REGULATION OF ANGIOGENESIS BY CELL-BOUND MATRIX PROTEOLYSIS

THE EFFECT OF HYPOXIA

PROEFSCHRIFT

ter verkrijging van
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Stellingen

1. Afhankelijk van de samenstelling van de extracellulaire matrix wenden endotheelcellen verschillende proteolytische systemen aan voor gecontroleerde matrix afbraak.
(dit proefschrift)

2. Hypoxie stimuleert de vorming van capillair-achtige structuren door humane endotheelcellen *in vitro* zonder betrokkenheid van autocriene vascular endothelial growth factor (VEGF) productie.
(dit proefschrift)

3. Binnen één populatie endotheelcellen bestaat een duidelijke heterogeniteit in urokinase-plasminogeen activator (u-PA) receptor expressie.
(dit proefschrift)

4. De betrokkenheid van een haem protein in de regulatie van de u-PA receptor door hypoxie in humane microvasculaire endotheelcellen is niet waarschijnlijk.
(dit proefschrift)

5. Hypoxie bezorgt endotheelcellen geen ademnood.
(Lee et al.,Circ Res 1987, 60: 653-658)(Graven et al., J Cell Physiol 157, 544-554)

6. Hypoxia inducible factor (HIF) is een veelbelovende factor in de therapeutische toepassing van angiogenese-stimulatie, maar kan door zijn rol in apoptose ook een oncologische toepassing hebben.
(Semenza et al., Adv. Exp. Med Biol. 2000;475:123-130)(Carmeliet et al., Nature 1998,394:485-490)

7. De extracellulaire matrix biedt houvast voor invasie van cellen tijdens angiogenese, maar is tevens het substraat waaruit angiogenese remmers gevormd kunnen worden.
(Eliceiri et al., Cancer J Sci Am 2000,6:S245-9)(Miosge et al. FASEB J 1999,13:1743-1750)(Colorado et al, Cancer Res 60:25202526)

8. Door de flexibiliteit van de natuur dient men zeer voorzichtig te zijn met het interpreteren van conclusies uit studies gebaseerd op knock-out muizen.

9. De kwaliteit van het endotheel kan een belemmerende factor zijn bij de behandeling van perifeer vaatlijden met angiogene groeifactoren.
(Rivard et al., Circulation 1999,99:111-120)(Rivard et al., J Biol Chem 2000: 275:29643-7)

10. Aangezien het groene hart een ideale plek is om te wonen, zal het niet lang groen meer zijn.

11. De digitale snelweg is een goed alternatief voor rekeningrijden.

12. Een AIO project kan levensvullend zijn, alleen de promotieplicht maakt er een vervroegd einde aan.

Leiden, 15 maart 2001

Marielle Kroon

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GENERAL INTRODUCTION

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1. Angiogenesis

The formation of new capillaries from existing blood vessels, angiogenesis, plays an important role in the development of the cardiovascular system. In adult life angiogenesis contributes to the maintenance of the functional and structural integrity of the organism. It occurs in wound healing, in conditions of ischemia and in the female reproductive system [1]. In these situations neovascularization is regulated by the metabolic demands of the tissue concerned. Angiogenesis also occurs in an uncontrolled manner in numerous pathological conditions. Examples of such conditions are rheumatoid arthritis [2], diabetic retinopathy [3], and solid tumor growth and metastasis [4].

Angiogenesis is a multistep process, highly regulated by a balance between positive and negative factors [5;6]. Examples of these factors are growth factors and cytokines and environmental factors such as the composition of the matrix, presence of different cell types and the surrounding oxygen tension. When positive regulators of angiogenesis are in favor, endothelial cells become activated. Local vasodilatation, increased vascular permeability and subsequent accumulation of extravascular fibrin, and proteolytic degradation of the basement membrane of the parent vessel follow this activation. Cytoplasmic protrusions are then extended from the activated endothelial cells, and a directed migration occurs into the surrounding matrix towards the angiogenic stimulus. Migrating endothelial cells elongate and align with one another to form a capillary sprout, besides that cell division occurs proximal to the region of the sprout and anastomose at their tip to form functional capillary loops. Vessel maturation and stabilization is accomplished by the interaction of the capillary endothelium with pericytes and reconstitution of the basement membrane [7] (Figure 1).

The following paragraphs will introduce processes/factors which regulate the process of angiogenesis. The proteolytic systems involved in the degradation of the extracellular matrix by endothelial cells, the influence of the composition of the extracellular matrix, angiogenic growth factors and the regulation of angiogenesis by oxygen tension are discussed. Finally, the outline and the aims of the thesis are given.

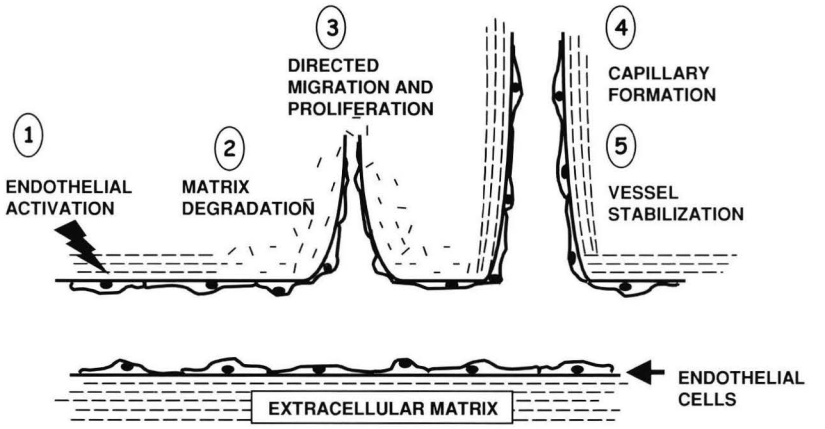


Figure 1: Schematic representation of the angiogenic process. Following activation of resting endothelial cells in an existing capillary, the basement membrane and extracellular membrane are degraded and endothelial cells migrate into the surrounding interstitium. The cells proliferate, elongate and lumens are generated. Finally, vessel stabilization is achieved by interaction with pericytes and reconstitution of the basement membrane.

2. Regulation of angiogenesis

Angiogenesis is expected to be mediated through a balance of angiogenic promotors and inhibitors, acting on different steps of the angiogenic process. The degradation of the extracellular matrix is under control of proteolytic enzymes and their inhibitors. The balance between proteases and their inhibitors determines if controlled lysis, leading to angiogenesis, can occur. The composition of the extracellular matrix is another important factor, facilitating or impairing angiogenesis. For example, fibrin degradation products in addition to ECM fragments provide stimuli to migrating cells and induce angiogenesis [8].

During the process of angiogenesis, endothelial cells are exposed to numerous growth factors and cytokines produced by non-endothelial cells, e.g. macrophages, and by endothelial cells themselves. These factors can be both stimulators or inhibitors of angiogenesis. Promotors include angiogenic growth factors like fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), and inflammatory mediators like transforming growth factor α and tumor necrosis factor (TNF)α [6]. Under certain conditions, however, the cytokine TNFα can also inhibit angiogenesis [9;10]. Other inhibitory factors include interferon-α/β [11], interleukin 12 [12], transforming growth factor β [10], platelet factor 4 [13], thrombospondin-1 and -2 [14;15], the fragment of plasminogen angiostatin and a C-terminal fragment of collagen XVIII, endostatin [16;17].

Often the driving force for angiogenesis is the insufficient oxygen supply to tissues.

The surrounding oxygen tension is therefore an important regulator of angiogenesis [18].

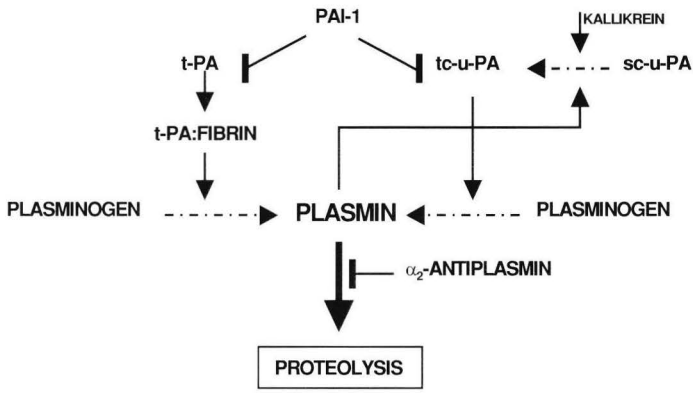
3. Role of proteases during angiogenesis

Basement membrane degradation, extracellular matrix invasion, and capillary lumen formation are essential processes of the angiogenic process. They all are dependent on a cohort of proteases and protease inhibitors produced by endothelial and non-endothelial cells. The most relevant proteolytic enzymes, involved in the process of angiogenesis, belong to two families: the serine proteases, in particular the plasminogen activator/plasmin system, and the matrix metalloproteinases [19;20]. Many mechanisms are involved in regulation of extracellular proteolysis (transcriptional and translational control). However, spatial localization is especially relevant in cell migration. Spatial and temporally localization restricts proteolytical activity to the immediate pericellular environment and is achieved by two mechanisms: first, by binding of the proteolytic enzymes to cell surface receptors, and second, by the coproduction of protease inhibitors [21]. A precise protease-anti-protease equilibrium allows controlled pericellular matrix degradation during cell migration, while it protects the extracellular matrix against inappropriate destruction [22].

3.1 The plasminogen activator/plasmin system

Plasmin is primarily responsible for the proteolysis of a fibrin clot [23] but can also degrade a variety of other proteins in the extracelullar matrix, such as laminin, fibronectin and collagens [24-26]. Plasmin is secreted by hepatocytes in an inactive single-chain form, plasminogen, and has to be cleaved to a two-chain form to become proteolytical active [27]. Tissue-type plasminogen activator (t-PA) and urokinase type-plasminogen activator (u-PA) are able to catalyze the conversion of inactive plasminogen to the active protease plasmin [28]. While t-PA functions mostly intravascular, u-PA acts predominantly in extravascular spaces as a major player in cell migration and matrix remodeling [29]. As plasmin, t-PA and u-PA are secreted as single-chain zymogens. The activity of t-PA is low in the absence of fibrin, binding to fibrin markedly enhances its catalytic efficacy [30]. This is the case for both single-chain and two-chain t-PA, which have comparable activities. In contrast to t-PA, single-chain u-PA is inactive and has to be activated to two-chain u-PA by plasmin or kallikrein [31;32] to obtain proteolytical activity. The resulting two-chain u-PA has an activity that is at least several hundreds fold higher than single-chain u-PA (pro-u-PA). The plasminogen activator system is regulated by controlled activation.

Inhibition of single- as well as two-chain t-PA and two-chain u-PA occurs by plasminogen activator inhibitors, of which PAI-1 is the predominant physiological inhibitor. PAI-1 is secreted by platelets [33], hepatocytes [34], smooth muscle cells and endothelial cells [35]. In addition to PAI-1, PAI-2 is predominantly active at sites of inflammation, related to the presence of monocytes [36]. Plasmin is inhibited by α_2-antiplasmin [37] (Figure 2).



Figuur 2: Schematic representation of the plasminogen activator system.
Abbreviations: u-PA: urokinase-type PA, sc-u-PA: single-chain u-PA, tc-u-PA: two-chain u-PA, t-PA: tissue-type PA, PAI: PA-inhibitor.

Plasminogen, pro-u-PA and u-PA bind with high affinity to cell-surface receptors present on endothelial cells. Plasminogen binds with high capacity to the plasminogen receptor [38;39]. Both the binding of plasmin and u-PA to their specific receptors accelerates u-PA-induced plasmin formation and in addition protects plasmin from inactivation by α_2-antiplasmin [40]. Pro-u-PA and u-PA bind to a specific u-PA receptor (u-PAR) on the surface of many celltypes [41;42]. The binding of u-PA to this receptor leads to an accelerated generation of plasmin activity compared to the activation in the pericellular environment [43]. In addition, cell-surface localized plasmin accelerates the conversion of pro-u-PA to u-PA on the u-PAR [44]. Therefore, an interplay exists between receptor-bound u-PA and receptor-bound plasmin(ogen), and plasmin formation is likely to happen [43;45]. These two cell surface receptors play a central role in pericellular proteolysis.

3.2 The u-PA receptor (u-PAR)

The u-PAR is a highly glycosylated cell surface receptor with an apparent molecular weight of 50-65 kDA [46] and is attached to the cell surface by a glycosyl-phospatidylinositol (GPI) anchor [47]. It has three repeated domains. The first N-terminal domain (domain 1) contains the u-PA binding site [46;47] as well as the

interaction site with the extracellular matrix compound vitronectin [48;49]. The two other domains (domains 2 and 3) are not directly involved in these interactions but are needed for high affinity interactions. This synergistic effect of domain 1 and domain 2+3 on u-PA binding is thought to result from a conformational change [50;51]. The u-PAR is found on many cell types, including endothelial cells, and is localized in focal attachment sites, where integrin-matrix interactions occur, and in cell-cell contact areas [52;53].

3.2.1 Interactions of u-PAR with u-PA and PAI-1

The u-PAR acts both as a site for focal pericellular proteolysis by u-PA and as a clearance receptor for the u-PA:PAI-1 complex. Both pro-u-PA and active u-PA bind reversible to u-PAR by the amino residues 5-49 (the growth factor like domain) in the amino-terminal fragment (ATF) [42], but only active u-PA complexed with PAI-1 is internalized together with u-PAR. The u-PA:PAI-1 complex is degraded in the lysosomes, while the u-PAR is recycled to the cell surface [54] (Figure 3). Other receptors co-operate in this internalization process. In monocytes and hepatocytes a role for the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α_2-MR/LRP), glycoprotein 330 and the very low density lipoprotein (VLDL) receptor has been demonstrated [55;56]. In endothelial cells, however, other yet unknown mechanisms are also involved [57; 58] (Figure 3).

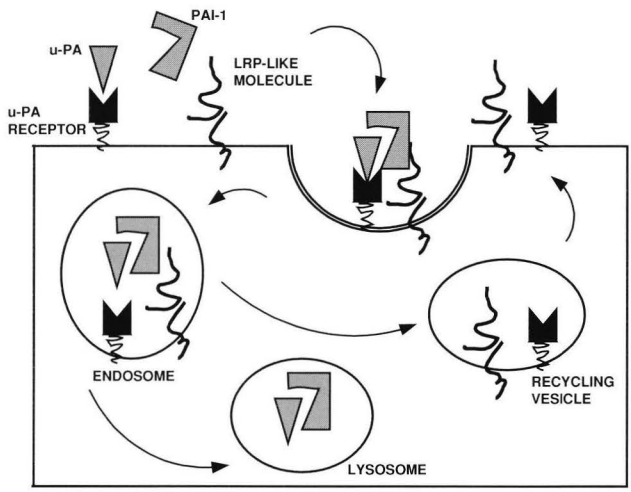


Figure 3: Schematic representation of u-PA:PAI-1 endocytosis via the u-PA receptor. Receptor bound u-PA is complexed with PAI-1 and the resulting u-PA:PAI-1 complex is recognized by a LRP-like molecule, which mediates the endocytosis and endosomal /lysosomal degradation of the u-PA:PAI-1 complex. The u-PAR and LRP-like molecule then recycle to the cell surface.
(modified from Bu et al. [65]).

The internalization and recycling of the u-PAR is a mechanism to regulate u-PAR density on the cell surface and thus u-PA activity. Another transcriptionally-independent mechanism to regulate this density is the cleavage of the u-PAR from

the cell membrane [59]. The GPI-anchor of u-PAR can be cleaved by GPI-specific phospholipases resulting in a soluble form of u-PAR. In addition the cleavage of domain 1 can be mediated by u-PA [60] or by other proteases like trypsin, elastase [61] and MMP-like proteases (Koolwijk et al., 2000, submitted)[62].

The expression of u-PAR is regulated on transcriptional level by several angiogenic growth factors such as FGF-2 and VEGF-A [63;64].

3.2.2 Interactions of u-PAR with vitronectin and integrins

Besides localizing proteolytical activity at the cell surface, the u-PAR is also involved in cell-matrix interactions. Reversible cell binding to vitronectin is mediated by high affinity association with u-PAR [48;49;66]. The binding affinity of multimeric vitronectin for u-PAR is increased in the presence of u-PA, whereas active PAI-1 abrogates u-PAR-vitronectin interaction [49;66]. This effect is due to direct competition for binding of u-PAR to the amino- and carboxy-terminal regions of vitronectin [67]. Therefore, u-PAR will not interact with vitronectin in a high PAI-1 milieu, on the other hand an excess of u-PA will prevent cell anchorage by efficient pericellular proteolysis. Activation of plasmin can also lead to the cleavage of the D1-domain of the u-PAR and the PAI-1 binding site on vitronectin, thereby modulating that interaction and leading to dissociation of u-PAR or PAI-1 from vitronectin [48].

Beside vitronectin, integrins have also been found to interact with u-PAR. At vitronectin-rich extracellular matrix sites, a co-localization is observed of u-PAR together with different integrins in focal adhesion areas [52;68].

Integrins are ubiquitous transmembrane adhesion receptors that mediate the physical linkage for cell-cell and cell-extracellular matrix contacts and thereby link the extracellular environment with the intracellular cytoskeleton in virtual all cells [69]. u-PAR is co-precipitated with the β2- integrin subunit [70] and cooperation with β1- and β3-integrin subunits has also been observed [66;71]. Due to direct interactions with β1-integrins, u-PAR may regulate cellular adhesion with respect to vitronectin rich matrices [66]. In addition, vitronectin-mediated cell migration via $\alpha_v\beta_5$ was favored by u-PA-binding to u-PAR [72]. From these data it appears that u-PAR is able to regulate the function of different integrins in various ways.

3.2.3 Signal transduction via u-PAR

After the binding of u-PA, u-PAR is able to transduce signals into the cell. u-PA, independently of its cell surface proteolytical activity, acts as an effector in the stimulation of migration [73], proliferation [74] and the activation of monocytes and neutrophils [75;76]. The u-PAR does not traverse the cell membrane, which means

that this receptor must interact with transmembrane proteins to achieve signal transduction into the cell. Integrins are examples of such adapter proteins. The first link between u-PAR, integrins and signal transduction was provided by data showing that in a u-PAR transfected carcinoma cell line, u-PA binding induced an association of u-PAR with β1-integrins leading to tyrosine phosphorylation of $p125^{fak}$ and $p130^{cas}$ [77]. In addition, the u-PAR has been shown to transduce signals that induce transcription factors like c-myc, c-fos, and c-jun [78-80], increase diaglycerol (DAG) generation [81] and induce phosphorylation of Erk 1 and 2 [82] and several proteins of the Jak/Stat pathway [83].

3.3 The role of u-PA/u-PAR in cell migration

It has been consistently observed that u-PA expression is quite low in resting endothelial cells [84;85]. The expression of the enzym is induced in endothelial cells during neovascularization [86] and during inflammation [87], situations in which endothelial cell migration is induced. Pepper *et al.* [88] reported an increased expression of u-PA and u-PAR in migrating bovine endothelial cells. This increase was mediated by endogenous production of the angiogenic growth factor FGF-2. Besides regulation of u-PAR and u-PA transcription by FGF-2 [64;89], u-PAR and u-PA expression is also enhanced by the endothelial specific angiogenic factor VEGF [63;90]. These growth factors enhance u-PA production in bovine endothelial cells, but in human endothelial cells the exposure to the cytokine tumor necrosis factor α (TNFα) is required for u-PA induction [91]. The results of several studies point to an important role for the u-PA/u-PAR-system in both cell migration as the process of angiogenesis. For example, renal tumor capillary endothelial cells overexpress u-PAR, whereas resting vascular endothelial cells of a normal kidney do not [92]. In addition, angiogenesis occurring in tumors derived from melanoma, prostate and colon cancer cells *in vivo* is reduced by administration of a catalytic inactive u-PA, which retains receptor binding and thus competes for binding of native u-PA [93;94] or by gene transfer of the amino-terminal fragment of u-PA [95].

3.4 MMP system

The matrix metalloproteinases (MMPs) constitute a large family of zinc-dependent extracellular endopeptidases that exhibit substrate specifities for multiple extracellular matrix proteins. MMPs have a great affinity for fibronectin, laminins, elastin and collagens which are the major extracellular matrix components found in endothelial cell basement membrane and interstitial spaces [96]. However, some MMPs act effectively as fibrinolysins through a plasminogen activator independent

pathway [97]. In addition, MMPs are able to activate or inactivate growth factors [98-100]. Most MMPs (with exception of the membrane-type MMPs (MT-MMPs) which are transmembrane proteins) are secreted from the cell as latent enzymes that require cleavage of their amino-terminal propeptide to become active. Plasmin is a potent activator of most MMPs, whereas several active MMPs can also activate latent MMPs [101]. The regulation of MMPs occurs at the transcriptional level, proenzyme activation and inhibition by specific inhibitors, the TIMPs [102].

3.5 The role of MMPs in cell migration

The observation that TIMPs and synthetic MMP inhibitors can significantly reduce the process of *in vitro* endothelial cell tube formation pointed to the importance of MMPs in the process of angiogenesis [103-105]. Endothelial cells have been reported to produce MMP-1 (interstitial collagenases), MMP-3 (stromelysin-1), MMP-2 and MMP-9 (gelatinase A and B) and MT1-MMP [106;107]. The extent of expression of each of these MMPs depends on the growth factors and cytokines present and the vascular bed of which the endothelial cells originated [107]. Both MMP-2 and MT1-MMP are key participants in the switch to an invasive phenotype in multiple cell types [108]. MT-MMPs are able to localize extracellular matrix proteolysis to specific domains of the migrating cell. In tumor cells MT1-MMP and MMP-2 have been localized to the invadopodia of the migrating cells [109]. Furthermore, there is evidence that other cell surface receptors than MT-MMP, such as $\alpha_v\beta_3$-integrin, may bind MMP-2 and catalyze its activation by presenting latent MMP-2 to adjacent MT-MMPs [110;111] (Figure 4).

In contrast to the stimulatory action of MMPs on angiogenesis, these proteinases also have an inhibitory role in this process. MMPs are able to cleave off the ATF-domain of u-PA, disabling the binding to its receptor [112]. In addition, plasminogen can be inactivated by MMPs [113] and furthermore the angiogenesis inhibitor angiostatin is formed by cleavage of plasminogen by MMPs [114;115]. It becomes more and more clear that angiogenesis related proteolysis is a complex interplay between different proteolysis systems (Figure 4).

4. Involvement of matrix composition

The extracellular matrix (ECM) consist of numerous proteins such laminin, collagen, vitronectin, fibronectin and hyaluoronic acid. In different pathological settings, the ECM has a different composition. Furthermore, this composition can change in time in particular pathological settings like wound healing. It has been shown that fibrin depositions persist in wounds up to 5 days after injury [116], whereas 7 day old

wounds have a substantial organized collagen fiber network and very little fibrin [117;118]. The composition and organization of the matrix in the direct environment plays important roles in directing dynamic responses of capillary endothelial cells as during angiogenesis [119-121].

Endothelial cells posses a variety of receptors for different components of the extracellular matrix, in particular a variety of members of the integrin family (Table 1). The signals transduced through these receptors can influence the phenotypic expression of endothelial cells. Fibrin has been shown to be a stimulatory factor for angiogenesis [122;123], but the mechanism of this angiogenic effect is not fully clarified. Dejana *et al.* [124;125] reported that fibrinogen induced endothelial cell adhesion, microfilament reorganization and migration. Furthermore, fibrin stimulates FGF-2 and u-PA secretion by bovine endothelial cells [126]. Adherence of activated endothelial cells to fibrin involves predominantly the expression of α_v-integrins. $\alpha_v\beta_3$-integrins not only adhere the endothelial cells to the extracellular matrix but also activate signaling pathways involved in the cell survival and angiogenesis [127;128].

In addition, collagen fibrils have also been shown to have a stimulatory effect on endothelial cells, probably by the interaction with cellular integrins. Endothelial cells cultured on interstitial collagens (collagen types I and III) undergo proliferation [129], this in contrast to cells cultured on basement membrane collagen (types IV and V). In addition, the main collagen-binding integrin $\alpha_2\beta_1$ is able to regulate the expression of MMP-1 in cells grown on a collagen matrix [130]. These data underline

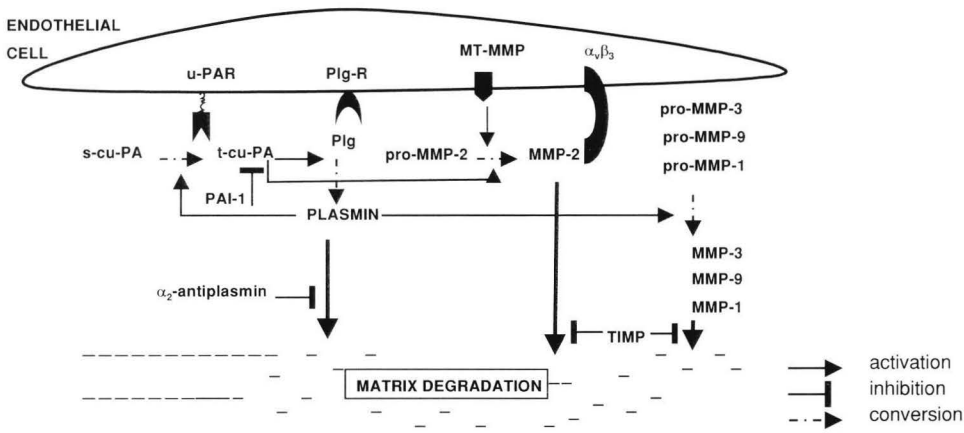


Figure 4:Schematic representation of the interaction between the u-PA/plasmin system and the presumed activation of matrix degrading metalloproteases (MMPs). Abbreviations: u-PA: urokinase-type PA, sc-u-PA: single-chain u-PA, tc-u-PA: two-chain u-PA, u-PAR: u-PA receptor, Plg: plasminogen, Plg-R: Plg receptor, PAI: PA inhibitor, $\alpha_v\beta_3$: $\alpha_v\beta_3$-integrin, MT-MMP: membrane bound MMP, TIMP: tissue inhibitor of MMP.

the concept that integrins are receptors that interact with components of the ECM by transducing specific signals from the matrix into the cell, thereby being responsible for modifying the three-dimensional structure of the surrounding ECM.

It can be concluded that depending on the composition of the matrix, different signals are transduced into the endothelial cell and a different angiogenic phenotype will be displayed.

TABLE 1: INTEGRINS EXPRESSED BY ENDOTHELIAL CELLS

TYPE	LIGANDS
$\alpha_1\beta_1$	Laminin, collagen
$\alpha_2\beta_1$	Laminin, collagen, fibronectin
$\alpha_3\beta_1$	Laminin, collagen, fibronectin
$\alpha_5\beta_1$	Fibronectin, fibrinogen
$\alpha_6\beta_1$	Laminin
$\alpha_6\beta_4$	Laminin
$\alpha_v\beta_1$	Fibrinogen
$\alpha_v\beta_3$	Vitronectin, fibrinogen, fibronectin, von Willebrand factor, thrombin, thrombospondin, tenascin, laminin, osteopontin , degraded collagen
$\alpha_v\beta_5$	Vitronectin, fibrinogen, fibronectin, von Willebrand factor, thrombin, thrombospondin, osteopontin, degraded collagen

5. Angiogenic growth factors and cytokines

5.1 Fibroblast growth factor (FGF)

Among the angiogenic growth factors, FGF-2 and VEGF are the best characterized. The FGF gene family comprises nine members. The FGF prototypes FGF-1 (acidic FGF) and FGF-2 (basic FGF) are unique because neither possesses a classical signal sequence to direct its secretion through the endoplasmic reticulum (ER)-Golgi

apparatus. Since FGF-1 and -2 are released from cells, it appears to follow an unconventional release pathway [131;132]. FGF family members have a high affinity for heparan sulfates, and the induction of signal transduction pathways is dependent on the presence of heparan sulphates or exogenous heparin as a cofactor [133]. In addition, because extracellular FGF binds to heparan sulfate proteoglycans in the matrix and at the cell surface, this binding is thought to protect cells. This reservoir of FGF becomes available after a trauma that might require a rapid mobilization of growth factor activity (like angiogenesis). FGF-1 and FGF-2 mediate their biological effects by binding to and activating a family of specific high-affinity cell-surface receptors (FGFR1 to 4) with protein tyrosine kinase activity. FGF-FGFR interactions are extremely complex, with each receptor subtype having the capacity to bind multiple ligands with similar affinity [134], and multiple receptor isoforms of FGFR1, FGFR2 and FGFR3 have been identified [135-137]. Both FGF-1 and FGF-2 stimulate the proliferation of multiple cell types and in addition exert a broad spectrum of other biological activities [138]. For example, *in vitro*, FGF-1 and FGF-2 promote cell migration, and proteoglycan synthesis [139;140] and exhibit potent angiogenic activity towards endothelial cells [141], in part by increasing the u-PAR in bovine and human endothelial cells [64;91].

5.2 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor-A (VEGF-A) is a homodimeric protein with great homology with placental-derived growth factor and the other members of the VEGF family VEGF-B, C and D [142;143]. Four forms of VEGF-A arise from alternative splicing of the mRNA from a single gene, coding for proteins of 121, 165, 189, and 206 amino acids. The two larger forms apparently stay membrane bound, presumably by their high affinity towards cell surface heparan sulfate proteoglycans. $VEGF_{165}$ is the predominant form, secreted by a variety of normal and transformed cells. All VEGF-A isoforms act on endothelial cells and transduce signals, alter gene transcription, increase microvascular permeability and promote angiogenesis [144-146]. Disruption of one of the two alleles of the gene encoding VEGF results in a lethal phenotype because of defects in the cardiovascular system [147;148]. This observation indicates that vessel formation depends on the generation of precise VEGF concentration gradients.

Three VEGF-specific tyrosine kinase receptors are known till now: VEGFR-1 (flt-1), VEGFR-2 (kdr) and VEGFR-3 (flt-4). Although these receptors are found primarily on endothelial cells, some studies have identified these receptors on non-endothelial cells such as melanoma cells and monocytes [149;150]. Signal transduction cascades induced by VEGFR-1 and 2 are somewhat different. Activation of the VEGFR-2 by

VEGF results in a mitogenic response as well as migration [151;152], whereas the VEGFR-1 has been shown to be important for cell migration but not for mitogenesis [152;153]. Both receptors are, however, important in vasculogenesis although they differ in temporal importance to the process. Disruption of the genes encoding VEGFR-2 and VEGFR-1 results in severe abnormalities of the blood vessels. Mice deficient of VEGFR-2 die before birth, because differentiation of endothelial cells does not take place and blood vessels do not form [154]. Disruption of the VEGFR-1 gene had no effect on differentiation of endothelial cells but the development of functional blood vessels was severely impaired [155].

Besides an important role in vessel development, VEGF is also involved in pathological angiogenesis. An increase in VEGF expression has been observed in the vast majority of human tumors [10;156-158], in diabetic retinopathy [159;160] and psoriasis [161]. In these conditions, VEGF expressioin is clearly associated with the stimulation of angiogenesis, in the case of cancer leading to a poor prognosis [10;162]. The importance of VEGF during angiogenesis is further underlined by its regulation at the transcriptional and posttranscriptional levels under conditions of hypoxia, a major driving force for angiogenesis [163-167].

Recent clinical studies carried out in patients with peripheral artery occlusion underscore the potential usefulness of VEGF and FGF-2 in treatment of ischemic limb and heart disease [168-171]. However, potential risks must not be forgotten. For example, VEGF-induced angiogenesis in an atherosclerotic plaque may weaken the plaque, the rupture of which may cause thrombosis and lead directly to myocardial infarction [172] and angiogenesis induced in a slumbering tumor may induce the spreading of metastasis.

5.3 Inflammatory mediators

Angiogenesis and chronic inflammation are codependent in pathological conditions. Inflammation involves proliferation, migration and recruitment of tissue and inflammatory cells [173]. The healing or inflamed proliferating tissue contains an abundance of inflammatory cells, angiogenic blood vessels and derived inflammatory mediators. Many cells are capable of producing angiogenic factors when their environment becomes hypoxic or inflammatory, including tumor cells, keratinocytes, synovial fibroblasts, monocytes, and macrophages. Almost every growth factor and cytokine known to regulate angiogenesis can be produced by macrophages [174]. Inflammatory mediators can both, directly or indirectly, promote angiogenesis, and inhibit angiogenesis. Prostaglandins E1 and E2, TNFα, interleukins 1, 6, and 8, and nitric oxide are examples of inflammatory mediators that have been shown to induce angiogenesis *in vivo* [175-180].

6. Regulation by oxygen tension

6.1 Hypoxia and angiogenesis

The process of angiogenesis is triggered in those places where it is most needed. A prime example of neovascularization representing a positive feedback response to insufficient perfusion is the development of collateral blood vessels in the ischemic myocardium or leg. Collateral development is mostly not restricted to the formation of microvessels but larger vessels are also formed. Formation of a collateral network seems to include both "classical" angiogenesis and the recruitment of preexisting vessels, i.e. arteriogenesis. The latter process requires the formation of new connections through vascular fusions [169;181] and in addition involves the increase in vessel diameter (thus leading to improved flow) through interstitial growth of preexisting vessels. Interstitial vessel growth might be promoted by ischemia-induced factors as well as by changes in sheer-stress due to diversion of flow to these vessels. The development of collateral vessels in a hypoxic situation does not occur spontaneously but requires the presence of angiogenic growth factors [182].

Tissue-ischemia usually develops as a result of vascular injury in wounds. Hence, ischemia-driven angiogenesis is a major factor in neovascularization associated with wound-healing. Indeed, a hypoxic tissue gradient is mandatory for wound-healing angiogenesis, and when the hypoxic gradient is destroyed, capillary growth ceases [183]. Numerous cell-types are able to respond to low oxygen levels so that they can survive short or even prolonged hypoxia [184]. Macrophages are preferentially recruited to hypoxic regions of the wound [185] and are a major source of angiogenic stimuli such as FGF [186;187], VEGF [164;188;189] and interleukin-8 [190]. In addition, endothelial cells are also able to respond to hypoxia and influence angiogenesis in a direct way.

For the adaptation of cells to low oxygen tensions several genes are upregulated. Examples of hypoxia-induced proteins are erythropoietin, which controls erythropoesis leading to a higher oxygen delivery to hypoxic tissues [191], and proteins important in glycolysis and glucose transport [192;193], inflammation [194], and cell adhesion [195;196]. Furthermore, angiogenic factors like VEGF [163;197], platelet derived growth factor B [198], FGF-2 [199;200] and TGF-β1 [201] are also enhanced in hypoxia. Besides an effect of hypoxia on the transcriptional level, proteins like erythropoeitin and VEGF are also post-transcriptionally influenced. The half-life times of erythropoeitin and VEGF mRNA are significantly prolonged in reponse to hypoxia [202;203]

6.2 Oxygen sensing

The mechanism by which cells sense differences in oxygen levels is still not fully elucidated. It can be concluded from the data from numerous studies that the mechanism by which hypoxia is sensed at the molecular level is highly conserved and tightly regulated. Generally, molecular interactions with oxygen are of two types; redox-based systems in which oxygen acts as a electron acceptor, and the reversible liganding of dioxygen to metal ions as occurs in oxygen carrying proteins such as cytochromes and many oxidases. It is very unlikely that these two systems function separately of each other; the involvement of heme-containing groups in more complex redox based sensing mechanisms is a very plausible option for oxygen sensing [204-206]. The chemical signals generated by the mechanisms, mentioned above influence transcription factors that regulate oxygen-responsive genes.

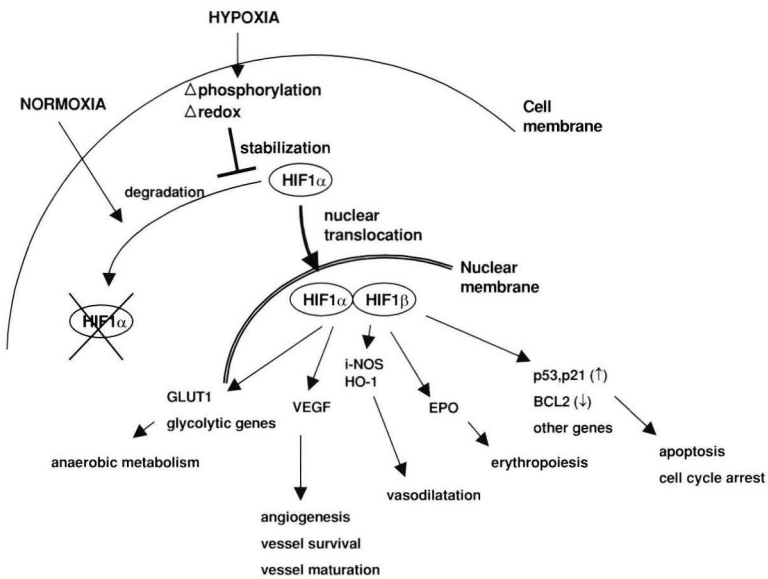


Figure 5: Current view of hypoxia signal transduction. Low oxygen levels, sensed by an unknown oxygen sensor (possibly a heme protein) activate HIF-1α via three independent mechanisms, indicated by bold arrows: stabilization, translocation to the nucleus and enhancing transcriptional activity. The activate HIF complex then regulates its target genes. Abbreviations: HIF: hypoxia-inducible factor, VEGF:vascular endothelial growth factor, i-NOS: inducible nitric oxide synthase, HO-1:heme-oxygenase-1, EPO: erythropoietin. *(Modified from Dor and Keshet [221])*

A whole range of transcription factors have been reported to be involved in the response to hypoxic stress. Examples are AP-1 and NF-κB [207]. A transcription factor called HIF-1 (hypoxia-inducible factor-1) was also identified to play an important role (Figure 5). HIF-1 is a dimeric protein consisting of HIF-1α and HIF-1β

subunits [208]. HIF-1β or ARNT is a common subunit for several other transcription factors. The HIF-1α protein is highly regulated by the cellular oxygen concentration and determines the level of HIF-1 activity [209;210]. In normoxia HIF-1α is degraded by the proteasome pathway and is stabilized under hypoxia, and is translocates from the cytosol to the nucleus [211] where it dimerizes with HIF-1β to form the active HIF complex [210]. HIF-1 transcriptionally activates a wide range of hypoxia-inducible genes [212]. Recently it has been recognized that HIF-1 not only plays a role in the adaptive response of hypoxic tissues, but is also involved in embryo development [213;214], tumor growth and metastasis [215;216], and in apoptosis [217]. A related protein, EPAS or HIF-2α, shares 40% overall amino acid identity with HIF-1α and dimerizes with HIF-1β. HIF-2α may be restricted to a more limited number of cell types, especially endothelial cells [218-220]. HIF-2α may therefore be important in the adaptive response of endothelial cells to hypoxia.

7. Aims of the study

The stimulation of the angiogenic process may be of benefit in pathological situations like wound healing and fracture repair. In addition, it becomes clear from experimental data that therapeutical angiogenesis may be beneficial for the treatment of ischemia [168;171;222]. Two settings that may benefit from this form of therapy are coronary artery disease and peripheral arterial occlusive disease, in which the objective is to reduce tissue hypoxia in areas of low blood flow by local stimulation of angiogenesis by the administration of growth factors. The success of this treatment depends on a number of factors. Examples are the activation state of the endothelial cells, the composition of the extracellular matrix and the oxygen tension at the pathological site.

Regulating the angiogenic process in a controlled manner depends partly on the knowledge about the effects of growth factors and environmental factors on the angiogenic potential of endothelial cells.

The aim of the present studies was to investigate the role of proteolytic processes in growth factor-induced *in vitro* angiogenesis in both normoxic as hypoxic conditions. The *in vitro* model used in these studies consisted of a three-dimensional matrix composed of fibrin, collagen type I or mixtures of fibrin and collagen. On top of these matrices human microvascular endothelial cells were seeded, which formed capillary-like structures after stimulation with a growth factor and the cytokine TNFα.

The degradation of fibrin depends mostly on proteolytical activity generated by the PA-system. In chapter two, the importance of the u-PAR in controlling the

availability of u-PA was investigated and furthermore it was assayed whether u-PAR and u-PA are site-specifically localized in newly formed microvascular structures.
In chapter three, the signal transduction pathway via which the growth factors FGF-2 and VEGF regulate the u-PA receptor expression in endothelial cells was investigated. Understanding the regulation of u-PAR, an important determinant during angiogenesis in a fibrin matrix as revealed in chapter two, offers possibilities to therapeutically regulate angiogenesis.

Angiogenesis in a hypoxic situation was studied in chapter four. Hypoxia in combination with a growth factor and a cytokine is a strong stimulus for angiogenesis. In this chapter the effects of hypoxia on the *in vitro* angiogenesis model and the mechanism via which hypoxia induces angiogenesis were studied. Growth factors, the PA-system and integrins play important roles in angiogenesis, therefore an effect of hypoxia on the expression of these factors was expected.

In chapter five, the angiogenic effect of hypoxia on endothelial cells was studied in more detail. Hypoxia is known to activate numerous signal transduction cascades in different cell types. The aim of this chapter was to reveal hypoxia-induced signal transduction pathways, which could be involved in hypoxia-induced angiogenesis.
In chapter six, another environmental factor was studied; the composition of the extracellular matrix. In the *in vivo* situation, the extracellular matrix consists of multiple proteins, and does not always consist of the same components. Since endothelial cells are able to detect a change in matrix composition, the angiogenic response of these cells very likely depends on the composition of the matrix. Angiogenesis on matrices consisting of fibrin, collagen and both fibrin and collagen was studied. The involvement of the PA-system and MMP-system during angiogenesis on matrices of different composition was determined.

In chapter seven the general conclusions that can be drawn from this work are discussed.

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2

ROLE AND LOCALIZATION OF UROKINASE RECEPTOR IN THE FORMATION OF NEW MICROVASCULAR STRUCTURES IN FIBRIN MATRICES

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Abstract

Fibrin or a fibrinous exudate can facilitate angiogenesis in many pathological conditions. *In vitro* the outgrowth of capillary-like structures in fibrin can be mimicked by exposing human microvascular endothelial cells (hMVECs) to an angiogenic growth factor and tumor necrosis factor (TNF)-α. Urokinase-type plasminogen activator (u-PA) and plasmin activities are required for this angiogenic process. This study focuses on the role and localization of the uPA receptor (u-PAR) in newly formed microvascular structures.

The u-PAR-blocking monoclonal antibody (mAb) H-2 completely inhibited the formation of capillary-like tubular structures induced by exposure of hMVECs to basic fibroblast growth factor and TNF-α. This was accompanied by a several-fold increase in u-PA accumulation in the conditioned medium. The effect of mAb H-2 was not caused by blocking cellular activation by u-PA/u-PAR interaction, as the aminoterminal fragment (ATF) of u-PA, which also activates u-PAR, prevented tube formation. In addition, the inhibition by mAb H-2 was not due to an effect of the antibody on u-PAR-vitronectin binding. These data show that inhibition of tube formation can be caused not only by inhibition of u-PA or plasmin activities but also by unavailability of the u-PAR for cell-bound proteolysis.

Immunohistochemical analysis showed that in *in vitro* angiogenesis u-PAR and u-PA were localized on the invading, tube-forming hMVECs and not on the endothelial cells that are located on top of the fibrin matrix. u-PAR and u-PA were also prominently expressed on endothelial cells of neovessels present in an atherosclerotic plaque. These data may give more insight into the role of u-PAR in repair-associated angiogenesis.

Introduction

In the adult, angiogenesis is usually associated with pathological conditions. It is an important factor in the growth of many solid tumors and the proliferative ingrowth of pannus tissue in rheumatoid arthritis and it is the underlying cause of proliferative diabetic retinopathy [1-3]. Angiogenesis can also restore blood flow to ischemic tissue and recanalise a mural thrombus [4;5]. Four sequential steps can be distinguished: the degradation of the basement membrane and interstitial matrix, endothelial cell migration, endothelial cell proliferation, and the formation of tubular structures with a lumen and a new basement membrane [6]. Three of these steps critically depend on proteolytic activity, generated in particular by the plasminogen activator (PA)/ plasmin system and the matrix metalloproteinases (MMPs) [7-9]. Recent studies showed that several of these proteolytic activities are localized to specific cellular receptors, which control their activities in space and time [10;11].

In conditions in which neovascularisation occurs in adults, frequently a fibrinous exudate is formed, which facilitates the angiogenesis process [12]. The formation of capillary-like structures can be mimicked *in vitro* in a model consisting of a three-dimensional fibrin matrix covered by microvascular endothelial cells (MVECs). It was shown to depend critically on the urokinase-type PA (u-PA)-mediated activation of plasminogen [13;14]. Although in bovine MVECs angiogenic factors basic fibroblast growth factor (bFGF or FGF-2) and vascular endothelial growth factor (VEGF) by themselves are able to induce angiogenesis *in vitro* [13;15], human (h)MVECs require the additional exposure to tumor necrosis factor (TNF)-α for the induction of u-PA [16] and the formation of capillary-like structures [14]. In both cell types bFGF and VEGF induce the expression of a cellular u-PA receptor (u-PAR) [7;14;17]. The u-PAR is a glycosyl phosphatidyl inositol-anchored glycoprotein [18]. It has been shown that u-PAR plays an essential role in endothelial cell migration [7] and in the invasion of tumor cells in tissues [19-21]. Several studies also have suggested that it plays a role in angiogenesis [14;21;22].

The u-PAR plays an important role in controling u-PA activity. The single-chain proenzyme u-PA can be activated on the u-PAR more efficiently [19]. In the cell environment active two-chain u-PA is quickly inhibited by the plasminogen activator inhibitor-1 (PAI-1) [23], but bound to u-PAR, u-PA displays a higher activity [24]. To ensure the continuous availability of free u-PAR, the u-PA:PAI-1 complex is internalized together with u-PAR. The u-PA:PAI-1 complex is degraded in the lysosomes, while the u-PAR is recycled to the cell surface [25]. This internalization process is aided by other receptors, in particular the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α_2-MR/LRP), glycoprotein 330, and the

very low density lipoprotein (VLDL) receptor, in various cell types [26;27], but in endothelial cells other yet unknown mechanisms are also involved [28].

Beside a function in controling u-PA activity, u-PAR is also found to play a role in activating cell signaling pathways, including diacylglycerol formation [29], activation of a serine kinase [30], protein tyrosine phosphorylation and the activation of the JAK/Stat pathway [31] and focal adhesion kinase [32]. Furthermore, it enforces cellular interaction with the extracellular matrix, in particular by binding to the matrix protein vitronectin [33;34].

In this study we investigated to what extent the u-PAR controls the availability of u-PA and u-PA-dependent *in vitro* angiogenesis in fibrin matrices and whether u-PAR and u-PA are site-specifically localized in newly formed human microvascular structures.

Materials and Methods

Materials

Medium 199 (M199) supplemented with 20 mmol/L HEPES and penicillin/streptomycin were obtained from Biowitthaker (Verviers, Belgium); newborn calf serum (NBCS) was obtained from Gibco (Grand Island, NY). Tissue culture plastics were from Costar (Cambridge, MA, USA) and L-glutamine from ICN (Costa Mesa, CA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine hypothalamus as described by Maciag *et al* [35]. Human serum was obtained from a local bloodbank and was prepared from freshly obtained blood from 10-20 healthy donors, pooled, and stored at 4 °C. Trypsin was purchased from Gibco (Grand Island, NY), heparin and thrombin from Leo Pharmaceutic Products (Weesp, The Netherlands), and human fibrinogen from Chromogenix AB (Mölndal, Sweden). Factor XIII was generously provided by Dr. H. Keuper (Centeon Pharma GmbH, Marburg, Germany). Human vitronectin came from Collaborative Research (Bedford, MA). bFGF was purchased from Pepro Tech EC (London, UK) and human recombinant 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 (Basel, Switzerland). Receptor-associated protein (RAP) was produced in *Escherichia coli* strain DH5α as a fusion protein with glutathione S-transferase (RAP-GST) and purified by gluthatione-Sepharose chromatography as described previously [36]. The amino-terminal fragment (ATF) from u-PA came from our laboratory; it was produced by Chinese hamster ovary (CHO) cells transfected with an adenoviral shuttle vector containing the ATF fragment of human u-PA, aminoacids 1 to138 and the 11 carboxyl-terminal amino acid residues of u-PA which contain the stop codon. Rabbit polyclonal anti-u-PA antibodies were also prepared in our laboratory [37] as were anti-FITC monoclonal antibodies (mAbs). The monoclonal u-PAR-blocking antibody H-2 came from Boehringer Mannheim, Penzberg, Germany [38], and LM609 was purchased from Chemicon, Temecula, CA. Horseradish peroxidase (HRP) conjugates of goat anti-rabbit IgG, rabbit anti-mouse IgG, and rabbit anti-von Willebrand factor IgG were from DAKO (Glostrup, Denmark). Technovit 8100 was obtained from Heraeus Kulzer (Wehrheim, Germany).

Other materials used in the methods described below have been specified in detail in related references or in the text or were purchased from standard commercial sources.

Cell culture

Human foreskin microvascular endothelial cells (hMVECs) were isolated, cultured, and characterized as previously described [39;40]. HMVECs were cultured on gelatin-coated dishes in M199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% human serum, 10% heat -inactivated NBCS, 150 µg/ml crude ECGF, 2 mmol/L L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C under 5% CO_2/ 95% air atmosphere. Experiments were performed after they reached confluency (0.7 x 10^5 cells/cm^2) and had been cultured without growth factor for at least 24 hours.

In vitro angiogenesis model

Human fibrin matrices were prepared by the addition of 0.1 U/ml thrombin to a mixture of 5 U/ml factor XIII (final concentrations), 2 mg/ml fibrinogen, 2 mg/ml sodium-citrate, 0.8 mg/ml NaCl, and 3 µg/ml plasminogen in M199 medium, and 300-µl aliquots of this mixture were added to the wells of 48-wells plates. After clotting at room temperature, the fibrin matrices were soaked with M199 supplemented with 10% (vol/vol) human serum and 10% (vol/vol) NBCS for 2 hours at 37 °C to inactivate the thrombin. Highly confluent endothelial cells (0.7 x 10^5 cells/cm^2) were detached and seeded in a 1.25:1 split ratio on the fibrin matrices and cultured for 24 hours in M199 medium supplemented with 10 % human serum, 10% NBCS, and penicillin/streptomycin. The endothelial cells were then stimulated with the mediators for the time indicated. Every second day, the culture medium was collected and fresh medium was added. Invading cells and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix were analyzed by phase contrast microscopy, and the total length of tube-like structures of six randomly chosen microscopic fields (7.3 mm^2/field) was measured using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software, and expressed as mm/cm^2.

Cell attachment assay

Cell attachment assays were performed in bacteriological 96-wells plates (Greiner, Frickenhausen,Germany, Elisa plates) coated with vitronectin or fibrin as described previously [41]. In brief, for vitronectin, 10 µl of vitronectin (100 µg/ml) was applied, resulting in a protein-coated area of 0.12 cm^2. After 16 hours at 4 °C, the fluid was removed, and 100 µl of 60 % methanol was added to each well for 2 hours at 4 °C. The methanol was removed, and wells were washed for 30 minutes at 4 °C with blocking buffer (50 mmol/L Tris-HCl (pH 7.8), 110 mmol/L NaCl, 5 mmol/L $CaCl_2$, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1% bovine serum albumin, and 0.1 µmol/L sodium azide) to block unbound sites on the plastic. After the removal of blocking buffer, the wells were washed three times with M199 containing 0.03 % human serum albumin.

For coating with fibrin, fibrin matrices were prepared as described above, 100 µl of fibrin was added to each well. Matrices were soaked with M199 supplemented with 10% (vol/vol) human serum and 10% (vol/vol) NBCS for 2 hours at 37 °C before use.

HMVECs (90 % confluent) were precultured in M199 medium supplemented with 10 % human serum, 10% NBCS, and penicillin/streptomycin. For attachment assays, hMVECs were detached, resuspended in M199 medium containing 0.03 % human serum albumin and

seeded at a concentration of 5000 cells/150 µl on the matrix-coated 96-wells. The 96-wells dishes were incubated at 37 °C under 5% CO_2/ 95% air atmosphere for 90 minutes. Each well was washed three times with serum free medium, and attached cells were fixed for 20 minutes with 80 % methanol at 4 °C. Cells attached to vitronectin were stained with amido black, and cells attached to fibrin were stained with toluidine blue. Attached cells were counted using using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software.

Determination of specific u-PA binding and u-PA:PAI-1 degradation

Diisopropylfluorophosphate-treated u-PA (Ukidan; DIP-u-PA) was radiolabeled using $Na^{125}I$ by using the Iodogen procedure (Pierce Chemical Co., Rockford, IL) Binding of ^{125}I-labeled DIP u-PA to hMVECs was determined at 0 °C. The cells were placed 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 M199 medium and incubated with 8 nmol/L of ^{125}I-labeled DIP-u-PA in endothelial-cell-conditioned medium (M199 medium supplemented with 1% human serum albumin, conditioned for 24 hours) for 3 hours. Incubation was performed in endothelial-cell-conditioned medium to exclude residual binding of u-PA to cell-associated PAI-1. In parallel incubations, a 50-fold excess of DIP-u-PA was included to assess non-specific binding. After the incubation period, unbound ligand was removed by extensive washing with ice-cold M199 medium. Cell-bound ligand was solubilised with 0.3 mol/L NaOH, and the radioactivity was determined in a γ-counter (Cobra Auto gamma, Packard, Meriden, CT). Specific binding was calculated by subtraction of non-specific binding from the total binding.

For determining u-PA:PAI-1 degradation, hMVECs were incubated for 5h at 37 °C in the presence of 8 nmol/L ^{125}I-labeled u-PA in endothelial-cell-conditioned medium. Because of the excess of PAI-1 present ^{125}I-labeled u-PA was rapidly converted to the ^{125}I-labeled u-PA:PAI-1 complex. After the incubation period an aliquot of the supernatant fluid was taken and the degradation products were determined as the supernatant's radioactivity obtained after protein precipitation by trichloroacetic acid (final concentration 10%) and corrected for trichloroacetic acid radioactivity in the initial preparation.

u-PA ELISA

u-PA antigen was measured as previously described [14]. In short 96-well microtiter plates were coated with a mixture of two mAbs, UK2.1 and UK 26.15, recognizing different epitopes on the u-PA antigen. These mAbs recognize single-chain u-PA, two-chain u-PA, and the u-PA:PAI-1 complex with comparable efficiency. The next day, the plates were blocked with casein, and serial dilutions of standard u-PA (UKIDAN (Serono, Aubonne, Switzerland), assuming that one unit, as determined by the manufacturer, is 10 ng protein) or culture supernatants were added. Finally, an incubation was done with a HRP conjugate of anti-u-PA antibody (LMW 11.1), and then tetramethylbenzidine substrate was added to react. The reaction was stopped with H_2SO_4 after 15 minutes incubation at room temperature. The extinction at 450 nm was measured with a multichannel spectrophotometer (Titertek multiscan, Flow Labs, McLean, VA)

Biological immunoassay (BIA) for plasmin-activatable sc-u-PA and tc-u-PA activity

This assay was performed as described by Binnema *et al.* [42]. In short, u-PA irrespective of its molecular form is extracted from the conditioned media by a mixture of two mAbs, UK2.1

and UK 26.15, immobilized on microtiter plates. In the next step, the PA activity of the immuno-immobilized material is measured before (active tc-u-PA) and, in parallel wells, after a 30-minute activation step of the immuno-immobilized material with human plasmin (active tc-u-PA plus plasmin-activatable sc-u-PA). The PA activity was assayed in a two-step cascade with plasminogen and S-2251 as described [42].

Calculation and assay of u-PA:PAI-1 complex

The amount of u-PA:PAI-1 complex was calculated from the difference between total u-PA antigen and sc-u-PA antigen (no active tc-u-PA was detectable in the collected samples). The validity of this subtraction was verified by ELISA of the u-PA:PAI-1 complex using anti-PAI-1 IgG as catching antibody and anti-u-PA IgG as tagging antibody. Comparable values were obtained in the two assays.

Immunohistochemistry

Fibrin matrices were fixed at 4°C for 3 hours in 2 % *p*-formaldehyde in phosphate-buffered saline (PBS, pH 7.4), washed for 3 hours in 6% sucrose, dehydrated in a graded series of ethanol and finally embedded in Technovit 8100, according to the manufacturer's recommendations (Heraeus Kulzer).

Serial sections were cut (4 µm) perpendicular to the surface of the matrix sheet. For morphologic evaluation the sections were stained with 0.25 % phloxin. For immunohistochemistry, the sections were treated for 15 minutes with 0.1 % trypsin (wt/vol) in 0.1 % $CaCl_2$ (wt/vol) in 0.1 mol/L Tris/HCl, pH 7.8, at 37 °C and subsequently washed in PBS. Incubation with the anti-u-PA receptor mAb H-2 (10 µg/ml in PBS with 0.1 % casein (wt/vol)) or polyclonal anti u-PA (0.04 µg/ml in PBS with 0.1 % casein (wt/vol)) was done for 2 hours at 37 °C. After 4 washes in PBS, HRP-conjugated rabbit anti-mouse or goat anti-rabbit antibodies (1: 40 in PBS with 0.1 % casein (wt/vol) and 0.1 % normal human serum) were added and incubated for 1 hour at room temperature.

The peroxidase activity was developed with 3-amino-9-ethyl-carbazole as substrate (10 mg 3-amino-9-ethyl-carbazole dissolved in 2.5 ml dimethylformamide in 50 ml 0.1 mol/L sodium acetate buffer, pH 5; after filtration, 50 µl of H_2O_2 was added). The sections were counterstained with Mayer's hematoxylin and mounted in Kaiser's glycerin (7 g of gelatin in 42 ml distilled water).

Human atherosclerotic plaques were embedded in paraffin and cut in sections of 4 µm. The cross sections were prewashed in PBS containing 1% NaN_3 and 1% of a 30% H_2O_2 solution. Subsequently, they were incubated for 15 minutes in a "block"-buffer (150 mmol/L NaCl, 10 mmol/L Tris-HCl, 5 mmol/L EDTA, 0.25 % (wt/vol) gelatin, 0.05 % (vol/vol) Tween-20) to reduce background staining. After 3 wash steps in PBS, the first antibody (H-2, 6 µg/ml; anti-u-PA, 0.01 µg/ml; or anti-von Willebrand Factor, 1.2 µg/ml) was added in PBS supplemented with 0.05 % Tween-20 and 0.1 % bovine serum albumin, followed by an overnight incubation at 4 °C. The next day, the sections were washed in PBS and HRP-conjugated rat anti-mouse or goat anti-rabbit (1:300 in PBS supplemented with 0.05 % Tween-20, 0.1% bovine serum albumin and 0.1% normal human serum) was added and incubated for 1 hour at 37 °C. Hereafter the sections were washed and developed as described above.

Data are expressed as the mean ± SD.

Results

The anti-u-PAR mAb H-2 inhibits u-PA binding to endothelial cells

It has been shown before that the mAb H-2 specifically recognizes u-PAR on a Western blot of lysates of monocytes at a molecular mass of 55 kd [38]. We show here that mAb H-2 prevents u-PA binding to its receptor on endothelial cells. mAb H-2 completely inhibited the specific binding of ^{125}I-Labeled DIP-u-PA to hMVECs, in which u-PAR expression had been enhanced (1.8±0.03-fold) by preincubation with bFGF and TNF-α or strongly enhanced (6.0±0.005-fold) by preincubation with 10^{-8} mol/L phorbol myristate acetate for 24 hours (Figure 1).

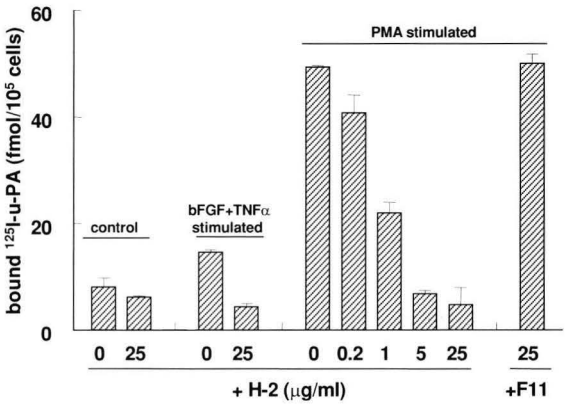

Figure 1: Inhibition of u-PA/u-PAR interaction by mAb H-2. HMVECs were preincubated for 24 hours in M199 supplemented with 10 % human serum with or without phorbol myristate acetate (10^{-8} mol/L) or bFGF and TNF-α (20 ng/ml each). Subsequently, the cells were cooled on ice, and the specific binding of ^{125}I-labeled DIP-u-PA to hMVECs was determined in the presence of the antibody H-2 (0, 0.2, 1, 5 or 25 μg/ml) or the non-blocking antibody F11 (25 μg/ml) as described in Materials and Methods. The data represent mean ± SD of triplicate wells of a representative experiment. Similar results were obtained in two independent experiments.

This inhibition was detectable at 0.2 μg/ml and maximal at 5 μg/ml (Figure 1). The nonblocking anti-u-PAR mAb F11 did not have an effect on specific binding of ^{125}I-labeled DIP-u-PA to hMVECs. Similar results were obtained using human umbilical vein or iliac artery endothelial cells.

The anti-u-PAR mAb H-2 prevents formation of tubular structures by hMVECs in a fibrin matrix

The mAb H-2 was used to establish the involvement of the u-PAR in the formation of capillary-like tubular structures by hMVECs in a three-dimensional fibrin matrix. When hMVECs were cultured without growth factors or cytokines, the confluent monolayer on top of the fibrin matrix remained unaltered (Figure 2a). Stimulation of the cells with the combination of bFGF and TNF-α (bFGF/TNF-α) induced the formation of tubular structures, which developed in a period of 3 to 8 days (Figure 2b).

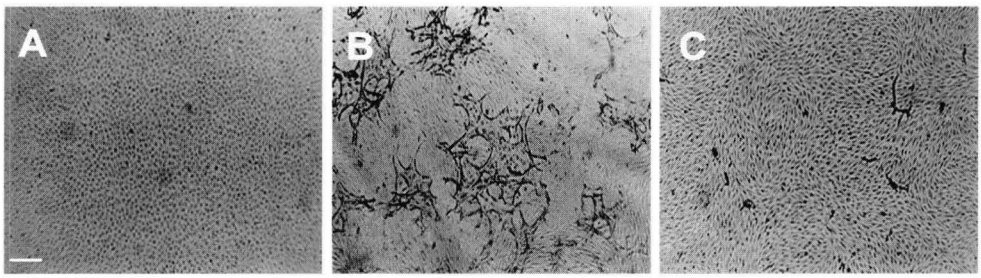

Figure 2: Capillary-like tube formation is inhibited by blocking u-PAR antibodies. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% human serum and 10% NBCS and were not stimulated (**A**) or stimulated with 20 ng/ml bFGF and 20 ng/ml TNF-α (**B**), or with bFGF and TNF-α in combination with 5 μg/ml of mAb H-2 (**C**). After 8 days of culture, non-phase photomicrographs were taken. Bar, 300 μm.

Simultaneous incubation of the bFGF/TNF-α-stimulated cells with the mAb H-2 inhibited tube formation (Figure 2c) in a dose-dependent manner (Figure 3a), up to 88±4 % at 5 μg/ml H-2. A similar extent of inhibition was observed when anti-u-PA IgG or the plasmin inhibitor aprotinin were used (Figure 3a).

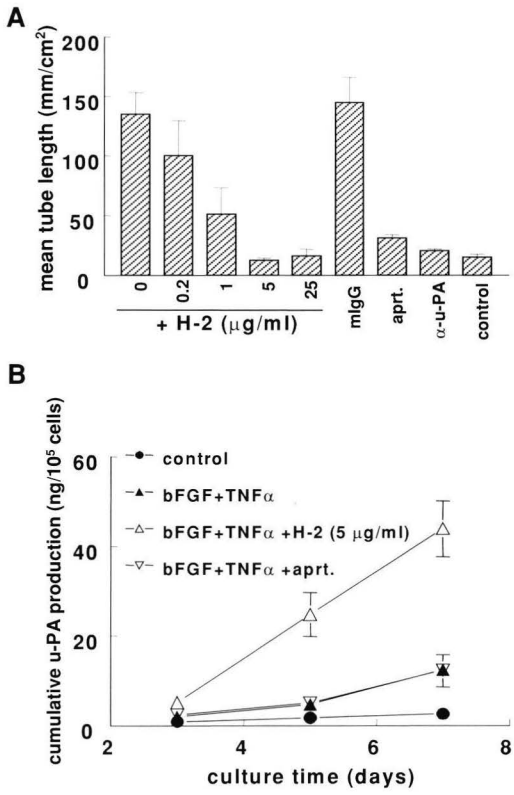

Figure 3: Dose-dependent inhibition of capillary-like tube formation by blocking u-PAR antibodies. A: HMVECs were cultured on the surface of a three-dimensional fibrin matrix in M199 supplemented with 10% human serum and 10% NBCS and not stimulated (control) or stimulated with 20 ng/ml bFGF and 20 ng/ml TNF-α in the presence of 0, 0.2, 1, 5 or 25 μg/ml mAb H-2, 25 μg/ml mouse IgG (α-FITC), 100 U/ml aprotinin (aprt.), or polyclonal anti-u-PA IgG (100 μg/ml). After 8 days of culturing total tube length /cm² ± SD of triplicate wells was measured as described and the medium was collected. Similar results were obtained in three independent experiments. **B**: u-PA antigen levels in the collected media were determined by ELISA as described and expressed as cumulative production of u-PA (ng/10⁵ cells). The data represents mean± SD of triplicate wells. Similar results were obtained in three independent experiments.

The presence of mAb H-2 caused an increased u-PA accumulation in the conditioned medium of bFGF/TNF-α-stimulated hMVECs (Figure 3b).

Interestingly, when the binding of u-PA to its receptor was competed by the amino-terminal fragment of u-PA (ATF) a complete prevention of the formation of tubular structures was observed (Figure 4). The ATF lacks the proteolytically active domain, but is still able to bind to the receptor and to activate u-PAR-dependent signal transduction [32]. Therefore, complete prevention of tube formation by mAb H-2 is unlikely primarily due to blockage of u-PAR-dependent cell signaling.

An effect of mAb H-2 or ATF on cell proliferation is also highly unlikely. It was shown by staining with the proliferation marker bromodeoxyuridine that hardly any proliferation occured in our model in the presence of TNF-α (data not shown).

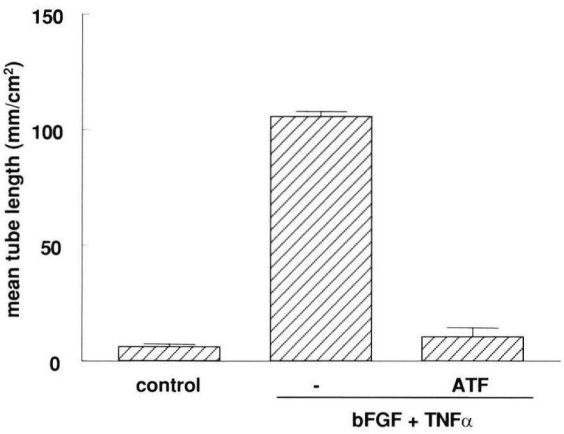

Figure 4: ATF inhibits capillary-like tube formation. HMVECs were cultured on the surface of a three-dimensional fibrin matrix in M199 supplemented with 10% human serum and 10% NBCS and not stimulated (control) or stimulated with 20 ng/ml bFGF and 20 ng/ml TNF-α in the presence of conditioned media of CHO cells, which were mock transfected or were transfected with an ATF construct ([ATF]=6.25 μg/ml). After 8 days of culturing total tube length /cm ^{2} ± SD of triplicate wells was measured as described. Similar results were obtained in three independent experiments.

u-PAR-vitronectin interaction is not required for tube formation in fibrin

In addition to a role in localizing u-PA proteolytical activity to the cell surface, the u-PAR also interacts with vitronectin and thus facilitates cell-matrix interaction and cell spreading. When hMVECs were seeded in vitronectin-coated dishes, cell adherence was completely prevented by the mAb LM609 that blocks the vitronectin receptor $\alpha_v\beta_3$-integrin (Figure 5a). The mAb H-2 reduced the adhesion of hMVECs to vitronectin-coated dishes by 52±8 %. When hMVECs were seeded in fibrin-coated dishes, neither mAb LM609 nor mAb H-2 reduced cell attachment (Figure 5a). mAb LM609 also did not significantly reduce the formation of tubular structures or invasion of hMVECs in a fibrin matrix, while mAb H-2 completely inhibited the formation of tubular structures in parallel dishes (Figure 5b). This data suggests that direct interaction of the endothelial cells with binding sites on fibrin dominated in the formation of tubular structures, and that the action of mAb H-2 on this process was not due to the reduction of u-PAR-vitronectin interaction.

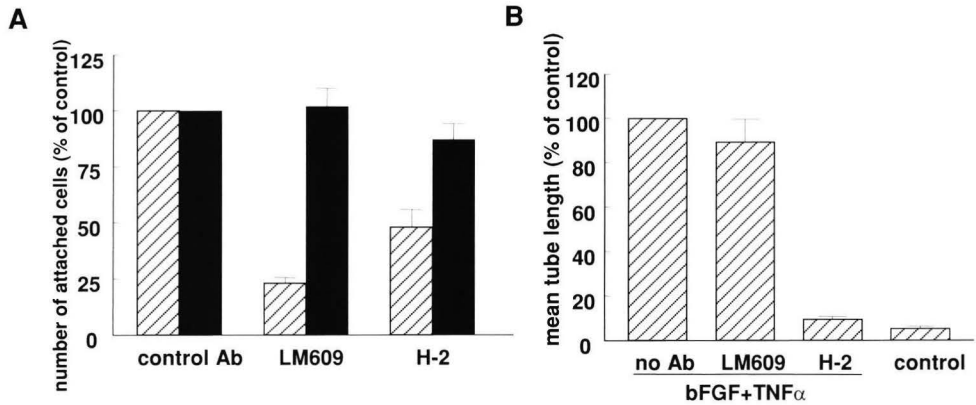

Figure 5: mAb H-2 inhibits adhesion of hMVECs to vitronectin but not to fibrin. **A**: Cell adhesion assay on vitronectin or fibrin was performed as described in Material and Methods. HMVECs were allowed to adhere to vitronectin (hatched bars) or fibrin (closed bars) for 90 minutes in the presence of a control antibody anti-FITC (10 µg/ml), LM609 (10 µg/ml) or H-2 (10 µg/ml). Each condition was performed in sixfold, and experiments were repeated two times. **B**: HMVECs were cultured on the surface of a three-dimensional fibrin matrix in M199 supplemented with 10% human serum and 10% NBCS and not stimulated (control) or stimulated with 20 ng/ml bFGF and 20 ng/ml TNF-α alone (no Ab) or in the presence of LM609 (10 µg/ml) or H-2 (10 µg/ml). After 8 days of culturing the mean total tube length/cm^2 ± SD of triplicate wells was measured as described. The effects of LM609 and H-2 are expressed as the mean percentage of control ± SD of three independent experiments.

Accumulation and cellular uptake of u-PA is modified by mAb H-2

During incubation u-PA is internalized as a u-PA:PAI-1 complex and degraded. mAb H-2 prevents this internalization by blocking the u-PA:u-PAR interaction. As a consequence, the amount of u-PA antigen that accumulated in the conditioned medium increased (Figure 6).

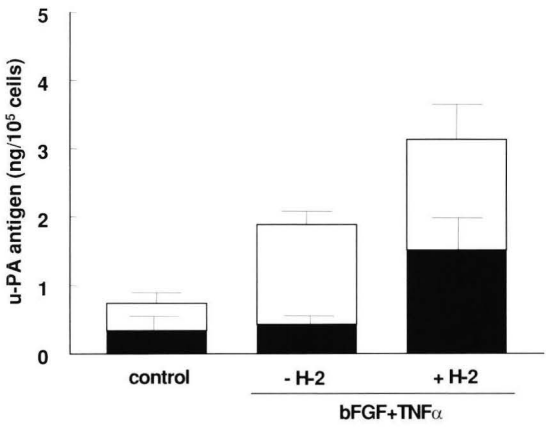

Figure 6: mAb H-2 increases the amount of sc-u-PA compared to total u-PA in conditioned media of hMVECs. Confluent hMVECs were cultured for 24 hours in M199 medium supplemented with 10% human serum and stimulated without (control) or with bFGF in combination with TNF-α (20 ng/ml each) in the absence or presence of 5 µg/ml H-2 antibody. After incubation, conditioned media were collected and plasmin-activatable u-PA (■) and total u-PA antigen (■+□) were assayed by BIA and ELISA, respectively, as described in Materials and Methods. In the samples, tc-u-PA activity (data not shown) was below the detection limit (0.1 ng/ml). The data represent the mean ± SD of three independent experiments.

The accumulated u-PA antigen consisted of single-chain u-PA (sc-u-PA or pro-urokinase) and a complex of u-PA and PAI-1; no active u-PA was detectable in the conditioned media. When the amounts of sc-u-PA and u-PA:PAI-1 complexes were determined in bFGF/TNF-α -stimulated endothelial cells, we found that in the presence of mAb H-2 equal amount of pro-urokinase (sc-u-PA) and u-PA:PAI-1 complex were produced (Figure 6). In the absence of mAb H-2, the amount of sc-u-PA was considerably less, whereas that of u-PA:PAI-1 complex was unaltered. Because only the u-PA:PAI-1 complex is internalized, this suggests that the missing amount of sc-u-PA is activated on the uPAR, and subsequently inactivated and internalized. The u-PA:PAI-1 encountered in the conditioned medium apparently derives from u-PA that is activated independent of receptor binding. Furthermore, the data indicate that prevention of tube formation by the presence of mAb-2 is not due to a limitation of the amount of u-PA that is available in the conditioned medium but to a lack of focally exposed u-PA, i.e., bound to and activated on the cellular u-PAR.

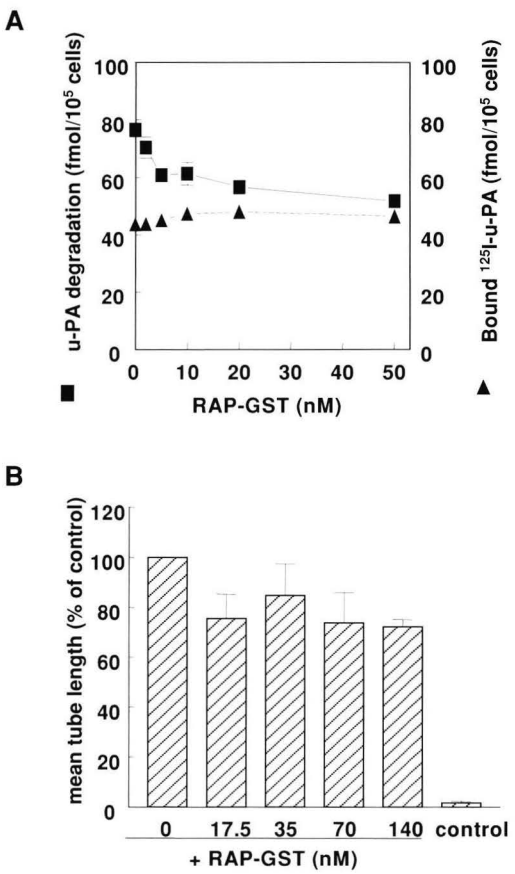

Figure 7: Effect of RAP on u-PA:PAI-1 degradation and capillary-like tube formation in hMVECs. **A**: Confluent hMVECs were cultured for 24 hours in M199 medium supplemented with 10% human serum and stimulated with phorbol myristate acetate (10^{-8} mol/L). Subsequently, cells were cooled on ice and the specific binding of ^{125}I-labeled DIP-u-PA was determined in the presence of 0, 2, 5, 10, 20 and 50 nmol/L RAP-GST or cells were incubated for 5 hours at 37 °C in the presence of ^{125}I-labeled u-PA:PAI-1 and 0, 2, 5, 10, 20 and 50 nmol/L RAP-GST. u-PA:PAI-1 degradation was determined as described in Material and Methods. The data represent the mean ± SD of triplicate wells. **B**: HMVECs were cultured on the surface of a three-dimensional fibrin matrix in M199 supplemented with 10% human serum and 10% NBCS and not stimulated (control) or stimulated with 20 ng/ml bFGF and 20 ng/ml TNF-α in the presence of 0, 17.5, 35, 70 or 140 nmol/L of RAP-GST. After 8 days of culturing the mean total tube length/cm² ± SD of triplicate wells was measured as described. The effect of RAP is expressed as the mean percentage of control ± SD of four independent experiments

RAP-GST partly reduces u-PAR availability and formation of capillary-like tubular structures

Internalization of the u-PA:PAI-1/u-PAR complex is needed to recover unoccupied u-PAR for the subsequent new binding and activation of u-PA. The u-PAR is a glycosyl phosphatidyl inositol-linked protein and needs accessory membrane proteins to become internalized. In other cell types, LRP and VLDL-receptors, proteins of the LDL receptor class, have been identified as such accessory proteins [26;27]. These proteins are inhibited by the receptor-associated protein RAP.

To evaluate whether interference with u-PAR internalization by blocking the complexation of the occupied u-PAR to accessory membrane proteins reduced the formation of capillary-like tubular structures, RAP-GST was incubated with the cells. RAP-GST reduced the internalization of ^{125}I-labeled DIP-u-PA:PAI-1 complex by EC with 34±2 % (Figure 7a). This was accompanied by a reduction of 23±5% of the formation of tubular structures (Figure 7b). These data support the suggestion that a decreased availability of free u-PAR reduces the formation of capillary-like structures.

Localization of the u-PA receptor and u-PA in capillary-like tubular structures

To investigate whether the u-PAR and its ligand are localized at specific sites in our *in vitro* angiogenesis model, we studied the localization of the expression of these factors in cross-sections of the fibrin matrix by immunohistochemistry.

Immunostaining for u-PAR showed that under control conditions the u-PAR could only be detected on a few distinct cells in the monolayer (Figure 8b). When the hMVECs were incubated in the presence of bFGF and TNF-α for 8 days, tubular structures were clearly visible underneath the endothelial monolayer (compare Figure 8a with 8c,f). These capillary structures consisted of hMVECs surrounding a lumen. The u-PAR is prominently expressed on the endothelial cells of the tubular structures (Figure 8, d and g). u-PA was also almost exclusively localized on endothelial cells forming the tubular structures, in particular, on cells in the deepest and probably most recently formed tubular structures (Figure 8, e and h). Under control conditions (unstimulated cells) no positive immunostaining for u-PA was detectable (data not shown). Negative controls (same staining procedure but without the primary antibody) showed no immunostaining.

Localization of the u-PA receptor and u-PA in an atherosclerotic plaque

To compare our *in vitro* model with an *in vivo* condition, sections of atherosclerotic plaques with organized thrombi were studied. The neointima with incorporated thrombus contained new vascular structures, which could be visualized by

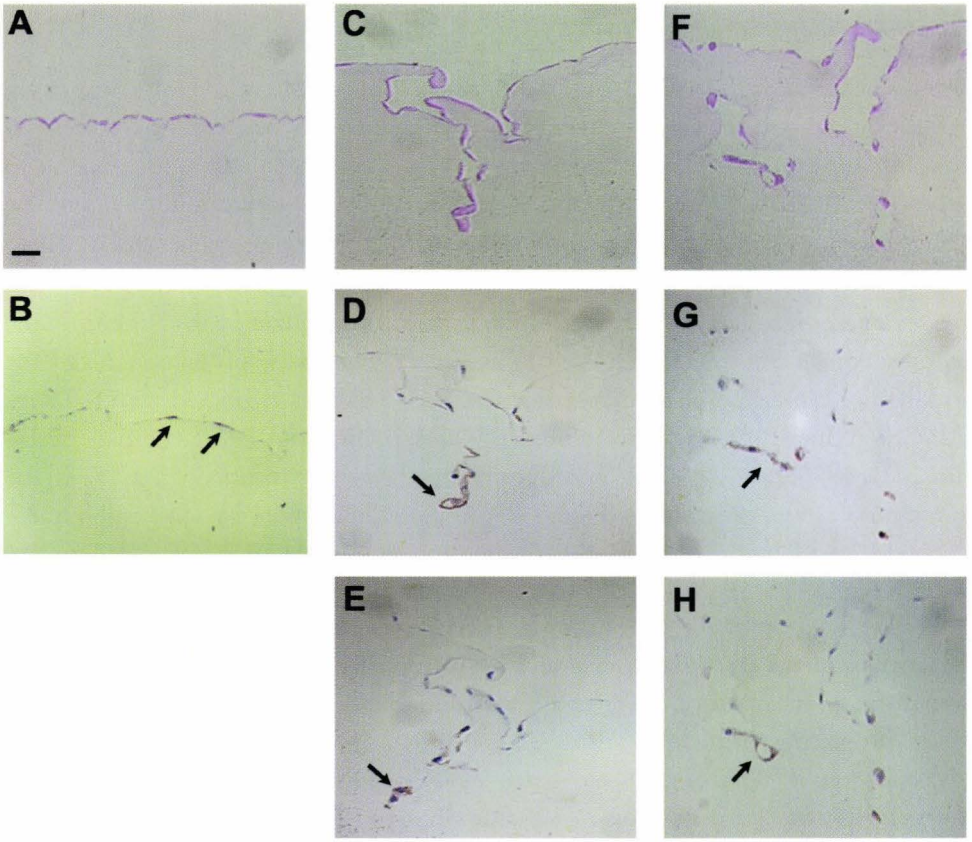

Figure 8: Localization of u-PAR and u-PA in capillary-like tubular structures of hMVECs in fibrin matrices. HMVECs were cultured on the surface of a three-dimensional fibrin matrix in M199 supplemented with 10% human serum and 10% NBCS and stimulated without or with 20 ng/ml bFGF and 20 ng/ml TNF-α. After 8 days of culture, the fibrin matrices were fixed and embedded as described and 4-μm cross sections perpendicular to the matrix surface were cut. Immunohistochemistry was performed as described. **A**, **C**, and **F**: Phloxin staining of unstimulated (**A**) and of stimulated (**C** and **F**) cells. **B**, **D**, and **G**: Immunostaining for u-PAR on unstimulated cells (**B**) and immunostaining for u-PAR on stimulated cells (**D** and **G**). **E** and **H**: Immunostaining for u-PA on stimulated cells. Positive cells are indicated by a red endproduct (arrowheads). Bar, 50 μm.

immunohistochemistry using antibodies against von Willebrand factor (Figure 9a). Staining with antibodies against u-PAR revealed that the endothelial cells of the neovessels expressed high levels of u-PAR (Figure 9b). This anti-u-PAR staining was more prominent than that in the endothelial cells lining the original vessel wall (data not shown). In addition to the endothelial cells, smooth muscle cells also stained positive for the u-PAR (Figure 9b). Immunostaining for u-PA was found on part of the endothelial cells of the newly formed vessels. A few smooth muscle cells of the neointima were also positive for u-PA antigen but to a lesser extent than for u-PAR

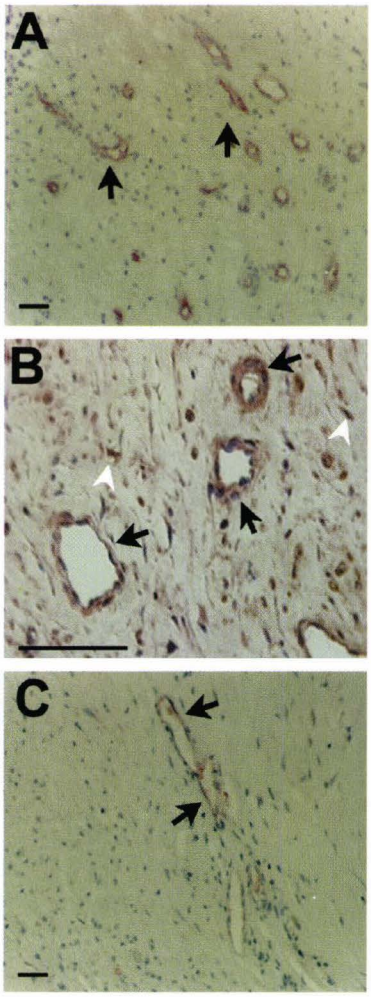

Figure 9: Localization of u-PAR and u-PA in neovessels formed in a human atherosclerotic plaque. Immunohistochemistry was performed on paraffin sections of an atherosclerotic plaque that had incorporated a mural thrombus, as described in Material and Methods. **A**: The endothelial cells of the newly formed microvessels are visible after immunostaining for von Willebrand Factor. B and C: Immunostaining for u-PAR (**B**) and immunostaining for u-PA (**C**). Black arrows indicate examples of positive endothelial cells; white arrowheads indicate examples of positive smooth muscle cells. Bar, 50 µm. Results are representative for organized plaques of two patients.

antigen (Figure 9c). Negative controls (same staining procedure but without the primary antibody) showed no positive staining.

Discussion

In this report we have provided additional evidence for the involvement of the u-PAR in the formation of capillary-like tubular structures by hMVECs in a fibrin matrix and have shown the specific localization of the u-PAR and u-PA in the endothelial lining of newly formed tubular structures *in vitro* and microvessels *in vivo*.

The u-PA/u-PAR system enables endothelial cells to degrade the basement membrane directly or indirectly and to invade the fibrin matrix. In previous

experiments it was shown that scavenging u-PA by anti-u-PA antibodies or soluble u-PAR inhibits tube formation in fibrin matrices almost completely [14]. We have extended these studies and showed that specific blockage of the u-PAR also prevented the formation of tubular structures by hMVECs in a fibrin matrix, despite the fact that sufficient u-PA was present in the conditioned medium around the cells.

On the basis of present knowledge, three mechanisms can be anticipated that may contribute to the requirement of u-PA:u-PAR interaction in angiogenesis. First, the u-PAR localizes u-PA activity to specific sites on the cell surface. On these sites proteolysis of matrix proteins occurs, which is kept in balance with the formation of new cell-matrix interactions. This mechanism fits well with the previous observations that *in vitro* angiogenesis is also inhibited by blocking u-PA and plasmin activities [14;43].

Secondly, binding of u-PA to its receptor causes activation of a signal transduction pathway in the cell [29;30]. This cellular activation by u-PAR is also mediated by the binding of the amino-terminal fragment (ATF) of u-PA [32], which lacks the catalytic domain but binds to the u-PAR similarly to u-PA. However, in contrast to u-PA, ATF is unable to stimulate tube formation in our model [14;44]. Furtermore, it can block tube formation, probably by competing with u-PA for binding to its receptor (our own unpublished data). Therefore, it is unlikely that u-PAR-dependent signal transduction plays an important role in the formation of tubular structures by hMVECs in fibrin matrices. A role for u-PAR in cell proliferation as mentioned by Fibbi *et al.* [45] is also unlikely, because the proliferation rate of hMVECs is extremely low in the conditions in which we assayed the formation of tubular structures, due to the continuous presence of TNF-α (checked by the proliferation marker bromodeoxyuridine).

Finally, the u-PAR affects integrin function [46] and interacts directly with the matrix protein vitronectin [33;34]. The interaction between vitronectin and u-PAR is enhanced by binding of u-PA and reduced by PAI-1 [47-49]. Because vitronectin associates with fibrin [50], one may suggest that the u-PAR:vitronectin interaction may be involved in endothelial migration in a fibrin matrix. In our study the attachment and cell spreading of hMVECs to vitronectin was inhibited partially by mAb H-2, but these processes were unaffected when hMVECs were seeded on fibrin-coated dishes. This suggests that the fibrin matrix itself contains sufficient interaction sites for hMVECs. In particular, the RGD sequence in the α-chains and possibly the Bβ chains participate in this interaction [51-53]. Also, when hMVECs formed tubular structures in the fibrin matrix, an effective $\alpha_v\beta_3$-blocking antibody, LM609, did not affect the outgrowth of tubular structures. It should be noted that in our *in vitro* model a pure fibrin matrix has been used, whereas a fibrinous exudate *in vivo* also contains other plasma proteins. Because vitronectin may not penetrate sufficiently

into the pure fibrin matrix, it is possible that in our model the potential contribution of vitronectin is underestimated. Nevertheless, the data indicates that in our experimental conditions the reduction of angiogenesis by inhibition of u-PA binding to its receptor is not primarily caused by affecting the interaction between vitronectin and hMVECs.

Taken together, our data show that inhibition of tube formation can be caused not only by inhibition of u-PA or plasmin activities, but also by the unavailability of u-PAR to localize cell-bound proteolysis. This observation is in accordance with the finding that angiogenesis occurring in certain tumors *in vivo* can be reduced by administration of a catalytic inactive u-PA, which retains receptor binding and thus competes for binding of native u-PA [21;22].

The concomitant binding of u-PA and plasminogen to the cell surface accelerates the plasminogen activation cascade [24;54]. Because u-PA activity is rapidly inactivated by PAI-1, the continuous regeneration of unoccupied u-PAR from the u-PA:PAI-1-receptor complex is needed. Our data show that a reduction of the extent of internalization of these complexes causes a reduction of available u-PAR on the cell surface and is accompanied by a decrease in the extent of tube formation by hMVECs.

If u-PA and u-PAR are critically involved in *in vitro* angiogenesis in a fibrin matrix, one would expect that these proteins are present in the invading capillary-like structures. Indeed, it was found that the u-PAR is strongly expressed on the endothelial cells of the tubular structures. The localization of u-PA was confined to the cells located deep in the fibrin matrix. These cells probably represent the structures that have been formed most recently, and probably are proteolytically most active. Although the localization of u-PA does not necessarily mean that u-PA is synthesized by the cells to which it is allocated (compare ref. 55 [55]), the more general expression of the u-PAR as compared to u-PA suggests that u-PA is produced in higher amounts at the sites where it is encountered. The difference in u-PAR expression between the cells remaining on top of the fibrin matrix and those lining the tubular structures is striking. Apparently, cells that are invading the fibrin gel express a different gene repertoire, which includes u-PAR and probably the $\alpha_v\beta_3$-integrin [56]. Alternatively, the fact that a few cells in the unstimulated monolayer are positive for u-PAR may reflect the existence of a subpopulation of cells that are more able to invade the fibrin matrix if adequate stimuli, which induce u-PA expression, are provided. Evidence for heterogeneous populations of endothelial cells has also been proposed by Asahara *et al.* [57]. These investigators isolated putative progenitor endothelial cells for angiogenesis on the basis of their specific surface expression of CD34.

It should be noted that our study is confined to neovascularisation in a fibrin

matrix. The condition of the *in vitro* system used in this study can best be compared with pathological conditions in which neovascularisation occurs upon fibrin degradation [12]. Recanalization of a mural thrombus associated with an atherosclerotic plaque is such a condition. Indeed, the u-PAR was clearly expressed in the neovessels present in an organized fibrin-rich neointima. In some of the endothelial cells of such neovessels, u-PA was also present. Our findings are in agreement with the co-localization of factors of the plasminogen activator system as observed in other studies on atherosclerotic plaques [58;59]. The confined localization and expression of u-PAR and u-PA in endothelial cells actively involved in angiogenesis may explain how these cells are capable of realizing a localized fibrinolytic activity which is sufficiently limited to prevent complete lysis of the scaffold matrix and cell detachment. Understanding the balance between detachments and the formation of new cell matrix attachment sites, may provide leads as to how angiogenesis can selectively be influenced in pathological conditions.

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3

VASCULAR ENDOTHELIAL GROWTH FACTOR ENHANCES THE EXPRESSION OF UROKINASE RECEPTOR IN HUMAN ENDOTHELIAL CELLS VIA PROTEIN KINASE C ACTIVATION

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Abstract

Among other proteolytic enzymes, the urokinase-type plasminogen activator (u-PA)/plasmin cascade contributes to cell migration and the formation of capillary-like structures in a fibrinous exudate. The u-PA receptor (u-PAR) focuses proteolytical activity on the cell surface of the endothelial cell and hereby accelerates the pericellular degradation. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2 enhance u-PA receptor expression in human endothelial cells. In this paper we show that the protein kinase C (PKC) inhibitors Ro31-8220 and GF109203X inhibit $VEGF_{165}$-induced u-PAR antigen expression in human endothelial cells, whereas PKC inhibition had no effect on FGF-2-induced u-PAR antigen enhancement. In addition, inhibition of PKC activity had no effect on $VEGF_{165}$- or FGF-2-induced proliferation in human endothelial cells. We conclude that $VEGF_{165}$ induces u-PAR via a PKC-dependent pathway, whereas proliferation is induced via a different pathway probably involving tyrosine phosphorylation of proteins downstream of the VEGF receptors.

Introduction

In several pathological settings quiescent vascular endothelial cells can be induced by angiogenic factors to form new capillaries. Angiogenesis involves a sequential process of basement membrane degradation, endothelial cell migration and proliferation, and finally generation and stabilization of tubular structures and formation of a new basement membrane. The sequential steps of angiogenesis require concerted detachments and renewals of cell-cell and cell-matrix interactions, which involve integrins, in particular $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins [1], and the activities of matrix-degrading proteases like plasminogen activators (PA), plasmin and matrixmetalloproteases [2-4]. *In vivo* and *in vitro* studies have demonstrated that cell-bound PAs, in particular urokinase type PA (u-PA), play an important role in endothelial cell migration, invasion and angiogenesis in a fibrin matrix [2;5;6].

The activity of u-PA is localized on the cell surface by the expression of a specific u-PA receptor (u-PAR), predominantly at focal attachment sites and cell-cell contacts [7]. Upon secretion single chain u-PA (sc-u-PA) binds with its growth factor domain to the receptor, on which it can be activated . Receptor-bound two-chain u-PA (tc-u-PA) can be inhibited by plasminogen activator inhibitor (PAI)-1 [8], albeit at a slower rate than in solution [9]. Sc-u-PA and tc-u-PA are not internalized, but once a complex with PAI-1 is formed, the u-PA:PAI-1 complex is internalized and degraded in the lysozomes [10].

The synthesis of u-PA and u-PAR in endothelial cells is regulated by several factors, including the angiogenic growth factors vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2 [6;11;12]. VEGF is a growth factor highly specific for endothelial cells [13] and interacts with endothelial cells primarily by two tyrosine kinase receptors VEGFR-1 and 2 [14;15]. The physiological importance of VEGF has been demonstrated both by the association of VEGF expression and various angiogenesis-related diseases, such as diabetic retinopathy [16], rheumatoid arthritis [17], psoriasis [18] and various types of malignancies [19].

Despite this interest in VEGF, its cellular signaling is only partially understood. In addition to tyrosine phosphorylation associated with the VEGF receptors, VEGF can increase the cytoplasmic Ca^{2+} and inositol-triphosphate concentration [20], induce phosphorylation of several protein kinases [21-23] and activate PKC [24;25]. However, via which signal pathway VEGF induces the synthesis of PAs and u-PAR in endothelial cells has not been resolved. Here we report on the effects of VEGF and FGF-2 on the synthesis of the u-PAR in human endothelial cells, and demonstrate that the induction of u-PAR expression by VEGF proceeds via the activation of protein kinase C.

Material & methods

Materials

Cell culture reagents were purchased as described by Kroon *et al.* [26]. FGF-2 was purchased from Pepro Tech EC (London,England), $VEGF_{165}$ was a kind gift of Dr. H. Weich (GFB, Braunschweig, Germany). The monoclonal u-PAR-blocking antibody H-2 was a kind gift from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany)[27]. GF109203X came from Biomol (Plymouth Meeting, PA) and Ro31-8220 from Roche Products (Welwyn, Garden City, USA). Tyrphostin A47 was obtained from LC Laboratories (Woburn, MA).

cDNA probes: The following cDNA fragments were used as probes in the hybridization experiments: a 585 bp BamHI fragment of the human u-PAR cDNA (a kind gift from Dr. F. Blasi, Milano, Italy), a 1.9 kb Bgl II fragment of the human t-PA cDNA and a 1200 bp PST I fragment of a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA kindly provided by Dr. R. Offringa.

Other materials used in the methods described below have been specified in detail in related references, in the text , or were purchased from standard commercial sources.

Cell culture

Human aorta endothelial cells (HAECs) [28], human umbilical vein endothelial cells (HUVECs) and human foreskin microvascular endothelial cells (hMVECs) were isolated, cultured and characterized as previously described [29;30]. Cells were cultured on gelatin-coated dishes in M199 supplemented with 20 mM HEPES (pH 7.3), 10% human serum, 10% heat -inactivated NBCS, 150 µg/ml crude ECGF, 2 mM L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C under 5% CO_2/ 95% air atmosphere, unless mentioned otherwise. Experiments were performed with confluent cells (0.7×10^5 cells/cm^2), which had been cultured without growth factor for at least 24 hours.

ELISAs

u-PA, t-PA and PAI-1 antigen determinations were performed by commercially available immunoassay kits: uPA EIA HS Taurus (Leiden, The Netherlands); Thrombonostika t-PA (Organon-Teknika, Turnhout, Belgium); IMULYSE® PAI-1 (Biopool, Umea, Sweden).

Incorporation of ^{3}H-thymidine

Incorporation of ^{3}H-thymidine in DNA was determined as previously described [6]. In short, confluent cultures of endothelial cells were detached by trypsin/EDTA solution and seeded at a density of 10^4 cells/cm^2 on gelatin-coated dishes in M199-HEPES medium supplemented with 10% heat-inactivated newborn calf serum and penicillin/streptomycin and incubated with the factors indicated in the text. After a preincubation period of 48h, a tracer amount of ^{3}H-thymidine (0.5 µCi per 2cm^2 well, added in a 10 µl volume) was added, and the cells were incubated for another 8 hours period. Subsequently, the cells were washed with cold phosphate buffered saline and fixed in 100% methanol; ^{3}H-labeled DNA was precipitated in 5% trichloroacetic acid, dissolved in 0.3 M NaOH and counted in a liquid scintillation counter.

Determination of specific u-PA binding

Determination of specific u-PA binding was determined as previously described by Kroon *et*

al. [26]. In short, cells were incubated for 10 minutes on ice with 50 mmol/L glycine/HCl buffer (pH 3.0) to remove receptor-bound endogenous u-PA. Subsequently, the cells were incubated on ice with 8 nmol/L ^{125}I-labeled DIP-u-PA in endothelial-cell conditioned medium (M199 supplemented with 1% human serum albumin, conditioned for 24 hours) for 3 hours. After the incubation period, unbound ligand was removed by extensive washing with ice-cold M199. Cell-bound ligand was solubilized with 0.3 mol/L NaOH, and the radioactivity was determined in a gamma counter (Cobra Auto gamma, Packard, Meriden, CT). Specific binding was calculated by the substraction of nonspecific binding from the total binding.

RNA Isolation and Northern Blots

Total RNA was isolated as described by Chomczynski and Sacchi [31] and Northern blots were performed as described by Lansink *et al.* [32].

Statistical analysis

All experiments were repeated at least three times with similar findings, and data are expressed as the mean ± SD. The unpaired *t*-test was used for comparison of groups with equal variance and normal distribution. A P-value < 0.05 was considered statistically significant.

Results

Effect of VEGF$_{165}$ and FGF-2 on u-PAR expression

The specific binding of u-PA to different endothelial cell types was studied. Human macrovascular endothelial cells, i.e. umbilical cord endothelial cells (HUVECs) and aorta endothelial cells (HAECs), and human microvascular endothelial cells (hMVECs) were used. Both VEGF$_{165}$ and FGF-2 increased the specific binding of ^{125}I-DIP-u-PA in all three cell types (Figure 1). In agreement with previous publications, the PKC activator PMA strongly increased this binding (Figure 1).

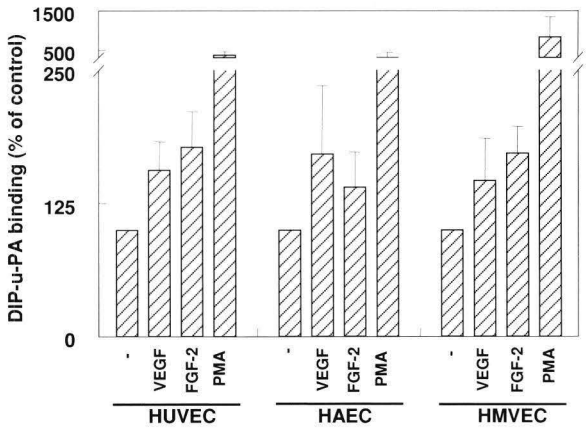

Figure 1: Effect of VEGF$_{165}$, FGF-2 and PMA on u-PA binding to endothelial cells. HUVECs, HAECs or hMVECs were cultured for 24 hours in M199 supplemented with 10% HS and not stimulated or stimulated with 100 ng/mL VEGF$_{165}$, 10 ng/mL FGF-2 or 10^{-8} mol/L PMA for 24h. Subsequently, the cells were cooled on ice, and the specific binding of ^{125}I-labeled DIP-u-PA to the endothelial cells was determined as described and expressed as a percentage of non-stimulated cells (6.4 ± 3.2 fmol/10^5 cells for HUVECs, 4.4 ± 0.7 fmol/10^5 cells for HAECs and 2.6 ± 1.3 fmol/10^5 cells for hMVECs). Data represent mean ± SD of at least three independent experiments performed in duplicate wells.

The effect of VEGF on u-PA binding was concentration-dependent both in the absence and presence of FGF-2 (Figure 2). $VEGF_{165}$ increased t-PA production two-fold both in basal conditions and in the presence of FGF-2 (Figure 3A), whereas $VEGF_{165}$ and FGF-2 did not affect the production of PAI-1 (Figure 3B).

On u-PAR mRNA level, $VEGF_{165}$ and FGF-2 induced a small increase in u-PAR mRNA in HUVECs, while PMA increased the u-PAR more extensively (Figure 4). The effect of FGF-2 on the u-PAR mRNA expression was additive to that of $VEGF_{165}$ (Figure 4B), similarly as found in the binding studies. t-PA mRNA was considerably

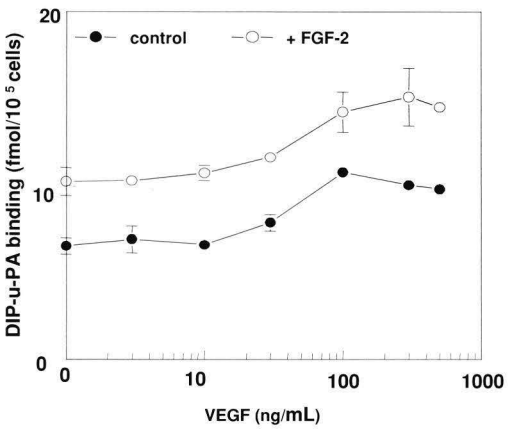

Figure 2: u-PAR antigen expression is increased by $VEGF_{165}$ and FGF-2. HUVECs were cultured for 24 hours in M199 supplemented with 10% HS and not stimulated or stimulated with $VEGF_{165}$ (0-300 ng/mL)(control) or with a combination of $VEGF_{165}$ (0-300 ng/mL) and FGF-2 (20 ng/mL)(+ FGF-2). Subsequently, the cells were cooled on ice, and the specific binding of ^{125}I-labeled DIP-u-PA to the endothelial cells was determined as described and expressed as fmol ^{125}I-DIP-u-PA/10^5 cells. Data represent mean ± SD of three independent experiments performed in duplicate wells.

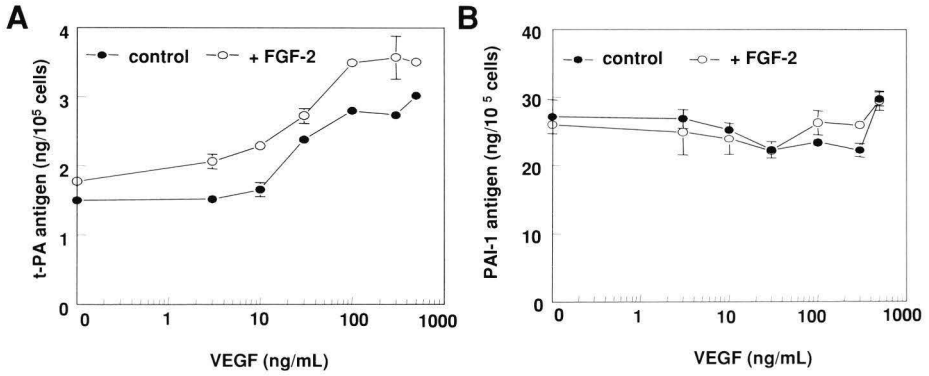

Figure 3: Effect of $VEGF_{165}$ and FGF-2 on t-PA and PAI-1 antigen levels. HUVECs were cultured for 24 hours in M199 supplemented with 10% HS and stimulated with $VEGF_{165}$ (0-300 ng/mL)(control) or with a combination of $VEGF_{165}$ (0-300 ng/mL) and FGF-2 (20 ng/mL)(+ FGF-2). After 24h, (**A**) t-PA antigen levels and (**B**) PAI-1 antigen levels were determined in the conditioned media and expressed as ng antigen/10^5 cells. Data represent mean ± SD of three independent experiments performed in duplicate wells.

enhanced by $VEGF_{165}$ and the combination of FGF2 and $VEGF_{165}$ (Figure 4B). u-PA mRNA was hardly detectable in these experimental conditions (data not shown).
On the u-PA antigen level $VEGF_{165}$ as well as FGF-2 reduced the u-PA antigen level in hMVECs significantly as compared to unstimulated control hMVECs ($VEGF_{165}$, 36 ±18% reduction, p=0.009, n=3 ; FGF-2, 70±17% reduction, p=0.001, n=3) (Figure 5). The same effect was found in HUVECs (data not shown). It has previously been demonstrated that an increase in cellular uptake of the u-PA:PAI-1 complex as a resultant of increased u-PAR expression can cause a reduction of u-PA accumulation in the medium [26;33]. Indeed, when a blocking monoclonal antibody to the u-PAR

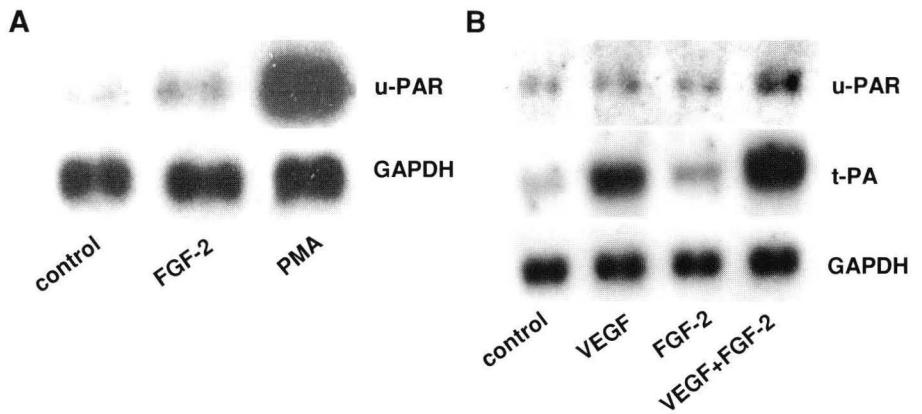

Figure 4: Effect of $VEGF_{165}$, FGF-2 and PMA on u-PAR and t-PA mRNA expression. HUVECs were cultured for 24 hours in M199 supplemented with 10% HS and not stimulated or stimulated with (**A**) 20 ng/mL FGF-2 or 10^{-8} mol/L PMA or stimulated with (**B**) 100 ng/mL $VEGF_{165}$ or 20 ng/mL FGF-2 or a combination of $VEGF_{165}$ (100 ng/mL) and FGF-2 (20 ng/mL). After 24 hours, total RNA was isolated and analyzed by Northern blotting using [α-^{32}P]CTP-labeled cDNA probes for u-PAR and t-PA. Equal loading of RNA was verified by hybridization with GAPDH.

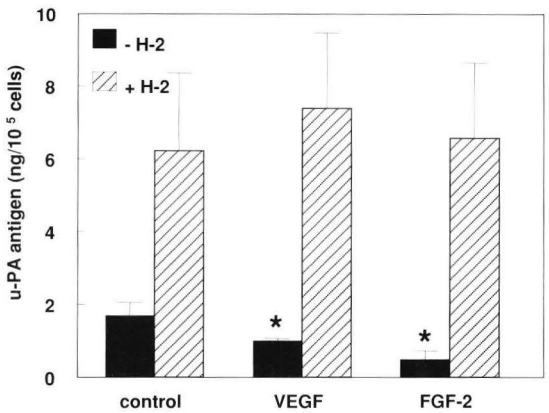

Figure 5: $VEGF_{165}$ and FGF-2 decrease u-PA levels in the conditioned media of hMVECs. HMVECs were cultured in M199 supplemented with 10% HS and were not stimulated (control) or stimulated with 50 ng/mL $VEGF_{165}$ or 20 ng/mL FGF-2 in the absences or presence of 5 µg/mL Mab H-2. After an incubation period of 24 hours, u-PA antigen levels were determined in the conditioned media by ELISA and expressed as ng u-PA/10^5 cells. Data represent mean ± SD of three independent experiments performed in duplicate wells.

(Mab H-2) was added, the decrease of u-PA in the conditioned media was completely abolished (Figure 5). Thus, the total production of u-PA was not significantly altered by $VEGF_{165}$ or FGF-2 stimulation in our experimental conditions (Figure 5).

$VEGF_{165}$ and FGF-2 induce mitosis and u-PAR expression via different pathways

$VEGF_{165}$ and FGF-2 are both mitogenic factors for human endothelial cells. Maximal ^{3}H-thymidine incorporation was reached at 2 ng/ml $VEGF_{165}$ and 3 ng/ml FGF-2, much lower concentrations than were necessary to induce u-PAR, as is shown for HUVECs in Figure 6A. This suggests that different signal pathways are involved in mitosis and the induction of u-PAR. This suggestion is further strengthened by the observation that the ^{3}H-thymidine incorporation and cell proliferation induced by $VEGF_{165}$ was inhibited by the tyrosine kinase inhibitor tyrphostin A47 (Figure 6B), whereas the induction of u-PAR was not affected by this inhibitor (Figure 6C).

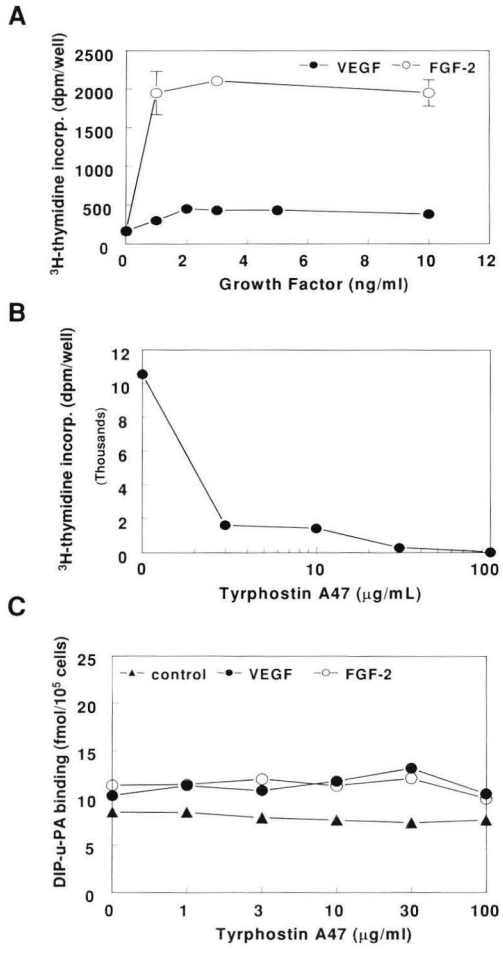

Figure 6: Induction of mitosis and u-PAR expression by $VEGF_{165}$ and FGF-2 proceed via different pathways. (**A**) Subconfluent HUVECs were cultured in M199 supplemented with 10% NBCS and stimulated with $VEGF_{165}$ (0-10 ng/mL) or FGF-2 (0-10 ng/mL). After 48 hours, a tracer amount of ^{3}H-thymidine was added to the medium and the incubation continued in the same medium for another 6 hours and ^{3}H-thymidine incorporation was determined as described and expressed as dpm/well. (**B**) Subconfluent HUVECs were cultured in M199 supplemented with 10% NBCS and stimulated with 100 ng/mL $VEGF_{165}$ in the presence of tyrphostin A47 (0-100 µg/mL). After 48 hours, ^{3}H-thymidine incorporation was determined as described above. (**C**) HUVECs were cultured in M199 supplemented with 10% HS and not stimulated (control) or stimulated with 10 ng/mL FGF-2 or 100 ng/mL $VEGF_{165}$. After 24 hours, the cells were cooled on ice, and the specific binding of ^{125}I-labeled DIP-u-PA to the endothelial cells was determined as described and expressed as fmol ^{125}I-DIP-u-PA/10^5 cells. Data represent mean ± SD of three independent experiments performed in duplicate wells

$VEGF_{165}$ increases u-PAR by a PKC-dependent mechanism

u-PAR mRNA expression is increased by both the protein kinase C activator PMA and the growth factors $VEGF_{165}$ and FGF-2. To evaluate if protein kinase C activation plays a role in the $VEGF_{165}$- and FGF-2-induced increase of u-PAR, the protein kinase C inhibitors Ro31-8220 and GF109203X were used. $VEGF_{165}$- and PMA-induced u-PAR expression in HUVECs was concentration-dependently inhibited by Ro31-8220, as shown by a decreased u-PA binding to these cells (Figure 7A). In contrast, the FGF-2-induced and basal u-PAR antigen level was not significantly influenced by Ro31-8220. Ro31-8220 and GF109203X also inhibited PMA and $VEGF_{165}$-induced specific u-PA binding to hMVECs (Figure 7B). As in HUVECs, these PKC-inhibitors did not influence FGF-2-induced u-PA binding. HA-1004, an inactive PKC inhibitor had no effect on u-PA binding (data not shown).

Endothelial t-PA production, is markedly increased by PKC activation [34]. Indeed, $VEGF_{165}$- and PMA-induced t-PA production was also inhibited by Ro31-8220 and GF109203X (data not shown).

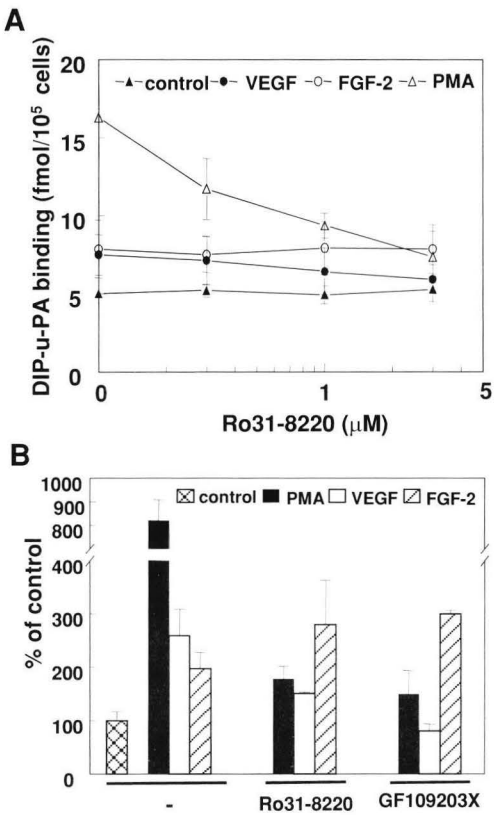

Figure 7: Inhibition of PKC activity inhibits $VEGF_{165}$-induced u-PAR expression. (**A**) HUVECs were cultured in M199 supplemented with 10% HS and not stimulated (control) or stimulated with, 100 ng/mL $VEGF_{165}$, 10 ng/mL FGF-2 or 10^{-8} mol/L PMA in the presence Ro31-8220 (0 to 3 μM). Subsequently, the cells were cooled on ice, and the specific binding of ^{125}I-labeled DIP-u-PA to the endothelial cells was determined as described in the Methods section and expressed as fmol ^{125}I-DIP-u-PA/10^5 cells. Data represent mean ± SD of three independent experiments performed in duplicate wells. (**B**) HMVEC were cultured in M199 supplemented with 10% HS and not stimulated (control) or stimulated with 10^{-8} mol/L PMA, 100 ng/mL $VEGF_{165}$ or 10 ng/mL FGF-2 in the absence or presence of 1μM Ro31-8220 or 3μM GF109203X. Subsequently, the cells were cooled on ice, and the specific binding of ^{125}I-labeled DIP-u-PA to the endothelial cells was determined and expressed as a percentage of control (6 fmol DIP-u-PA/10^5 cells). Data represent mean ± SD of two independent experiments performed in duplicate wells.

VEGF$_{165}$- and FGF-2-induced mitosis proceeds via a PKC-independent pathway

To verify the hypothesis that VEGF$_{165}$-induced u-PAR expression and cell proliferation proceed via different pathways, the effect of Ro31-8220 and GF109203X on endothelial cell proliferation was tested. GF109303X did not inhibit ^{3}H-thymidine incorporation induced by VEGF$_{165}$ or FGF-2, whereas Ro31-8220 inhibited cell proliferation in unstimulated cells by 55 ± 5% , in VEGF$_{165}$-stimulated cells by 46 ± 5 % and in FGF-2-stimulated cells by 38 ± 10 % (Figure 8). In the same experiments tyrphostin A47 inhibited cell proliferation almost completely.

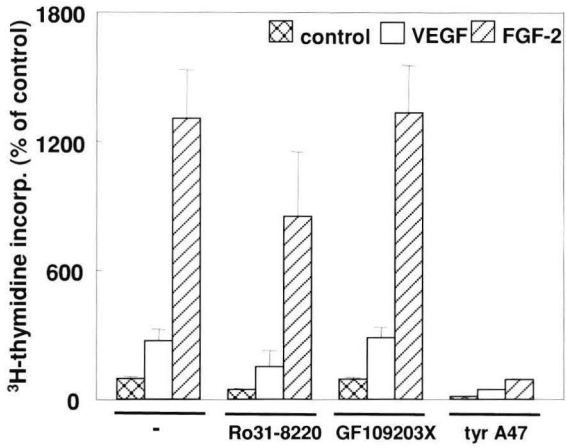

Figure 8: Inhibition of PKC activity does not influence VEGF$_{165}$- or FGF-2-induced mitosis. Subconfluent hMVECs were cultured in M199 supplemented with 10% NBCS and non-stimulated (control), stimulated with VEGF$_{165}$ (6.25 ng/mL) or FGF-2 (2.5 ng/mL) in the absence or presence of Ro31-8220 (1 μM), GF109203X (3 μM) or tyrphostin A47 (10 μg/mL). After 48 hours, a tracer amount of ^{3}H-thymidine was added to the medium and the incubation continued in the same medium for another 6 hours and ^{3}H-thymidine incorporation was determined as described and expressed as dpm/well. Data represent mean ± SD of three independent experiments performed in duplicate wells.

Discussion

In the present paper we have described that the growth factors VEGF$_{165}$ and FGF-2 enhance the expression of the u-PAR on human macro- and microvascular cells and that VEGF$_{165}$-induced u-PAR synthesis requires protein kinase C activity, whereas FGF-2 acts by a different mechanism. Both mechanisms are independent of the mitogenic effects of the growth factors.

Our experiments confirm previous studies showing that angiogenic growth factors enhance u-PAR on endothelial cells [2;12]. They extend these observations by showing that the u-PAR mRNA is enhanced rapidly and that an increase in u-PAR is accompanied by an increased uptake of u-PA. This mechanism explains the decrease in u-PA accumulation observed in the conditioned medium of human endothelial cells incubated with VEGF$_{165}$ or FGF-2.

The increase in u-PAR induced by both VEGF$_{165}$ and the protein kinase C-activating phorbol ester PMA was inhibited by the protein kinase C inhibitors Ro31-8220 and

GF109203X in both HUVECs and hMVECs, whereas the basal- and FGF-2 induced u-PAR synthesis were not. This not only indicates that $VEGF_{165}$-induced u-PAR synthesis requires protein kinase C activity, but also that $VEGF_{165}$ and FGF-2 act by different mechanisms. This is in line with observations that the effect of $VEGF_{165}$ and FGF-2 on endothelial u-PA-binding are additive [6;35](this study).

Both $VEGF_{165}$ and FGF-2 have pleiotropic effects on endothelial cells and act on these cells via several receptors. At present, three tyrosine-kinase receptors, specific for $VEGF_{165}$, have been identified on endothelial cells; VEGFR-1, 2 and 3 [14;15;36]. Whereas VEGFR-3 is expressed in lymph vessels and endothelial cells of the venous system [36;37], VEGFR-1 and 2 are expressed in the human microvascular and macrovascular endothelial cells used in this study [38]. The signal transduction cascades induced by VEGFR-1 and 2 are not identical. Activation of the VEGFR-2 (and not VEGFR-1) is required for mitogenesis [21], whereas VEGFR-1 plays a role in cell migration [39]. Under our experimenal conditions VEGFR-2 is in particular important in the formation of capillary-like tubular structures (Koolwijk *et al.*, submitted).

Signal transduction cascades activated by VEGFR-2 are relatively better described than VEGFR-1 activated transduction proteins. The activation of PKC via VEGFR-2 has been described in several reports. In primary endothelial cells, $VEGF_{165}$-induced activation of Raf-MEK-MAP kinase and DNA synthesis are mainly mediated by a PKC-dependent pathway [40;41]. $VEGF_{165}$-induced eNOS upregulation via activation of the VEGFR-2 proceeds via a downstream PKC pathway [42] and $VEGF_{165}$-induced migration is dependent on PKC activation [43]. The role for PKC in $VEGF_{165}$-induced proliferation is not clear. Although a role for PKC in $VEGF_{165}$-induced proliferation has been described [24], other investigators suggested that other protein kinases than PKC were involved [44]. In our experimental setting, $VEGF_{165}$-induced proliferation in endothelial cells also did not depend on PKC activity. FGF-2-induced proliferation did not depend on PKC activity, as is described before [25].

This finding leads to the conclusion that $VEGF_{165}$-induced u-PAR expression proceeds via a different pathway than the induction of proliferation. This conclusion is also supported by the fact that maximum proliferation was found at lower concentrations of $VEGF_{165}$ than needed for u-PAR induction. Furthermore, tyrphostin A47 was able to block proliferation but not u-PAR induction. These last two statements also apply to FGF-2, indicating that FGF-2-induced proliferation also proceeds via a different pathway than u-PAR induction. It is not known which specific tyrosin phosphorylations are inhibited by tyrphostin A47. As the VEGF-receptors undergo multiple autophosphorylations, we can not exclude the possibility that phosphorylations relevant for proliferation are inhibited, while other phosphorylations are not affected. However, it is much more likely that tyrphostin

A47 affects a tyrosine phosphorylation, involved in proliferation, downstream of the VEGF-receptors.

As mentioned before, $VEGF_{165}$ is important in the process of neovascularization of ischemic tissues. The inhibition of PKC was sufficient to block the $VEGF_{165}$-induced neovascularization of the retina and the neovascularization of tumors [45;46]. In contrast, FGF-2-induced tube formation is not dependent on PKC activation [47]. Furthermore, the neovascularization of tumors has been shown to be dependent on u-PAR availability [5;48]. These papers are in agreement with our results that PKC activity is required for $VEGF_{165}$-induced u-PAR expression. This report provides further insight into the mechanism by which $VEGF_{165}$ induces u-PAR expression and how it exerts its angiogenic action.

Acknowledgments

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4

UROKINASE RECEPTOR EXPRESSION ON HUMAN MICROVASCULAR ENDOTHELIAL CELLS IS INCREASED BY HYPOXIA; IMPLICATIONS FOR CAPILLARY-LIKE TUBE FORMATION IN A FIBRIN MATRIX

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Abstract

Hypoxia stimulates angiogenesis, the formation of new blood vessels. This study evaluates the direct effect of hypoxia (1% oxygen) on the angiogenic response of human microvascular endothelial cells (hMVECs) seeded on top of a three-dimensional fibrin matrix. HMVECs, stimulated with fibroblast growth factor-2 (FGF-2) or vascular endothelial growth factor (VEGF) together with tumor necrosis factor-α (TNF-α) formed 2- to 3-fold more tubular structures under hypoxic than in normoxic (20% oxygen) conditions. In both conditions the in-growth of capillary-like tubular structures into fibrin required cell-bound urokinase-type plasminogen activator (u-PA) and plasmin activities. The hypoxia-induced increase in tube formation was accompanied by a decrease in u-PA accumulation in the conditioned medium. This decrease in u-PA level was completely abolished by u-PA receptor-blocking antibodies. During hypoxic culturing u-PA receptor activity and messenger RNA (mRNA) were indeed increased. This increase and, as a consequence, an increase in plasmin formation, contribute to the hypoxia-induced stimulation of tube formation. A possible contribution of VEGF-A to the increased formation under hypoxic conditions is unlikely, because there was no increased VEGF-A expression detected under hypoxic conditions and the hypoxia-induced tube formation by FGF-2 and TNF-α was not inhibited by soluble VEGFR-1 (sVEGFR-1) or by antibodies blocking VEGFR-2. Furthermore, although the α_v-integrin subunit was enhanced by hypoxia, blocking antibodies against $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins had no effect on hypoxia-induced tube formation. Hypoxia increases u-PA association and the angiogenic response of human endothelial cells in a fibrin matrix; the increase in u-PA receptor is an important determinant in this process.

Introduction

In pathological disorders, low oxygen tension or hypoxia often occurs. Especially in the course of tissue damage, a loss of adequate blood supply causes hypoxia [1]. Angiogenesis, or the formation of new vessels, is strongly induced by hypoxic conditions [2;3]. A number of cell types respond to hypoxia and produce angiogenic factors, such as vascular endothelial growth factor-A (VEGF-A) [4], platelet derived growth factor-B (PDGF-B) [5;6] and fibroblast growth factor (FGF)-2 [7;8]. Macrophages in particular are extremely sensitive to hypoxia. They may act as oxygen sensors and initiate the process of vessel renewal by secreting a number of angiogenic factors [9-11]. On the other hand, endothelial cells, which are the dominant cell type in microvessels, play a key role in the formation of new vessel networks. Endothelial cells are able to survive severe hypoxic conditions [12;13] and are potential vectors of hypoxia-induced angiogenesis.

During the onset of angiogenesis, endothelial cells degrade their basement membrane, migrate into the interstitial matrix, proliferate, and form new microvascular structures. Matrix remodeling proteases of the plasminogen activator/plasmin- and matrix-degrading metalloproteinase (MMP) cascades together with their receptors and inhibitors play pivotal roles in several of these steps [14]. Depending on the composition of the matrix proteins in the area of angiogenesis, different groups of proteases are involved. In pathological angiogenesis of the adult, angiogenesis is often accompanied by vascular leakage and by the formation of a fibrinous exudate [15;16]. The fibrin matrix in this exudate facilitates cell migration by providing additional scaffolding for invading leukocytes and endothelial cells. Endothelial invasion into a fibrin matrix and the subsequent formation of capillary structures require cell-bound urokinase-type plasminogen activator (u-PA) and plasmin activities [17-19]. The u-PA is secreted as a single-chain inactive pro-enzyme, which binds to its cellular receptor u-PAR (CD87) and is proteolytically activated. Active u-PA converts plasminogen into plasmin, a broadly acting protease that degrades several matrix proteins and can activate latent MMPs [16]. Both plasmin and u-PA are rapidly inactivated, the latter by PA- inhibitor type-1 (PAI-1). The u-PA:PAI-1 complex is internalized together with u-PAR and degraded, while the unoccupied u-PAR returns to the plasma membrane [20].

It has been shown in a number of *in vitro* as well as *in vivo* studies that the binding of u-PA to u-PAR is essential for its action in angiogenesis [19;21;22]. Beside a role in localizing u-PA activity, the u-PAR can also play a role in cell adhesion by interacting with vitronectin and integrins [23-25]. This cell-matrix interaction is modified by the binding of u-PA and PAI-1 [26;27]. In addition, the occupied u-PAR is also involved in cellular signal transduction [28;29].

Hypoxia may stimulate angiogenesis by several mechanisms. It increases the transcription of VEGF in a number of cells [4;10;30]. In endothelial cells hypoxia-induced VEGF production is described [31], although the resistance of endothelial cells to hypoxic stimulation with regard to VEGF production can also be observed [32;33]. Similarly, conflicting data have been reported regarding the effect of hypoxia on the expression of VEGF receptors in endothelial cells [33-35]. In addition to this, hypoxia also induced an increase in the expression of $\alpha_v\beta_3$ - and $\alpha_v\beta_5$-integrins in retinal endothelial cells [36], integrins that may play a critical role in pathological angiogenesis [37]. Furthermore, hypoxia alters the fibrinolytical potential of endothelial cells [38;39]. While hypoxia induced a decrease in u-PA production in endothelial cells [38], it increased the expression of u-PAR [39]. As both u-PA and u-PAR are required during angiogenesis in a fibrin matrix [18;19], the effects of hypoxia on u-PA and u-PAR are expected to counteract each other.

In the present study we have evaluated the effect of hypoxia on the formation of capillary-like tubular structures by human microvascular endothelial cells in a three-dimensional fibrin matrix. In particular the regulation and involvement of the plasminogen activator system, VEGF, and $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins were studied.

Materials and Methods

Materials

Medium 199 (M199) supplemented with 20 mmol/L HEPES and penicillin/streptomycin were obtained from Biowitthaker (Verviers, Belgium); newborn calf serum (NBCS) from Gibco (Grand Island, NY). Tissue culture plastics were purchased from Costar (Cambridge, MA, USA) and Falcon (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and L-glutamine from ICN (Costa Mesa, CA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine brain as described by Maciag *et al.* [40]. Human serum was obtained from a local bloodbank and was prepared from freshly obtained blood from 10-20 healthy donors, pooled and stored at 4 °C. Heparin was obtained from Leo Pharmaceutic Products (Weesp, The Netherlands) and thrombin from Organon Technika (Boxtel, The Netherlands); human fibrinogen and S-2251 from Chromogenix AB (Mölndal, Sweden) . Factor XIII was generously provided by Dr. H. Boeder and Dr. P. Kappus (Centeon Pharma GmbH, Marburg, Germany). FGF-2 was purchased from Pepro Tech EC (London, England), $VEGF_{165}$ and soluble-VEGFR-1 (sVEGFR-1) was a kind gift of Dr. H. Weich (GFB, Braunschweich, Germany). Human recombinant 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). Rabbit polyclonal antibodies to u-PA and t-PA were prepared in our laboratory [41]. The monoclonal u-PAR-blocking antibody H-2 was a kind gift from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany) [42]. Monoclonal $\alpha_v\beta_3$ - and $\alpha_v\beta_5$-integrin-blocking antibodies (clone LM609 and clone P1F6, respectively) were purchased from Chemicon (Temecula, CA, USA). Vitronectin came from GibcoBRL® (Life Technologies, Breda, The Netherlands) and plasminogen from Boehringer Mannheim (Penzberg,

Germany).

cDNA probes: The following cDNA fragments were used as probes in the hybridization experiments: a 1023 bp fragment of the human u-PA cDNA (a kind gift from Dr. W-D. Schleuning, Schering AG, Berlin, Germany) [43], a 585 bp BamHI fragment of the human u-PAR cDNA (a kind gift from Dr. F. Blasi, Milan, Italy) [44] and a 1200 bp Pst I fragment of hamster actin cDNA [45].

The human α_v and β_5 cDNA probe were prepared in our laboratory by RT-PCR. In short, 1 µg of total RNA from HUVECs was reverse transcribed using oligo-dT (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in a PCR reaction using specific primers for α_v and β_5 integrin (α_v: forward: 5'-CTTCAACCTAGACGTGGACAGT-3'; reverse: 5'-TTGAAATCTCCGACAGCCACAG-3' [36]; β_5: forward: 5'-CATAGGTGAACATCATGACGC-3'; reverse: 5'-GCAGCTGCAACCAGTGCTCCTG-3'). PCR cycles were as follows: 96°C, 3 minutes (1x); 96°C, 45 seconds; 54°C, 1 minutes; 72°C, 90 seconds (35 x); 72°C, 7 minutes (1x). Subsequently, the PCR product was purified using an extraction kit (Qiagen, GmbH, Hilden, Germany) and then used as a cDNA probe.

Other materials used in the methods described below have been specified in detail in related references, in the text, or were purchased from standard commercial sources.

Cell culture

Human foreskin microvascular endothelial cells (hMVECs) were isolated, cultured and characterized as previously described [46;47]. HMVECs were cultured on gelatin-coated dishes in M199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% human serum, 10% heat -inactivated NBCS, 150 µg/mL crude ECGF, 2 mmol/L L-glutamine, 5 U/mL heparin, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37 °C under 5% carbon dioxide (CO_2)/ 95% air atmosphere, unless mentioned otherwise. Experiments were performed with confluent cells (0.7 x 10^5 cells/cm^2), which had been cultured without growth factor for at least 24 hours.

Establishment of hypoxic culture conditions

For culturing in hypoxic conditions, hMVECs were placed in a NAPCO® incubator (serial number 7101-C1,Precision Scientific Inc, Chicago, USA) that controls the oxygen concentration by flushing with nitrogen (N_2). Oxygen levels in the incubator were monitored by an internal oxygen sensor as well as by external calibration using Dräger Tubes 6728081 (Drägerwerk Ag, Lübeck, Germany). Hypoxic condition is defined as culturing at 37 °C under 1% O_2 /5% CO_2 atmosphere.

In vitro angiogenesis model

Human fibrin matrices were prepared by the addition of 0.1 U/mL thrombin to a mixture of 2.5 U/mL factor XIII (final concentrations), 2 mg/mL fibrinogen, 2 mg/mL Na-citrate, 0.8 mg/mL NaCl and 3 µg/mL plasminogen in M199 medium (mixture pH 7.4). 300 µl aliquots of this mixture were added to the wells of 48-wells plates. After clotting at room temperature, the fibrin matrices were soaked with M199 supplemented with 10% (vol/vol) human serum and 10% (vol/vol) NBCS for 2 hours at 37 °C to inactivate the thrombin. Confluent endothelial cells (0.7 x 10^5 cells/cm^2) were detached and seeded in a 1.25:1 split ratio on the fibrin matrices to form a highly confluent monolayer. After 24 hours culturing in M199 medium supplemented with 10 % human serum, 10% NBCS, and

penicillin/streptomycin, the endothelial cells were stimulated with the mediators for the time indicated. At the end of the culturing period the media were collected and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix was analyzed by phase contrast microscopy and the total length of tube-like structures of six randomly chosen microscopic fields (7.3 mm^2/field) was measured using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software, and expressed as mm/cm^2.

Enzyme-linked immunosorbent assays

u-PA, t-PA, PAI-1 and VEGF antigen determinations were performed by commercially available immunoassay kits: uPA EIA HS Taurus (Leiden, The Netherlands); Thrombonostika t-PA (Organon-Teknika, Turnhout, Belgium); IMULYSE® PAI-1 (Biopool, Umea, Sweden) and VEGF ELISA (R&D system, Minneapolis, USA). Antibodies used in the u-PA ELISA recognize single-chain-u-PA, two-chain-u-PA and the u-PA:PAI-1 complex with the same efficiency. The PAI-1 ELISA detects active and latent forms of PAI-1 whereas t-PA:PAI-1 and u-PA:PAI-1 complexes are not recovered. The t-PA ELISA recognizes both t-PA and t-PA:PAI-1 complexes.

Determination of specific u-PA binding

Diisopropylfluorophosphate-treated u-PA (Ukidan®) (DIP-u-PA) was radiolabeled using $Na^{125}I$ by using the Iodogen® procedure (Pierce Chem. Co.). The binding of ^{125}I-DIP-u-PA to hMVECs was determined at 0 °C. The cells were placed 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 M199 medium and incubated with 8 nmol/L ^{125}I-DIP-u-PA in endothelial cell-conditioned medium (M199 medium supplemented with 1% human serum albumin, conditioned for 24 hours) for 3 hours. Incubation was performed in endothelial cell-conditioned medium to exclude the residual binding of u-PA to cell-associated PAI-1. In parallel incubations, a 50-fold excess of DIP-u-PA was included to assess non-specific binding. After the incubation period, unbound ligand was removed by extensive washing with ice-cold M199 medium. Cell-bound ligand was solubilized with 0.3 mol/L NaOH and the radioactivity was determined in a γ-counter (Cobra Auto gamma, Packard). Specific binding was calculated by subtraction of non-specific binding from the total binding.

RNA Isolation and Northern Blots

Total RNA was isolated as described by Chomczynski and Sacchi [48] and electrophoresed in a 1.2% (wt/vol) agarose gel under denaturing conditions using 1 mol/L formaldehyde. It was transferred to Hybond-N filter by blotting, and the filters were hybridized overnight at 63 °C in 7% (wt/vol) sodium dodecyl sulfate (SDS), 0.5 mol/L Na_2HPO_4/NaH_2PO_4 buffer (pH 7.2), 1 mmol/L ethylenediamine tetraacetic acid (EDTA) containing a 3 ng/mL of [α-^{32}P]CTP-labeled probe. The probes were labeled by a Megaprime kit (Amersham), yielding an average activity of 0.0074 MBq/ng (0.2 μCi/ng) DNA. After hybridization of the filters, they were washed twice with 2xSSC (1x SSC being: 0.15 mol/L NaCl, 0.015 mol/L sodium citrate-dihydrate, pH 7.0), 1%SDS and twice with 1xSSC, 1% SDS for 20 minutes time periods at 63 °C. The filters were exposed to a Fuji imaging plate type BAS-MP (Fuji Photo Film, Tokyo, Japan) and quantification of relative amounts of transcribed mRNA was performed using a Phosphor–imager BAS-reader (Fuji Fujix Bas 1000, Fuji).

Cell attachment assay

Cell attachment assays were performed in bacteriological 96-wells plates (Greiner, Frickenhausen, Germany, ELISA plates) coated with vitronectin (10 µg/mL) as described previously [19].

Assay of cell-associated plasmin formation

We cultured hMVECs untill confluency in 96-wells culture plates and stimulated them for 72 hours with the factors indicated in the text. The cells were washed 3 times with ice-cold 0.05 mol/L Tris-HCl buffer (pH 7.4) supplemented with 0.03% human serum albumin. We added 40 µl substrate mix, containing plasminogen at a final concentration of 200 nmol/L and chromogenic substrate S-2251 at a final concentration of 0.3 mmol/L in 0.05 mol/L Tris-HCl + 0.03% human serum albumin (pH 7.4), was added. The culture plates were placed at 37 °C and absorbance was monitored at 405 nm using a multichannel spectrophotometer (Titertek multiscan, Flowlabs, McLean, VA) producing the expected increase for p-nitroanilide from plasmin production.

Statistical analysis

The data are expressed as the mean ± SD. The unpaired *t*-test was used for comparison of groups with equal variance and normal distribution. A P-value < 0.05 was considered statistically significant.

Results

Effect of hypoxia on cell viability and capillary-like tube formation

The viability of hMVECs cultured in hypoxic condition (1% oxygen atmosphere) was comparable to that in standard oxygen atmosphere (20% oxygen, further indicated as normoxic), as determined by trypan blue exclusion. When confluent hMVECs had been incubated for 72 hours in normoxic and hypoxic condition, without the addition of growth factors, their viabilities were 98.3±0.1% and 97.0±1.5% (mean ± SD of 3 experiments in duplicate, p=0.81). The monolayers of hypoxic hMVECs showed a very regular cobblestone appearance on gelatin-coated dishes (data not shown) and on a three dimensional fibrin matrix in the absence of angiogenesis stimulating factors (Figure 1A,B).

In previous studies we have shown that hMVECs cultured on top of a three-dimensional fibrin matrix can be induced to form capillary-like structures by simultaneous exposure to FGF-2 and TNF-α (FGF-2+TNF-α) or $VEGF_{165}$ and TNF-α ($VEGF_{165}$+TNF-α) [18;19;49]. An ambient 1% oxygen atmosphere further enhanced the extent of tube formation (compare figure 1C with 1D). The total tube length of FGF-2+TNF-α-induced tubular structures was increased by a factor 3.2 ± 0.3 (n=5, p<0.001) during a 72 hours incubation period, while the $VEGF_{165}$+TNF-α-induced tubular structures increased 2-fold (2.0 ± 0.2, n=5, p=0.001) (Figure 2). The stimulation of tube formation by hypoxia could not be mimicked by cobalt chloride, nickel chloride or deferoxamine, agents that point to the involvement of a heme protein in the oxygen-

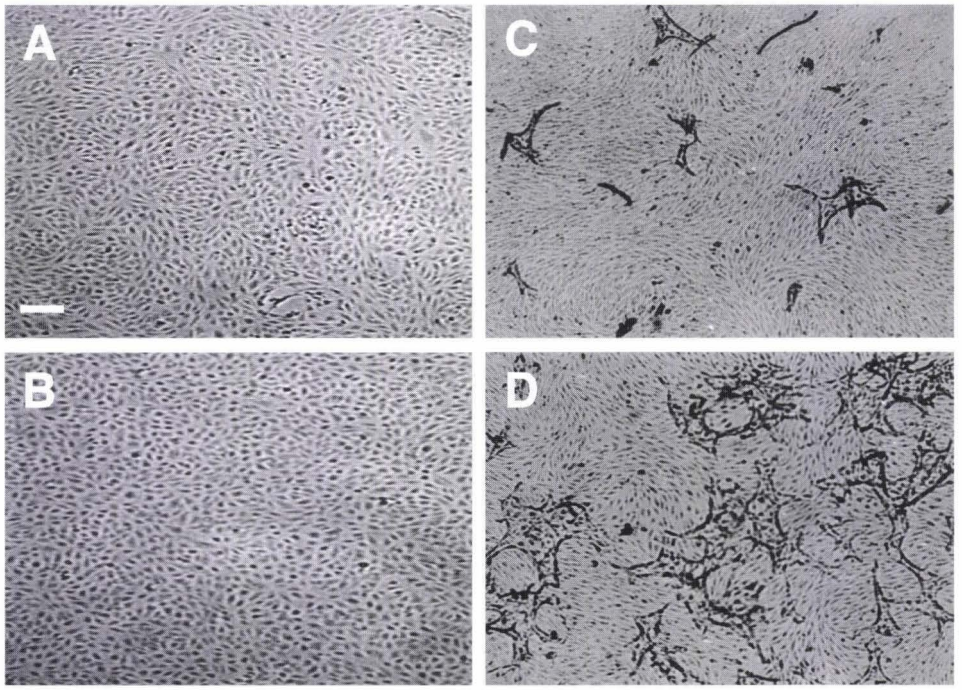

Figure 1: Capillary-like tube formation is increased under hypoxic conditions. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS under normoxic (**A/C**) or hypoxic culture conditions (**B/D**) and were not stimulated (**A/B**) or stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α (**C/D**). After 3 days of culturing, non-phase photomicrographs were taken. Bar, 300 μm.

sensing mechanism (data not shown).

FGF-2+TNF-α-induced capillary-like tube formation under hypoxia was inhibited by the addition of antibodies directed against u-PA, u-PAR and the plasmin inhibitor aprotinin, (86±1 %, 89±3% and 89±1% inhibition (n=3)), respectively (Figure 3). These degrees of inhibition did not differ significantly from those observed after the addition of these inhibitors in normoxic culture condition (94±0.4%, 84±0.3% and 86±4% inhibition (n=3)). Antibodies against t-PA did not affect tube formation under hypoxic conditions, which is a similar finding to that previously found in normoxic conditions [19;49] (Figure 3). This data indicates that the hypoxia-enhanced in-growth of hMVECs in fibrin matrices also requires cell-bound u-PA and plasmin activities.

Hypoxia decreases u-PA levels in the conditioned media of hMVECs

To determine the effect of hypoxia on the fibrinolytic activity of hMVECs, we determined the u-PA, t-PA and PAI-1 antigen levels by ELISA in the conditioned

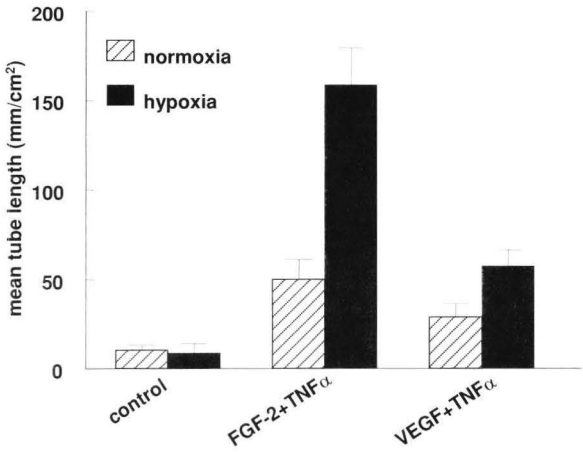

Figure 2: Capillary-like tube formation is increased under hypoxic conditions. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS under normoxic or hypoxic culture conditions and were not stimulated (control) or stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α (FGF-2+TNF-α), or with 25 ng/mL $VEGF_{165}$ and 10 ng/mL TNF-α ($VEGF_{165}$+TNF-α). After 3 days of culturing, mean tube length (mm/cm²) was measured as described. The data represent mean ± SD of five independent experiments performed in duplicate wells.

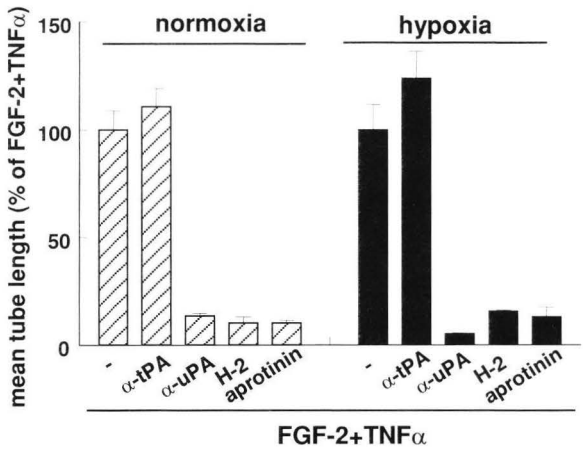

Figure 3: Inhibition of capillary-like tube formation by u-PAR antibodies. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS under normoxic or hypoxic culture conditions and were stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α (FGF-2+TNF-α) in the presence of polyclonal anti-t-PA (100 µg/mL), polyclonal anti-u-PA (100 µg/mL), Mab H-2 (anti u-PAR; 5 µg/mL) or aprotinin (100 U/mL). After 3 days of culturing, mean tube length (mm/cm²) was measured as described and expressed as a percentage of FGF-2+TNF-α. The data represent mean ± SD of three independent experiments performed in duplicate wells.

media of normoxic and hypoxic hMVECs. Under hypoxic culture conditions u-PA antigen was significantly decreased by 67 % in non-stimulated cells, 74 % in TNF-α-stimulated cells and 77 % in FGF-2+TNF-α-stimulated cells, as compared to normoxic culture conditions (Table 1). The amounts of PAI-1 and t-PA produced by the cells was comparable in hypoxic and normoxic cells (Table 1). The effect of hypoxia on u-PA levels in the conditioned media of hMVECs could not be mimicked by incubation with cobalt chloride, nickel chloride or deferoxamine (data not shown). This decrease in fibrinolytic potential in response to hypoxia was not expected, since the formation of capillary-like structures requires the presence of cell-bound u-PA (previous paragraph).

Table 1

Addition	Normoxia	Hypoxia	% inhibition by hypoxia
	u-PA (ng/10^5 cells)		
control	0.09 ± 0.03	0.03 ± 0.01#	67
TNF-α	0.83 ± 0.10	0.22 ± 0.03#	74
FGF-2 + TNF-α	2.23 ± 0.18	0.51 ± 0.02#	77
	t-PA (ng/10^5 cells)		
control	1.82 ± 0.14	1.35 ± 0.47	26
TNF-α	1.82 ± 0.23	1.67 ± 0.18	8
FGF-2 + TNF-α	2.70 ± 0.10	2.56 ± 0.25	5
	PAI-1 (ng/10^5 cells)		
control	136 ± 54	115 ± 54	15
TNF-α	599 ± 60	608 ± 87	-2
FGF-2 + TNF-α	560 ± 81	524 ± 85	6

Table 1: Effect of hypoxia on u-PA, t-PA and PAI-1 antigen levels. HMVECs were cultured on gelatin-coated wells for 72 hours in normoxic and hypoxic conditions in M199 supplemented with 10 % HS and were stimulated with 10 ng/ml TNF-α or 10 ng/ml FGF-2 and 10 ng/ml TNF-α (FGF-2+TNF-α) or not (control). After the incubation period u-PA, t-PA and PAI-1 antigen levels were determined by ELISA as described and expressed as ng/10^5 cells. Data represent mean ± SD of 9 cultures in three independent experiments. # indicates $p<0.05$, which is significantly different from normoxia.

u-PAR expression is upregulated in hypoxic conditions

The u-PA level in the conditioned medium of cells is the resultant of its production and internalization by the cellular receptor u-PAR. The effect of hypoxia on the u-PAR expression was assayed. Hypoxic conditions increased u-PAR mRNA in non-stimulated cells as well as in TNF-α- or FGF-2+TNF-α-stimulated hMVECs (Figure 4A/B). The u-PAR mRNA signal was normalized for actin mRNA and quantified (Figure 4B). Compared to the normoxic situation, hypoxia stimulated u-PAR mRNA expression by 139 %, 144 %, 130 % in non-stimulated cells, TNF-α-, and FGF-2+TNF-α-stimulated cells, respectively. The u-PA mRNA levels were also determined (Figure 4A,B); these signals were very weak but clearly enhanced after hypoxic treatment: 101%, 141%, 124% in non-stimulated cells, TNF-α-, and FGF-2+TNF-α-stimulated cells, respectively (Figure 4B).

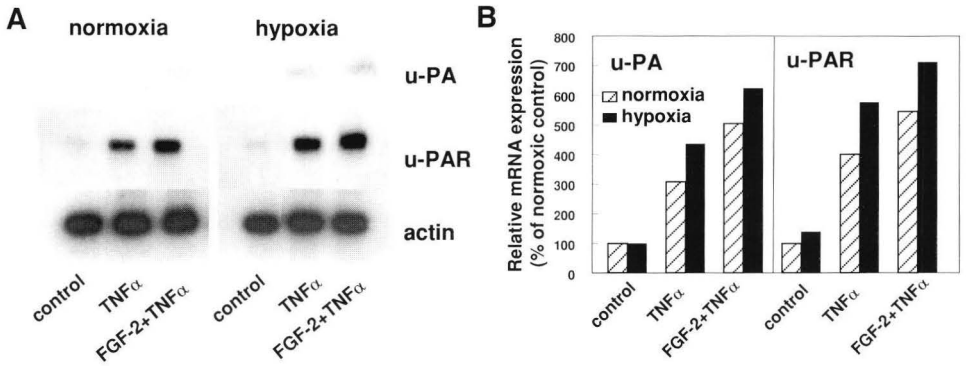

Figure 4: Effect of hypoxia on u-PA and u-PAR mRNA expression. (**A**) HMVECs were cultured for 72 hours in normoxic and hypoxic conditions in M199 supplemented with 10 % HS and not stimulated (control) or stimulated with 10 ng/mL TNF-α or 10 ng/mL FGF-2 and TNF-α (FGF-2+TNF-α). After 72 hours, total RNA was isolated and analyzed by Northern blotting using [α-^{32}P]CTP-labeled probes for u-PA, u-PAR and actin. (**B**) The signals for u-PA and u-PAR mRNA were quantified by phosphorimager analysis and adjusted for the corresponding actin mRNA. Data are expressed as a percentage of normoxic control cells. Similar results were obtained in three independent experiments.

The increase in u-PAR mRNA expression in hypoxic conditions was accompaniedby an enhanced u-PAR antigen level, compared to normoxic conditions. The increase in specific bound ^{125}I-labeled-DIP-u-PA was evident in non-stimulated hMVECs (138 ± 38 %, n=4, p=0.2) and significantly increased in cells stimulated with FGF-2+TNF-α (142 ± 17 %, n=4, p=0.02) or with the strong inducer of u-PAR expression, phorbol myristate acetate (164 ± 11 %, n=4, p=0.03) (Figure 5).

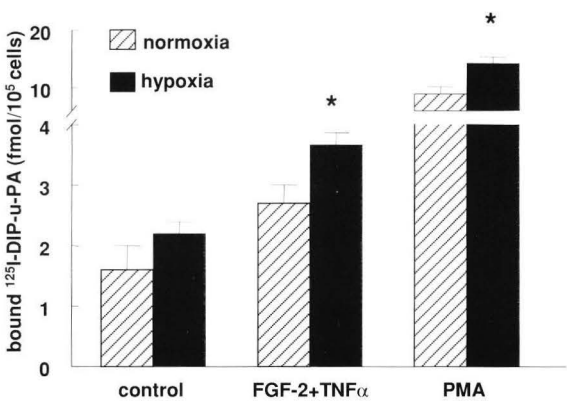

Figure 5: Hypoxia increases u-PAR antigen levels. HMVECs were cultured for 72 hours in normoxic and hypoxic conditions in M199 supplemented with 10 % HS and not stimulated (control) or stimulated with 10 ng/mL FGF-2 and TNF-α (FGF-2+TNF-α) or with 10^{-8} mol/L phorbol myristate acetate (PMA). Subsequently the cells were cooled on ice, and the specific binding of ^{125}I-labeled DIP-u-PA to hMVECs was determined and expressed as fmol ^{125}I-u-PA/10^5 cells. Data represent mean ± SEM of four independent experiments performed in duplicate wells. * indicates $p<0.05$, which is significantly different from the normoxic counterpart.

When the u-PA:u-PAR binding was prevented by the blocking mAb H-2 against u-PAR, the decrease in u-PA antigen level observed under hypoxic conditions was completely abolished in non-stimulated as well as in FGF-2+TNF-α-stimulated cells (Figure 6). After incubation with mAb H-2, 5 times more u-PA accumulated in the media of control as well as FGF-2+TNF-α stimulated normoxic hMVECs. In non-stimulated and FGF-2+TNF-α-stimulated hypoxic cells, 6 and 24 times more u-PA was accumulated in the conditioned media, respectively.

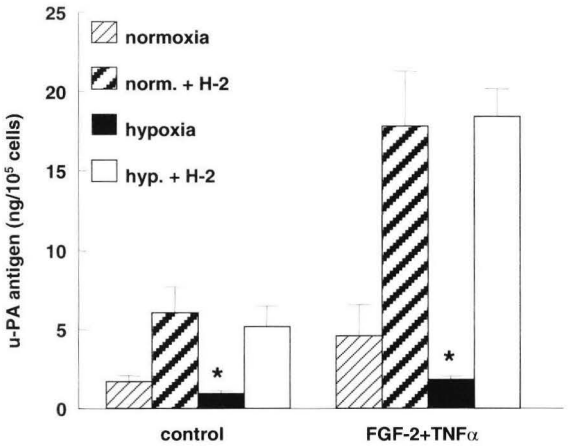

Figure 6: Hypoxia does not decrease u-PA production. HMVECs were cultured for 72 hours in normoxic and hypoxic conditions in M199 supplemented with 10 % HS and not stimulated (control) or stimulated with 10 ng/mL FGF-2 and TNF-α (FGF-2+TNF-α) in the presence of 0 or 5 μg/mL Mab H-2. After the incubation period, u-PA antigen levels were determined in the conditioned media by ELISA as described and expressed as ng u-PA/10^5 cells. Data represent mean ± SD of three independent experiments performed in duplicate wells. * indicates $p<0.05$, which is significantly different from the normoxic counterpart.

Plasmin generation is enhanced in hypoxic culture conditions

Stimulation of hMVECs with FGF-2+TNF-α resulted in an increased plasmin formation (Figure 7) as compared to unstimulated cells. The plasmin generation was completely blocked by aprotinin. Parallel to the increased u-PAR expression, hypoxia further increased plasmin formation by FGF-2+TNF-α-stimulated hMVECs, compared to their normoxic counterparts ($p<0.002$). This plasmin formation was completely inhibited by the addition of the blocking antibody H-2 against u-PAR, indicating that solely receptor bound u-PA was responsible for plasmin formation (Figure 7). Antibodies against t-PA did not influence plasmin formation (data not shown), meaning that only u-PA was responsible for the activation of plasminogen.

$VEGF_{165}$ is not involved in hypoxia-induced tube formation by hMVECs in a fibrin matrix

It has been reported that $VEGF_{165}$ expression is enhanced in hypoxic conditions in a number of cell types, including endothelial cells [31] by an increased transcriptional activation [50] as well as by mRNA stabilization [51;52]. Furthermore it has been shown in HUVECs and hMVECs that $VEGF_{165}$ can upregulate u-PAR expression

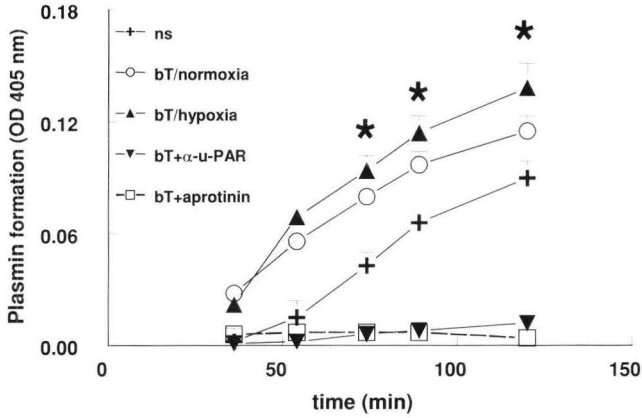

Figure 7: Plasmin formation is increased in hypoxia. HMVECs were cultured in M199 supplemented with 10 % HS and 10% NBCS and not stimulated (ns) or stimulated with 10 ng/mL FGF-2 and TNF-α (FGF-2+TNF-α) in normoxia (bT/normoxia) or hypoxia (bT/hypoxia). Normoxic hMVECs were cultured in the presence of Mab H-2 (5 μg/ml) or aprotinin (200U). After 72 hours, plasmin formation was measured as described in material and methods. H-2 and aprotinin were also present during the plasmin formation assay. Data are expressed as mean ± SD. Each condition was performed eightfold. * indicates $p<0.002$, which is significantly different from normoxic FGF-2+TNF-α-stimulated cells.

[18;53]. Therefore, it was investigated whether endogenous synthesis of $VEGF_{165}$ might play a role in the observed increase in tube formation of hMVECs in a fibrin matrix under hypoxic conditions.

$VEGF_{165}$ could not be detected by ELISA (detection limit of 5 pg/mL) in the conditioned media of both normoxic and hypoxic hMVECs. In addition, $VEGF_{165}$ mRNA was not detected by Northern blot analysis at 8, 24 or 72 hours nor by RT-PCR (data not shown). The addition of 29 nmol/L sVEGFR-1 (a 50-fold excess over the $VEGF_{165}$ concentration used in the *in vitro* angiogenesis experiments) did not affect the increased tube formation of FGF-2+TNF-α-stimulated hMVECs in hypoxic conditions (Figure 8). In the same group of experiments sVEGFR-1 completely inhibited $VEGF_{165}$+TNF-α-induced tube formation in normoxic conditions, indicating the ability of sVEGFR-1 to neutralize all $VEGF_{165}$ from the media. In addition, a blocking antibody to VEGFR-2 was not able to block FGF-2+TNF-α-induced tube formation in normoxic as well as in hypoxic conditions (data not shown).

Involvement of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrin in hypoxia-induced tube formation

Besides the importance of proteolytic activity, the ability of the endothelial cells to reattach to the matrix is essential for cell migration and angiogenesis. The importance of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins in angiogenesis and their regulation by hypoxia have been shown in a number of studies [36;54-56].

After 16 hours of hypoxic culturing, hMVECs showed an increase of α_v-subunit mRNA expression compared to normoxic conditions (Figure 9A). This increase was evident at both control (4-fold induction compared to normoxia) and FGF-2+TNF-α-stimulated cells (2.6-fold induction). At this time-point, no effect of hypoxia on the β_5-

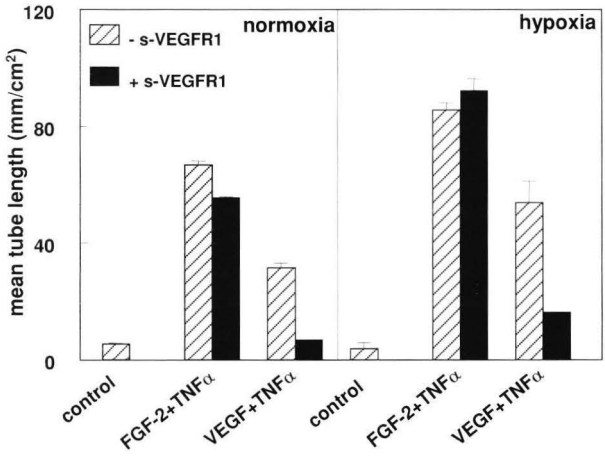

Figure 8: sVEGFR-1 does not inhibit FGF-2 + TNF-α induced tube formation in hypoxia. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS under normoxic or hypoxic culture conditions and were not stimulated (control) or stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α (FGF-2+TNF-α) or with 25 ng/mL $VEGF_{165}$ and 10 ng/mL TNF-α ($VEGF_{165}$+TNF-α) in the presence of 0 or 29 nmol/L sVEGFR-1. After 3 days of culturing, mean tube length (mm/cm^2) was measured as described. The data represent mean ± SD of three independent experiments performed in duplicate wells.

subunit mRNA could be detected on both the control and FGF-2+TNF-α-stimulated cells (Figure 9A). The β_3-integrin mRNA remained below the detection limit by Northern blot assay.

The involvement of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins in hypoxia-induced tube formation was investigated by adding blocking mAbs, directed against the $\alpha_v\beta_3$- and $\alpha_v\beta_5$- integrins. When hMVECs were seeded in vitronectin-coated dishes, cell adherence was significantly inhibited by the Mab LM609 ($p<0.001$), which blocks the $\alpha_v\beta_3$-integrin, and by the mAb P1F6 ($p<0.001$), which blocks the $\alpha_v\beta_5$-integrin The strongest inhibition was seen after incubation with both antibodies (Figure 9B). No significant reduction was observed in capillary-like tube formation (Figure 9C) by either antibody or a combination of the two antibodies.

Discussion

In this report we have shown that hypoxia strongly enhances the formation of capillary-like tubular structures by human MVECs in a three-dimensional fibrin matrix. Our data indicate that enhanced expression of u-PAR by hypoxia, at least in part, explains the increased angiogenic response of endothelial cells. In our experimental model, which only contains endothelial cells, the expression of α_v-integrin, but not that of $VEGF_{165}$, also was enhanced by hypoxia. However, neither $VEGF_{165}$ expression nor the expression of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins significantly contributed to the hypoxia-induced increase in tube formation.

Hypoxia is a potent stimulus for angiogenesis [2;3]. *In vivo* macrophages are highly

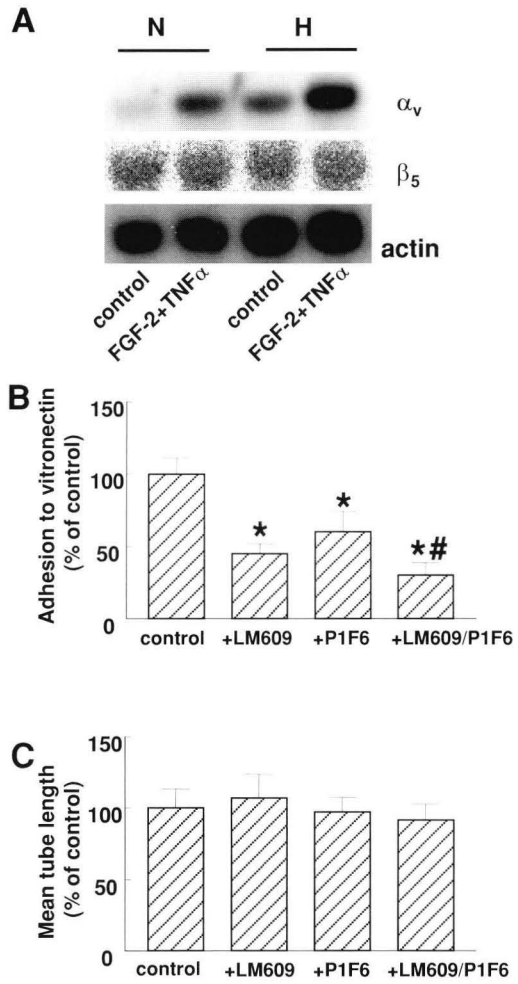

Figure 9: Hypoxia increases α_v-integrin mRNA expression. (A) HMVECs were cultured for 16 hours in normoxic (N) and hypoxic (H) conditions in M199 supplemented with 10 % HS and not stimulated (control) or stimulated with 10 ng/mL FGF-2 and TNF-α (FGF-2+TNF-α). After the incubation period, total RNA was isolated and analyzed by Northern blotting using [α-^{32}P]CTP-labeled probes for α_v, β_5-integrin and actin. (B) Cell adhesion assay was performed as described in Materials and methods. HMVECs were allowed to adhere to vitronectin for 90 minutes in the presence of control antibody anti-FITC (10 μg/mL), LM609 (10 μg/mL), P1F6 (10 μg/mL) or a combination of LM609 and P1F6 (10 μg/mL each). Data are expressed as mean percentage of control ± SD. Each condition was performed fourfold. The different groups were compared by a One-Way ANOVA. * indicates $p<0.001$, which is significantly different from control, # indicates $p=0.005$, which is significantly different from P1F6. (C) HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS under normoxic or hypoxic culture conditions and were stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α (control) alone or in the presence of $\alpha_v\beta_3$ blocking Mab LM609 (10 μg/mL), $\alpha_v\beta_5$ blocking Mab P1F6 (10 μg/mL) or a combination of these antibodies (LM609/P1F6). After 3 days of culturing, the mean tube length (mm/cm^2) was measured as described. The effect of LM609 and P1F6 is expressed as the mean percentage of FGF-2+TNF-α-stimulated cells ± range of two independent experiments performed in duplicate wells.

sensitive to hypoxia and are induced by it to produce angiogenic factors such as VEGF [9-11]. VEGF is also induced in other tissue cells, such as stroma cells and smooth muscle cells [4;10;30]. In these cells hypoxia can act both on gene transcription and mRNA stability [50;57]. In this report we have shown that hypoxia increases the formation of capillary-like tubular structures in a model in which endothelial cells invade a fibrin matrix. No accessory cells were present indicating that hypoxia directly affected endothelial cells. This affect appears independent of an increased $VEGF_{165}$ production, because $VEGF_{165}$ mRNA was not detectable in the

cells, $VEGF_{165}$ antigen was not detectable by ELISA, and the effect could not be inhibited by soluble-VEGFR-1 or anti-VEGFR-2 IgG. While Namiki *et al.* [31] reported the induction of $VEGF_{165}$ in human endothelial cells, other investigators [33] could not detect any increase in $VEGF_{165}$ by hypoxia, similar to our findings. In line with this latter study, our data does not support an autocrine stimulation of $VEGF_{165}$-induced angiogenesis by hypoxia and provides information regarding a mechanism additional to the presently established paracrine stimulation of $VEGF_{165}$-induced angiogenesis by hypoxia. A hypoxia-induced upregulation of $VEGF_{165}$ receptors in endothelial cells may be of interest in this context [33-35]. Such mechanisms may contribute to hypoxia-enhanced $VEGF_{165}$+TNF-α-stimulated formation of capillary-like tubes. While the latter process is completely inhibited by soluble-VEGFR-1, soluble-VEGFR-1 had virtually no effect on hypoxia-enhanced FGF-2+TNF-α-dependent angiogenesis in our model. This suggests that other mechanisms not related to the expression of and the response to $VEGF_{165}$ contribute to this hypoxia-induced capillary-like tube formation.

Human endothelial cells invade a fibrin matrix after exposure to an angiogenic growth factor, $VEGF_{165}$ or FGF-2, and the cytokine TNF-α [18]. This process requires cell-bound u-PA activity, which depends on the availability of the u-PAR [19]. Wojta *et al.* [38] reported a decrease in u-PA activity after hypoxic treatment of endothelial cells due to an increased production of PAI-1. In the present study, we also found a dramatic decrease in the amount of u-PA antigen that accumulated in the conditioned medium. However, no increase in PAI-1 antigen was detected and no decrease in u-PA mRNA either. By the use of a Mab that prevented the interaction of u-PA with its receptor it was demonstrated that the decrease in u-PA accumulation was caused by a u-PAR-mediated process. Probably it reflects the cellular uptake of the u-PA:PAI-1 complex [58]. The increase in u-PAR expression by hypoxia found in our hMVECs closely agrees with similar findings in other cell types, i.e. throphoblasts, umbilical vein endothelial cells and breast carcinoma cells [39;59]. An increased binding of u-PA to its receptor and thus a higher internalization rate of u-PA can efficiently deplete the conditioned medium of u-PA [19]. Because u-PA is taken up by the cell only after activation and subsequent inhibition by PAI-1 [60], the decrease in u-PA not only refllects the cellular consumption of u-PA, but also reflects the fact that u-PA had been active, probably on the cell surface. This is also proven by an increased plasmin formation in hypoxic conditions. In line with our previous observations that u-PA:u-PAR interaction is required for tube formation in a fibrin matrix and that modulation of u-PAR expression influences the rate of this process [18], the higher expression of u-PAR and consequently a higher plasmin formation are likely to contribute to the increased angiogenic response of hMVECs under hypoxic conditions. In pericellular proteolysis the u-PA/plasmin and MMP cascades

often cooperate. One may expect that hypoxia alters the expression of MMPs. Under our experimental conditions we observed no difference in the expression of the mRNA of MMP-1 and MMP-3 as compared to the normoxic conditions. Similarly the expression of MMP-2 and MMP-9 remained unaltered as revealed by gelatin zymography (our unpublished data). However, these data do not exclude the possibility that another proteolytic enzyme or unknown proangiogenic factor is influenced by hypoxia.

The mechanism by which hypoxia increases the expression of u-PAR, is not yet known. Cobalt chloride, nickel chloride and deferoxamine, could not mimic the hypoxic response in our system, indicating that a heme protein is not involved. This finding is in contradiction to the studies of Graham *et al.* [39;59], who suggested the involvement of a heme protein in u-PAR induction. The difference in these results is not easy to explain. Probably they are caused by the use of different celltypes or the different time scale in which the experiments were performed (24 hours compared to 72 hours in this study). In addition to the involvement of a heme protein, redox-based signaling is indicated as a possible oxygen-sensing mechanism [61;62]. The activity of HIF-1, a well-studied hypoxia-induced transcription factor, has been linked to the redox state of the cell [63]. Three potential HIF-1 binding sequences can be found in the 5'-flanking region of the u-PAR gene [39], indicating that hypoxia can directly influence u-PAR transcription. In addition or alternatively to its effect on gene transcription, hypoxia also may increase the stability of u-PAR expression. Because hypoxia also affects the expression of other proteins, such as $VEGF_{165}$ [57] and erythropoietin [64] by increasing mRNA stability, this is a plausible option.

In addition to matrix degradation, the ability of the endothelial cells to re-adhere to this matrix is also indispensable for invasion. $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins are matrix-receptors which bind to vitronectin and other matrix proteins [37]. These integrins play essential roles in cell migration [65] and angiogenesis [55;66]. It has previously been shown in bovine retinal endothelial cells that the expression of α_v-,β_3- and β_5-sububits is increased in reponse to hypoxia [36] and in this manner may promote cell migration. In human MVECs the α_v-subunit mRNA expression is enhanced after 16 hours of hypoxia, while the expression of β_5-integrin subunits is not altered by hypoxia. The β_3-integrin mRNA was not detectable by Northern blot, probably due to its low expression in human endothelial cells [47]. Although the α_v-subunit is regulated by hypoxia and may lead to a higher expression of integrins containing the α_v-subunit, hypoxia-induced tube formation was not influenced by $\alpha_v\beta_3$- or $\alpha_v\beta_5$-blocking antibodies. The possibility that other integrins present on endotelial cells interact with fibrin can not be excluded.

In conclusion, this study shows that endothelial cells by themselves are able to increase their angiogenic potential in response to hypoxia. In our experimental

conditions, the increase of u-PAR expression by hypoxia appears an important determinant in this process. We would like to stress that angiogenesis in different conditions is regulated by a number of regulators, and that our findings reflect angiogenesis in the temporary repair matrix fibrin. In other conditions, such as development and bone repair, the role of u-PA and u-PAR may be less prominent and other proteins, such as MMPs, play a dominant role. We conclude that cell-bound u-PA is an important determinant in capillary like tube formation in fibrin matrices. In particular, it is anticipated to act in the recanalization of fibrin clots and fibrous exudates, in addition to the paracrine effects of hypoxia involving the induction of $VEGF_{165}$ by adjacent tissue cells.

Acknowledgments

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HYPOXIA IN COMBINATION WITH FGF-2 INDUCES TUBE FORMATION BY HUMAN MICROVASCULAR ENDOTHELIAL CELLS IN A FIBRIN MATRIX. INVOLVEMENT OF AT LEAST TWO SIGNAL TRANSDUCTION PATHWAYS

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Abstract

Hypoxia in combination with a growth factor is a strong inducer of angiogenesis. Among several effects, hypoxia can activate endothelial cells directly, but the mechanism by which it acts is not fully elucidated. *In vitro*, human microvascular endothelial cells (hMVECs) form capillary-like tubules in fibrin solely after stimulation with a combination of fibroblast growth factor (FGF)-2 or vascular endothelial growth factor (VEGF) and the cytokine tumor necrosis factor (TNF)-α. We show in this paper that in hypoxic conditions, FGF-2-stimulated hMVECs form tube-like structures in a fibrin matrix in the absence of TNF-α. Hypoxia/FGF-2-stimulated cells express more urokinase-type plasminogen activator (u-PA) receptor than normoxia/FGF-2-stimulated cells and display a slightly higher turnover of u-PA. This small increase in u-PA activation probably cannot fully explain the hypoxia/FGF-2-induced tube formation. Hypoxia activated at least two signal pathways that may contribute to the enhanced angiogenic response. In hypoxia/FGF-2-stimulated hMVECs the transcription factor p65 was activated and translocated to the nucleus, whereas in normoxia/FGF-2-stimulated cells p65 remained inactive. Furthermore, in hypoxic conditions, the amounts of phosphorylated mitogen-activated protein kinases ERK1/2 were increased compared to normoxic conditions. We conclude that hypoxia is able to activate different signal pathways in FGF-2-stimulated human endothelial cells, which may be involved in hypoxia-induced angiogenesis.

Introduction

The growth of new blood vessels or angiogenesis takes place in response to angiogenic factors by a series of discrete, but overlapping, phases, including vascular dilation and increased vascular permeability, endothelial cell activation, degradation of the endothelial cell basal lamina, endothelial cell migration and proliferation, lumen formation and capillary stabilization [1]. Analysis of the array of angiogenesis stimulators strongly suggests that there must be several different mechanisms for stimulating capillary growth. Not only angiogenic growth factors and cytokines, but also environmental factors such as matrix composition and oxygen tension, influence the angiogenic response.

In a wound area, fibrin exudates are formed as a temporary matrix and stimulate angiogenesis [2]. Previously we have shown that human microvascular endothelial cells (hMVECs) form capillary-like tubular structures in a fibrin matrix after stimulation with an angiogenic growth factor (fibroblast growth factor (FGF)-2 or vascular endothelial growth factor (VEGF)) in combination with the cytokine tumor necrosis factor (TNF)-α [3]. These angiogenic factors induce, among other effects, the enhancement of components of the plasminogen activator system, namely urokinase-type plasminogen activator (u-PA) and its cell-bound receptor, u-PA receptor (u-PAR), thereby increasing proteolytic capacity and allowing hMVECs to degrade the extracellular matrix [3].

Hypoxia is a common feature of many of the pathological conditions in which neovascular growth is observed. Different cell types are able to sense the drop in oxygen level and react in various ways. Macrophages secrete growth factors like VEGF [4], FGF-2 [5] and platelet derived growth factor [6] in response to hypoxia. Endothelial cells enhance the expression of specific integrins [7] and enhance their proteolytic activity [8;9]. The mechanism by which endothelial cells sense the oxygen level is not yet known, but it has been shown that hypoxia influences the activation of several components of signal transduction pathways. The transcription factor nuclear factor κB (NF-κB) as well as the members of the mitogen-activated protein kinase (MAPK) superfamily, consisting of extracellular-related kinase (ERK)1/2, c-jun NH2-terminal kinase (JNK) 1/2 and p38, have been shown to be activated by hypoxia [10-13]. Phosphorylation and thus activation of these MAPK after stimulation with growth factors such as FGF-2 and TNF-α, has been correlated with the process of angiogenesis; NF-κB and NF-κB-like sites are located in the promoter region of target genes in endothelial cells, whose expression is critical to the initiation of capillary formation by stimulated endothelial cells [14;15]. ERK activation has been implicated in FGF-2-mediated angiogenesis in the chorioallantoic membrane [16]. The function

of the stress-activated kinase cascades in endothelial cell activation is not well understood. A role of p38 in the migration of human umbilical vein endothelial cells through actin reorganization is, however, shown by Rousseau *et al.* [17].

Hypoxia can induce angiogenesis both *in vitro* and *in vivo* situations and the angiogenic response is even more pronounced when a growth factor is present [18;19]. After delivering of FGF-2 [20-22] or VEGF [18;23] to ischaemic legs or myocardia, improved blood flow in these regions has been reported. The mechanism by which the endothelial cells are activated in such situations is not fully understood. In order to understand this mechanism, capillary-like tube formation in fibrin matrices by human microvascular endothelial cells, stimulated with hypoxia in combination with FGF-2, was studied. We focused on the proteolytic capacity of the cells and the induced signal transduction pathways in hypoxic culture conditions.

Materials & Methods

Materials

Cell culture materials were purchased as described by Kroon *et al.* [24]. Human fibrinogen was obtained from Chromogenix AB (Mölndal, Sweden); Factor XIII was generously provided by Dr. H. Boeder and Dr. P. Kappus (Centeon Pharma GmbH, Marburg, Germany) and thrombin came from Organon Technika (Boxtel, The Netherlands). FGF-2 was purchased from Pepro Tech EC (London, England), $VEGF_{165}$ and soluble-VEGFR-1 was a kind gift of Dr. H. Weich (GFB, Braunschweig, Germany). Human recombinant TNF-α was a gift from Dr. J. Travernier (Biogent, Gent, Belgium) and contained 2.45×10^7 U/mg protein and less than 40 ng lipopolysaccharide per mg protein. Aprotinin was purchased from Pentapharm Ltd. (Basel, Switzerland). Rabbit polyclonal anti-u-PA antibodies were prepared in our laboratory [25] as well as the rabbit polyclonal anti-t-PA antibodies. The monoclonal u-PAR-blocking antibody H-2 was a kind gift from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany) [26]. The polyclonal antibodies recognizing p65 and IκBα came from Santa Cruz (Santa Cruz, CA). Polyclonal antibodies recognizing phospho-p44/p42 (Erk1/2) MAP Kinase and phospho-p38 came from New England Biolabs (Beverly, MA), polyclonal anti-active™ JNK antibodies from Promega (Madison, WI). Horseradish-conjugated goat-anti-rabbit antibodies were bought at Roche Diagnostics Nederland BV (Almere, The Netherlands) and FITC-conjugated swine-anti-rabbit antibodies came from DAKO (Glostrup, Denmark). PD98059 and SB203580 were purchased from Alexis Biochemicals (Läufelfingen, Switzerland).

cDNA probes: The following cDNA fragments were used as probes in the hybridization experiments: a 1023 bp fragment of the human u-PA cDNA (a kind gift from Dr. W-D. Schleuning, Schering AG, Berlin, Germany) a 585 bp BamHI fragment of the human u-PAR cDNA (a kind gift from Dr. F. Blasi, Milan, Italy) and a 1200 bp Pst I fragment of hamster actin cDNA.

Cell culture

Human foreskin microvascular endothelial cells (hMVECs) were isolated, cultured and characterized as previously described [27;28]. HMVECs were cultured on gelatin-coated

dishes in M199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% human serum (HS), 10% heat -inactivated new born calf serum (NBCS), 150 μg/ml crude endothelial cell growth factor, 2 mmol/L L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C under a 5% CO_2/ 95% air atmosphere, unless mentioned otherwise. Experiments were performed with confluent cells (0.7 x 10^5 cells/cm^2), which had been cultured without growth factor for at least 24 hours.

Establishment of hypoxic culture conditions

For culturing in hypoxic conditions, hMVECs were placed in a NAPCO® incubator (serial number 7101-C1, Precision Scientific Inc, Chicago, USA), which controls the oxygen concentration by flushing with N_2. Oxygen levels in the incubator were monitored by an internal oxygen sensor as well as by external calibration using Dräger Tubes 6728081 (Drägerwerk Ag, Lübeck, Germany). Hypoxic condition is defined as culturing at 37°C under 1% O_2/5% CO_2 atmosphere.

In vitro angiogenesis model

Human fibrin matrices were prepared by the addition of 0.1 U/ml thrombin to a mixture of 2.5 U/ml factor XIII (final concentrations), 2 mg/ml fibrinogen, 2 mg/ml Na-citrate, 0.8 mg/ml NaCl and 3 μg/ml plasminogen in M199 medium (mixture pH 7.4). 300 μl aliquots of this mixture were added to the wells of 48-wells plates. After clotting at room temperature, the fibrin matrices were soaked with M199 supplemented with 10% (vol/vol) HS and 10% (vol/vol) NBCS for 2 hours at 37°C to inactivate the thrombin. Confluent endothelial cells (0.7 x 10^5 cells/cm^2) were detached and seeded in a 1.25:1 split ratio on the fibrin matrices to form a highly confluent monolayer. After 24 hours culturing in M199 medium supplemented with 10 % HS, 10% NBCS, and penicillin/streptomycin, the endothelial cells were stimulated with the mediators for the time indicated. At the end of the culture period the media were collected and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix was analyzed by phase contrast microscopy and the total length of tube-like structures of six randomly chosen microscopic fields (7.3 mm^2/field) was measured using an Olympus CK2 microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software, and expressed as mm/cm^2.

For histochemistry, matrices were fixed in 2% (wt/vol) p-formaldehyde in phosphate-buffered saline (PBS)(0.15 mol/L NaCl/10 mmol/L Na_2PO_4/1.5 mmol/L KH_2PO_4, pH 7.4) embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany), sectioned at 4 μm and stained with phloxin.

ELISAs

u-PA, t-PA, PAI-1 and VEGF antigen as well as fibrin degradation products (FDP) determinations were performed by commercially available immunoassay kits: uPA EIA HS Taurus (Leiden, The Netherlands), Thrombonostika t-PA (Organon-Teknika, Turnhout, Belgium), IMULYSE® PAI-1 (Biopool, Umea, Sweden), VEGF ELISA (R&D system, Minneapolis, USA), and Fibrinostika® FDP ELISA (Organon-Teknika, Turnhout, Belgium). MCP-1 ELISA was previously described by Peri *et al.*[29].

Determination of specific u-PA binding

Determination of specific binding of diisopropylfluorophosphate-treated u-PA (Ukidan®) (DIP-u-PA) to hMVECs was described previously by Kroon *et al.* [24].

RNA Isolation and Northern Blots

Total RNA was isolated as described by Chomczynski and Sacchi [30] and electrophoresed in a 1.2% (wt/vol) agarose gel under denaturing conditions using 1 mol/L formaldehyde. Northern blotting was performed as described by Lansink *et al.* [31].

NF-κB (p65) immunofluorescence staining

HMVECs were cultured to confluency on glass cover-slips (14mm diameter). Cells were treated with different stimulators and incubated for the time indicated. After the incubation, cells were fixed for 5 minutes in ice-cold methanol and air-dried. After 3 washes with PBS containing 0.1% bovine serum albumin (BSA), the cover-slips were incubated for 20 minutes in a block-buffer (PBS + 10% NBCS). The cells were washed 3 times and incubated with the first antibody (anti NF-κB p65, 1:100 in PBS + 0.1% BSA). After an incubation of 1 hour and 3 washes, the cells were incubated with the second antibody (FITC-conjugated swine-anti-rabbit, 1:50 in PBS + 0.1% BSA). Finally, the cells were dehydrated in graded steps of ethanol and attached to a slide with a drop of Vectashield (Vector Laboratories, Burlingame,CA).

Western Blots

Equal numbers of hMVECs, cultured on gelatin-coated wells (5cm^2 wells/condition), were washed with ice-cold PBS and lysed in a lysis buffer containing 10% glycerol (vol/vol), 3% SDS (wt/vol), 0.125 mol/L Tris-PO_4 pH 6.7 and 10% β-mercaptoethanol (vol/vol). Protein was separated on a 10% SDS-polyacrylamide gel and electrophoretically transferred onto polyvinylidene difluoride membrane (Amersham, Uppsala, Sweden) in a buffer of 192 mmol/L glycine, 25 mmol/L Tris (pH 8.3) and 20% (vol/vol) methanol. The filters were blocked with 5% (wt/vol) non-fat milk in 137 mmol/L NaCl, 20 mmol/L Tris (pH 7.6) and 0.25% Tween 20 (TBST) for 1 hour, followed by overnight incubation at 4°C with the primary polyclonal antibodies (anti-IκBα 1:10000, anti-phospho-ERK1/2 1:1000, anti-active JNK1/2 1:2000, anti-phosho-p38 1:1000) in TBST + 5% non-fat milk + 0.5 mmol/L sodium orthovanadate. Subsequently, the blots were washed 3 times with TBST and incubated for 1 hour at room temperature with horseradish-conjugated goat-anti-rabbit antibodies (1:5000) in TBST + 5% non-fat milk + 0.5 mmol/L sodium orthovanadate, as a conjugate. The bands were visualized with ECL (Sigma, St Louis, Missouri).

Statistical analysis

Data are expressed as the mean ± S.D. The unpaired *t*-test was used for comparison of groups with equal variance and normal distribution. A P-value < 0.05 was considered statistically significant.

Results

Hypoxia induces tube formation in FGF-2-stimulated human microvascular endothelial cells

Under 20% oxygen atmosphere (further indicated as normoxic conditions), hMVECs required the exposure to an angiogenic growth factor (FGF-2) and the cytokine tumor necrosis factor (TNF)-α to form capillary-like tubular structures in the fibrin

matrix (Figure 1A) [3]. When the experiments were performed under hypoxic conditions (1% oxygen atmosphere), an increase in the extent of tube formation was observed at all FGF-2 concentrations used (5-50 ng/ml) (Figures 1B, 2). Normoxic hMVECs, grown on a fibrin matrix for 5 days and stimulated with FGF-2 only, neither formed tubes nor sustained an intact monolayer (Figure 1C), probably due to excessive fibrin degradation induced by t-PA [32]. In cross-sections of these three-dimensional fibrin matrices, gaps were visible in the monolayer (indicated with an arrow in Figure 1E). In the presence of anti-t-PA IgG, gap formation in the monolayers was prevented, but tubes could still not be induced (data not shown). Interestingly, in the hypoxic condition the sole addition of ≥ 30 ng/ml FGF-2 induced tubular structures (indicated with an arrow in Figures 1D,1F; Figure 2). This difference was not due to an altered fibrin degradation, since no significant difference in fibrin degradation products was measured (15.1±1.3 µg/ml vs 14.2±1.4 µg/ml, respectively, n=3) in the conditioned media of normoxia/FGF-2- and hypoxia/FGF-2-stimulated cells.

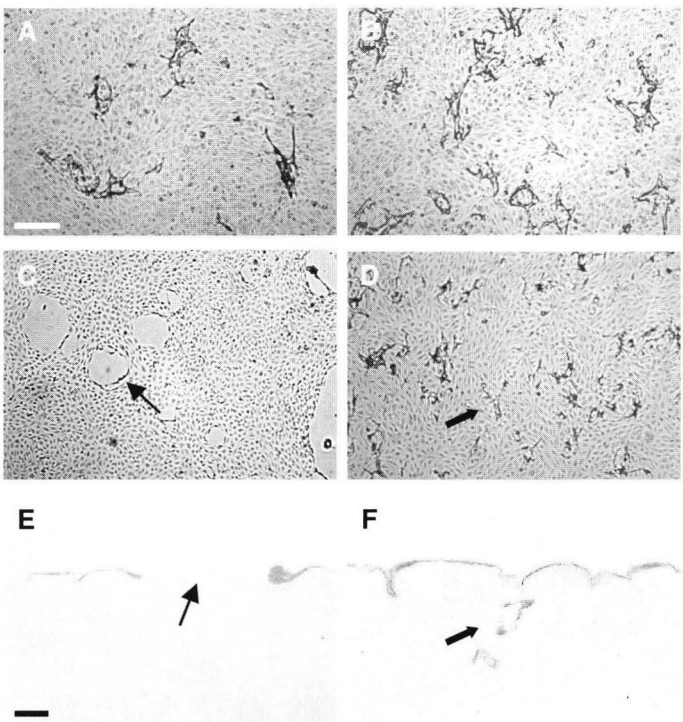

Figure 1: FGF-2 in combination with hypoxic conditions induces capillary-like tube formation. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS and stimulated with 10 ng/ml FGF-2 and 10 ng/ml TNF-α in normoxic (**A**) or hypoxic culture conditions (**B**), or stimulated with FGF-2 (50 ng/ml) in normoxia (**C**) or hypoxia (**D**). After 5 days of culture, non-phase photomicrographs were taken. Bar, 500 µm. In addition, FGF-2-stimulated cells on fibrin matrices in normoxic conditions (**E**) and hypoxic conditions (**F**) were fixed and embedded as described, cross-sections perpendicular to the matrix surface were cut and stained with phloxin. Bar, 100 µm. → indicates gap formation; ➡ indicates tube formation.

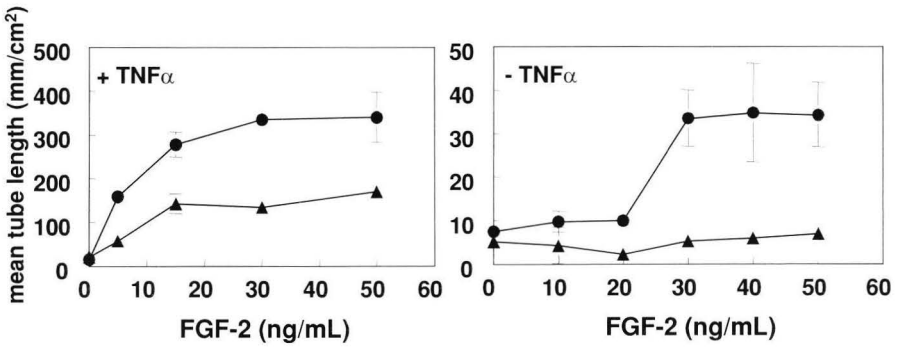

Figure 2: FGF-2 in combination with hypoxia induces capillary-like tube formation. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS and stimulated with increasing amounts of FGF-2 in the absence or presence of TNF-α (10 ng/ml) in normoxic (▲) or hypoxic (•) conditions. After 5 days of culture, mean tube length (mm/cm²) was measured as described and data were expressed as mean ± S.D. of three independent experiments performed in duplicate wells.

To investigate whether $VEGF_{165}$ induction contributed to the hypoxia/FGF-2-dependent tube formation, soluble-VEGFR-1 was added (29 nmol/L; a 50-fold excess over the $VEGF_{165}$ concentration used in the control experiments) to the medium in combination with FGF-2. Soluble-VEGFR-1 did not affect the tube formation of hypoxia/FGF-2-stimulated hMVECs (Figure 3A). In the same group of experiments soluble-VEGFR-1 inhibited the $VEGF_{165}$+TNF-α-induced tube formation by 74±3%, indicating the ability of soluble-VEGFR-1 to neutralize $VEGF_{165}$ in the media.

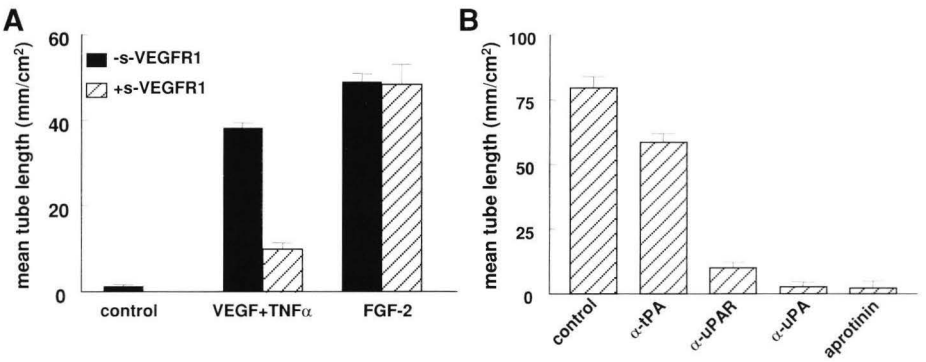

Figure 3: FGF-2-induced tube formation is inhibited by antibodies against u-PA and u-PAR but not by soluble-VEGFR-1. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS in hypoxic culture conditions and were stimulated with (**A**) 25 ng/ml VEGF and 10 ng/ml TNF-α or 50 ng/ml FGF-2 in the absence or presence of 100 μg/ml soluble VEGFR-1 or stimulated with (**B**) 50 ng/ml FGF-2 in the absence or presence of polyclonal anti-t-PA (100 μg/ml), MAb H-2 (anti u-PAR; 5 μg/ml), polyclonal anti-u-PA (100 μg/ml), or aprotinin (100 U/ml). After 5 days of culture, mean tube length (mm/cm²) was measured as described and data were expressed as mean ± S.D. of three independent experiments performed in duplicate wells.

The capillary-like structures, formed by hypoxia/FGF-2-stimulated hMVECs, were inhibited by antibodies against u-PA (93±4%;n=3;p=0.02), u-PAR (72±2%;n=3;p=0.01), the plasminogen inhibitor aprotinin (94±5%;n=3;p=0.01) but not significantly by antibodies against t-PA (26±5%;n=3;p=0.06)(Figure 3B), in agreement with our previous findings [32], indicating that t-PA does not play a significant role in tube formation in this *in vitro* system.

Hypoxia induces u-PAR in FGF-2 stimulated cells

Because cell-bound u-PA activity is required to induce capillary-like structures under our experimental conditions, we evaluated whether hypoxia affected the production of fibrinolytic proteins in non- and FGF-2-stimulated cells.

In hypoxia- and hypoxia/FGF-2-stimulated hMVECs, u-PAR mRNA was enhanced as compared to their normoxic counterparts (Figure 4A). In addition, u-PAR antigen was enhanced in hypoxic hMVECs as measured by specific ^{125}I-DIP-u-PA binding. This increase was significant for hypoxia/FGF-2-stimulated hMVECs (138±11%, p=0.03, n=3 performed in duplicate wells) (Figure 4B). In addition, u-PAR antigen was measured by a u-PAR ELISA. An increase of 146±2% (n=3) in u-PAR antigen was found in FGF-2/hypoxia-stimulated cells as compared to their normoxic counterparts.

In hypoxic conditions less u-PA antigen accumulated in the conditioned media (Figure 4C). However, when the cellular uptake of the u-PA:PAI-1 complex was prevented by blocking the interaction of u-PA with its receptor u-PAR by the blocking antibody H-2, the decrease in u-PA accumulation in hypoxic conditions was completely abolished and comparable amounts of u-PA were produced (Figure 4C). In addition, no difference in u-PA production was detected between control and FGF-2-stimulated cells. As only the u-PA:PAI-1 complex is internalized, this indicates that u-PA had been activated. A significant increase in the amount of relatively internalized u-PA was observed in hypoxic/FGF-2-stimulated cells as compared to their normoxic counterparts (6±0.3% increase; n=3; p=0.03). The amounts of t-PA produced by FGF-2-stimulated hMVECs were comparable in hypoxic and normoxic culture conditions (1.5±0.1 vs. 1.4±0.3 ng t-PA/10^5 cells, respectively, n=3). This was also the case for the PAI-1 levels in the conditioned media (162±16 vs. 164±13 ng PAI-1/10^5 cells, respectively, n=4).

Hypoxia activates the transcription factor NF-κB (p65)

To evaluate whether hypoxia induces an activation of endothelial cells similar to that mediated by TNF-α, the activation of NF-κB was investigated. To this end, translocation of NF-κB to the nucleus was determined via immunofluorescence staining of the p65 subunit of NF-κB. In control hMVECs a diffuse

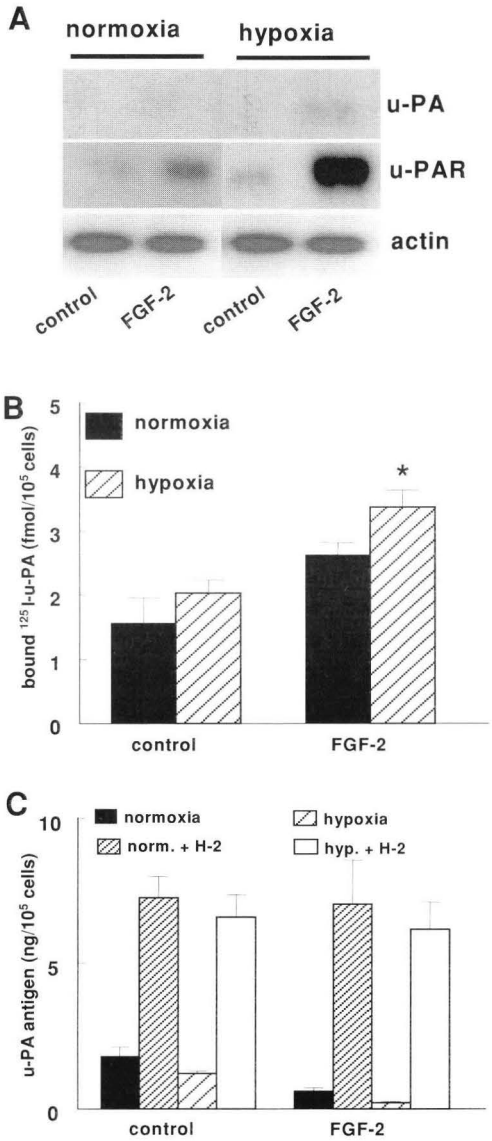

Figure 4: Hypoxia increases u-PAR expression. HMVECs were cultured for 72 hours in normoxic and hypoxic conditions in M199 supplemented with 10 % HS and not stimulated (control) or stimulated with 50 ng/ml FGF-2. (**A**) After 72 hours, total RNA was isolated and analyzed by Northern blotting using [α-^{32}P]CTP-labeled cDNA probes for u-PA, u-PAR and actin. (**B**) After 72h, in parallel experiments hMVECs were cooled on ice, and the specific binding of ^{125}I-labelled DIP-u-PA to hMVECs was determined and expressed as fmol ^{125}I-DIP-u-PA/10^5 cells. Data represent mean ± S.E.M. of three independent experiments performed in duplicate wells. * significantly different (p=0.03) from normoxic counterpart. Similar results were obtained in three independent experiments. (**C**) HMVECs were cultured for 72 hours in normoxic and hypoxic conditions in M199 supplemented with 10 % HS and not stimulated (control) or stimulated with 50 ng/ml FGF-2 in the presence of 0 or 5 μg/ml MAb H-2 (anti-u-PAR). After the incubation period, u-PA antigen levels were determined in the conditioned media by ELISA as described and expressed as ng u-PA/10^5 cells. Data represent mean ± S.D. of three independent experiments performed in duplicate wells.

immunofluorescence staining was observed in both normoxic and hypoxic cells (Figures 5A, B). Translocation of p65 was observed in several cells, probably due to endothelial cell activation caused by culturing on glass cover-slips. After stimulation with TNF-α in normoxic or hypoxic conditions for 8 hours, all p65 was translocated to the nucleus (Figures 5C, D). In normoxic conditions, stimulation with FGF-2 for 8 hours did not result in the translocation of p65 (Figure 5E). However, in hypoxia/FGF-2-stimulated cells a considerable part of p65 was translocated to the nucleus (Figure 5F). After 16 hours of incubation this translocation was no longer

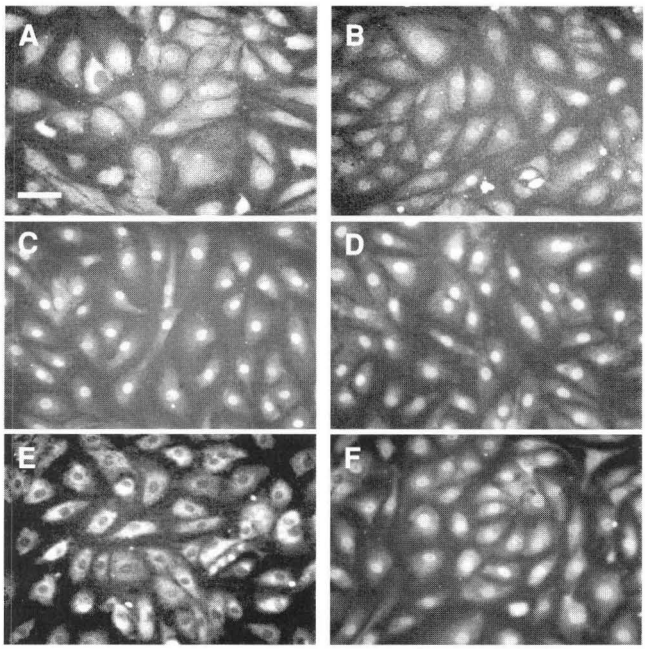

Figure 5: Hypoxia in combination with FGF-2 activates NF-κB subunit p65. HMVECs were cultured in M199 supplemented with 10% HS and 10% NBCS in normoxia (**A/C/E**) or hypoxia (**B/D/F**) and not stimulated (control) (**A/B**), stimulated with 10 ng/ml TNF-α (**C/D**) or 50 ng/ml FGF-2 (**E/F**). After 8 hours of incubation, hMVECs were fixed and immunofluorescence with antibodies against NF-κB subunit p65 was performed as described. Bar, 100 μm. Similar results were obtained in two independent experiments.

observed in hypoxia/FGF-2-stimulated cells, in contrast to TNF-α-stimulated hMVECs (data not shown).

Because nuclear translocation of NF-κB was found after 8 hours of hypoxia, we investigated whether the cellular content of IκBα was altered by hypoxia. Figure 6 shows that the amount of IκBα-antigen did not change at various times of exposure to hypoxia (1-16 hours), compared to normoxic exposure.

A sensitive reflection of endothelial activation is the production of monocytic chemoattractant protein (MCP)-1. Hypoxia did not significantly alter MCP-1 production in non-stimulated cells. However, hypoxia/FGF-2-stimulated hMVECs produced significantly more MCP-1 after an incubation period of 8 hours than their normoxic counterparts (2.0±0.9-fold, p=0.002, n=3 performed in duplicate wells) (Figure 7).

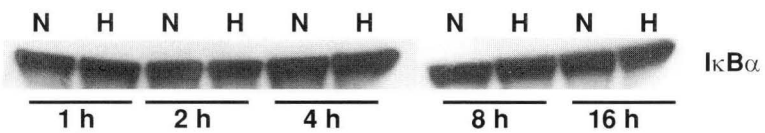

Figure 6: Hypoxia in combination with FGF-2 does not influence IκBα antigen expression in hMVECs. HMVECs were cultured in M199 supplemented with 10% HS and 10% NBCS and stimulated with 50 ng/ml FGF-2 in normoxic (N) and hypoxic (H) conditions. After 1, 2, 4, 8 and 16 hours of incubation cell lysates were prepared and IκBα antigen was determined by Western blotting as described. Similar results were obtained in two independent experiments.

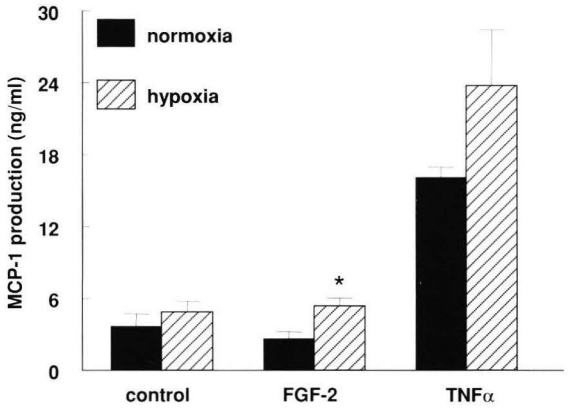

Figure 7: MCP-1 production is enhanced at hypoxic conditions. HMVECs were cultured in M199 supplemented with 10% HS and 10% NBCS in normoxia or hypoxia and not stimulated (control), stimulated with 50 ng/ml FGF-2 or 10 ng/ml TNF-α. MCP-1 production was determined by ELISA as described and expressed as mean ng MCP-1/ml ± S.D. of three independent experiments performed in duplicate wells. * significantly different from normoxic counterpart (p=0.002).

Hypoxia/TNF-α-stimulated cells also produced more MCP-1 than normoxia/TNF-α-stimulated cells, but this did not reach statistical significance (1.5±0.5-fold, p=0.066, n=3 performed in duplicate wells).

Hypoxia does not influence JNK 1/2 and P38 phosphorylation, but induces ERK 1 and ERK2 activation

Subsequently the effect of hypoxia/FGF-2-stimulation on the activity of members of the MAPK family (JNK 1/2, p38 and ERK 1/2) was evaluated. In non-stimulated or hMVECs stimulated for 4 hours with FGF-2, hardly any phosphorylated JNK1/2 and p38 were detected in normoxic and hypoxic conditions, whereas TNF-α induced phosphorylation of JNK1, JNK2 and p38 both in normoxic and hypoxic conditions (Figure 8). These data were confirmed by the observation that hypoxia/FGF-2-induced tube formation was not influenced by SB203580, a specific inhibitor of p38 MAPK (Figure 9).

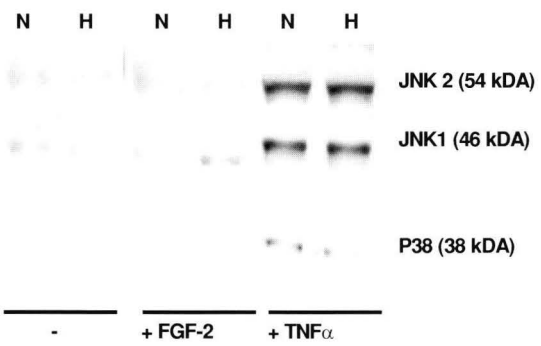

Figure 8: JNK1/2 and p38 are not induced by hypoxia. HMVECs were cultured in M199 supplemented with 10% HS and 10% NBCS and not stimulated (-), stimulated with 50 ng/ml FGF-2 or 10 ng/ml TNF-α in normoxic or hypoxic conditions. After 4 hours of incubation cell lysates were prepared and phosphorylated JNK1/2 and p38 was determined by Western blotting as described. Similar results were obtained in three independent experiments.

FGF-2+TNF-α-induced tube formation was partly blocked by SB203580 (58±8%, two independent experiments, data not shown). On the contrary, PD98059, a specific inhibitor of ERK1/2 phosphorylation, was able to inhibit hypoxia/FGF-2-induced tube formation completely (Figure 9).

In normoxic conditions, treatment of hMVEC with FGF-2 or TNF-α enhanced phosphorylation of ERK1 and ERK2 (Figure 10A), FGF-2 being a stronger stimulator than TNF-α. After 4 hours of hypoxia, phosphorylation of ERK1 as well as ERK 2 was increased as compared to normoxia, both under basal (176±21%, n=3) and FGF-2- stimulated (141±14%, n=3) or TNF-α-stimulated conditions (119±7%, n=3)(Figure 10A). No difference in phosporylation between ERK1 and 2 was detected. The same results were found with cells cultured on a gelatin or fibrin coating (data not shown). In addition, PD98059 was able to almost completely inhibit hypoxia/FGF-2-induced ERK 1/2 phosphorylation, while SB 203580 had no effect on ERK1/2 phosphorylation (Figure 10B).

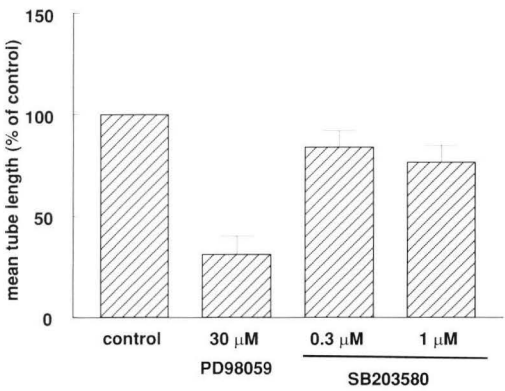

Figure 9: Inhibition of p38 activity does not inhibit FGF-2-induced capillary-like tube formation in hypoxic conditions. HMVECs were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS under hypoxic culture conditions and were stimulated with 50 ng/ml FGF-2 in the absence or presence of 30 μM PD98059 or in the absence or presence of SB203580 (0.3 or 1 μM). After 5 days of culture, mean tube length (mm/cm^2) was measured as described and data were expressed as mean ± S.D. of three independent experiments performed in duplicate wells.

Discussion

In normoxic culture conditions both a growth factor and the cytokine TNF-α are required for the formation of capillary-like structures by human microvascular endothelial cells (hMVECs) in a fibrin matrix [3;24]. In the current report we have shown that in hypoxic conditions, stimulation solely with the growth factor FGF-2 is sufficient to induce *in vitro* angiogenesis in a fibrin matrix. Hypoxia activates at least two signal transduction pathways in FGF-2-stimulated cells; the NF-κB pathway and the ERK1/2 pathway. Both pathways may be involved in the stimulating effect of hypoxia on tube formation.

Several studies have shown that the administration of angiogenic growth factors, such as VEGF and FGF-2, can result in an improvement of blood flow in ischaemic

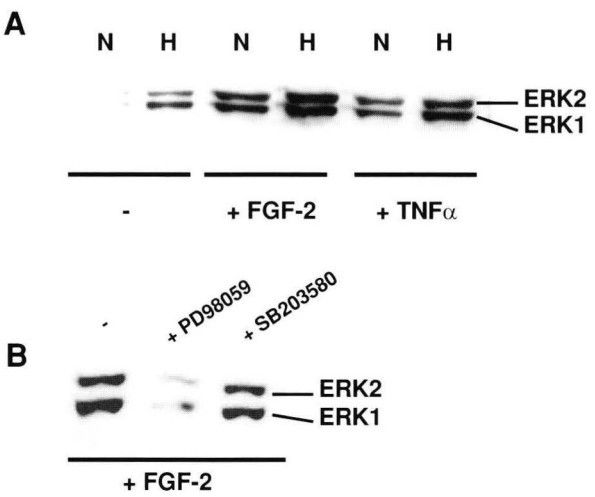

Figure 10: ERK1 and 2 phosphorylation is enhanced by hypoxia. HMVECs were cultured in M199 supplemented with 10% HS and 10% NBCS and (**A**) not stimulated (-), stimulated with 50 ng/ml FGF-2 or 10 ng/ml TNF-α in normoxic (N) or hypoxic (H) conditions or (**B**) stimulated with 50 ng/ml FGF-2 in the absence or presence of PD98059 (10 μM) or SB203580 (1 μM) in hypoxic conditions. After 4 hours of incubation cell lysates were prepared and phosphorylated ERK1/2 was determined by Western blotting as described. Similar results were obtained in three independent experiments.

limbs and myocardia [18-22]. Our observation that FGF-2 induces capillary-tubes in hypoxic conditions, but not in normoxic conditions agrees with these findings. In contrast, FGF-2 induced the formation of capillary-like structures in bovine microvascular endothelial cells under normoxic conditions [33] This induction was mediated by the induction of autocrine VEGF expression [34]. In human MVEC, FGF-2 only induced the formation of capillary-like structures if an additional factor such as TNF-α or hypoxia was present [3](this study). In this study, we demonstrated that under our experimental conditions the FGF-2/hypoxia-induced tube formation did not depend on the endogenous production of VEGF. This may be related to the fact that hMVECs do not produce VEGF (as quantified by RT-PCR; our own observations).

Several mechanisms may contribute to the hypoxia/FGF-2-enhanced formation of capillary-like structures. FGF-2 and hypoxia may affect matrix-degrading proteases [33;35;36]. Proteolytic enzymes may stimulate the invasion by stimulating controlled pericellular proteolysis, or prevent invasion of endothelial cells by excessive lysis of the fibrin matrix [37]. When hMVECs were stimulated with FGF-2 in the absence of TNF-α, the intact monolayer of hMVECs could not be sustained on a fibrin matrix, due to excessive fibrin degradation by t-PA-dependent plasmin activation [32]. However, although titration of anti-t-PA antibodies reduced the lysis in a concentration dependent way and restored monolayer integrity, no capillary-like tubular structures were formed [32]. Therefore, it is unlikely that the fibrin lysis per se is the only factor that prevents the ingrowth of endothelial tubes. Induction of cellular signaling pathways and additional factors by hypoxia apparently underlies

the hypoxia/FGF-2-stimulated tube formation.

Hypoxia/FGF-2-stimulated hMVECs had comparable overall production of u-PA, but expressed higher levels of u-PAR than their normoxic counterparts. Similar to previous observations in normoxic FGF-2/TNF-α-stimulated conditions, the hypoxia/FGF-2-induced tube formation requires cell-bound u-PA and plasmin activities [3;24]. Although FGF-2 affects the production of t-PA and PAI-1 [33;38], hypoxia did not alter the overall production of u-PA, t-PA and PAI-1 in FGF-2-stimulated cells, but significantly increased the relative uptake of u-PA by hypoxia/FGF-2-stimulated cells. It is unlikely that an accompanying moderate increase in u-PAR-dependent pericellular fibrinolysis in FGF-2-stimulated hMVECs is the major contributor to the induction of tubes by hypoxia. Alternatively, u-PAR may enforce cell attachment, in particular in relation to the vitronectin-binding integrins [39] and thus contribute to the maintenance of the cell monolayer on fibrin. In this context it is of interest to note that α_v-integrin is upregulated in endothelial cells by hypoxia [7;9].

Hypoxia has multiple effects on endothelial cells and multiple signal transduction pathways important for the process of angiogenesis can be triggered. Our data imply that hypoxia in combination with FGF-2 induces the activation of the subunit p65 of NF-κB and the activation of members of the MAPK superfamily. The transcription factor NF-κB regulates a number of genes involved in angiogenesis (such as TNF-α, IL-2, IL-6, VCAM-1 and u-PA) [14;15;40], which indicates that activation of NF-κB may be of importance in tube formation by hypoxia/FGF-2-stimulated hMVECs. Although NF-κB is involved in the regulation of the u-PA gene, u-PA expression in hMVECs was not enhanced by hypoxia. It has been described that u-PA expression is regulated by the Rel-A subunit of NF-κB [41], but also by C-Rel transcription factor [42;43]. The complexity of the regulation of the u-PA gene might explain why hypoxia per se is not sufficient to increase in u-PA expression in hMVECs.

Koong *et al.* [44] reported activation of NF-κB upon exposure to severe hypoxia (0.02% oxygen). This activation was accompanied by phosphorylation, dissociation and degradation of IκB [44;45]. Under our experimental conditions we did not detect a decrease in the cellular IκBα content, but this does not exclude a transiently reduced IκB activity at 1% oxygen tension. The low oxygen tension probably lowers the nitric oxide (NO) production in endothelial cells [46]. Because NO reduces NF-κB activation [47;48] by stabilizing IκB [49], a reduced NO production will facilitate NF-κB activation and its subsequent nuclear translocation.

Other signal transduction pathways are also affected by hypoxia [50], the members of the MAPK superfamily, p38, JNK1/2 and ERK1/2, have all been reported to be influenced by hypoxia [10-12]. In our experimental setting, p38 and JNK1/2 phosphorylation was only induced by TNF-α-stimulation and was not changed by

hypoxic conditions. In addition, SB203580, a specific inhibitor of p38, did not inhibit hypoxia/FGF-2-induced tube formation. These data indicate that p38 and JNK1/2 activity are not essential for hypoxia-induced tube formation by human MVEC in a fibrin matrix.

In contrast to p38 and JNK1/2, ERK1/2 phosphorylation in hMVECs was increased by hypoxia, as shown by other groups [51;52]. There are several possible ways in which ERK1 activation can lead to increased angiogenesis in hypoxic conditions. Tanaka *et al.* showed that PD98059, a specific inhibitor of ERK1/2 phosphorylation, can inhibit induction of ETS-1 mRNA, a prototype of ets family transcription factors [53]. ETS-1 converts endothelial cells to an invasive phenotype by inducing the expression of proteases including u-PA, MMP-1, -3 and -9 as well as β-3 integrin subunit as target genes in endothelial cells [54]. Recently, it has been shown by several groups that ERK activation by hypoxia is involved in the activation of the hypoxia-inducible factor (HIF)-1α [51;55] as well as the activation of HIF-2α [12]. HIF-1α regulates many genes, some of which are involved in the organization of vascular networks [56]. Active ERK1/2 induced a rapid phosphorylation of HIF-1α, whereas p38 and JNK were not capable of phosphorylating HIF-1α [55].

The activation of NF-κB and ERK1/2 is observed very soon after hypoxic incubation. A rapid and short-lasting activation of NF-kB by hypoxia has previously been observed [13;44] and MAPK activation can be found as early as 5 to 15 minutes after hypoxic stimulation [52]. Despite the quick and often short activation of these factors, their actions are described as important in such lengthy processes as angiogenesis [53;57]. Obviously, the activation of transcription factors like NF-κB and ERK1/2 are important for the initiation of the angiogenic response, for example by mediating the expression of factors, which can propagate the angiogenic response. These factors could be essential for the angiogenic potential of the endothelial cells themselves or could indirectly influence the angiogenic response in hypoxic conditions. For example, NF-κB regulates the expression of MCP-1 [58]. It has been suggested that MCP-1 is a chemokine that recruits leukocytes to sites of inflammation, neovascularization, and vascular injury. In hMVECs, MCP-1 production was stimulated by TNF-α, but also by exposure to hypoxia in combination with FGF-2. Hypoxic induction of MCP-1 expression could be of importance because of the attraction of inflammatory cells, which are capable of producing angiogenic factors in hypoxic conditions, and in this manner indirectly contribute to hypoxia-induced angiogenesis.

In conclusion, this study shows that hypoxia in combination with a growth factor induces at least two pathways in hMVECs that might be responsible for the induction of tube-like formation, via direct activation of the endothelial cells or via indirect mechanisms. Understanding the effect of hypoxia in combination with

growth factors on endothelial cells may be useful for improving the treatment of patients with chronic ischaemia by administering growth factors.

Acknowledgments

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6

COLLAGEN TYPE 1 RETARDS TUBE FORMATION BY HUMAN MICROVASCULAR ENDOTHELIAL CELLS IN A FIBRIN MATRIX

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Manuscript in preparation

Abstract

Angiogenesis, or the formation of new microvessels, is often encountered in pathological situations. A fibrinous exudate often acts as temporary matrix for these new microvessels. This matrix consist mainly of fibrin, but is mingled with other plasma components and collagen fibers. *In vitro,* capillary-like tube formation can be mimicked by exposing human microvascular endothelial cells (hMVECs), seeded on top of a three-dimensional fibrin matrix, to an angiogenic growth factor (e.g. fibroblast growth factor (FGF)-2) and the cytokine tumor necrosis factor (TNF)-α. Plasmin activity is required in this process. We investigated whether the angiogenic potential of hMVECs was dependent on the matrix composition. The addition of collagen to fibrin matrices dose-dependently inhibited tube-formation. Tube-formation in these fibrin/collagen matrices by hMVECs required MMP activity, as well as plasmin activity. On a pure collagen type I matrix, hMVECs were not able to form tube-like structures in the matrix but formed sprouts. This sprouting required on MMP activity and was, in contrast to the tube-like structures in a fibrin matrix, not influenced by hypoxia. In addition, expression of MMP-1, 3, 9 and TIMP-2 mRNA was not altered by the matrix composition or hypoxia. These data indicate that the interaction between endothelial cells and different matrix components is of importance for the angiogenic potential of these cells.

Introduction

In pathological conditions, the formation of new microvessels (angiogenesis) is an essential process. During the progress of angiogenesis, dynamic mutual interactions between endothelial cells and the extracellular matrix take place.

In vivo, the extracellular matrix (ECM) consists of a number of proteins, such as laminin, fibrin, collagen, vitronectin, fibronectin and hyaluoronic acid. The composition of the ECM can be different in various pathological settings, and can change in time in one particular pathological setting. It has been shown that fibrin clots persist in wounds up to 5 days after injury [1], whereas 7 day old wounds have a substantial organized collagen fiber network and little, if any, fibrin [2;3]. Furthermore, if a fibrinous exudate is formed interstitially, the fibrinous matrix is precipitated around already existing collagen fibers and other matrix proteins. As a consequence, ingrowing endothelial cells will encounter different extracellular matrix proteins. Cellular receptors for different matrix components can transduce signals into the cell and thereby alter the angiogenic response of capillary endothelial cells, e.g. by altering expression of proteases [4]. Different proteases are involved in the degradation of the ECM, depending on the types of matrix proteins present.

The invasion of endothelial cells into a matrix requires proteolytic activity. Plasmin is the major protease for fibrin degradation [5] while various types of matrix metalloproteases (MMPs) are involved in the degradation of collagens. Plasmin is secreted as the pro-enzym plasminogen and is very efficiently converted to plasmin by the tissue type plasminogen activator (t-PA) or the urokinase-plasminogen activator (u-PA), which is usually bound to its cell surface receptor (u-PAR) [6]. In fibrin matrices, cell-bound u-PA activity has been shown to be essential for the ingrowth of neovessels *in vitro* [7]. Besides its direct fibrinolytical activity, plasmin also plays a role in the activation of another class of proteases. The MMPs constitute a large family of zinc-dependent endoproteinases that exhibit substrate specificities for multiple extracellular matrix proteins [8]. Plasmin notably activates interstitial collagenase (MMP-1), stromelysin-1 (MMP-3) and gelatinase B (MMP-9) [9]. Synthetic MMP-inhibitors have been successfully used to inhibit blood vessel growth in animal models of growth factor-induced angiogenesis [10;11], indicating the involvement of MMPs in angiogenesis.

The expression of components of the plasminogen activator system and the MMPs are influenced by several factors. Growth factors, such as fibroblast growth factor (FGF)-2 and vascular endothelial growth factor (VEGF) and cytokines, like tumor necrosis factor (TNF)-α, can both influence the expression of u-PA, u-PAR and MMPs in endothelial cells [12-16]. In addition, environmental factors are of importance. Hypoxia, a strong inducer of angiogenesis, was shown to increase u-PAR expression

in endothelial cells and to enhance *in vitro* angiogenesis [17;18]. A consistent effect of hypoxia on the expression of MMPs, however, has not been reported [19-22].

The aim of this work was to determine whether the additional presence of collagen affected the formation of tubular-like structures by human microvascular endothelial cells in a fibrin matrix. Capillary-like tube formation was studied in three-dimensional fibrin, collagen and mixed fibrin/collagen matrices and the involvement of the plasmin/plasminogen activator- and the MMP-system was determined in these different experimental conditions.

Materials and Methods

Materials

Cell culture materials were purchased as described by Kroon *et al.* [7]. Human fibrinogen was obtained from Chromogenix AB (Mölndal, Sweden); Factor XIII was generously provided by Dr. H. Boeder and Dr. P. Kappus (Centeon Pharma GmbH, Marburg, Germany) and thrombin came from Organon Technika (Boxtel, The Netherlands). Rat tail collagen type I was prepared in our laboratory. FGF-2 was purchased from Pepro Tech EC (London, England), $VEGF_{165}$ was a kind gift of Dr. H. Weich (GFB, Braunschweig, Germany). Human recombinant TNF-α was a gift from Dr. J. Travernier (Biogent, Gent, Belgium) and contained 2.45×10^7 U/mg protein and less than 40 ng lipopolysaccharide per mg protein. Aprotinin was purchased from Pentapharm Ltd. (Basel, Switzerland). Rabbit polyclonal anti-u-PA antibodies were prepared in our laboratory [23] as well as the rabbit polyclonal anti-t-PA antibodies. The monoclonal u-PAR-blocking antibody H-2 was a kind gift from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany) [24]. BB-94 was a kind gift of Dr. R.V. Martin (Roche Bioscience, Pato Alto, CA, USA).

cDNA probes: The following cDNA fragments were used as probes in the hybridization experiments: full-length cDNA probes for human MMP-1 and rat MMP-3 were kindly supplied by Dr. P. Angel [25] and Dr. LM Matrisian [26], respectively. The MMP-9 and TIMP-2 probe were obtained by reverse transcriptase PCR, and a 1200 bp Pst I fragment of hamster actin cDNA was used [27].

Cell culture

Human foreskin microvascular endothelial cells (hMVECs) were isolated, cultured and characterized as previously described [28;29]. HMVECs were cultured on gelatin-coated dishes in M199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% human serum (HS), 10% heat -inactivated new born calf serum (NBCS), 150 μg/mL crude endothelial cell growth factor, 2 mmol/L L-glutamine, 5 U/mL heparin, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C under 5% CO_2/ 95% air atmosphere, unless mentioned otherwise. Experiments were performed with confluent cells (0.7×10^5 cells/cm^2), which had been cultured without growth factor for at least 24 hours.

Establishment of hypoxic culture conditions

For culturing in hypoxic conditions, hMVECs were placed in a NAPCO® incubator (serial number 7101-C1,Precision Scientific Inc, Chicago, USA) which controls the oxygen concentration by flushing with N_2. Oxygen levels in the incubator were monitored by an

internal oxygen sensor as well as by external calibration using Dräger Tubes 6728081 (Drägerwerk Ag, Lübeck, Germany). Hypoxic condition in our experiments was defined as culturing at 37°C under 1% O_2/5% CO_2 atmosphere.

In vitro angiogenesis model

Human fibrin matrices were prepared by the addition of 0.1 U/mL thrombin to a mixture of 2.5 U/mL factor XIII (final concentrations), 2 mg/mL fibrinogen, 2 mg/mL Na-citrate, 0.8 mg/mL NaCl and 3 µg/mL plasminogen in M199 medium (mixture pH 7.4). 300 µl aliquots of this mixture were added to the wells of 48-wells plates. After clotting at room temperature, the fibrin matrices were soaked with M199 supplemented with 10% (vol/vol) HS and 10% (vol/vol) NBCS for 2 hours at 37°C to inactivate the thrombin. For collagen matrices, 7 volumes of rat tail collagen type I (Type I collagen was solubilized by stirring adult rat tail tendons for 48 hours at 4°C in a sterile 1:1000 (vol/vol) acetic acid solution (300 mL for 1 g collagen). The resulting solution of 3 mg/mL collagen type I was extensively dialyzed against 1:10000 (vol/vol) acetic acid and stored at 4°C [30]) were mixed with 1 volume of 10xM199 and 2 volumes of 2% (wt/vol) Na_2CO_3 (mixture pH 7.4). 300 µl aliquots were added to each well and allowed to gelate at 37°C in the absence of CO_2. For fibrin/collagen-matrices, the fibrinogen/FXIII mixture was added to the collagen/10xM199/ Na_2CO_3-mixture in the ratio as indicated in the text. Finally, thrombin (0.1 U/mL) and aliquots of 300 µl were added to each well. After gelation at 37°C, the gells were soaked with M199 supplemented with 10% (vol/vol) HS and 10% (vol/vol) NBCS for 2 hours at 37°C to inactivate the thrombin.

Confluent endothelial cells (0.7 x 10^5 cells/cm^2) were detached and seeded in a 1.25:1 split ratio on the fibrin, collagen or fibrin/collagen matrices to form a highly confluent monolayer. After 24 hours culturing in M199 medium supplemented with 10% HS, 10% NBCS, and penicillin/streptomycin, the endothelial cells were stimulated with the mediators for the time indicated. At the end of the culture period the media were collected and the formation of tubular structures was analyzed by phase contrast microscopy and the total length of tube-like structures of six randomly chosen microscopic fields (7.3 mm^2/field) was measured using an Olympus CK2 microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software, and expressed as mm/cm^2.

For histochemistry, matrices were fixed in 2% (wt/vol) *p*-formaldehyde in phosphate buffered saline (PBS)(0.15 mol/L NaCl/10 mmol/L Na_2PO_4/1.5 mmol/L KH_2PO_4, pH 7.4) embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany), sectioned at 4 µm and stained with phloxin.

Substrate gel analysis

Gelatinolytic activities of secreted MMPs were analyzed by zymography on gelatin-containing polyacrylamide gels as described [31]. Using this technique both active and latent species can be visualized. Samples were made in 2% (wt/vol) SDS and 10% (vol/vol) glycerol and applied to 10 % (wt/vol) polyacrylamide gels co-polymerized with 0.2% (wt/vol) gelatin. After electrophoresis the gels were washed twice for 15 minutes in 50 mmol/L Tris/HCl, pH 8.0, containing 5 mmol/L $CaCl_2$, 1 µmol/L $ZnCl_2$ and 2.5% (wt/vol) Triton X-100 to remove the SDS, followed by two washes of 5 minutes in 50 mmol/L Tris/HCl, pH 8.0, containing 5 mmol/L $CaCl_2$, and incubated overnight at 37°C. The gels were stained with Coomassie Brilliant Blue R-250.

RNA Isolation and Northern Blots

HMVECs were cultured on a collagen or fibonectin coating. Total RNA was isolated as described by Chomczynski and Sacchi [32] and electrophoresed in a 1.2% (wt/vol) agarose gel under denaturing conditions using 1 mol/L formaldehyde. Northern blotting was performed as described by Lansink *et al.* [33].

Results

Addition of collagen type I to a fibrin matrix inhibits tube formation

To mimic the major components of an interstitial fibrinous exudate, we prepared three-dimensional matrices consisting of mixtures of fibrin and rat tail collagen type I. In matrices consisting of separate layers of collagen and fibrin, a haematoxiline von Giesson staining stained the fibrin layer more intense than the collagen layer (Figure 1). In mixed matrices the collagen and fibrin were mixed homogeneously and no distinction in staining was observed between fibrin and collagen.

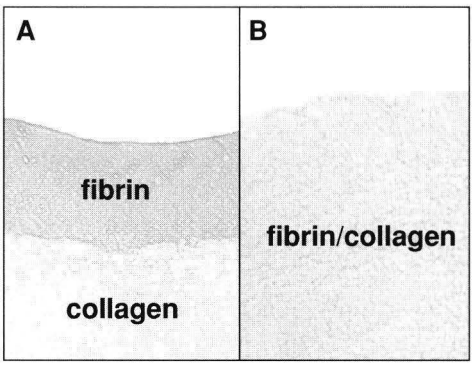

Figure 1: Fibrin and collagen mix homogeneously. (**A**) A layer of fibrin was clotted on top of a three-dimensional collagen matrix and stained with a haematoxiline von Giesson. The separate layers are clearly visible. (**B**) A fibrin/collagen matrix was prepared as described in Methods and stained with a haematoxiline von Giesson staining.

Capillary-like tube formation induced by FGF-2 and TNF-α in pure fibrin matrices was compared to that in matrices containing increasing amounts of collagen type I. A concentration dependent decrease in the extent of tube formation was seen when hMVECs were seeded on top of matrices containing 0-20% of 3 mg/mL collagen type I homogeneously mixed with fibrin. The addition of 5,10,15 and 20% collagen inhibited tube formation by 24±7%, 36±6%, 58±9% and 92±4% (n=3), respectively, as compared to tube formation in pure fibrin matrices (Figure 2A). The morphology of the tubes was not visibly altered (data not shown). The addition of 20% of the solvent of the collagen solution to the fibrin matrix did not inhibit tube formation (data not shown). Parallel to the inhibition of capillary-like tube formation by the addition of collagen to fibrin matrices, less fibrin degradation products (FDP) were found in the conditioned media of hMVECs grown on matrices containing fibrin as well as collagen (Figure 2B). In matrices containing 5,10,15 and 20% collagen, respectively 79,

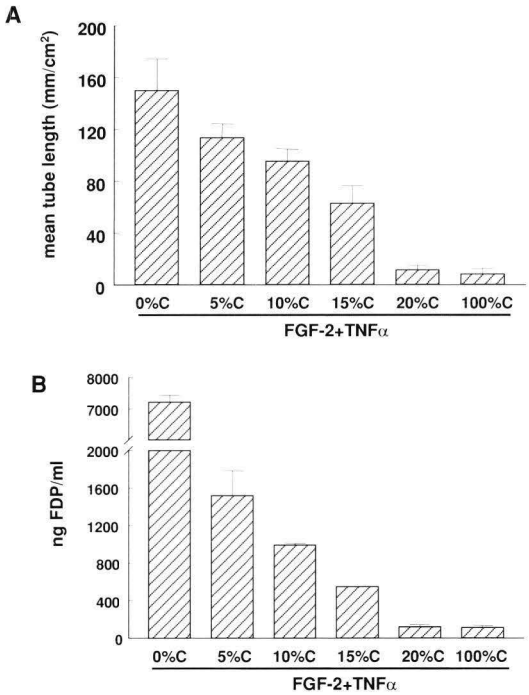

Figure 2: Addition of collagen to fibrin inhibits tube formation. (**A**) HMVECs were cultured on fibrin matrices containing respectively 0, 5, 10, 15 and 20% collagen (c) or on a 100% collagen matrix and stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α in M199 supplemented with 10 % HS and 10% NBCS. After 7 days of culturing, mean tube length was measured by image analysis as described and expressed as mean tube length/cm² ± SD of three independent experiments. (**B**) In addition, the conditioned media were collected and fibrin degradation products (FDP) were measured by ELISA as described and expressed as ng FDP/mL ± SD of three independent experiments.

86, 92 and 98% less FDP were measured.

Tube formation in a fibrin/collagen matrix depends on both u-PA and MMP activities

Tube formation in a fibrin matrix requires plasmin generation on the cell surface [7]. FGF-2/TNF-α-induced capillary-like tube formation in a fibrin matrix was inhibited by antibodies to u-PA or u-PAR and by the plasmin inhibitor aprotinin (85±1%, 91±4% and 77±2% respectively (n=3)) (Figure 3A). BB-94, an inhibitor of MMP activity, did not inhibit tube formation (Koolwijk *et al.*, submitted). The importance of these protease systems in tube formation in matrices consisting of 90% fibrin and 10% collagen was investigated. As shown in Figure 3B, FGF-2/TNF-α-induced capillary-like tube formation was partly inhibited by the addition of antibodies against u-PA (44±8% inhibition, n=3) and by aprotinin (39±14% inhibition, n=3). BB94 almost completely inhibited tube formation (85±7% inhibition, n=3). BB94 in combination with antibodies to u-PA inhibited tube formation to the same extent as BB94 alone (81±10% inhibition, n=3). In the conditioned media of FGF-2/TNF-α-stimulated cells the same amount of FDP were found as in the conditioned media of cells treated with FGF-2/TNF-α in combination with α-u-PA and BB94 (data not shown).

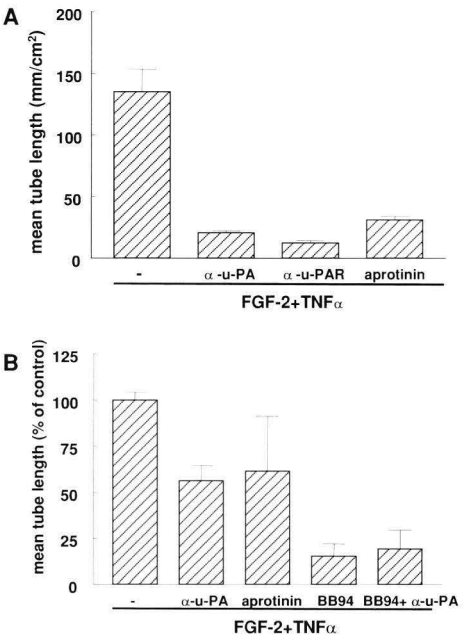

Figure 3: Tube formation in a fibrin/collagen matrix is dependent on both u-PA and MMP activity. HMVECs were cultured in M199 supplemented with 10% HS and 10% NBCS and stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α on top of a three-dimensional fibrin matrix (**A**) in the absence (-) or presence of polyclonal anti-u-PA (100 μg/mL), monoclonal anti u-PAR (Mab H-2; 5 μg/mL), aprotinin (200 U/mL) or on top of a three-dimensional fibrin matrix containing 10% collagen (**B**) in the absence (-) or presence of polyclonal anti-u-PA (100 μg/mL), aprotinin (200 U/mL), BB-94 (10 μg/mL) or a combination of BB-94 and anti-u-PA (BB-94+α-u-PA). After 7 days of culturing, mean tube length was measured by image analysis as described and expressed as mean tube length/cm² ± SD of three independent experiments (**A**) or expressed as % of control of three independent experiments (**B**).

Tube formation is not induced on a collagen matrix

As shown in the previous paragraphs, collagen has a profound effect on the angiogenic response of human endothelial cells. In addition to tube formation on matrices consisting of fibrin and collagen tube formation on pure collagen matrices was assayed. On a three-dimensional rat tail collagen matrix, hMVECs formed a less quiescent monolayer as compared to the monolayer on a fibrin matrix (compare Figs 4A, B). When hMVECs on a collagen matrix were stimulated for 7 days with FGF-2 or TNF-α alone, "sprouting" was observed (data not shown). After stimulation with FGF-2/TNF-α, considerably more sprouting was observed than after stimulation with FGF-2 or TNF-α alone (Figure 4D). In cross sections of FGF-2/TNF-α-stimulated cells on a collagen matrix, a few cells were found just beneath the monolayer (indicated with an arrow in Figure 4F). These structures consisted rarely of more than one cell and a lumen was only sporadically observed. This in contrast to the multicellular capillary-like structures formed in a fibrin matrix (Figure 4E). Sprouting of hMVECs on a collagen matrix was also induced by stimulation with VEGF (25 ng/mL) in combination with TNF-α (10 ng/mL) and after stimulation with phorbol myristate acetate (10 nmol/L) (data not shown).

Hypoxia, known to participate in the process of angiogenesis, highly stimulated FGF-2/TNF-α-induced capillary-like tube formation in a fibrin matrix compared to the normoxic situation (compare Figs 5A and B) [18]. Sprouting on a collagen matrix was, however, not significantly influenced by hypoxia (23±4 mm/cm² in normoxia vs 25±6 mm/cm² in hypoxia, n=2) (compare Figs 5C and D).

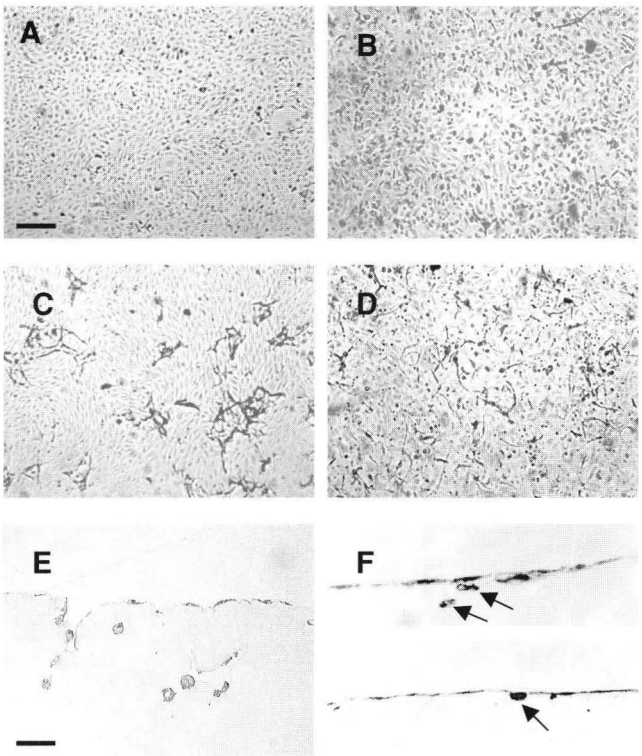

Figure 4: Tube formation in fibrin and collagen matrices. HMVECs were cultured on top of a three-dimensional fibrin matrix (**A/C/E**) or collagen matrix (**B/D/F**) in M199 supplemented with 10% HS and 10% NBCS and not stimulated (**A/B**) or stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α (**C/D**). After 7 days of culture, non-phase photomicrographs were taken. Bar, 300 μm. In addition, hMVECs on fibrin (**E**) and collagen (**F**) matrices were fixed and embedded as described, cross-sections perpendicular to the matrix surface were cut and stained with phloxin. Bar, 50 μm.

Similar results were found on matrices of bovine collagen type I (Vitrogen®, Cohesion Technologies Inc), although the amount of sprouting by hMVECs was considerably less than on rat tail collagen type I. In contrast to hMVECs, FGF-2/TNF-α-stimulated bovine arterial endothelial cells were able to form tubular structures in both matrices of rat tail collagen and bovine collagen type I (data not shown).

Sprouting on a collagen matrix requires MMP activity

In contrast to tube formation on a pure fibrin matrix, sprouting of cells on a collagen matrix was not significantly inhibited by antibodies directed to u-PA or by aprotinin (6±2%, 1±1% inhibition respectively, n=3)(Figure 6). However, BB-94 inhibited sprouting of hMVECs for 54±5% (n=3), a combination of BB94 and antibodies to u-PA caused an even stronger inhibition (78±3%, n=3). The total length of the sprouts formed on a collagen matrix was several times less than the total length of the tubes formed in the fibrin matrix.

Sprouting of endothelial cells on a collagen matrix seems to be mediated via MMP-activity. It was investigated if culturing of hMVECs on a collagen coating induces the expression of some MMPs, involved in collagen type I degradation. MMP-1 mRNA expression was not regulated by stimulation with FGF-2, TNF-α or FGF-2/TNF-α and

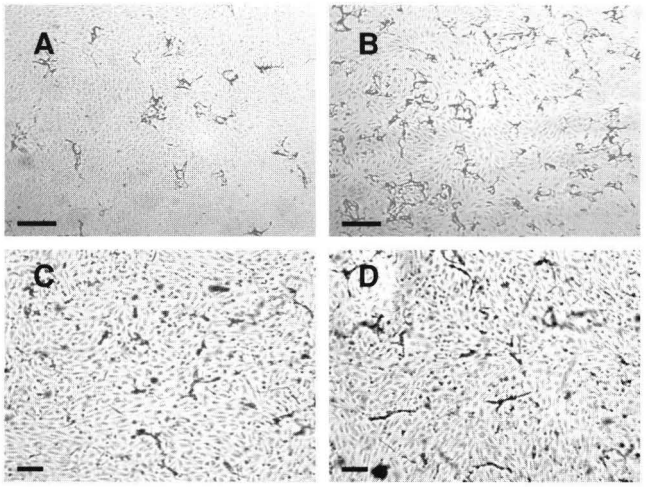

Figure 5: Effect of hypoxia on endothelial sprouting and tube formation. HMVECs were cultured on top of a three-dimensional fibrin matrix (**A/B**) or collagen matrix (**C/D**) in M199 supplemented with 10% HS and 10% NBCS and stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α in normoxic (**A/C**) or hypoxic (**B/D**) conditions. After 7 days of culture, non-phase photomicrographs were taken. Bar, 300 μm.

in addition no difference in expression was observed between hMVECs seeded confluently on a human fibronectin or rat tail collagen coating 24 hours prior to stimulation (Figure 7). HMVECs hardly expressed MMP-3 mRNA at control conditions and after stimulation with FGF-2 for 24 hours. Stimulation with TNF-α or FGF-2/TNF-α enhanced MMP-3 mRNA expression several times (Figure 7). No difference in MMP-3 expression was detected between hMVECs cultured on a fibronectin or collagen coating. MMP-9 mRNA was not detected in these cells after stimulation with FGF-2, TNF-α or FGF-2/TNF-α (data not shown). In addition to MMP-1 and MMP-3 mRNA expression, TIMP-2 mRNA expression was not influenced by the type of matrix (Figure 7).

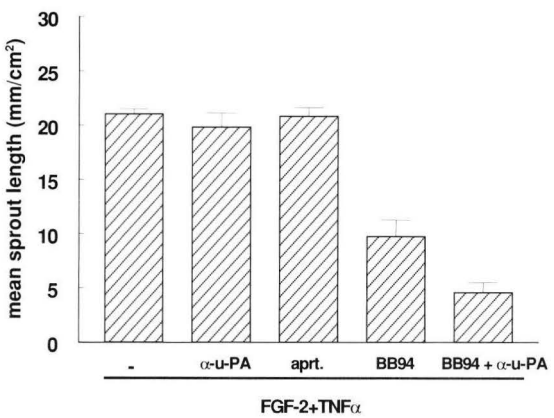

Figure 6: Sprouting of hMVECs on a collagen matrix is dependent on MMP activity. HMVECs were cultured on top of a three-dimensional collagen matrix in M199 supplemented with 10% HS and 10% NBCS and stimulated with 10 ng/mL FGF-2 and 10 ng/mL TNF-α in the absence (-) or presence of polyclonal anti-u-PA (100 μg/mL), aprotinin (aprt., 200 U/mL), BB-94 (10 μg/mL) or a combination of BB94 and anti-u-PA (BB94+α-u-PA). After 7 days of culturing, mean sprout length was measured by image analysis as described and expressed as mean sprout length/cm² ± SD of three independent experiments.

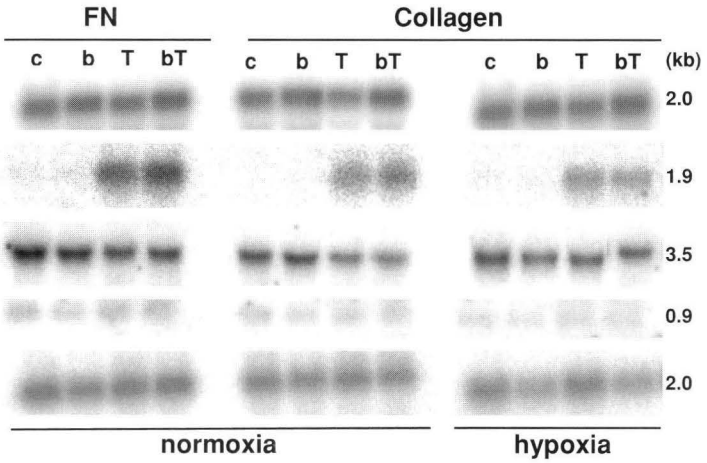

Figure 7: MMP expression by hMVECs on fibronectin and collagen coatings. HMVECs were cultured in M199 supplemented with 10% HS and were not stimulated (c) or stimulated with FGF-2 (10 ng/mL; b), TNF-α (10 ng/mL; T) or FGF-2 and TNF-α (10 ng/mL each; bT) on fibronectin (Fn) or collagen coatings in normoxic (A) or on collagen coating in hypoxic conditions (B). After 24 hours total RNA was isolated as described and analyzed by Northern blotting using [α-^{32}P]dCTP-labeled cDNA probes for MMP-1, MMP-3 and TIMP-2. Equal loading was verified by hybridization with actin cDNA. The same results were observed in two independent experiments.

Culturing for 24 hours in hypoxic conditions did not influence the expression of MMP-1, MMP-3 and TIMP-2 mRNA. This was observed both on a fibronectin (data not shown) and on a collagen coating (Figure 7). Gelatin zymography of the conditioned media of non-stimulated or FGF-2/TNF-α-stimulated hMVECs showed a band for latent MMP-2 (72 kDA) and a minor band of 55 kDA revealed the presence of latent MMP-1 and/or latent MMP-3 (Figure 8). Stimulation with the phorbol ester PMA induced MMP-9 expression in hMVECs and in addition the activation of MMP-2 (Figure 8). No difference in this pattern was observed between the conditioned media from cells cultured on a fibronectin and collagen coating. MMP expression, as revealed by gelatin zymography, of conditioned media of hypoxic cells did not differ from normoxic cells. This was observed both on a fibronectin (data not shown) and on a collagen coating (Figure 8).

The expression of MMP-1,3, 9 and TIMP-2 mRNA in hMVECs cultured on a fibrin/collagen matrix did not differ from expression in hMVECs cultured on a fibronectin or collagen coating (data not shown).

Discussion

Stimulation of human microvascular endothelial cells with FGF-2 and TNF-α results in the formation of capillary-like tubes in three-dimensional fibrin matrix. The

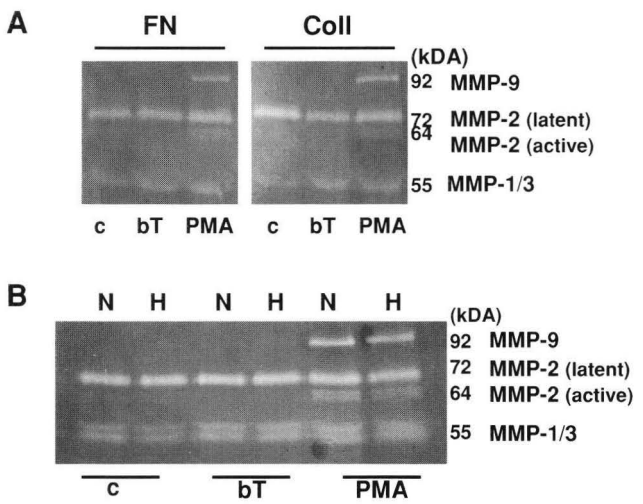

Figure 8: MMP expression by hMVECs on a fibronectin and collagen coating. HMVECs were cultured in M199 supplemented with 0.03 % human serum albumin and were not stimulated (c) or stimulated with FGF-2 and TNF-α (10 ng/mL each; bT) or PMA (10 nmol/L) on fibronectin (FN) or collagen (Coll) coatings in normoxic (**A**) or on collagen coatings in hypoxic conditions (**B**). After 48 hours the conditioned media were collected and substrate gel analysis was performed as described. The same results were observed in two independent experiments.

addition of collagen to fibrin matrices resulted in a strong inhibition of tube formation. Tube formation in these matrices depended both on the PA- and the MMP-system. When hMVECs were seeded on a matrix consisting of type I collagen, the induction of capillary-like tubes by FGF-2 and TNF-α was severely impaired. Multicellular structures were not formed as in fibrin matrices, but only sprouting of cells just beneath the collagen matrix occurred. This sprouting of endothelial cells was MMP-dependent.

In contrast to migration by hMVECs in fibrin matrices, in matrices consisting of both fibrin and collagen proteolysis by MMPs played a role in endothelial cell migration. The formation of tubes in these matrices was partly inhibited by addition of antibodies to u-PA, whereas BB-94 inhibited tube formation almost completely. These data indicate that hMVECs both display plasmin- as well as MMP-activity. Furthermore, it appears that PA-activity is not directly involved in matrix degradation but involved in the activation of MMPs. The co-existence of these two proteolytic systems in matrices containing fibrin is reported before. In pure fibrin matrices MMP activity is also present. However, due to the dominant role for plasmin in fibrin degradation, the PA system has first to be inhibited to detect MMP-mediated fibrin degradation. In plasminogen free conditions, MMPs can then act as pericellular fibrinolysins and induce neovascularization [34](Collen *et al.*, manuscript in preparation).

Addition of collagen type I to fibrin matrices resulted in a dose-dependent reduction in fibrin degradation and consequently in tube formation by hMVECs. *In*

vivo, both fibrin and collagen depositions can be often demonstrated in the same area. A transition of a fibrinous matrix to a more collagen-rich extracellular matrix has been described for skin wounds [1-3]. It is known that the formation of a fibrinous excudate is extremely favorable for the formation of neovessels [35;36]. The conversion from a matrix type, favorable for angiogenesis to a matrix, that is less angiogenic for endothelial cells, may force the endothelial cells to become quiescent and stimulate maturation of the vessel. This in order to avoid an excess of neovessels impairing, for example, the healing of a wound.

The mechanism of inhibition by collagen is not elucidated by this study. Several hypotheses can be posed. First, the fibrin structure could be altered by the addition of collagen. Fibrin structure can be altered by factors such as ionic strength and pH, and the concentrations of fibrinogen and thrombin [37-39]. In our experimental conditions, collagen/fibrin matrices were always compared to pure fibrin matrices to which the solvent of the collagen was added in the same percentage as in the collagen/fibrin matrices. By this way, a change in fibrin structure by a difference in ionic strength or pH can be neglected. Still it is possible that the collagen fibers by itselves somehow change the fibrin structure in such a way that it is less susceptible for degradation.

Secondly, it is known that ECM components can transduce signals into the cell. Both fibrin as collagen have been shown to have stimulatory effects on the angiogenic phenotype of endothelial cells [4;40]. However, hMVECs cultured on collagen did not enhance MMP-expression as compared to cells cultured on fibronectin. Langholz *et al.* [4] described a change in MMP-expression pattern when cells were cultured on collagen matrices. It is possible that hMVECs, used in our study, do not express collagen receptors, such as $\alpha_1\beta_1$- and $\alpha_2\beta_1$-integrins, to transduce stimulatory signals for matrix degradation.

Third, it is known that degradation products of collagen type XVIII and collagen type IV [41-43] can inhibit angiogenesis. The isolation of the collagen type I, used in this study, may contain tiny amounts of these products, which may contribute to angiogenesis inhibition.

In line with the inhibitory action of collagen on tube formation, hMVECs were not able to migrate deep into a pure collagen matrix but migrated on top of the matrix. This so-called sprouting was not dependent on plasmin activity. BB-94, a general inhibitor of MMPs, inhibited sprouting, indicating that this process is MMP-dependent. The fact that inhibition of the PA-system did not influence sprouting, points to a role of MMPs which can be activated in the absence of plasmin. Fully developed tubes were not formed in a collagen matrix. This may be explained by the absence of sufficient MMP-activity in FGF-2/TNF-α-stimulated cells. Although these cells express several MMP-types important in collagen degradation [44] (this study),

it is possible that these are mainly present in a latent form or complexed with TIMPs. In addition, the stimulation with FGF-2 and TNF-α might not induce the essential MMPs for the degradation of this type of collagen matrix. Bovine endothelial cells have been shown to form extensive tubes in a collagen matrix, dependent on MMP-activity [45]. This difference between human and bovine endothelial cells is probably due to their different expression of proteolytic enzymes. Hotary *et al.* [14] recently showed that invasion by an epithelial cell line in a collagen type I matrix depends on MT-MMP expression. MT1-MMP can activate proMMP-2 and since hMVECs have been shown to be poor activators of MMP-2 [44], is suggested that these cells express little MT1-MMP and therefore do not form fully developed capillary-like structures in a collagen matrix.

Hypoxia is one of the strongest inducers of angiogenesis. In response to hypoxia, endothelial cells gain a more angiogenic phenotype [18]. For example, the expression of u-PAR is enhanced in hypoxic endothelial cells [17;18], resulting in an increased tube formation in fibrin matrices [18]. Sprouting of hMVECs on collagen matrices was not enhanced in hypoxic conditions. In accordance, expression of the mRNA of MMP-1,3 and 9 as well as TIMP-2 by hMVECs was not influenced in hypoxia. The effects of hypoxia on MMP expression are not conclusive and differ among MMP species and different cell types. A downregulation of MMP-2 and MMP-3 has been reported in hypoxia epithelial cells and fibroblasts, whereas TIMP-1 expression was increased in epithelial cells [21;22]. On the other hand, recently MMP-13 was identified as a hypoxic-inducible gene in carcinoma cell lines [20].

This study shows that the composition of the matrix has implications for the angiogenic phenotype of human microvascular cells. Depending on the matrix components, the PA- and/or the MMP-system is active in matrix degradation. It seems, however, that FGF-2/TNF-α-stimulated hMVECs, cultured on a pure collagen matrix, do not display enough proteolytic capacity to develop mature vessels. By using matrices consisting of both collagen and fibrin, the *in vivo* situation was more approached. These data gain more insight in the role the different proteolytic systems in the modulation of a fibrinous exudate by endothelial cells.

Acknowledgments

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7

GENERAL DISCUSSION

General discussion

The major findings of the studies in this thesis are that angiogenesis in a fibrin matrix can only occur when the proteolytic activity, essential for matrix degradation, is localized to the cell surface. Especially cells situated in the tips of the newly formed capillary sprouts exhibit cell bound proteolytic activity. Furthermore hypoxia, a known feature of numerous pathological conditions, directly influences the angiogenic capacity of human microvascular cells by the activation of signal transduction pathways which, among other effects, lead to an enhanced proteolytic capacity.

By the use of matrices of different composition, it was found that the components of the matrix are a profound factor in determining which proteolytic systems are involved in matrix degradation during angiogenesis.

These results in this thesis are based on *in vitro* studies. Therefore it is of importance to discuss these findings in a broader context to understand the relevance of these findings for the *in vivo* situation.

Importance of u-PAR-localized proteolytic activity

In order to form neovessels endothelial cells have to invade the extracellular matrix, which depends on the degradation of this matrix. By expressing proteolytic enzymes, endothelial cells are able to degrade the matrix and create a way through the extracellular matrix along which migration is possible [1;2]. In conditions in which neovascularization occurs in the adult, frequently a fibrinous exudate is formed which facilitates the angiogenic process [3]. In fibrin matrices degradation is mediated by u-PA-dependent plasmin formation. In vitro, capillary-like tube formation in fibrin matrices only occurred when u-PA activity was localized to the u-PAR on the cell surface. After inhibition of active u-PA by PAI-1, the u-PA:PAI-1 complex is internalized and in this way u-PA is cleared from th extracellular space. Consequently, blocking the u-PAR resulted in u-PA accumulation in the pericellular space, providing sufficient plasmin activity for fibrin degradation. However, controlled proteolysis, needed for the forming of capillaries, was not achieved in this way. These findings are in line with the observation that excessive fibrin lysis, due to the lack of inhibitors or overexpression of proteolytic enzymes, does not result in the formation of capillary-like tubular structures [4-6]. Tube formation is only possible when controlled localized fibrin lysis occurs (this thesis). The presence of the u-PAR and plasminogen receptor [7;8] on the cell surface provides the cell with a tool for localized proteolytic activity. In addition to localization of the proteolytic activity, the u-PAR is able to regulate the amount of activity on the cell-surface by internalizing

inactive u-PA:PAI-1 complexes [9]. An upregulation of u-PAR by for example angiogenic factors or hypoxia results in an increase in binding sides for active u-PA, resulting in an enhanced plasmin formation (this thesis) [10-12].

Not all endothelial cells in a capillary sprout express the same gene expression pattern [13;14]. It was clear that the u-PAR was expressed more prominent by those cells localized at the tips of newly forming sprouts (this thesis). In this way proteolysis is localized at those places were matrix degradation is most needed for the development of the sprout. An interesting question is the origin of this difference in gene expression in endothelial cells. Besides the possibility of different activation by environmental factors due to their position in the new sprout, the occurrence of subpopulations of circulating endothelial cells, which are able to initiate angiogenesis, has been suggested [15]. The latter suggestion would mean that in the isolation of microvascular endothelial cells used in this study different subtypes of endothelial cells expressing different gene patterns should be present.

Results from the studies in this thesis indicate an essential role for localized u-PA activity in the process of angiogenesis. In accordance to these results [16-18], tumor angiogenesis *in vivo* can be inhibited by the administration of u-PAR antagonists. However, in contrast to these findings, mice deficient for u-PAR do not display an impairment in neovascularization after myocardial infarction [19], and in addition, even plasminogen deficient mice display no defects in angiogenesis [20]. These data suggest that in neovascularization of the myocardium other proteinase systems, as the MMP system, are involved in matrix degradation. In addition, it is very likely that a compensatory mechanism develops for plasmin dependent matrix degradation in animals deficient for u-PA, u-PAR or plasmin.

It is evident from recent literature that besides the PA-system the MMP-system is also able to provide controlled proteolyis to allow angiogenesis [21]. In our *in vitro* studies inhibition of MMP activity was sufficient to inhibit sprouting by human microvascular endothelial cells (hMVECs) in three-dimensional collagen matrices, and in addition to inhibit capillary-like tube formation in matrices composed of both fibrin and collagen. In contrast to in pure collagen matrices, in matrices consisting of both fibrin and collagen u-PA-activity was involved in tube formation. These data indicate that both the PA- as the MMP-system can co-operate in the process of angiogenesis. Tube formation in pure fibrin matrices seems to depend solely on degradation of the extracellular matrix by plasmin. However, in plasminogen free conditions MMP-mediated fibrin degradation is demonstrated in fibrin matrices and MMPs can then act as pericellular fibrinolysins and induce neovascularization [22].

By using matrices composed of more than one matrix component we showed that different proteolytic systems could co-operate in inducing angiogenesis. A temporary matrix in which angiogenesis occurs *in vivo* contains several different components. It

is very likely that in these physiological matrices both the PA-system and the MMP-system provide controlled matrix degradation in the process of angiogenesis. Only by understanding the molecular interplay between these two enzyme cascades we will be able to develop strategies to stimulate or inhibit angiogenesis without interfering with the integrity of the organism.

Matrix receptors

Besides the ability to remodel and degrade the extracellular matrix, the ability to make new contact-sites to the matrix is indispensable for the invasion of endothelial cells. Integrins are matrix-receptors, which are of importance for the adherence and migration of endothelial cells [23-25] Especially $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins play important roles in *in vivo* angiogenesis [26;27] not only by ligation of the endothelial cells to the surrounding matrix but also by activating signalling pathways that are involved in cell survival [28]. The inhibition of the $\alpha_v\beta_3$- and $\alpha_v\beta_5$-interactions with the extracellular matrix leads in several conditions to the inhibition of angiogenesis *in vivo* [23;27]. In endothelial cells *in vitro*, the expression of the α_v-subunit is enhanced in response to hypoxia (this thesis), and the induction of the β_3- and β_5-subunit has also been reported [29;30]. A higher expression of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins in hypoxia may promote hypoxia-induced angiogenesis by increasing matrix interactions and cell survival. However, in our model, using fibrin matrices, the $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins appeared not essential to normoxia- as well as hypoxia-induced tube formation. On fibrin matrices, which are expected also to contain serum-derived adhesive molecules such as fibronectin and vitronectin, other integrins or other types of matrix receptors that interact with the matrix components may be of more importance [31-33]. Recently, the $\alpha_5\beta_1$-integrin, that binds fibronectin and fibrinogen, has been indicated to control, together with the $\alpha_v\beta_3$-integrin, vacuolation and lumen formation by endothelial cells [34]. In additional studies, in which $\alpha_5\beta_1$-integrin is blocked, it should be investigated whether $\alpha_v\beta_3$-integrin indeed plays an additional role to $\alpha_5\beta_1$-integrin in the formation of capillary-like tubular structures in fibrin.

The discrepancy between the *in vivo* and *in vitro* situation, regarding the importance of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins, may be explained by the fact that a fibrinous exudate *in vivo* contains more diverse plasma proteins than *in vitro*. This difference in constitution may influence the kind of receptors endothelial cells use to adhere to the matrix.

Hypoxia-driven angiogenesis

As shown in this thesis and other studies [35-37], hypoxia is a strong stimulator of angiogenesis. In an *in vivo* situation, different cell types are present in the surrounding of the endothelial cells and secrete angiogenic growth factors in response to hypoxia [38-40]. Especially the angiogenic growth factor vascular endothelial growth factor (VEGF) is hypoxia-inducible and plays an important role in the mechanism of hypoxia-induced angiogenesis [36;41]. It is generally believed that monocytes are the prime cells that respond to hypoxia. In the studies of this thesis we find that also endothelial cells by themselves respond to hypoxia. Hypoxia did not induce capillary-like tube formation in non-stimulated human endothelial cells, but enhanced tube formation by cells, which were stimulated with one or more angiogenic factors. Hypoxia-induced VEGF expression has been reported in endothelial cells [42]. However, the human microvascular endothelial cells used in our studies showed an increased angiogenic response to hypoxia in the absence of autocrine VEGF production. It must therefore be concluded that, in addition to paracrine effects of hypoxia involving induction of growth factors from bystander cells, endothelial cells themselves are able to increase their angiogenic potential in response to hypoxia by a mechanism which does not involve autocrine production of VEGF.

Hypoxia has multiple effects on endothelial cells, ranging from effects on glycolysis to the expression of adhesion molecules [29;43-45] and components of the PA system [46-48]. The human microvascular endothelial cells used in the studies described in this thesis reacted to hypoxia by enhancing their u-PAR expression. This was shown to result in an increased plasmin activity at the cell surface. Consequently, capillary tube formation in fibrin matrices was enhanced. As mentioned before, hypoxia has numerous effects on endothelial cells. Therefore it is believed that the increase in plasmin activity was not the only factor involved in inducing tube formation in hypoxia.

The importance of matrix receptors has been described in the previous paragraph. Although hypoxia induced the α_v-integrin expression under our experimental conditions, inhibition of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins per se did not influence hypoxia-induced tube formation. If a combined interaction of $\alpha_v\beta_3$-integrin and other integrins , e.g. $\alpha_5\beta_1$-integrin, occured with fibrin, the effect of hypoxia on $\alpha_v\beta_3$-integrin-expression are apparently not rate limiting. It can not be excluded, however, that other adhesion molecules, not identified in our studies, are regulated by hypoxia and are of importance in hypoxia-induced angiogenesis in a fibrin rich environment.
Hypoxia did not enhance the angiogenic capacity of endothelial cells on a pure collagen matrix. Furthermore, hypoxia did not influence on the expression of some of

the MMPs involved in angiogenesis as is also reported by other groups [33;49;50]. It is therefore plausible that the mechanism by which hypoxia enhances the angiogenic capacity of human endothelial cells does not involve the MMP system.

From the result of the studies in this thesis, it appears that the induction of the PA-system in hypoxia plays a major role in hypoxia-induced tube formation in fibrin by human microvascular endothelial cells. The possibility that other proteolytic enzymes or angiogenic factors, till now unknown to be involved in agiogenesis, are influenced by hypoxia can not be excluded.

Mechanism of oxygen sensing

The mechanism by which cells are able to sense oxygen levels and transduce signals in response to this level has long been unclear. However, in this research field, great progress has been made the last decade. Although the exact nature of the oxygen sensor remains elusive, several signal transduction pathways are shown to be influenced by hypoxia.

In human microvascular endothelial cells, stimulated with angiogenic growth factors, at least two signal pathways, the nuclear factor-kappa B (NF-κB) and the mitogen activated protein kinase (MAPK) pathway were influenced by hypoxia. NF-κB was activated in hypoxic conditions, whereas ERK1 and 2, two members of the MAPK family, were induced in hypoxia. Besides these pathways, other groups have also described the activation by hypoxia of the stress activated protein kinases p38 and JNK in several cell types, but not in endothelial cells [51-55]. The finding that the stress activated protein kinases are not activated in endothelial cells (this thesis)[48] in hypoxia might indicate that endothelial cells do not experience hypoxia as stressful but as an activating physiological stimulus.

Both the activities of NF-κB and ERK 1/2 (members of the MAPK family) are associated with angiogenesis. The transcription factor NF-κB regulates for example TNFα, IL-2, IL-6, VCAM-1 and u-PA expression [56-58]. Tanaka et al. showed that ERK1/2 phosphorylation led to induction of the transcriptionfactor ETS-1 [59]. ETS-1 converts endothelial cells to an invasive phenotype by inducing the expression of proteases including u-PA, MMP-1, -3 and –9 as well as the β-3 integrin subunit [60]. The induction of these genes in hypoxia may contribute to hypoxia-induced angiogenesis.

Recently, the mitogen activated protein kinases (MAPK) pathway has been linked to the activity of the hypoxia-inducible transcription factor HIF [61;62]. The recognition of HIF explained the mechanism of induction by hypoxia of several proteins, including erothrypoietin and VEGF [63]. In the 5'-flanking region of the u-PAR gene three potential HIF-1 binding sequences are found [47], although a direct

link between hypoxic induction of u-PAR and HIF activity has not been reported till now.

From these data it can be hypothesized that in hypoxic human microvascular endothelial cells pro-angiogenic factors, as the u-PAR, are induced by hypoxia-inducible transcription factors, HIF-1 or 2, which are regulated by hypoxia inducible signal transduction pathways involving, among others, NF-κB and MAPK activities.

Perspectives for therapeutic applications

Hypoxia-driven angiogenesis is an adaptive response to a disturbance in the maintenance of oxygen supply. This disturbance can be caused by different reasons: atherosclerosis [64], occlusion of blood vessels and tissue damage (wound healing) [35]. The clinical significance of hypoxia-induced angiogenesis in ischemic disorders of the cardiovascular system is profound. For example, coronary artery collateral blood vessels created by the process of angiogenesis in response to progressive coronary artery stenosis and myocardial ischemia are the major determinants of the extent of necrosis after coronary artery occlusion [65;66]. Also in the context of peripheral vascular disease promotion of collateral vessel growth has been demonstrated to be feasible and therapeutic in animals [67-69] and humans [70-72].

Although angiogenesis is the physiological adaptation of a tissue to hypoxia, it seems that the compensatory response *in vivo* sometimes becomes insufficient. In situations like chronic limb ischemia or myocardial ischemia, the hypoxic stress is chronic and persistent, lasting for days or even months. In these situations hypoxic induction of angiogenic growth factors is markedly blunted [73]. Furthermore, advanced age results in endothelial dysfunction leading to a defect in VEGF expression [74;75]. Administration of growth factors becomes, therefore, essential for the induction of therapeutic angiogenesis. The administration of growth factors can be achieved by several different approaches. One approach is to administer VEGF in a fibrin plug to, for example, ischemic leg ulcers [76;77]. As shown in this thesis, the presence of fibrin creates a favorable environment for angiogenesis and the presence of administered growth factors and the hypoxic surrounding create an optimal setting for angiogenesis. It is hypothesized that this optimal setting is then, at least in part, achieved by the direct activation of the endothelial cells by the combination of angiogenic growth factors, fibrin and hypoxia.

It can be concluded that growth factor-induced angiogenesis in hypoxic conditions is a research field of relevance for therapeutical interference in peripheral vessel disease. Understanding the interactions between growth factors, extracellular matrix and hypoxic endothelial cells may provide the knowledge to improve treatment of peripheral vessel disease.

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8

SUMMARY

Summary

In **Chapter 1**, the process of angiogenesis is introduced. Angiogenesis is under regulation of numerous factors. Factors which have both a negative as a positive influence on this process. FGF-2 and VEGF are examples of angiogenic growth factors that can highly stimulate angiogenesis. In addition, environmental factors are described which have impact on angiogenesis. The surrounding oxygen tension and influence of matrix composition are highlighted. The angiogenic capacity of endothelial cells depends for a large part on the ability to degrade and remodel the extracellular matrix. The two most important proteolytic enzymes in angiogenesis are introduced, the PA system and the MMP system. Angiogenic growth factors and environmental factors influence both these systems.
In ischeamic diseases it is favorable to stimulate the formation of neovessels by administering growth factors like VEGF and FGF-2. In this thesis, special attention is given to the mechanism by which growth factors in normoxic and hypoxic conditions influence the angiogenic potential of endothelial cells. To this purpose an *in vitro* system was used consisting of a three-dimensional fibrin matrices on which human microvascular endothelial cells (hMVECs) were seeded. After stimulation with both a growth factor and a cytokine, capillary-like tubules were formed in these matrices.

In **Chapter 2**, the importance of cell-bound u-PA activity was assayed. It became clear that growth factor/cytokine-induced tube formation was inhibited by the inhibition of u-PA or plasmin, but also by blocking the u-PAR. This means that although an excess of u-PA is present around the cells, the u-PAR is essential in localizing the u-PA activity that makes controlled lysis of the matrix possible. The inhibition of tube formation by a blocking monoclonal antibody to u-PAR was not due to inhibition of the signal transduction pathways evoked by association of u-PA to the u-PAR, and also not by blocking the matrix-interaction function of u-PAR. Furthermore, it was shown that u-PAR and u-PA were localized primarily on the invading, tube-forming hMVECs and to a lesser extent on the quiescent endothelial cells located on top of the fibrin matrix. u-PA and u-PAR were also prominently expressed in neovessels in an atherosclerotic plaque. These findings indicate the importance of the u-PA/u-PAR system in repair-associated angiogenesis.
FGF-2 and VEGF both induce capillary-like structures in the *in vitro* model. The upregulation of u-PAR by these factors is an important determinant in the formation of capillary-like tubes. In **Chapter 3**, the mechanism of regulation of the u-PAR expression by FGF-2 and VEGF was studied. We focussed on the involvement of the protein kinase C (PKC). By using selective PKC inhibitors it became clear that VEGF enhances u-PAR antigen expression via a signal transduction pathway involving

PKC, whereas FGF-2 acts via a PKC-independent pathway. PKC inhibitors were not able to inhibit the mitogenic effect of VEGF and FGF-2. Tyrphostin A47, an inhibitor of tyrosine kinase, did inhibit FGF-2- and VEGF-induced proliferation whereas tyrphostin A47 did not influence u-PAR regulation. These data indicate that the effects of these growth factors on mitogenesis and regulation of u-PAR expression act via different pathways. Since regulation of u-PAR might be a tool for controlling angiogenesis the knowledge about the regulation of this receptor is of importance.

In **Chapter 4**, the influence of hypoxia on growth factor/cytokine-induced tube formation was studied. Hypoxia was shown to be a strong stimulator of the capillary-like tube formation in fibrin matrices. After 3 days of culture, 2 to 3 times more tubes were formed by hMVECs stimulated with FGF-2 or VEGF in combination with TNFα. The increase in tube formation was accompanied by a decrease in u-PA accumulation in the conditioned medium, which was found to be caused by the upregulation of u-PAR. The enhancement of u-PAR expression and consequently increased plasmin formation at the cell surface contributed to the increased tube formation in hypoxia. A possible contribution of hypoxia-induced VEGF production was unlikely, since no VEGF mRNA or antigen was found in the human endothelial cells. Furthermore, the possible role of integrins in hypoxia-induced tube formation was considered. Although the α_v-integrin was enhanced in hypoxia, blocking antibodies to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ did not inhibit tube formation in normoxia as well as in hypoxia. Probably other matrix-receptors then $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are used to adhere to a pure fibrin matrix.

The effect of hypoxia on angiogenesis is obvious but the mechanism by which hypoxia exerts these effects is not elucidated yet. In **Chapter 5**, the signal transduction pathways, which might be of importance for angiogenesis and are activated by hypoxia were studied. In normoxia, hMVECs only form tubes when stimulated with a growth factor and a cytokine. However, in hypoxic conditions the stimulation with FGF-2 is enough to induce tube formation. Hypoxia obviously had a cytokine-like effect on endothelial cells. Hypoxia/FGF-2 hMVECs displayed a higher expression of u-PAR and a higher u-PA turnover. However, the increase was quit small, so the effects of hypoxia cannot fully be explained by this small increase in proteolytic activity. Hypoxia activated at least two signal pathways, which may contribute to the enhanced angiogenic response. In contrast to the normoxic situation, the subunit of NF-κB p65 was translocated to the nucleus upon stimulation with FGF-2. Indicating that NF-κB may be involved in the induction of angiogenic factors. In addition hypoxia stimulated the activation of the mitogen-activated protein kinases ERK1/2. It was concluded that hypoxia activates different signal transduction pathways in FGF-2-stimulated human endothelial cells, which may be involved in hypoxia-induced angiogenesis.

In **Chapter 6**, the effects of another environmental factor, i.e. the matrix composition, on tube formation by hMVECs was studied. In pathological conditions, associated with angiogenesis, often a fibrinous exudate is formed which facilitates the ingrowth of neovessels. This temporary repair matrix consists mainly of fibrin but is mixed with other plasma proteins and collagen fibers. The formation of tubes on a pure collagen type I matrices and matrices consisting of mixtures of collagen and fibrin was compared to tube formation in pure fibrin. On collagen matrices hMVECs did not form tubes but migrated on top of the matrix, so called sprouting. This sprouting was dependent on the MMP-system in contrast to tube formation in a fibrin matrix, which is dependent on the PA system. In hypoxia this sprouting was not enhanced. In addition the expression of MMP-1, 3 and 9 mRNA expression was not altered by the matrix composition and also not by culturing in hypoxia. On matrices consisting of fibrin/collagen mature tubes were formed and the formation of these depends on both the PA- as MMP-system. The interaction between endothelial cells and the extracellular matrix is clearly of importance for their angiogenic capacity.

In **Chapter 7**, the results of the studies are summarized and discussed in a broader perspective.

Nederlandse samenvatting

Het onderzoek in dit proefschrift heeft zich gericht op de vorming van nieuwe capillairen (kleine bloedvaten) uit een bestaand bloedvat, een proces dat angiogenese wordt genoemd. Nieuwe bloedvaten worden alleen onder bepaalde omstandigheden gevormd in het lichaam van een volwassen persoon. Bij het helen van een wond is het bijvoorbeeld noodzakelijk dat, door middel van angiogenese, beschadigde bloedvaten worden vervangen en zo het herstellende weefsel van voedingsstoffen en zuurstof te voorzien. Maar ook tumoren gebruiken nieuw gevormde bloedvaten om te groeien en om uit te zaaien. Angiogenese treedt niet spontaan op, het is noodzakelijk dat het bestaande bloedvat geprikkeld wordt door het omliggende weefsel of tumor. Deze prikkels worden angiogene factoren genoemd. Angiogene factoren kunnen variëren van groeifactoren, ontstekingsmediatoren, tot de zuurstofspanning en de compositie van de omringende matrix.

In gebieden waar de zuurstofspanning in het weefsel daalt door eventuele schade aan de bestaande bloedvaten, wordt angiogenese gestimuleerd. Het bloedvat is blijkbaar in staat om een verschil in zuurstofspanning te detecteren, en adequaat te reageren om afsterven van het weefsel te voorkomen. Een bloedvat bestaat uit verschillende celtypen. De binnenkant van een bloedvat is bedekt met endotheelcellen. Het zijn deze cellen die, na prikkeling met angiogene factoren, het proces van angiogenese in gang zetten. Van groot belang in dit proces is het vermogen van de endotheelcellen om de omliggende matrix gecontroleerd af te breken door middel van hiervoor gespecialiseerde eiwitten (proteolytische enzymen) waardoor er ruimte ontstaat voor de cellen om zich voort te bewegen door deze matrix. Daarnaast is het ook van belang dat de endotheelcellen genoeg eiwitten tot expressie brengen om zich vast te houden aan deze matrix, zodat ze door de matrix kunnen migreren. Deze matrix eiwitten worden integrinen/adhesiemoleculen genoemd.

In veel pathologische condities speelt angiogenese een grote rol. In tumorgroei, oogaandoeningen bij diabetische patiënten en gewrichts-ontstekingen is de angiogenese een negatieve factor. Er zijn echter ook pathologische condities waar stimulering van angiogenese kan leiden tot een verbetering van de conditie. Voorbeelden daarvan zijn claudicatio intermittens (etalagebenen), hartinfarcten en diabetische wonden. In deze condities is de zuurstofspanning in de aangetaste gebieden erg laag en is een belangrijke factor in de vorming van nieuwe vaten. Het begrijpen van het werkingsmechanisme maakt het in de toekomst misschien mogelijk om angiogenese op een gecontroleerde wijze te stimuleren en te remmen en klachten van deze patiënten te reduceren. **Het doel van de studies, in dit proefschrift beschreven, was het bestuderen van de rol van proteolytische**

processen in door groeifactoren geïnduceerde angiogenese onder normale zuurstofspanning (normoxie) en lage zuurstofspanning (hypoxie). Voor deze studies werd een *in vitro* model systeem gebruikt, bestaande uit een drie-dimensionale matrix waarop humane endotheelcellen werden uitgezaaid. Na stimulering met angiogene factoren vormen deze cellen capillair-achtige structuren.

Zoals eerder genoemd is het vermogen van endotheelcellen om de omliggende matrix af te breken van groot belang voor angiogenese. Endotheelcellen produceren verschillende typen eiwitten die in staat zijn de omringende matrix gecontroleerd af te breken. In veel situaties waar angiogenese voorkomt bestaat de omringende matrix voornamelijk uit het eiwit fibrine. Fibrinogeen treedt uit de bloedvaten na bijvoorbeeld een verwonding en vormt daar een tijdelijke fibrine matrix, welke zeer geschikt is voor de ingroei van nieuwe bloedvaten. Endotheelcellen zijn in staat fibrine goed af te breken met behulp van het plaminogeen-activator/plasmine systeem. Plasminogeen activator, ook wel u-PA genoemd, wordt door de endotheelcellen zelf gemaakt en is een enzym dat andere enzymen kan activeren. Eén zo'n ander enzym is plasminogeen. Na activering door u-PA heet plasminogeen plasmine en dit plasmine is in staat om de omringende matrix af te breken. Zowel plasminogeen als u-PA kunnen aan het celoppervlak gebonden worden door eiwitten die receptoren heten. Deze u-PA receptoren zijn echter niet noodzakelijk voor de activering van plasminogeen. Een rustende endotheelcel brengt weinig u-PA en u-PA receptor tot expressie. Angiogene factoren zijn noodzakelijk om de expressie zodanig te verhogen dat er genoeg proteolytische activiteit ontstaat om de matrix af te breken. De angiogene groeifactoren, fibroblast groeifactor-2 (FGF-2) en vasculaire endotheel groeifactor (VEGF) kunnen de expressie van de u-PA receptor induceren. Daarnaast induceert de ontstekingsmediator/groeifactor tumor-necrosis-factor-α (TNF-α) u-PA in humane endotheelcellen. Stimulering van de endotheelcellen met een combinatie van ofwel FGF-2/TNF-α of VEGF/TNF-α leidt tot angiogenese in het *in vitro* model gebruikt in deze studies. In hoofdstuk twee van dit proefschrift is het belang van de binding van u-PA aan de u-PA receptor voor angiogenese in een fibrine matrix bestudeerd. Gecontroleerde matrix afbraak, resulterend in angiogenese, bleek alleen mogelijk als u-PA aan zijn receptor gebonden was. Blokkering van de u-PA receptor resulteerde in totale remming van de vaatvorming, alhoewel er genoeg proteolytische activiteit rond de cel aanwezig was. Naast de lokalisatie van u-PA heeft de u-PA receptor nog andere functies. De u-PA receptor kan onder andere interacties aangaan met zowel de matrix als met de integrinen en kan daarmee de migratie van de cel beïnvloeden. Verder kan de u-PA receptor signalen de cel insturen die leiden tot bijvoorbeeld een verhoogde groei van de cel. De blokkering van angiogenese bleek echter niet veroorzaakt door remming van

deze u-PA receptor-gemedieerde processen, maar puur door het ontbreken van gelokaliseerde proteolytische activiteit. Verder werd gevonden dat de endotheelcellen die het front vormen van het nieuwe bloedvaatje veruit de meeste receptoren voor u-PA expresseerden. Dit resultaat impliceert dat deze cellen het meest actief in matrix afbraak zijn en een weg banen voor de cellen die na hen komen.

Uit het voorgaande blijkt dat naast de expressie van u-PA, de mate waarin de endotheelcel de u-PA receptor expresseert een belangrijke factor is voor de angiogene capaciteit van de cel. De manier waarop de expressie van deze receptor gereguleerd wordt kan daarom een belangrijke factor zijn in het beïnvloeden van angiogenese. In hoofdstuk drie is onderzocht op welke manier FGF-2 en VEGF de u-PA receptor expressie in de endotheelcel reguleren. Voor deze studie werd gebruik gemaakt van specifieke remmers van bepaalde eiwitten die betrokken zijn bij het doorgeven van signalen naar de celkern. Het werd duidelijk dat VEGF de u-PA receptor induceert via een route waarin het eiwit protein kinase C (PKC) een rol speelt. De FGF-2-geïnduceerde u-PA receptor expressie werd niet beïnvloed door een remmer van PKC.

Zoals eerder genoemd is een lage zuurstofspanning (hypoxie) een stimulerende factor voor angiogenese. Via welk mechanisme hypoxie endotheelcellen aanzet tot een verhoogde angiogene activiteit is niet helemaal duidelijk. Het is bekend dat andere cel typen dan endotheelcellen ook op hypoxie kunnen reageren door bijvoorbeeld meer angiogene groeifactoren te produceren. VEGF is bijvoorbeeld een hypoxie-induceerbare groeifactor. Op deze manier kunnen het endotheel indirect, dus via andere cel typen, door hypoxie gestimuleerd worden. In hoofdstuk vier is het directe effect van hypoxie op de angiogene capaciteit van de endotheelcellen bestudeerd. In het *in vitro* model werden ongeveer drie keer zo veel vaatjes gevormd in fibrine onder hypoxische dan onder normoxische omstandigheden. De u-PA receptor expressie bleek te zijn verhoogd in hypoxie en dat was een van de belangrijkste redenen waarom endotheelcellen meer vaatjes vormden dan in normoxische condities. Het is echter niet alleen de afbraak van de matrix dat een grote rol speelt in angiogenese. Ook het vermogen van de endotheelcellen om de matrix los te laten en er weer aan te hechten is zeer van belang voor de migratie door de matrix. De expressie van sommigen integrinen/adhesiemoleculen werd verhoogd in hypoxie. Het blokkeren van deze integrinen had echter geen invloed op angiogenese in een fibrine matrix. Inductie van deze integrinen droeg daarom niet bij aan de verhoogde angiogene respons in hypoxie onder onze experimentele condities.

In hoofdstuk vijf is onderzocht op welke manier de endotheelcel het hypoxie-signaal door kan geven naar de celkern en dus zijn effect kan hebben. Zoals eerder genoemd vormen endotheelcellen alleen vaatjes in een fibrine matrix als ze gestimuleerd worden met een angiogene groeifactor (FGF-2 of VEGF) en de ontstekingsmediator/angiogene groeifactor TNF-α. Onder hypoxische omstandigheden bleek echter dat stimulering met TNF-α niet noodzakelijk meer was. In de aanwezigheid van alleen FGF-2 (maar niet in aanwezigheid van alleen VEGF) waren de endotheelcellen in staat nieuwe vaatjes te vormen in hypoxie. Blijkbaar heeft hypoxie een TNF-α-achtig effect op de endotheelcel. Een aantal signaal transductie eiwitten, eiwitten die een signaal van buiten de cel naar de kern transporteren zodat daar adequaat gereageerd kan worden, zijn betrokken bij de regulatie van genen die betrokken zijn bij angiogenese. In tegenstelling tot in normoxie, kon FGF-2 in hypoxische omstandigheden de signaaltransductie-eiwitten NF-κB en ERK 1/2 in endotheelcellen activeren. NF-κB en ERK 1/2 zijn signaaltransductie-eiwitten die de regulatie van een aantal angiogene factoren kunnen beïnvloeden. Hypoxie is dus in staat verschillende signaal transductie routes in FGF-2-gestimuleerde endotheelcellen te activeren, waardoor eiwitten worden geëxpresseerd die betrokken kunnen zijn bij de hypoxie-geïnduceerde angiogenese.

Naast de hoogte van zuurstofspanning hangt de angiogene capaciteit van de endotheelcel ook af van de samenstelling van de omliggende matrix. In de hoofdstukken twee tot en met zes is steeds fibrine gebruikt als extracellulaire matrix. In een *in vivo* situatie zal een matrix echter nooit uit één type eiwit bestaan maar zullen er naast fibrine ook andere plasma-eiwitten en collageen aanwezig zijn. In hoofdstuk zeven is de invloed van de matrixcompositie op de angiogene capaciteit van de endotheelcellen bestudeerd. Hiertoe werd angiogenese op een fibrine matrix vergeleken met angiogenese op matrixen bestaande uit zowel fibrine en collageen en met matrixen van puur collageen. Het toevoegen van steeds meer collageen aan fibrine matrixen resulteerde in een afname van de vaatvorming. Naarmate er meer collageen aanwezig was, was de matrix afbraak minder afhankelijk van het plasminogeen-activator/plasmine systeem. Andere proteolytische eiwitten die beter in staat zijn collageen af te breken, de zogenaamde matrix metalloproteasen, bleken nu verantwoordelijk voor de afbraak van de fibrine en collageen. Op een pure collageen matrix waren de endotheelcellen niet in staat vaatjes te vormen. Ook hypoxie had geen stimulerende werking op een collageen matrix. Mogelijk produceren de endotheelcellen, gebruikt in deze studie, niet de juiste proteolytische enzymen voor de afbraak van een pure collageen matrix. Het is duidelijk dat de samenstelling van de extracellulaire matrix van groot belang kan zijn voor de regulatie van angiogenese.

List of abbreviations

ATF	amino-terminal fragment
ECGF	endothelial cell growth factor
ECM	extracellular matrix
ERK	extracelullar-related kinase
FDP	fibrin degradation products
bFGF or FGF-2	basic fibroblast growth factor or fibroblast growth factor-2
FGFR	FGF receptor
GPI	glycosyl-phosphatidylinositol
GST	gluthathione-S-transferase
HAECs	human aortic endothelial cells
HIF	hypoxia-inducible factor
HMVECs	human microvascular endothelial cells
HRP	horseradish peroxidase
HS	human serum
HSA	human serum albumin
HUVECs	human umbilical vein endothelial cells
I-κB	inhibitor-κB
JNK	c-jun NH2-terminal kinase
LRP	lipoprotein receptor-related protein
mAb	monoclonal antibody
MAPK	mitogen activated protein kinases
MCP	monocytic chemoattractant protein
MMP	matrix metalloprotease
MT-MMP	membrane-type MMP
α2-MR	α2-macroglobulin
NBCS	newborn calf serum
NF-κB	nuclear factor-κB
NO	nitric oxide
PA	plasminogen activator
PAI	PA inhibitor
PKC	protein kinase C
PMA	phorbol 12-myristate-13-acetate
Plg	plasminogen
sc-u-PA	single-chain u-PA
tc-u-PA	two-chain u-PA
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor-α
t-PA	tissue-type PA
u-PA	urokinase-type PA
u-PAR	u-PA receptor
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VLDL	very low density lipoprotein

List of publications

Full papers

A Collen, P Koolwijk, ME Kroon and VWM van Hinsbergh. Influence of fibrin structure on the formation and maintenance of capillary-like tubules by human microvascular endothelial cells. *Angiogenesis 1998, 2: 153-165.*

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Curriculum Vitae

De auteur van dit proefschrift werd op 2 mei 1972 te s'-Gravenhage geboren. Na het behalen van het VWO diploma aan het Huygens Lyceum te Voorburg in 1990, begon zij met de studie Biologie aan de Rijksuniversiteit Leiden. Tijdens deze studie werd medisch celbiologisch onderzoek verricht aan het Leids Universitair Medisch Centrum, afdeling Endocrinologie & stofwisselingsziekten; Universiteit van Lund, Department of Animal Physiology, Lund (Zweden); TNO Preventie en Gezondheid, afdeling Vaat- en Bindweefselonderzoek, Leiden en tenslotte aan de Kent State Universiteit, Department of neurochemistry, Kent (Ohio, USA). Het doctoraalexamen van deze studie werd in januari 1996 cum laude behaald met als specialisaties Medische Biologie en Celbiologie.

Als assistent in opleiding verrichtte zij vanaf februari 1996 tot zomer 2000 onderzoek in het Gaubius Laboratorium, TNO Preventie en Gezondheid, binnen de afdeling Vaat- en Bindweefselonderzoek onder leiding van Prof. dr. V.W.M. van Hinsbergh en Dr. P. Koolwijk. De resultaten van dit onderzoek staan in dit proefschrift beschreven.

Vanaf september 2000 is zij aangesteld als postdoctoraal onderzoeker onder leiding van Dr. C.W.G.M. Löwik en Dr. G. van der Pluijm aan de afdeling Endocrinologie & stofwisselingsziekten, Leids Universitair Medisch Centrum. Hier wordt onderzoek gedaan naar de toepasbaarheid en werkingsmechanismen van endostatine en kringle-5 bij tumor regressie.

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