during in vivo tissue remodeling a functional overlap between the functions of the plasminogen/uPA system and of the MMP system occurs. It has been elegantly shown that wound healing is impaired both in Plg-deficient mice and in wild-type mice treated with the MMP inhibitor galardin whereas a complete arrest could only be achieved when Plg-deficient mice were treated with a MMP inhibitor.35

On the basis of present knowledge, different mechanisms by which TAFI may be involved in our in vitro model of capillary-like tube formation can be envisaged. First, in this model the Plg/uPA system localizes the proteolytic activity to specific sites on the cell surface, facilitating matrix degradation and the invasion into the matrix. Therefore, TAFI might inhibit tube formation by removing carboxy-terminal lysines in the plasma clot matrix, preventing the upregulation of plasminogen activation in the matrix and in this way decreasing proteolysis. This mechanism fits with our observations as increasing TAFI or CPB concentrations in the matrix impaired tube formation and decreased proteolysis of the matrix (decrease in FbDP release). Addition of a TAFIa inhibitor (PCI) or reduction of TAFI concentration in the matrix resulted in the acceleration of tube formation and of matrix proteolysis.

Second, it has been shown previously that the treatment of cells with pancreatic CPB35,36 and with TAFIa results in a striking inhibition of plasminogen binding to cells, which was mediated by carboxypeptidase activity. This binding of plasminogen relies on cell surface receptors or other cell surface proteins, which have as common characteristics their relatively low affinity (Kd≈1 μmol/L), high density (104 to 107 sites/cell), and requirement of free lysine binding sites of plasminogen. To investigate whether TAFI could be involved in the regulation of these receptors or other cell surface proteins we pretreated the hMVECs with CPB before seeding. Our results show that pretreatment of hMVECs efficiently inhibited tube formation and downregulated proteolysis. In addition, reduced accumulation of uPA in the stimulation medium was observed when hMVECs were pretreated with CPB. Although we cannot completely exclude that a small fraction of CPB may remain bound to the hMVECs, even after extensive washing, these results corroborate a direct effect of CPB on the the hMVECs, suggesting that the modulation of the cell-associated functions of the plasminogen system by TAFI may take place at several levels.

Third, Plg-deficient mice were found to have impaired wound healing with a decreased rate of keratinocyte migration34 and a disturbed keratinocyte migration was also reported in TAFI−/− mice.22 Decreasing TAFI concentration in the plasma clot matrix in our model also altered the magnitude of tube formation and the morphology of the capillary structures formed suggesting alterations of the migration pattern of the hMVECs. In agreement with in vivo results,21,22 TAFI seems to be able to modulate cell migration in our tube formation model probably by modulation of the Plg/uPA system (uPAR, uPA, and plasmin). In the wound assay, the migration of the hMVECs was inhibited in a dose-dependent manner by CPB and the Plg/uPA system was again shown to be involved. In addition, we observed a decreased accumulation of uPA in the conditioned medium when hMVECs were pretreated with CPB, which matches with the reduced hMVECs migration observed in the wound assay in the presence of CPB.

In the in vitro model of capillary-like tube formation, the 3D-plasma clot matrix was prepared by the addition of thrombin to plasma and thus one might suppose that TAFI activation was mediated by thrombin. However, a direct thrombin inhibitor was not able to abolish the effect of TAFI in this model, which points to additional pathways for TAFI activation. In fact, it seems more likely that the activation of TAFI may be induced by plasmin particularly in a cellular environment where this pathway is stimulated by glycosaminoglycans,37 but this has to be further explored.

It should be noted that our results are confined to neovascularization in a plasma-rich clot matrix. We can best
perceive this in vitro system as a model of wound healing angiogenesis and it can be compared with pathological conditions such as the neovascularization of a thrombus incorporated in an atherosclerotic plaque. Indeed, TAFI was clearly present both in the matrix and in the endothelial cell lining of the newly formed microvessels in the thrombus that we analyzed by immunohistochecmy.

In conclusion, our results provide evidence that TAFI is a skillful modulator of the cellular functions of the plasminogen/PA system. TAFI regulates at several levels the fine tuning of capillary tube formation and of matrix proteolysis by controlling the upregulation of plasminogen binding to the plasma clot matrix and to the cell surface and by controlling the migration of hMVECs.

Sources of Funding
This work has been supported by ZonMw (the Netherlands Organization for Health Research and Development) grant 902-17-090 (V.W.M.v.H. and N.L.) and a grant BGT.6733 of the Dutch Program for Health Research and Development (E.M.W.).

Disclosures
None.

References
TAFI and Pancreatic Carboxypeptidase B Modulate In Vitro Capillary Tube Formation by Human Microvascular Endothelial Cells
Ana H.C. Guimarães, Nancy Laurens, Ester M. Weijers, Pieter Koolwijk, Victor W.M. van Hinsbergh and Dingeman C. Rijken

Arterioscler Thromb Vasc Biol. 2007;27:2157-2162; originally published online August 2, 2007; doi: 10.1161/ATVBAHA.107.150144
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/10/2157

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/09/20/ATVBAHA.107.150144.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Text and Figures for article by Guimarães et al. “TAFI and pancreatic carboxypeptidase B (CPB) modulate in vitro capillary tube formation by human microvascular endothelial cells”.

Methods

Materials

Cell culture reagents were purchased as previously described. Human serum (HS) was obtained from a local bloodbank and was prepared from freshly obtained blood from 10-20 healthy donors, pooled and stored at 4°C. Newborn calf serum (NBCS) was obtained from Life Technologies (Grand Island, NY, USA). NBCS and HS were heat-inactivated before use. Basic fibroblast growth factor (bFGF) was purchased from Prepro Tech EC (London, UK) and human recombinant tumor necrosis factor-α (TNF-α) was a gift from Dr. J. Travernier (Biogent, Gent, Belgium) and contained 2.45 x 10^7 U/mg protein and less than 40 ng lipopolysaccharide per mg protein. Aprotinin was purchased from Pentapharm Ltd. (Basel, Switzerland). Heparin and bovine thrombin were obtained from Leo Pharmaceuticals Products (Weesp, the Netherlands) and potato carboxypeptidase inhibitor (PCI) was acquired from Calbiochem (La Jolla, CA, USA). Pancreatic carboxypeptidase B was purchased from Sigma-Aldrich (St. Louis, MO, USA) and TAFI was isolated as described previously. Citrated platelet-poor plasma from ten healthy volunteers was obtained from the blood bank, pooled and stored at -80°C. TAFI-depleted plasma was prepared using an anti-TAFI IgG sepharose column essentially as previously described. Refludan®-Lepirudin, a direct thrombin inhibitor was obtained from Pharmion (Amsterdam, The Netherlands). The monoclonal u-PA receptor (uPAR)-blocking antibody H-2 was a kind gift from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany). Rabbit polyclonal anti-u-PA antibodies were prepared in our laboratory. Horseradish peroxidase (HRP) conjugates of sheep anti-human TAFI IgG and rat anti-mouse IgG were from Affinity Biologicals (Hamilton, Ontario, Canada) and DAKO (Glostrup, Denmark), respectively. The CD31 murine IgG (clone 1A10) was from Monosan (Sanbio, Uden, The Netherlands).

Cell culture

Human foreskin microvascular endothelial cells (hMVECs) were isolated, cultured and characterized as previously described.

Preparation of the 3D-plasma clot matrix

Plasma clot matrices were prepared by the addition of 1 U/ml thrombin to citrated platelet-poor plasma. Immediately afterwards, 300 µl aliquots of this mixture were added to the wells of a 48-well plate. The plasma clot matrices were left at room temperature (RT) for at least 30 minutes and then equilibrated at 37°C, under humidified 5% CO₂/ 95% air atmosphere with serum-containing culture medium (Medium 199 - M199, supplemented with 10% (v/v) HS, 10% (v/v) NBCS, 100 IU/ml penicillin and 100 µg/ml streptomycin). During the next 24 hours, the matrices were thoroughly washed with serum-containing culture medium (3 to 4 times).

Pre-treatment of hMVECs with CPB

The hMVECs were detached by treatment with trypsin/EDTA and suspended in serum-containing culture medium. After washing, the hMVECs were incubated with 50 units/ml of pancreatic CPB in serum-containing culture medium at 4°C. After 30 min the CPB-containing medium was removed and the hMVECs were washed three times by centrifugation. These pre-
treated hMVECs were resuspended in serum-containing culture medium and seeded at a split ratio of 2:1 on top of the plasma matrices. Non-treated cells were submitted to the same procedure with serum-containing medium, in the absence of CPB.

In vitro tube formation assay
The formation of capillary-like tubes was evaluated essentially as previously described in fibrin matrices. Confluent hMVECs were seeded at a split ratio of 2:1 on top of the plasma matrices and cultured growth factor free for 24 hours in serum-containing culture medium. Then, the cells were stimulated with stimulation medium composed of serum-containing culture medium supplemented with bFGF (10 ng/ml) and TNF-α (10 ng/ml). The reagents to be tested were either added to plasma before preparing the matrix or added to the stimulation medium. Every second day the stimulation medium was collected and renewed. After seven days the formation of tubular structures was analyzed by phase-contrast microscopy. Quantification of the length of the structures formed was performed essentially as previously described, by measuring in six randomly chosen microscopic fields (7.3 mm²/field) using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software. For morphologic evaluation, fibrin matrices were fixed at 4°C for 3 hours in 2%(w/v) p-formaldehyde in phosphate buffered saline, washed for 3 hours in 6% (w/v) sucrose, dehydrated in a graded series of ethanol and finally embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany) and sectioned perpendicularly to the matrix surface (4 µm).

Wound assay
Confluent hMVECs were seeded on fibronectin-coated dishes of a 48-wells plate in serum-containing culture medium with 150 µg/ml crude endothelial cell growth factor and 5 U/ml heparin. Subsequently, hMVECs were growth factor deprived for 24 hours. A scratch was applied to the confluent monolayer and the cells were washed for three times with M199 to remove the detached cells. Immediately, the stimulation medium (serum-containing culture medium with 10 ng/ml bFGF and 10 ng/ml TNF-α) supplemented with the compounds of interest was added to the wells and the cells were incubated at 37°C, under humidified 5% CO₂/95% air atmosphere. During the following 24 hours, photographs of the wound were taken every 2 hours and wound analyses were performed by calculating the diameter of the wound for each time point (4 measurements/well) for duplicate wells.

Adhesion assay
Fibrin matrices were prepared in a 96-well plate by the addition of thrombin (0.1 U/ml) to fibrinogen (2 mg/ml) in M199. After clotting the matrices were equilibrated with 100 IU/ml penicillin, 0.1mg/ml streptomycin, 1%(w/v) L-glutamine, 10%(v/v) human serum and 10%(v/v) NBCS in M199 and incubated with 100 IU/ml penicillin, 0.1mg/ml streptomycin, 1%(w/v) L-glutamine, and 10%(v/v) NBCS in M199 overnight at 37°C. Confluent hMVECs were detached and resuspended in 1% (w/v) HSA in M199 supplemented with the compounds of interest, seeded at a split ratio of 1:4 on top of the fibrin matrices and allowed to attach for 4 hours at 37°C under 5% CO₂/95% air atmosphere. The wells were washed 3 times with M199 supplemented with penicillin/streptomycin and fixed with 2% (w/v) p-formaldehyde at 4°C, after which adhered cells were counted. The amount of attached cells was expressed as the mean percentage ± SEM of the results obtained in the control condition.
Proliferation assay
hmVECs were seeded 1:8 on a fibronectin-coated 24-well plate in serum-containing culture medium (0.5 ml/well). After 24 hours, the cells were stimulated with bFGF (10 ng/ml) alone or in combination with CPB (1, 10 or 100 U/ml) and incubated at 37°C under humidified 5% CO₂/95% air atmosphere for 96 hours. After the incubation period, the cells were washed for three times with M199 with 100 IU/ml penicillin and 0.1 mg/ml streptomycin and fixed for 10 minutes with 2% glutaraldehyde. The cell membrane was rendered permeable by fixation with 70% ethanol for 10 minutes. The cells were stained with crystal-violet for 2h, washed for four times with MilliQ and dried overnight at RT. Cell counting was performed by the computer program Optimas. The experiment was performed in duplicate and the data was based on six countings per well.

Fibrin degradation products (FbDP) EIA and uPA ELISA
The FbDP EIA was performed essentially as previously described. Calibration was performed using whole-blood clot lysate and pooled normal plasma was used as control. FbDP levels were calculated from duplicate measurements.

uPA antigen was measured by ELISA essentially as previously described.

Immunohistochemistry
Tissue sections (5 µm) of human atherosclerotic plaques with an incorporated mural thrombus were dewaxed by immersion in xylene and rehydrated in decreasing concentrations of ethanol. For HPS staining, the sections were counterstained with Mayer’s hematoxylin, phloxin and saffron. After dehydration in a reversed ethanol-xylene series, the sections were prepared for microscopy. Inhibition of endogenous peroxidase was accomplished by immersion in 1% hydrogen peroxidase in absolute methanol for 20 min. The sections were washed in deionized water and equilibrated in phosphate-buffered saline (PBS, pH 7.4). For antigen retrieval, sections were incubated in 0.1 M sodium-citrate in a microwave at 700 Watt until boiling point was reached, followed by a period of 10 min at 180 Watt. Subsequently, all the sections were blocked by 5% BSA in PBS to prevent nonspecific binding. Different antibodies were applied to the sections overnight at 4°C. After washes with PBS, the sections were exposed to the second antibody, the biotinylated horse anti-mouse IgG diluted in 1% BSA/PBS in a concentration of 1:400, for 1 hour at RT. Then after further washes with PBS, the sections were incubated for 30 min at RT with the HRP Avidin Biotinylated Complex (ABComplex). The signal was amplified by biotinylated tyramides for 10 min at RT, followed again by the HRP ABComplex for 30 min at RT. The sections were washed with PBS and stained with Novared for a period of 5 to 10 min. Finally, the sections were washed in aquadest, counterstained with hematoxylin, washed in running water and dehydrated in a reversed ethanol-xylene series and prepared for microscopy.

Statistical analysis
Tube formation results were expressed as the mean percentage ± SEM of the results obtained in the bFGF/TNF-α condition. For statistical analysis, we used one-way ANOVA followed by the Dunnett’s test as post-test. The Dunnett’s test is a modified t-test that takes into account multiple comparisons with a control condition. Statistical significance was accepted at \( P<0.05 \).
References


Supplemental Fig. I: Pancreatic carboxypeptidase B inhibits migration of hMVECs in a concentration-dependent way. Confluent hMVECs were seeded on a fibronectin coated well to obtain a cobblestone monolayer. (A) Scratches were made through the monolayer and photomicrographs of the migrating hMVECs were taken at regular intervals. (a), 0 hours; (b), 12 hours and (c), 24 hours (original magnification 20x). (B, C) Immediately after wounding, the cells were stimulated with bFGF (10ng/ml)/TNF-α (10ng/ml) (striped bars). (B) A negative control without stimulation was included (open bars). Some wells were exposed to combined stimulation with bFGF/TNF-α and increasing concentrations of CPB (B) 1 unit/ml (light grey bar), 10 units/ml (dark grey bar) and 100 units/ml (black bar) or (C) anti-uPA antibody (30µg/ml) (black
bar), anti-uPAR antibody (25µg/ml) (dark grey bar) and aprotinin (200U/ml) (light grey bar). Data represent mean ± SEM of 3 experiments performed in duplicate wells. P-values for comparisons between the different compounds tested and the bFGF/TNF-α condition were calculated by ANOVA - Dunnett’s test as post-test; * P<0.05 and ** P<0.01.

Supplemental Fig. II: Localization of TAFI and endothelial cell marker CD31 (PECAM-1) in neovessels formed in a human atherosclerotic plaque that had incorporated a mural thrombus. Immunohistochemistry was performed on paraffin sections as described under materials and methods. (A) Immunostaining of endothelial cells by CD31; arrows indicate examples of positive endothelial cells. (B,C) Two examples of immunostaining of TAFI with a polyclonal IgG, arrows indicate accumulation of TAFI. Results are representative for the organized plaques from two different specimens. Original magnification 400x