Fibrin matrix structure and angiogenesis

PROEFSCHRIFT

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Stellingen

1. De structuur van de fibrine matrix en de mate van cross-linking bepalen niet alleen de snelheid van vorming van capillair-achtige structuren maar ook de stabiliteit van de gevormde vaatjes.
(Dit proefschrift)

2. $\alpha_v\beta_3$ speelt geen essentiële rol bij de adhesie van endotheel cellen aan fibrine noch bij de vorming van capillair-achtige structuren in fibrine in vitro.
(Dit proefschrift)

3. De invloed van heparine op de proliferatie van endotheel cellen wordt bepaald door het soort vaatbed waaruit de cellen geïsoleerd zijn.
(Dit proefschrift)

4. De urokinase (u-PA)-receptor beschermt urokinase (u-PA) niet voor inactivering door trombine.
(Dit proefschrift)

5. Vascular endothelial growth factor (VEGF) speelt niet alleen een rol in de vaatbiologie maar ook tijdens de regeneratie van zenuwweefsel.
(Schratzberger P et al, Nat Med 6:405-41, 2000)

6. De u-PA receptor is zowel betrokken bij cel adhesie als bij cel migratie.
(Hoyer-Hansen G et al, FEBS Lett 420:79-85, 1997)

7. Therapeutische inductie van angiogenese in een ischemisch gebied veroorzaakt door atherosclerose kan leiden tot exacerbatie van de vaatlesie.
(Moulton KS et al, Circulation 99:1726-32, 1999)

8. Leptine vervult niet alleen een rol in de regulatie van de vetmassa maar ook in de regulatie van het afweersysteem, het stimuleren van angiogenese en de regulatie van de voedsel inname.
(Clement K et al, Nature 392:398-40, 1998; Lord GM et al, Nature 394:897-901, 1998; Sierra-Honigmann MR et al, Science 281:1683-1686, 1998; Bado A et al, Nature 394:790-793, 1998)

9. Tissue factor pathway inhibitor (TFPI) speelt een rol in cel migratie.
(Sato Y et al, Thromb Haemost 78:1138-41, 1997; Fischer EG et al, J Clin Invest 104:1213-21, 1999)

10. Beenmerg transplantatie van een gezonde donor naar een acceptor met psoriasis leidt vaak tot genezing van de huidziekte, en andersom tot het overdragen van de ziekte.
(Snowden JA et al, Arthritis Rheum 41:453-9, 1998; Snowden JA et al, Br J Dermatol 137:130-2, 1997)

11. Een volledig inhibitie van keratinocyten migratie kan alleen verwezenlijkt worden als zowel het plasminogeen activator/plasmine- als het matrix metalloproteinase-systeem geblokkeerd worden.
(Lund LR et al, EMBO J 18:4645-56, 1999)

Stellingen behorende bij het proefschrift: " Fibrin matrix structure and angiogenesis"

Leiden, 6 juni 2000 Annemie Collen

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chapter I

introduction

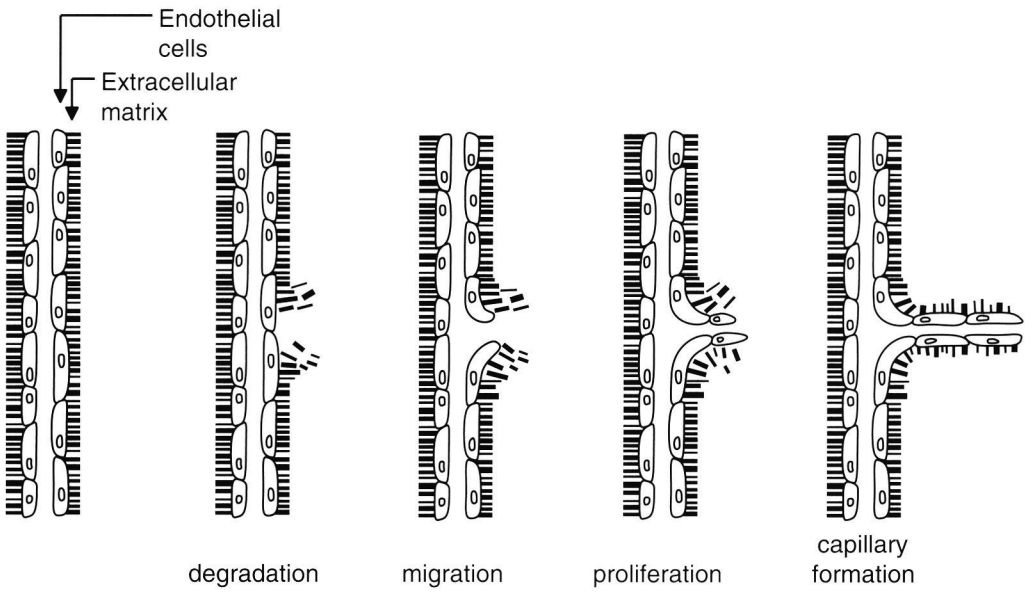
Introduction

Angiogenesis is multistep process, which leads to the formation of new capillaries from already existing vessels. This process occurs during embryo development when establishment and maintenance of the vascular tree is an absolute requirement for the growth of the embryo [1]. In the adult the vascular tree has fully developed, and controlled angiogenesis only occurs to a limited extent in the female reproductive system [2].

Angiogenesis in the adult is also associated with conditions such as wound repair and collateral formation in ischaemic areas, where the formation of new blood vessels is temporally and spatially strictly controlled and limited by the metabolic demands of the tissue. Uncontrolled angiogenesis contributes to the pathogenesis of a variety of diseases such as diabetic retinopathy [3,4], rheumatoid arthritis [5, 6], psoriasis [7], haemangiomas [8, 9], atherosclerosis [10], and the growth and metastasis of tumours [11].

The common features of angiogenesis in the adult, which distinguishes it from embryological angiogenesis, are the presence of vascular leakage [12], the deposition of fibrin [13], and the influx and presence of inflammatory cells [14]. Angiogenesis is driven by stimulated microvascular endothelial cells, which finally form patent endothelium-lined tube-like structures (figure 1). Endothelial cells of existing capillaries or postcapillary venules are activated by local angiogenic stimuli. Local vasodilatation, increased vascular permeability and accumulation of extravascular fibrin occurs, as well

Figure 1: Endothelial cells present in an existing capillary degrade the basement membrane and invade the surrounding interstitium. The elongated endothelial cells align to form a capillary sprout, in which gradually a lumen is formed.



as proteolytic degradation of the basement membrane of the parent vessel. The activated endothelial cells protrude cytoplasmatic processes and migrate into the surrounding matrix towards the angiogenic stimulus. The endothelial cells elongate and align with each other to form a capillary sprout (figure 1). The cells divide to further increase the number of cells and the length of the sprout. Subsequently, the cells organise and a lumen is gradually formed. Contiguous sprouts anastomose at their tips to form a capillary loop, into which blood flow is established [15]. Once endothelial cells are assembled into vascular channels they, become surrounded by smooth muscle cell (larger vessels) or pericytes (smaller vessels) that affect maturation of blood vessels not only by providing structural support but also by controlling endothelial cell proliferation, differentiation and vascular tone [17-18].

Specific features of pathological angiogenesis

Angiogenesis, which occurs in pathological conditions, is commonly accompanied by vascular leakage, the occurrence of inflammatory cells and the presence of fibrin. These factors are absent in angiogenesis during embryonic development. Inflammatory cells provide a number of factors favouring angiogenesis. Vascular leakage increases the recruitment of fibrinogen and vitronectin, which precipitate in the fibrinous exudate. The fibrin matrix is a repair matrix, which enforces the haemostatic plug and prevents further blood loss after wounding. In addition it provides a scaffolding for invading cells during the subsequent repair process.

Inflammatory cells

Angiogenesis *in vivo* involves other cell types besides endothelial cells. Macrophages are key angiogenesis effector cells by producing several growth stimulators and inhibitors and by secreting proteolytic enzymes and cytokines that influence the angiogenic cascade. They induce the formation of new blood vessels during tissue regeneration, and induce capillary regression of excessive vessels after tissue regeneration has been completed [19].

Macrophage progenitors originate from bone marrow and migrate into the blood stream as circulating monocytes [20]. Macrophages migrate into the wound, and undergo further differentiation and rapidly respond to environmental signals [21]. A number of environmental stimuli, such as low oxygen tension, lactate [22] and endothelial cell derived factors [23], induce the secretion of angiogenic factors from macrophages, as well as adhesion to and transmigration of leucocytes through the endothelium [24]. This activation process is tightly regulated and leads to increased phagocytosis, enhanced chemotactic activity, but also to a secretion of proteases, growth factors and cytokines. The secreted growth factors and cytokines attract endothelial cells and induce angiogenesis [25]. Among a number of factors that have been identified, TNFα [26], TGFα, TGFβ [27], and IL-8 [28] are potent mediators of angiogenesis *in vivo* and *in vitro*. The macrophages secrete proteases, such as plasminogen activators and

MMPs, which degrade the matrix. The accompanying fibrin degradation products are chemotactic for endothelial cells [29].
These inflammatory cells are present in a healing wound when the proliferative fase of the repair process is the most pronounced [30]. Their important role is demonstrated by the fact that depletion of monocytes results in a delayed wound repair [31], while the introduction of activated macrophages into healing wounds results in enhanced wound repair [32].
During the resolution phase many of the newly formed capillaries regress as tissue regeneration is completed. Macrophages play a role in the resolution phase by secreting substances inhibitory to endothelial growth and angiogenesis [33, 34].

Vascular leakage

Angiogenesis in pathological conditions is often accompanied by vascular leakage. This leakage is induced by the inflammatory cells and mediators involved [35] and by angiogenic factors, such as VEGF, also called vascular permeability factor [36]. VEGF induces both the formation of transendothelial channels [37] and dissociation of endothelial cell-cell contacts [38, 39]. Furthermore, it is likely that migration and proliferation of endothelial is also accompanied by the dissociation of cell-cell contacts between adjacent endothelial cells [40]. As a consequence of vascular leakage a fibrinous exudate or stroma is formed, which contains fibrin, vitronectin and other proteins.

The function of fibrin in angiogenesis

The formed fibrin matrix serves as a provisional matrix into which cells can infiltrate. It is formed after wounding of a vessel and composes a major part of the initial tumor stroma, providing a scaffolding for both invasive cancer cells and endothelial cells, thereby contributing to tumor growth and neovascularisation [12].
It is not only a passive matrix, but also induces morphological and biochemical changes in endothelial cells (Review Chapter 2). Endothelial cells in contact with fibrin undergo disorganisation [41] and become susceptible to growth factors [42]. Some of these factors not only induce proliferation of endothelial cells but also induce secretion of several proteins and proteases [43, 44].
Fibrin itself attracts inflammatory cells [45, 46]. These cells digest fibrin and generate degradation products that may be chemotactic for endothelial cells [47].
Endothelial cells interact with fibrin via a number of receptors ICAM-1, CD-44, VE-cadherin and integrins (Reviewed in chapter 2). The role of these receptors during angiogenesis is the best studied for integrins. Integrins are heterodimers consisting of two non-covalently associated transmembrane glycoproteins (an alpha and a beta unit) [48] that connect adhesive proteins in the extracellular matrix with the cytoskeleton. Endothelial cells express a number of integrins, of which $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are the

best studied in angiogenesis. Blocking antibodies against these integrins block angiogenesis in several *in vivo* and *in vitro* models (see chapter 2).

Fibrin formation

Fibrin accumulates in the extracellular milieu after injury and also as a consequence of vascular hyperpermeability, which is seen during inflammation and in tumours. Fibrin is formed from fibrinogen, which is present in plasma as a soluble protein. In the presence of thrombin fibrinogen molecules are transformed into an insoluble fibrin network.

Components of the coagulation system circulate as inactive enzymes in the circulation, and are activated by the coagulation cascade [49, 50]. The coagulation cascade can be initiated by the intrinsic pathway, via contact activation, or by the extrinsic pathway, via tissue factor/factor VII, which results in the formation of thrombin.

Thrombin cleaves both fibrinopeptides A (FPA) and fibrinopeptides B (FPB) from the amino-terminal part of the Aα- and Bβ-chains, respectively. This results in the formation of a fibrin monomer. Due

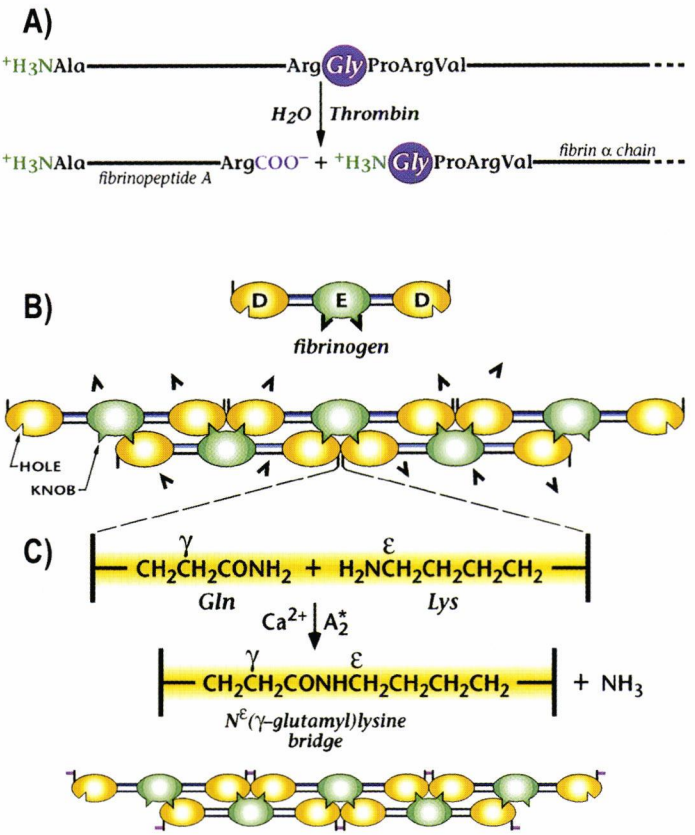


***Figure 2**: Thrombin cleaves off fibrinopeptides A(FPA) and B (FPB) (A) resulting in the formation of fibrin monomers (B). Fibrin monomers associate into dymers and fibrin oligomers which will form a 3-dimensional network. (B). Factor XIIIa covalently links adjacent fibrin molecules leadign to the stabilisation of the fibrin network. Reprinted from L. Lorand [236].*

to conformational changes, polymerisation sites, AA' and BB' are exposed at the amino-terminal end of the fibrin monomer. The complementary polymerisation site aa' is located at the carboxyl-terminal part of the γ-chain, while the complementary bb' site is located in the carboxyl-terminal part of the Aα-chain. The newly formed polymerisation sites AA' interact with complementary sites aa' on an adjacent fibrin monomer, forming half-staggered non-covalently linked dimers. Longer protofibrils or fibrin oligomers are formed by linear polymer formation between new AA' and aa' sites in neighbouring molecules. Interactions between BB' and bb' lead to lateral association, which will eventually lead to the formation of a three-dimensional fibrin network [51] (figure 2).

Blood coagulation factor XIII can, upon activation by thrombin [52], catalyse the formation of covalent cross-links, stabilising the structure. Intermolecular cross-linking occurs at the carboxyl-terminal regions of the γ-chains of fibrinogen [53, 54]. Cross-linking also involves α-chains, which is much slower [55]. γ-γ cross-linking results in the binding of monomers together into a protofibril, while α-α cross-linking results in the formation of bundles from protofibrils [56]. Factor XIIIa not only stabilises the fibrin network; it also binds matrix proteins to it such as fibronectin [57], von Willebrand Factor [58] and thrombospondin [59]. Cross-linking of fibrin results in a resistance to fibrinolysis, due to crosslinking of α_2-antiplasmin [60] and due to masking of high affinity binding sites for t-PA [61]. Fibrin cross-linking is also mediated by tissue transglutaminase, which covalently links two α-chains [62]. This delays the plasmin-mediated dissolution of the clot, ensuring its persistence until tissue repair is completed [63] (figure 2).

The architecture of the fibrin clot is determined by the conditions of polymerisation. Alterations in the fibrin structure not only have a profound influence upon the sensitivity of these matrices to plasmin dissolution but also on the spatial organisation of possible cell-binding sites [64] on the fibrin Aα-chain and possibly the Bβ–chain and γ-chain (see chapter 2). In 1949, Ferry and Morrisson correlated the optical properties of fibrin gels to their architecture. In a transparent network, the bundles are thin and arranged in a fine and rigid network, whereas in opaque fibrin, the bundles are thick and arranged in a porous, malleable network [65]. Fibrin architecture is influenced by the microenvironment conditions during polymerisation. The rate of fibrinogen activation is the most important factor in determining the final fibrin gel structure [66]. Kinetic factors such as ionic strength, pH [67], and concentrations of fibrinogen, thrombin and antithrombin [68] alter the final structure of the network. Plasma proteins [69] such as albumin [70], fibronectin [71], IgG [72] and complement factors [73] also influence the conformation of the network [51].

The presence of different cell types during polymerisation also influences the structure of the formed fibrin. The presence of erythrocytes [74], granulocytes [75] and platelets [76] modifiy the formed fibrin network. Glycosaminoglycans, such as hyaluronan [77] and chondroitin sulphate A [78] increase, while collagen [79] decreases the turbidity. Collagen type IV, on the other hand, increases the thickness of the bundles while laminin and dermatan sulphate decrease it [80]. Also several drugs, such as acetylsalicylic

acid [81], dextran [82], heparin [83, 84] and anti-diabetic drugs [85] have been reported to modify the fibrin network formed.
Altered fibrin structures have been reported in various pathological conditions, such as inflammation, multiple myeloma [86], factor XIII deficiency [87], diabetes [88] and proneness to myocardial infarction [89].

Fibrin degradation

When endothelial cells invade a fibrin matrix, they not only use it as a scaffolding, but also degrade it partially to acquire access into it and to provide space for the formation of capillary structures. The fibrin matrix is degraded by pericellular fibrinolytic activities associated with the invading cells. In addition fibrinolysis by plasma t-PA occurs to remove intravascular formed fibrin.

The plasminogen activator/plasmin system

Plasmin is the major protein involved in fibrin degradation. It is activated from its zymogen plasminogen by two types of plasminogen activators (PA), tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The activity of the plasminogen activators and plasmin is regulated by controlled secretion and activation, by interaction with receptors and by reaction with specific inhibitors. Inhibition of the fibrinolytic system may occur either at the level of the PAs, by specific plasminogen activator inhibitors (PAI), or at the level of plasmin, mainly by α_2-antiplasmin (α_2-AP) and possibly by tissue factor pathway inhibitor-2 (TFPI-2) (figure 3).

***Figure 3**: Scheme of the plasminogen activation system. Reprinted from P. Koolwijk and VWM van Hinsbergh [235]*

Abbreviations:

PA:	*plasminogen activator*
t-PA:	*tissue-type PA*
tcu-PA:	*two-chain urokinase-type PA*
scu-PA:	*single-chain urokinase-type PA*
PAI:	*PA inhibitor*
+:	*activation*
-:	*inhibition*

Plasminogen activators contribute to prevention and dissolution of an intravascular thrombus and participate in pericellular fibrinolysis involved in cell migration and invasion. The latter case involves cellular receptors, in particular the u-PA and plasminogen receptors.
Plasminogen, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) are serine proteases. The general structure of the serine proteases is similar. Serine proteases are

secreted as single chain proteins. Specific proteolytical cleavage converts the protein into a molecule composed of two chains, which are linked together with a disulphide bond. u-PA and plasminogen are secreted as inactive single-chain proteins, which are activated by proteolytic cleavage, while single chain t-PA is active as such. The carboxyl terminal part of the protein (B-domain or the light chain) contains the proteolytic active site, which is similar in all serine proteases. The amino-terminal part (A-domain or heavy chain) is more complex and contains the domains, which interact with receptors and matrix components. Serine proteases cleave their target proteins at the COOH-terminal side of Arg or Lys residues.

Plasminogen

Human plasminogen is secreted as a single chain 92 kDa glycoprotein by hepatocytes [90, 91] and is present at a concentration of 1.5-2 μM in blood. It is a 719 amino acid protein containing a preactivation peptide, five homologous triple loop structures (kringles 1-5) and a protease domain [92, 93]. Plasminogen is converted into plasmin by cleavage of the Arg561-Val562 bond by either u-PA or t-PA, giving rise to a two-chain molecule connected by two disulphide bonds. The A-chain (66 kDa) contains 5 kringles and mediates with its lysine and amino-hexyl binding sites the interaction of plasmin with α_2-antiplasmin and the specific binding of plasminogen to fibrin and cell surface receptors. The B-chain (24 kDa) contains the serine protease catalytic triad, His603-Asp646-Ser741.

Plasmin is a trypsin-like molecule with a broad spectrum of target proteins. It cleaves fibrin, several extracellular matrix components, such as laminin, thrombospondin, and collagens [94], coagulation factors (factor V, VIII and vWF), pro-MMPs [95] and plasminogen and plasmin itself.

Plasminogen binds to fibrin and cellular surfaces. Kringle 1 and 5 have a very high affinity for native fibrin, whereas kringle 4 is only capable of binding to partially digested fibrin surfaces [96].

Many cell types, including endothelial cells [97, 98], have cell surface localised plasminogen receptors. Plasminogen binds to cell-surfaces via kringle 1 and possibly also via kringle 5 [98]. Plasminogen binding is specific, saturable and reversible and plasminogen is bound with a high capacity, but a low affinity to cell surfaces (10^7 receptors per cell) [97, 99]. At a plasma concentration of 2 μM substantial occupancy of the receptors is accomplished [98, 100] Antibodies against candidate receptors can only partially block plasminogen binding, suggesting that more than a single component is involved in mediating binding [98, 101, 102].

Binding of plasminogen to a receptor enhances its activatability. The activation of plasminogen by u-PA and t-PA is enhanced [98, 103], although in comparison to plasminogen activation on a fibrin surface, the activation is still low. Cell-surface localised plasmin can efficiently activate single chain u-PA (scu-PA) bound to a u-PA receptor (u-PAR) in vicinity, leading to an amplification loop for plasminogen activation at the cell-surface [104, 105]. Plasmin, which is either bound to fibrin or to cell

surfaces is protected from inactivation by α_2-antiplasmin since the lysine binding sites, which interact with α_2-antiplasmin, are masked [99, 106].

t-PA mediated activation of plasminogen on fibrin and cell surfaces

In the absence of fibrin t-PA has low activity towards plasminogen, but the presence of fibrin markedly enhances its catalytic efficacy [107]. The fibrin binding part of t-PA is located in the so-called finger-like domain in the amino-terminal part of the molecule (amino acids 6-43) and in the kringle 2 [108, 109]. Fibrin provides a surface to which t-PA and plasminogen absorb in a sequential order to form a ternary enzyme-substrate-effector complex [107]. This enhances the affinity for t-PA-mediated plasminogen activation and leads to an efficient and localised activation [110]. On cell surfaces a ternary complex between t-PA, plasminogen and annexin II enhances plasminogen activation [102, 111] and provides a focal point for accelerated plasmin generation [112]. This process is involved in maintaining blood fluidity and a mechanism to produce local proteolysis.

The main goal of t-PA is to prevent excessive intravascular fibrin formation rather than removal of already existing clots. If fibrin is deposited at the luminal side of the endothelium, occlusion of the blood flow might occur, leading to distal ischemia and tissue damage. To prevent this endothelial cells are capable of instantaneously upregulating their fibrinolytic activity by secreting t-PA from an intracellular storage pool [113]. This secretion is restricted in time and space to prevent a systemic fibrinolytic activity [114].

u-PA/u-PAR mediated plasminogen activation

u-PA/u-PAR-mediated activation of plasminogen is involved in proteolytic processes during cell migration and matrix remodelling and only secondarily in removal of intravascular fibrin [115].

u-PA is a 55kDa [116] protein composed of 411 amino acids [117, 118] held together with 11 disulphide bonds. It has an epidermal growth factor like (EGF) domain (amino acids 5-49), a kringle domain (amino acids 50-131), a long connecting peptide (amino acids 132-158) and a protease domain (amino acids 159-411), containing the catalytic triad His 204-Asp255- Ser356 [119].

Single chain urokinase, scu-PA or pro-urokinase (HMW-u-PA) has little plasminogen activity and needs to be activated. Therefore, scu-PA binds to u-PAR and becomes activated by cleavage at residues Lys158-Ile159, which exposes the catalytic domain of u-PA [120]. Plasmin catalyses the activation of scu-PA to tcu-PA. In turn, tc-u-PA catalyses the activation of receptor bound plasminogen, which results in a positive feedback mechanism.

u-PA-receptor is a high affinity receptor for u-PA [121, 122] on different cell types [123], including endothelial cells [124]. u-PAR is a highly glycosylated 313 amino acid protein of 50-60 kDa [125], processed to a mature protein of 283 amino acids [126]. At the cell surface the polypeptide folds itself

into three extracellularly located domains: domain I (amino acids 1-92), domain II (amino acids 93-191) and domain III (amino acids 192-282) [127], which have a low sequence homology [128]. During posttranslational processing the COOH-terminal part of the nascent protein is removed and a glycosyl-phosphatidyl-inosityl moiety (GPI-anchor) is covalently linked to the new amino terminus, anchoring u-PAR to the cell membrane [129]. The receptor is homologous to a number of other GPI-anchored proteins from the Ly-6 family [128].
u-PA binds to its receptor by the amino residues 1-137 in the amino terminal domain (ATF domain, consisting of the EGF and kringle domain, in which the EGF domain is the most important [122]. Both single (inactive) and two chain (active) u-PA bind reversibly and saturably [130]. The binding sequence for u-PA on u-PAR is located at the amino acids 1-87 in the NH_2-terminal of domain I [131, 121, 122], but other domains have also been implicated in this process [132, 133].
The linker between domain I and II is susceptible to cleavage by several proteases such as trypsin, chemotrypsin, u-PA, plasmin [134] and thrombin [135]. It is an inactivation mechanism, since DII+DIII alone do not bind u-PA with a high affinity. Furthermore, removal of the EGF-domain from u-PA impairs binding of u-PA to the u-PA-receptor, limiting its activation [136]. Hereby pericellular plasmin activity is controlled. Thrombin cleaves Arg156-Phe157 [137, 138], yielding a u-PA, which is much slower activated by plasmin [139], suggesting a negative feedback of the coagulation cascade on plasminogen activation.

The inhibitors

The activity of the proteases of the fibrinolytic system is regulated by potent specific inhibitors, which are members of the serine protease inhibitor superfamily or SERPINS [140].

α_2-antiplasmin

Plasmin is instantaneously inhibited by α2-antiplasmin [141-143]. α2-antiplasmin binds to plasmin via its active site and via its lysine binding sites and specifically inactivates plasmin activity [144]. This reaction is attenuated when plasmin is bound to either fibrin or to its receptor [144, 145].

Plasminogen activator inhibitor (PAI)

The major inhibitors of PA activity are PAI-1 and PAI-2. While PAI-2 is active on monocytes and macrophages [146], PAI-1 is the major physiological inhibitor of t-PA and u-PA in blood and tissues [147]. It is synthesised by platelets [148], hepatocytes [149], smooth muscle cells and endothelial cells [150, 151]. PAI-1 is a 54 kDa polypeptide chain [152, 153]; it exists in two forms [154], an active form

that inhibits specifically both u-PA and t-PA [155], and a latent form, that cannot inactivate due to conformational changes [156].
PAI-1 interacts with PA at its reactive centre at amino acids 346-347 (P1-P1'). Potential binding sites comprise amino acids 1-80, amino acids 102-104, amino acids 350 [157]. PAI-1 very effectively inactivates single and two-chain t-PA and u-PA, but not scu-PA [158].

MMP-mediated fibrinolysis

Matrix metalloproteinases or MMPs are extracellular endopeptidases that selectively degrade components of the extracellular matrix [159] (table 1). They are produced by several cell types and by endothelial cells as inactive proenzymes that need to be activated. Plasmin generated by the u-PA/u-PAR system activates the proenzyme forms of MMP. This proteolytical cascade enhances pericellular proteolysis (figure 3). Furthermore, several active MMPs can activate other members of the MMP-system, resulting in a positive feedback mechanism. The regulation of MMP activity occurs at the level of gene transcription, pro-enzyme activation and inhibition by specific inhibitors, the TIMPs [161-163]. To date, the ability to mediate fibrinolysis has been attributed to the plasminogen activator plasmin system. Some active MMPs, such as MMP-3, MMP-7 and MT1-MMP are capable of degrading fibrin, while MMP-1, MMP-2, MMP-9 and MT2-MMP only degrade fibrin to a limited extent [163-165].

Role of pericellular proteolysis in cell migration and angiogenesis

Quiescent endothelial cells express t-PA, MMP-2 and minimally MMP-1 [166-168], but net proteolysis is prevented by coincident expression of PAI-1, TIMP-1 and TIMP-2 [169]. During migration and angiogenesis the proteases and protease inhibitors are concomitantly upregulated [170, 171]. A precise equilibrium between proteases and inhibitors is reached to allow localised pericellular matrix degradation, while protecting against excessive extracellular matrix degradation (figure 4). During this process existing cell-matrix interactions are disrupted and new attachments are made.

Involvement of u-PA/u-PAR in cell migration during angiogenesis

Migrating cells display u-PA activity confined to the cell surface. A marked, rapid and sustained increase of u-PAR suggests that u-PA/u-PAR is involved in cellular migration and invasion [172-176]. PAI-1 is expressed at a different location and time to allow a net increase in fibrinolytic activity [170]. After cessation of migration the expression of u-PA, u-PAR and PAI-1 return to normal.

After secretion u-PA binds to u-PAR, which localises u-PA on the cell surface. u-PA is converted from inactive scu-PA in active tcu-PA. The u-PA/u-PAR complexes are located at sites where plasminogen is bound to the cell surface. u-PA becomes activated at sites where plasminogen binds, and thereby limits the plasminogen activator activity to cellular protrusions during cell migration [177]. Plasminogen is converted in plasmin and proteolysis of the matrix occurs. On the cell surface the local microenvironment leads to an accelerated reciprocal activation of pro-u-PA and plasminogen [178, 179], which not only requires binding of u-PA to u-PAR but also binding of plasminogen to a "receptor" [104, 178]. Cell-associated plasmin is protected from inactivation by α_2-antiplasmin, leading to further activation of more scu-PA/u-PAR complexes.

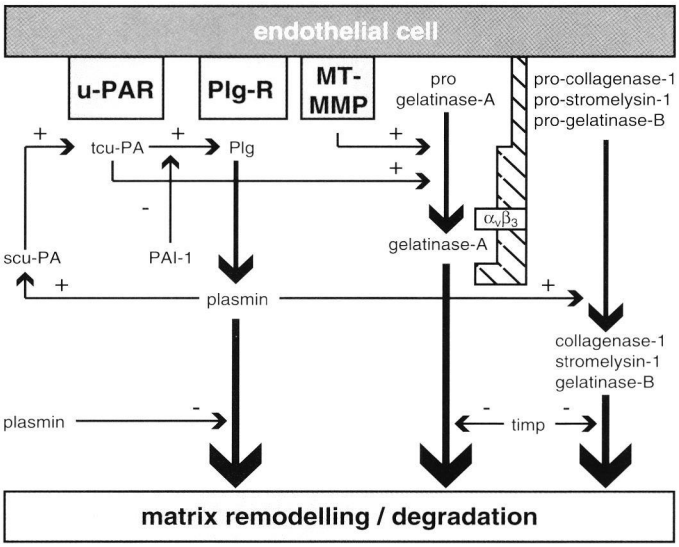


Figure 4. *Schematic representation of the interaction between the u-PA/plasmin system and the presumed activation of matrix-degrading metalloproteinases (MMPs). Reprinted from P. Koolwijk and VWM van Hinsbergh [235].*

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-1:	*PA inhibitor-1*
MT-MMP:	*membrane-bound MMP*
TIMP:	*tissue inhibitor of MMP*
+	*stimulation*
-	*inhibition*

Active u-PA on u-PAR is not internalised, while complexes with PAI-1, PAI-2 and PN-2 are internalised by the co-operation of α_2-macroglobulin receptor (VLDL-receptor or gp330) [180-182]. In endothelial cells it is still uncertain whether this process occurs via LRP or LRP-related protein [183]. After uptake u-PAR:u-PA:PAI-1 are dissociated. The u-PA:PAI-complexes are subsequently degraded in the lysosomes, while the free u-PAR is returned to the membrane.

A variety of cytokines and growth factors regulate the expression of proteinases. bFGF is a potent inducer of u-PA [184] and increases the expression of u-PAR on endothelial cells [185, 186]. VEGF-A upregulates u-PA, t-PA and u-PAR [187, 188]. TNFα induces u-PA and u-PAR in human monocytes [191, 192] and MMP-1, MMP-3 and MMP-8 in endothelial cells [168, 190]. TGF-β induces a decrease in u-PA and an increase in PAI-1 [184, 189], and stimulates the expression of TIMP-1.

u-PAR not only plays a role in localising u-PA to the cell surface and in catalysing the plasminogen activation at the cell surface [104]. The receptor is also involved in signal transduction. Binding of u-

PA to u-PAR induces specific phosphorylation of proteins [193]. Since u-PAR does not have a transmembrane nor intracellular domain, additional proteins are involved in the signalling.
u-PAR is also involved in cell-matrix interactions. On endothelial cells u-PAR is a major vitronectin binding protein. Vitronectin binds reversibly and with a high affinity [194, 195] to domain I on u-PAR [194, 196]. Presaturation of u-PAR by u-PA increases binding of VN [194-196], probably by inducing conformational changes favouring this binding [197, 198]. Vitronectin also binds PAI-1 and maintains it in an active conformation [199-201]. PAI-1 plays a decisive role in governing the balance between u-PAR mediated matrix binding and release [202]. Active PAI-1 abrogates the binding of vitronectin to u-PAR [194, 196] due to competition for binding in the NH_2-terminal somatomedin B-domain and COOH-terminal amino acids 364-380 of vitronectin [194, 201, 202]. u-PA/u-PAR, $\alpha_v\beta_3$ and vitronectin co-localise in focal adhesions [203, 204]. This distribution is triggered by cell adhesion to vitronectin, thus on sites where u-PA can be inactivated by PAI-1 [205, 197].

Involvement of MMP-system in cell migration during angiogenesis

Recent data suggest that remodelling of the extracellular matrix during the process of angiogenesis is accomplished largely through the action of MMP, of which the activity is the rate limiting step in ECM degradation [167, 206]. MMP inhibitors inhibit the angiogenesis process *in vivo* and *in vitro* [207-210].

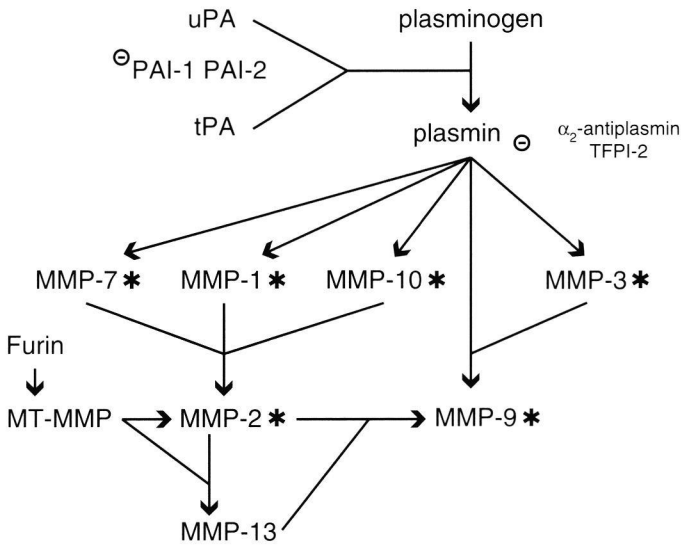


***Figure 5**: MMPs are secreted as inactive zymogens and are converted into active enzymes. In vitro plasmin can activate components of the MMP-system. Several active MMPs activate other members of the MMP-system.*

Endothelial cells can produce several MMPs, MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-14 and MMP-19. Of all MMPs secreted by endothelial cells, the regulation and substrate specificites of MMP-1, MMP-2, MMP-3 and MMP-9 are best characterised (table 1). MMPs can, in concert, degrade most

Group	*Members*	*MMP nr*	*Main substrates*	*Activation*
Collagenases	Interstitial collagenase	MMP-1	Fibrillar collagens (types I, II, III, VII and X) gelatin, proteoglycans, aggrecans, serpins	MMP-2
	Neutrophil collagenase	MMP-8	Fibrillar collagens (types I, II, III) proteoglycans	Not determined
	Collagenase-3	MMP-13	Fibrillar collagens (types IV, IX, X, XIV), serpins, aggrecan, fibronectin, laminin, tenascin, gelatin	MMP-2; MMP-9
	Collagenase-4	MMP-?	Not known	
Gelatinases	Gelatinase A	MMP-2	Gelatin, Collagen (types IV, V, VII, X), fibronectin	MMP-9; MMP-13
	Gelatinase B	MMP-9	Gelatin, Collagen (types IV, V, VII, X), fibronectin	Not determined
Stromelysins	Stromelysin-1	MMP-3	Laminin, non fibrillar collagens (II, IV, V, IX) fibronectin, elastin, gelatin, proteoglycan, aggregan, nidogen	MMP-1; MMP-8; MMP-9; MMP-13
	Stromelysin-2	MMP-10	Laminin, non fibrillar collagens (II, IV, V, IX) fibronectin, elastin, gelatin, proteoglycan, aggregan, nidogen	MMP-1; MMP-9
	Stromelysin-3	MMP-11	α_1 proteinase inhibitor (serpin), gelatin, fibronectin, proteoglycans	Not determined
	Matrilysin	MMP-7	Laminin, non fibrillar collagen (type IV), fibronectin, gelatin, elastin	MMP-2
MT-MMPs	MT1-MMP	MMP-14	Pro-MMP-2, collagens, gelatin	MMP-2; MMP-13
	MT2-MMP	MMP-15	Pro-MMP-2, collagens, gelatin	
	MT3-MMP	MMP-16	Pro-MMP-2, collagens, gelatin	
	MT4-MMP	MMP-17	Pro-MMP-2, collagens, gelatin	
Others	Metalloelastase	MMP-12	Elastin	
	Enamelysin	MMP-?	Not known	
	Xenopus MMP	MMP-?	Not known	
	Not known	MMP-19	Aggrecan	

of the components of the extracellular matrix [95], including fibrin [164, 165]. MMP-1 or collagenase is a 55 kDa protein, which degrades collagen I, II, III, VII and X. It has a basal expression in endothelial cells and is inducible. MMP-2 or gelatinase A is a 72 kDa protein which degrades collagen IV, V, VII, X, XI, gelatin, fibronectin and elastin. It has a basal expression but is not inducible. MMP-9 or gelatinase B, is a 92 kDa enzyme with the same spectrum as gelatinase A, it has no basal expression but is inducible. MMP-3 or stromelysin-1 degrades collagen I, II, III, IV, IX, X, laminin and fibronectin, gelatins and proteoglycans. It has a basal expression and is inducible.

MMPs are secreted as inactive zymogens and are converted into active enzymes by proteinases of different specificities, including trypsin, cathepsin G, plasmin and MMP itself [211] (figure 5).

in vitro studies have demonstrated that MMP activation can occur at the cell surface by the action of the pericellular localised u-PA/plasmin system. The activity of a number of MMPs, such as proMMP-1, proMMP-3, proMMP-9, proMMP-10 and proMMP-13, is controlled by the u-PA/plasmin system [212-214]. The concept of a pericellular activation mechanism of MMPs is further strengthened by the presence of membrane-associated MMPs on the cell surface [215]. Of the four known MT-MMPs, MT1-MMP, MT2-MMP and MT3-MMP can activate proMMP-2 as well as pro-MMP-13 [216, 217]. This cell surface activation mechanism, which is similar to the activation mechanism known for u-PA, localises the activity of the MMP to the pericellular area. A complex activation mechanism may be established as active MMPs are able to activate other MMP proenzymes. This activation cascade is tightly regulated by the inhibitors of MMPs, TIMPS [95] (figure 4 & 5).

The activity of MMPs also controls the PA system. MMPs cleave the receptor binding ATF-domain of u-PA, disabling binding to its receptor. Futhermore, MMPs can cleave plasminogen in such a way that it is no longer cleavable. This results in a decreased plasminogen activation [218, 219]. It is of interest to note that angiostatin, a potent inhibitor of angiogenesis is formed from plasminogen by MMPs [220-222]. This suggests a complex relationship between both systems involved in degradation of the extracellular matrix. This is also stressed by the fact that the same growth factors which induce the expression of u-PA and u-PAR, such as bFGF and VEGF, also upregulated the expression of MMPs in endothelial cells.

MMP-2 associates with $\alpha_v\beta_3$, which colocalises with the u-PAR in the focal adhesion sites, suggesting a direct link between the protease activity and the cell-matrix interaction, analogue to the u-PAR/u-PA/VN complex [223].

In vitro angiogenesis model

Angiogenesis in a fibrinous exudate can be mimicked in an *in vitro* angiogenesis model. Bovine microvascular endothelial cells, seeded on top of a fibrin matrix can be stimulated to form capillary-like tubular structures in the fibrin matrix by phorbol ester [224] and by the angiogenic growth factors bFGF and VEGF [188]. A direct correlation has been demonstrated between expression of the plasminogen activator/plasmin system and the formation of capillary sprouts. The formation of tube-like structures is critically dependent upon the u-PA-mediated plasminogen activation confined to the cell surface by u-PAR and restricted by the concomittant expression of PAI-1. bFGF induces the protease u-PA and its protease inhibitor PAI-1, resulting in a net positive balance towards proteolysis [225]. Similar to bFGF, VEGF alone can evoke an angiogenic response [226, 227] by inducing u-PA as well as u-PAR. The effect of VEGF may be attributed to the release of endogenous bFGF since bFGF-neutralising antibodies result in an increase of PAI-1 and a decrease of u-PA. This results in a net antiproteolytic balance and impaired angiogenesis [228]. bFGF and VEGF have a potent synergistic effect [188], suggesting that the nature of an angiogenic response by a cytokine depends on the

presence and concentration of other cytokines in the microenvironment of the responding endothelial cell.

During angiogenesis, tightly controlled proteolytic degradation of the extracellular matrix by invading microvascular endothelial cells is necessary. Excessive plasminogen activation causes the formation of endothelial cysts in the fibrin matrix [229], while enhancement of PA inhibition limits the extent of capillary tube formation [230]. TGF-β induces PAI-1 resulting in an antiproteolytic response inhibiting sprout formation [230]. Similarly to TGFβ, leukemia inhibiting factor (LIF) inhibits angiogenesis due to a decreased extracellular proteolysis by decreasing u-PA and increasing PAI-1 [231].

In agreement with the results of Montesano and Pepper, we were able to induce formation of tubular structures in a model composed of human microvascular endothelial cells seeded on top of a human fibrin matrix. In human microvascular endothelial cells, bFGF upregulates u-PAR [185], but for the upregulation of u-PA and PAI-1 TNFα is a necessary component [232-234]. The cooperative effect of a growth factor and a cytokine results in the formation of tubular structures. This process is also dependent upon u-PA/u-PAR interactions [234, 183], leading to pericellular plasminogen activation and localised fibrinolytic activity.

Aims of the study

The angiogenic response is critical for progression of wound healing and in certain angiogenesis-dependent diseases. The fibrin matrix, which is formed in pathological conditions, facilitates the invasion of cells and neovascularisation. Understanding of fibrin-associated angiogenesis can help to identify regulatory processes that can control angiogenesis, or to stimulate it in areas where it is progressing inadequately. To unravel the basic mechanism involved in extracellular matrix-endothelial cell interactions during angiogenesis, angiogenesis was mimicked *in vitro* by a three-dimensional angiogenesis model, as described above.

This thesis investigates whether the structure, the composition and the cross-linking of the fibrin matrix is a significant modulator of the angiogenic process in fibrin and in fibrinous exudates. This was investigated by manipulating the fibrinous matrix used in the *in vitro* angiogenesis model. The fibrin matrix was altered by physicochemical means, by biological agents, such as heparins and a fibrin matrix formed from a congenital dysfibrinogenemia was used. The interaction of endothelial cells with fibrin plays a pivotal role in angiogenesis in a fibrin and fibrinous matrix. Therefore, we have surveyed the current data on this interaction in chapter 2.

In the third chapter, the fibrin structure was physically modulated by altering the pH of the polymerisation buffer during coagulation. Polymerisation conditions determined the structure of the fibrin network formed. Low pH favoured the formation of an opaque matrix consisting of thick fibrin bundles in a porous, plasmin-sensitive network, while high pH favoured the formation of a transparent matrix, composed of thin fibrin bundles in a fine, tight and rigid network (Ferry and Morisson 1947).

The influence of the fibrin network architecture on the initiation, progression and stability of the capillary-like tubular structure formation was evaluated.

In the fourth chapter, the fibrin structure was changed by adding biologically active agents, such as heparins, during the polymerisation of the fibrin network.

In the fifth chapter the importance of $\alpha_v\beta_3$ on cell adhesion and *in vitro* angiogenesis was studied by using fibrinogen from a newly identified dysfibrinogenemic patient. The fibrinogen of this patient, called fibrinogen Nieuwegein, lacks a large part of the carboxyl-terminus of the Aα-chain of the molecule. This part of the molecule harbours an RGD-sequence, which is involved in integrin binding. The effect of this mutation on the formation of tubular structures in a fibrin matrix was evaluated. The deleted part of the fibrinogen molecule is also involved in cross-linking of the fibrin monomers by tissue transglutaminase, and in a much slower extent the cross-linking by factor XIIIa. Firstly, the effect of cross-linking of the fibrin matrix by tissue transglutaminase and factor XIIIa on the outgrowth of capillary-like tubular structures was evaluated. In this view, the effect of the absence of the cross-linking site in fibrinogen Nieuwegein was investigated.

In chapter six the presence of MMPs in capillary-like tubular structures was investigated. *In vitro* endothelial cells produce several components of the MMP-system, which are dependent upon plasmin to acquire activity. In the context of a possible contribution of MMPs in the ingrowth of capillary-like tubular structures in a fibrin matrix, we evaluated the effect of plasminogen-depletion on the formation of capillary-like tubular structures in our *in vitro* model

Finally, because u-PA and u-PAR are required in the angiogenic response in fibrin, we have evaluated the effect of thrombin, the protease involved in fibrin formation, on the availability of cell-bound u-PA in human endothelial cells.

Together these data provide evidence that the structure of the fibrin matrix is an important determinant for the rate of ingrowth and stability of capillary-like tubular structures in a fibrin matrix.

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chapter II

Interaction of endothelial cells with fibrin

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Introduction

Fibrinogen is a native plasma protein secreted by the liver reaching a concentration of 2 to 4 mg/ml. Cells in contact with the blood flow, such as platelets, leucocytes and endothelial cells are continuously exposed to fibrinogen. Fibrinogen present in the plasma plays an important role in facilitating platelet-platelet interactions during thrombus formation, endothelial cell-leucocyte interactions during inflammation, and endothelial cell-platelet interaction in haemostasis.

At sites of vascular injury or leakage, induced by tumours or inflammation, fibrinogen is converted in fibrin. Fibrin forms the cohesive network of haemostatic plugs and thrombi, and it provides an initial temporary matrix to support healing. Fibrin harbours specific features for adhesion and activation of endothelial cells. Fibrin formed at the luminal side of the endothelium activates endothelial cells, which causes the rapid release of several proteins and other products. When fibrin is formed at the basolateral side of the cells, it serves as a temporary matrix to adhere to, proliferate on and migrate into.

Interaction of endothelial cells with a fibrin matrix

Vascular injury disrupts the integrity of the vessel wall, leading to the exposure of the subendothelial matrix and the deposition of fibrin on the exposed thrombogenic surface. During fibrin formation new epitopes become exposed on the fibrinogen molecule that facilitate cell interaction. Binding of fibrinogen to artificial surfaces also causes exposure of these neo-epitopes by partial denaturation of the parent molecule. This should be taken into account when interpreting data on the interaction of endothelial cells with fibrinogen. Fibrin and fibrinogen-coated surfaces induce endothelial cell attachment, spreading and migration [1, 2, 3] and supports angiogenic responses. During tissue repair endothelial cells invade in the fibrinous matrix and interact with the fibrin network by different types of cellular receptors.

Four (super)families of cell adhesion receptors have been identified: integrins, members of the immunoglubulin-like superfamily, cadherins and selectins. In addition proteoglycans can be involved in such interactions. Interaction of cells with matrix proteins occurs via selective interaction of one or more of these cellular receptors with specific domains on the matrix molecules to which the cell adheres. Adherence and invasion of endothelial cells (in)to fibrin involves different cellular receptor molecules, namely the integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$, the proteoglycan CD44 and VE-cadherin. While the $\alpha_5\beta_1$-integrin and VE-cadherin are present in non-activated endothelial cells *in vivo*, the expression of the α_v-integrins and CD-44 is induced by exposure to inflammatory mediators, such as TNFα. The following sections describe these receptor molecules in more detail.

The vitronectin receptor and other α_v-integrins

Localisation and expression of $\alpha_v\beta_3$

$\alpha_v\beta_3$ belongs to the family of integrin receptors. Integrins are non-covalent linked heterodimers consisting of an α-unit and a β-unit. The family comprises 15 α and 8 β-units, which can heterodimerise in more than 20 combinations. Integrins bind RGD-sequences in different proteins, such as vWF, fibrinogen, vitronectin laminin and collagen, and binding can be inhibited by RGD-containing peptides. This suggests that binding is mediated by the common mechanism involving the broadly distributed family of membrane-associated glycoproteins that selectively recognise RGD-sequences in a variety of proteins. Different integrins can recognise the same protein and one integrin can bind more than one ligand [4].

The ligand binding pocket is composed of segments in the NH_2-terminal part (amino acids 139-167) and the cation binding sites (amino acids 312-349) in of the α_v-subunit and the midsegment of the β_3-subunit (amino acids 61-203) [5].

$\alpha_v\beta_3$ is a two-chain protein, which is synthesised as a single precursor. Prior to the processing of the oligosaccharide chains in the Golgi apparatus, the α– and β-chains form a complex. The $\alpha_v\beta_3$ is a heavily glycosylated transmembrane receptor. In the α-subunit there are 13 possible N-glycosylation sites present, while the β-subunit contains 6 possible N-glycosylation sites. The α-subunit is composed of 1018 amino acids of which 860 are located in the heavy chain (120 kDa) and 158 amino acids are located in the light chain (25 kDa). The heavy chain is located extracellularly and the light chain of 25 kDa has a transmembrane region and a 32 amino acid long cytoplasmatic tail [6]. Disulphide bridging occurs between neighbouring cysteines, the heavy chain contains 15 cysteines while the light chain contains only 3 cysteines. The β-subunit is composed of 762 amino acids of which 90% is localised extracellularly. The β-subunit has four cysteine rich tandem repeats of 40 amino acids, a single transmembrane sequence and a cytoplasmatic tail of 50 amino acids [6]. In the cytoplasmatic tail two potential tyrosine phophorylation sites, possibly used for integrin signalling, and a NPLY sequence influencing integrin activation are present.

$\alpha_v\beta_3$ is expressed by different vascular cells, such as smooth muscle cells, fibroblasts, leucocytes and tumour cells. On endothelial cells the expression and behaviour is dependent on the origin of the cells. On cultured HUVEC $\alpha_v\beta_3$ is present not only at the luminal side of the cells but also at their abluminal side and in the cell-cell and cell-matrix contacts [2, 7, 8, 9, 10, 11, 12]. $\alpha_v\beta_3$ is localised in the adhesion plaques of endothelial cells seeded on fibrinogen [13, 14], vitronectin and von Willebrand factor [15]. It not only localises in focal attachment sites but also at cell-cell contacts [10, 16]. The distribution of the antigen is regulated by the extent of reciprocal contacts among the cells. This antigen associates with the ventral adhesion sites when cells are not in close contact and plays a role in cell-cell adhesion when they are in close contact [7]. In non-confluent endothelial cells $\alpha_v\beta_3$ displays a discrete well-

organised distribution at the ventral side of the membrane, corresponding to the cell-adhesion structures. It colocalises with vinculin-rich focal contacts found at the insertions of the microfilamentous f-actin bundles of the stress fibre type. In a confluent monolayer stress fibres and focal attachment sites disappear, and the $\alpha_v\beta_3$ is localised at cell-cell contacts, representing the actin-rich peripheral reciprocal contacts between adjacent cells.

Binding of matrix components

The vitronectin receptor or the $\alpha_v\beta_3$ receptor on endothelial cells can interact with fibrin as well as surface-bound fibrinogen [2, 8, 9, 17, 18]. This receptor binds to vitronectin [19, 20] and other matrix proteins, such as vWF [8, 9, 18, 21, 22], fibronectin [8, 9, 10, 18, 23], thrombospondin [24], tenascin [25], laminin [26], osteopontin and degraded collagen [27]. Other molecules than matrix components, such as basic fibroblast growth factor (bFGF) and prothrombin can interact with $\alpha_v\beta_3$ [28, 29]. Furthermore, the non-catalytic site of active matrix metalloproteinase-2 adheres to $\alpha_v\beta_3$, which directs the collagenolytic activity directly to the cell surface and facilitates pericellular degradation. This pericellular degradation can be inhibited by PEX, a breakdown fragment of MMP-2 [30, 31].

The affinity of $\alpha_v\beta_3$ for a specific ligand is determined by the lipid composition of the plasma membrane of the cells. By this means a promiscuous receptor can have specific ligand binding affinity dependent upon the state of the cell [11].

The binding of endothelial cells to fibrin is specific, time and concentration dependent. Fibrin binding is partially mediated by an RGD mechanism since RGD-containing peptides and snake venoms can inhibit the binding dose-dependently, to a maximum of 75%. Monoclonal inhibiting antibodies against $\alpha_v\beta_3$ also can only partially inhibit binding of endothelial cell to fibrin. This suggests that not only $\alpha_v\beta_3$, but also other receptors are involved. During the conformational changes of fibrinogen to fibrin other neo-epitopes than Aα 572-574 and other RGD sequences might be exposed, which modulate the attachment of endothelial cells [17].

Role of $\alpha_v\beta_3$ in developmental angiogenesis

During blood vessel development $\alpha_v\beta_3$ is expressed on the surface of angioblasts and addition of blocking monoclonal antibodies disrupt the normal pattern of blood vessel development. In this process the ligation of $\alpha_v\beta_3$ to the surrounding matrix seems to be critical for cell differentiation and vessels maturation. Blocked cells remain in clusters and round, lacking the normal cellular protrusions. This leads to impairment of lumen formation and vessel patterning [32]. Although $\alpha_v\beta_3$ blocking studies have pointed to a major importance of this receptor in angiogenesis, the deletion of the β_3 gene does not seem to affect vascular developmental formation but induces thrombotic problems

resembling Glanzmann's disease [33]. Deletion of the α_v did not inhibit early vasculogenesis and angiogenesis. However, it impaired proper vascular development at late gestation, although some mice are born viable [34]. The small percentage of viable mice die soon after birth. This is due to extensive intestinal and brain blood vessel abnormalities, leading to massive haemorraghes. Other organs have a normal vasculature, which suggests that other receptors mediate vessel formation in the absence of α_v-integrins.

Role of $\alpha_v\beta_3$ in repair-associated and pathological angiogenesis

Angiogenesis participates in the progression of angiogenesis-dependent diseases, such as tumour growth and metastasis, proliferative diabetic retinopathy, rheumatoid arthritis and hypoxia-induced retinal neovascularisation. After tissue injury, granulation tissue, which is composed of capillaries invading the temporary fibrin matrix, is formed. In resting vessels endothelial cells do not express this receptor, while endothelial cells present in newly formed vessels have a high expression of $\alpha_v\beta_3$ [35, 36, 37, 38, 39, 40, 41, 42, 43]. Focal ischaemia, induced by ligation of the middle cerebral artery, increases the expression of $\alpha_v\beta_3$ in the ischemic area where fibrin deposition also occurs [44]. Not only $\alpha_v\beta_3$ is enhanced during angiogenesis in an hypoxic retina, but also the expression of $\alpha_v\beta_5$ is increased [45].

During angiogenesis silent endothelial cells become activated to an angiogenic phenotype. This switch can be induced by growth factors, usually in interplay with other cytokines. These angiogenic growth factors often induce ETS-1, a transcription factor, which is a principal regulator involved in the conversion of endothelial cells to the angiogenic type. ETS-1 is involved in the regulation of gene-expression of MMP-1, MMP-3 and MMP-9, giving the endothelial cells an invasive capacity. Furthermore, ETS-1 upregulates the expression of the β_3-gene [46].

Ligation of endothelial cells to the surrounding matrix is a prerequisite for endothelial cell survival and for the maturation of newly formed vessels. Ligation of $\alpha_v\beta_3$ not only adheres the endothelial cell to the surrounding matrix, but also activates cellular signalling pathways that are involved in cell survival. Ligation of $\alpha_v\beta_3$ suppresses p53 blocked p21WAF/CIP1 pathway and increases the Bcl2/Bax ratio promoting survival [49]. Inhibition of endothelial cell attachment to its surrounding matrix induces apoptosis [47, 48, 49, 50]. It results in the activation of p53 and the Bax cell death pathway [51]. The integrin-mediated cell survival also involves regulation of nuclear factor kappa B, NFκB [52]. Ligation of β3 induces an increase in NFκB activity. This activation depends on the small GTP-binding protein RAS and the tyrosine kinase SRC. Finally, integrins can activate the RAS/MAP kinase pathway. During the initial process of angiogenesis induced by bFGF on the choroid allantois membrane of the egg (CAM), mitogen activated protein kinase (ERK) activity is induced which is refractory to integrin antagonists, whereas the sustained activation depends on the ligation. Inhibition of this ligation by $\alpha_v\beta_3$

antagonists blocked the ERK activation and the formation of new capillaries [53]. These data indicate that in a number of conditions $\alpha_v\beta_3$ plays a pivotal role in angiogenesis and cell survival.

The specific and selective expression pattern of $\alpha_v\beta_3$ in vessels during the process of angiogenesis makes this molecule a possible target for the development of inhibitors of angiogenesis. The binding of $\alpha_v\beta_3$ to its substratum can be blocked either by administration of inhibiting monoclonal antibodies against $\alpha_v\beta_3$ or by peptides containing RGD-sequences. RGD-peptides are used to inhibit adhesion, migration and angiogenesis, since RGD is a general motif found in many ligands. It should be noted that RGD-peptides not only induce apoptosis by inhibiting endothelial cell attachment, but they can also enter the cell and bind directly to a potential RGD-binding motif in procaspase-3. This induces a conformational change and leads to autoprocessing and enzymatic activation of procaspase-3, one of the death executors of the apoptosis pathway [54].

In different animal models angiogenesis can be inhibited by the administration of RGD-containing peptides as well as by the administration of monoclonal antibodies, without affecting the existing blood vessels [39, 40, 45, 48, 55, 56, 57]. Since the endothelium is relatively easy accessible for gene-therapy, specific antibodies targeting the upregulated receptors can be delivered to the activated endothelium [58]. New desintegrins, such as Accutin, an antagonist of $\alpha_v\beta_3$, are developed. Accutin not only blocks adhesion of HUVEC to surface-coated fibrinogen, fibronectin and vitronectin, but also inhibits dose-dependently tube-formation in matrigel and angiogenesis in the CAM-assay by inducing apoptosis [59].

A major drawback of anti-angiogenesis strategies is that repair-associated angiogenesis may also be inhibited. Indeed, angiogenesis during the formation of granulation tissue can be inhibited by antibodies against $\alpha_v\beta_3$ [35, 42] and/or $\alpha_v\beta_5$ [47].

The unique expression pattern of $\alpha_v\beta_3$ has resulted in the development of a novel non-invasive diagnostic techniques to identify neovessels. Magnetic resonance can be used to visualise a paramagnetic contrast agent, which is coupled to LM 609©, a monoclonal antibody directed against $\alpha_v\beta_3$ [60].

The role of $\alpha_v\beta_3$ in the formation of capillary-like tubular structures in three-dimensional matrices *in vitro* remains controversial. Endothelial cells plated on a matrigel matrix form capillary-like structures, which can be inhibited with polyclonal antibodies against $\alpha_v\beta_3$ [61]. While Shue et al reported that this process can be inhibited with antibodies against $\alpha_v\beta_3$, other authors reported that the formation of capillary-like tubular structure formation in a fibrin matrix could not be inhibited [62, 63, 64]. It is of interest to note that, while an anti-$\alpha_v\beta_3$ and $\alpha_v\beta_5$ antibodies did not affect the formation of capillary-like tubular structures, the RGD-containing snake venom echistatin strongly inhibited this process (our unpublished results). This suggests that the antibodies were unable to react in the fibrin matrix or that other RGD-binding integrins were involved.

Localisation and expression of $\alpha_5\beta_1$

$\alpha_5\beta_1$ is the major fibronectin receptor on endothelial cells [10, 13, 14, 65]. It is an RGD-binding receptor [14, 65], which is structurally and functionally related to the fibronectin receptor isolated from placenta. It is a heterodimer composed of a 150 kD and a 125 kD polypetide [14].
This receptor is localised in the focal adhesion sites at the basolateral side of endothelial cell, colocalising with vinculin [14]. In addition it is also encountered at the luminal side of the cell, similarly as $\alpha_v\beta_3$ [11]. Adhesion of endothelial cells to fibronectin induces protein tyrosine phosphorylation of focal adhesion tyrosine kinase p125FAK. This results in the assembly of focal adhesions and actin fibres [66] and increases the level of cytoprotective Bcl-2 protein [67]. Inhibition of endothelial cell adhesion to fibronectin by a fibronectin-derived peptide induces apoptosis in these cells [67, 68].

Binding of $\alpha_5\beta_1$ to fibrinogen

The integrin $\alpha_5\beta_1$ is a major integrin on human microvascular endothelial cells [69]. Although a role in fibrin/fibronectin binding complexes is likely, little information is available at present regarding the role of $\alpha_5\beta_1$ in endothelial cell binding to fibrin. Studies on the interaction of fibrinogen with endothelial cells suggest such an interaction.
Endothelial cells in suspension bind to fibrinogen with $\alpha_5\beta_1$ [70], which is the major receptor for fibronectin [71]. This binding is supported by the presence of Mn^{2+}, but is inhibited by the presence of Ca^{2+}. The binding site in fibrinogen for $\alpha_5\beta_1$ is the RGD-sequence which is localised in the carboxyl-terminus Aα 572-574. $\alpha_5\beta_1$ and $\alpha_v\beta_3$ bind to the same side in fibrinogen, but under different circumstances. When fibrinogen is present as an immobilised substratum, $\alpha_5\beta_1$ can recognise it in the presence of Ca^{2+}. The binding of endothelial cells to immobilised fibrinogen can be blocked by antibodies against $\alpha_5\beta_1$ and $\alpha_v\beta_3$, but complete inhibition was only established by both antibodies and by RGD-containing peptides. This suggests that other RGD-containing integrins such as $\alpha_v\beta_1$ and $\alpha_v\beta_5$, which are also present on HUVEC, are potential candidates for fibrinogen binding [72].
It is of importance to determine if $\alpha_5\beta_1$ distinguishes between soluble fibrinogen, immobilised fibrinogen and if it binds fibrin.

The role of $\alpha_5\beta_1$ in angiogenesis

Integrin binding to fibrinogen and fibrin does occur in different circumstances. During wound healing integrin-expressing endothelial cells are in contact with fibrinogen and fibrin present on the exposed subendothelium. By binding and migrating over the fibrin(-ogen), endothelial cells can easily and quickly recover this surface. A temporary fibrin matrix, which is formed after vascular injury, is not only composed of fibrin but also of other plasma proteins, such as fibronectin. Endothelial cells can use

different integrins to adhere to and migrate in the matrix. $\alpha_5\beta_1$ may facilitate migration by binding as well to fibronectin as fibrin(-ogen). This mechanism of multiple ligand recognition is also known for $\alpha_v\beta_3$, which can bind vitronectin, fibrin, and other matrix proteins.

The β1-integrin plays a role in the formation of blood vessels in the embryo. The formation of a complex vasculature in beta-1 null embryoid bodies is significantly delayed and of poor quality [73]. To what extent β1-integrins are involved in pathological angiogenesis is poorly investigated. However, as indicated above, it is likely that $\alpha_5\beta_1$ integrins contribute to the interaction of endothelial cells with fibrin.

Similar as for $\alpha_v\beta_3$, $\alpha_5\beta_1$ may trigger an intracellular signalling pathway. The extracellular matrix controls the sensitivity of endothelial cells for growth factors and cytokines [74]. Interaction of the cell with its matrix by integrins provide a means to translate information from the matrix into the cell by integrin-mediated cell signalling.

The CD44-related chondroitin sulfate proteoglycan

During angiogenesis in the formation of granulation tissue and tumor stroma, the migrating endothelial cells increase the synthesis of proteoglycans [75, 76]. This increased synthesis is correlated with cell proliferation, migration and collagen synthesis [77]. This synthesis returns to basal levels once migration has ceased.

CD44 is implicated in cell-cell as well as in cell-substratum interactions, suggesting a role in adhesion and migration [78]. It is a cell surface receptor, which interacts with extracellular matrix proteins and participates in cell migration and invasion. CD44 is a major surface glycoprotein, produced by numerous cell types, including endothelial cells [79]. It contains an extracellular domain, binding a variety of extracellular matrix proteins, such as the glycosaminoglycan hyaluronan [78, 80, 81, 82], fibronectin [83, 84], collagen type I [84, 85] and fibrinogen [86], a transmembrane domain and a cytoplasmatic domain. It is associated with the cytoskeleton [87, 88, 89]. The intracellular domain of CD44 binds to GTP, thereby playing an important role in promoting interaction between CD44 and the cytoskeleton. Chondroitin sulphate proteoglycans is covalently coupled to the extracellular domain of the core CD 44 protein [85, 90].

CD44 antibodies inhibit the invasion of endothelial cells into a fibrin matrix. This suggests that it directs migration into a fibrin matrix. A coordinate interaction of different receptors on endothelial cells for binding to fibrin is suggested by the fact that CD44 antibodies could only inhibit 50% of the binding to fibrin [86].

VE-cadherin

Localisation of VE-cadherin

VE-cadherin receptor is an endothelial specific adhesive transmembrane protein, which belongs to the family of cadherins. It is organised in clusters at cell-cell contacts and connects cells with a complex network of cytoskeletal proteins via their cytoplasmatic domain [91] to strengthen cell-cell adhesion and transfer signals.

The cadherins form dimers, which associate with dimers on the opposite cell. The extracellular domain of VE-cadherin consists of five homologous repeats of 110 amino acids, which have Ca^{2+} binding sites. It mediates Ca^{2+}-dependent homophilic interaction between adjacent cells. Inside the cell it associates via its short cytoplasmatic region with three armadillo proteins, β-catenin, plakoglobin and P120, which links the receptor to the actin cytoskeleton [92].

Adherens junctions can transfer signals between adjacent cells in different ways. Kinases, such as src, lyn and yes, tyrosine phosphatases, components of the Ras signalling pathway, and small GTP-binding proteins are localised closely together at these cell-cell contacts, thereby facilitating their reciprocal interaction. The adherens junctions also control the levels of β-catenin and plakoglobin, which can act as signalling molecules when released into the cytosol and translocated to the nucleus. These proteins bind transcription factors and thereby control gene transcription [93]. Also, when endothelial cells attach to one another, signalling occurs through the release of growth factors and cytokines from subendothelial matrix.

The formation of adherence junctions is dependent on the functional state of the cells, the cadherin-catenin complex is highly dynamic and its composition changes rapidly. In highly confluent endothelial cells the majority of VE-cadherin, β-catenin and p120 molecules are unphosphorylated and VE-cadherin is associated with plakoglobin and the actin skeleton at cell-cell contacts. The association with p120 and β-catenin is reduced in the cadherin complex. In migrating endothelial cells VE-cadherin is highly tyrosine phosphorylated and linked to P120 and β-catenin, which are also highly phosphorylated [94, 95].

Not only in the transition of resting cells to migratory cells the composition of the junctions alters, but also during an increase in permeability and shear stress. Endothelial cells submitted to an increased shear stress exhibit profound changes in cell shape, requiring disassembly/assembly of adherens junction mediating cell-cell adhesion. Under static conditions the staining for VE-cadherin, α-catenin, β-catenin and plakoglobin is intense and peripheral forming a continuos band around the cells, near cell-cell junctions [96, 97].

Binding to fibrin

Several reports suggest that endothelial cells interact with the Bβ 15-42 sequence in fibrin, which not only allows binding but also triggers fibrin-mediated responses in endothelial cells [98]. Binding of endothelial cells can be prevented by the β 15-42-peptide coupled to ovalbumin and by antibodies against VE-cadherin, but not by RGDS or antibodies against the known integrins [62].

Role in developmental angiogenesis

The synthesis of VE-cadherin at the time of endothelial cell differentiation and its ubiquitous distribution along the vascular tree suggest a role in determining the correct organisation of the vasculature [99]. Endothelial cells differentiating *in vitro* from embryoid bodies, in which the VE-cadherin gene was deleted, are indeed capable of forming cell aggregates but they can not form correct vascular structures [100]. Deletion of the VE-cadherin during mouse development does also not impair assembly of endothelial cells in vascular plexi, but has a profound influence on the remodelling and maturation of the plexus [101, 102]. This points to an important role in the later steps of angiogenesis. It suggests that cadherins play an important role in cell-cell recognition and tissue segregation.

Role in adult angiogenesis

To form capillary-like tubular structures endothelial cells detach from the existing vessel, invade the surrounding tissue and form tubes, branch and organise into anastomic networks. During the first steps the endothelial junctions need to become looser to allow migration and proliferation. VE-cadherin needs to be phosphorylated on tyrosine residues, upon which it detaches from the cytoskeleton and plakoglobin. This can be induced by several growth factors, such as VEGF-A, epithelial growth factor and hepatocyte growth factor, and probably others [103]. Placental growth factor, which only binds to VEGFR1 (flt-1) can not alter the junctions, suggesting that this VEGF-mediated process involves VEGFR2. When the vascular network is formed, migration and proliferation need to cease by establishing cell-cell contacts [104]. The intercellular junctions engage VE-cadherin of which the intercellular domain interacts with cytoplasma proteins (catenins). These catenins contribute to the anchorage of VE-cadherin to the actin cytoskeleton and transmit the adhesion signal. Seeding of cells onto wells coated with the N-terminal VE-cadherin fragments inhibits proliferation, suggesting that VE-cadherin homotypic engagement at the junctions participates in density dependent inhibition of cell growth [105]. Truncation of the cytoplasmatic domain of VE-cadherin, which interacts with catenins, does not affect the binding of cells but impairs the inhibition of proliferation [104].
VE-cadherin is essential for tube formation and maintenance of the architecture of a formed capillary-like network in an underlying fibrin matrix and in other matrices such as matrigel and collagen.

Endothelial cells in contact with fibrin at their apical surface develop into capillary-like tubular structures [106] via the interaction with the Bβ 15-42 domain of fibrin. This domain is exposed after cleavage of fibrinopeptide B from fibrinogen by thrombin [107]. Antibodies against VE-cadherin, β 15-42-peptides coupled to ovalbumin and gamma linolic acid (which prevents the formation of VE-cadherin and catenin complexes) impair capillary-like tubular structure formation, suggesting that the interaction of the receptor with fibrin is important [62, 108]. The architecture of formed capillary-like tubular structures in different matrices is disrupted by the addition of antibodies against VE-cadherin [62, 108]. It should be noted that addition of VE-cadherin antibodies also impairs tubular structure formation and architecture in a matrigel and collagen matrix. This suggests that other mechanisms than interference with fibrin binding are involved [62, 109, 110].

Addition of VE-cadherin antibodies during the process of angiogenesis and after a network has been formed probably leads to disruption of cell-cell contacts and induces apoptosis of endothelial cells. Indeed, VE-cadherin mediated signalling is important in transmission of endothelial survival by VEGF-A to Akt kinase and Bcl-2 via VEGFR-2, which forms complexes with β-catenin, VE-cadherin and phosphoinositide 3-kinase. Deletion of VE-cadherin abolishes transmission of the VEGF survival signal, suggesting that the VE-cadherin/β-catenin complex is important in controlling endothelial cell survival [110]. Initiation of apoptosis correlates with cleavage and disassembly of intra- and extracellular components of the adherence junctions. Subsequently, membrane blebbing, cell rounding and loss of cell-cell and cell-matrix components occurs. Extracellularly located VE-cadherin is shedded by proteolytic cleavage by metalloproteinases, which leads to a dramatic decrease of the surface-located VE-cadherin. Intracellularly localised caspases, such as CPP32 and Mch2, proteolyticaly process β-catenin, plakoglobin and α-catenin. As a consequence β-catenin can not bind α-catenin anymore, disrupting not only the adherens junction but also interrupting signalling required for cell survival [111]. Increased permeability of an endothelial cell monolayer can be induced by antibodies against VE-cadherin [110, 112, 113, 114] or by VEGF [103, 115]. Both induce a reorganisation of the actin cytoskeleton, loss of junctional VE-cadherin from the interendothelial cell junctions and loss of cell-cell adhesion, resulting in an increased permeability. Destruction of the homotypic interactions between the extracellular domains of VE-cadherin by antibodies induces a rapid resynthesis of VE-cadherin to restore the endothelial cell-cell contact, establishing a critical role for VE-cadherin not only in the maintenance but also the restoration of the monolayer integrity.

Endothelial responses mediated by fibrin Bβ 15-42

To better understand the interactions, which occur between endothelial cells and fibrin at sites of vascular injury, and to characterise the responses that were induced by these interactions, fibrin surfaces exposing different epitopes have been prepared. Fibrinogen can be converted to fibrin by different clotting enzymes. Thrombin cleaves of fibrinopeptide A (FPA, Aα 1-16) from the Aα-chain of

fibrinogen at position Arg16-Gly17, as well as fibrinopeptide B (FPB, Bβ 1-14) from the Bβ-chain of fibrinogen at position Arg14-Gly15, generating two new amino termini. Reptilase or the snake venom ancrod only releases FPA. This results in the formation of fibrin without FPB cleavage. Thrombin-formed fibrin is called fibrin II, Reptilase formed fibrin is fibrin I. Contortrix is another venom which only removes FPB. Specific proteolytical cleavage of fibrinogen by protease III, a snake venom, removes Bβ 1-42, forming fibrinogen$_{325}$. This can be used to form fibrin with either clotting enzyme. These structurally different fibrin matrices, as well as fibrin formed from patients suffering from dysfibrinogenemia can be used to study fibrin-endothelial cell interactions.

The structural requirement for fibrin to activate endothelial cells is the exposure of an heparin-binding domain after cleavage of FPB. The conformational changes induced by cleaving FPB exposes hidden amino acids sequences important in modulating specific cellular responses. In their native conformation β 15-42 sequences are not available for binding to endothelial cells. Thrombin-induced exposure of β 15-42 is required for the binding by a heparin-dependent, RGD-independent mechanism at the new NH_2-terminus of fibrin [107]. Bach et al reported that on endothelial cells the interaction with this region is mediated by VE-cadherin [62]. This specific structural feature of the temporary fibrin matrix formed at sites of vascular injury modulates the responses of the endothelial cells. This property is counteracted by plasmin, which cleaves off this specific sequence.

Induction of migration, spreading, proliferation and formation of capillary-like tubular structures by fibrin

Spreading of endothelial cells on a fibrin matrix is mediated by a different mechanism than binding of endothelial cells. Spreading requires initial adhesion through an integrin, which is followed by cytoskeletal reorganisation. Stress fibber bundles composed of actin filaments are formed. They are connected to the plasma membrane via cytoplasmatic proteins, which bind to the intracellular segment of the integrin receptor located at the focal contacts between the cell and the matrix. Subsequently changes occur such as extrusion of laemellipodia, relocation of the centrosome and polarised elongation of the cell towards the site of injury.

A fibrin matrix prepared by Reptilase, which only cleaves of fibrinopeptide B, supports endothelial cell adhesion but not spreading, while a matrix prepared by thrombin supports both adhesion and spreading. Fibrinogen$_{325}$, lacking the amino acids 1-42 of the fibrinogen Bβ-chain clotted with either thrombin or Reptilase is unable to support spreading. This suggests that the cleavage of FPB (amino acids 1-14) and the subsequent exposure of amino acids 15-42 are essential for spreading [116].

Endothelial cells can bind to the amino acids 15-42 in the Bβ-chain of fibrin with a non-integrin 130 kDa receptor [117]. Recently this receptor has been characterised as VE-cadherin [62].

Endothelial cells exposed to fibrinogen also undergo spreading [7, 10]. The endothelial cells interact with fibrinogen through $\alpha_v\beta_3$, and spreading occurs. This process requires the synthesis and secretion

of fibrinonectin as well as the clustering of $\alpha_5\beta_1$ in the focal adhesion [7]. Spreading of endothelial cells on a fibrinogen matrix is not dependent on the binding of amino acids Bβ 15-42 since fibrinogen$_{325}$ supports the spreading of cells. The spreading depends on the RGD-recognition specificity and the interaction of $\alpha_v\beta_3$, since antibodies against the integrin can block the spreading [7]. The discrepancy between the mechanisms for spreading on a fibrinogen and a fibrin matrix can be explained by the fact that coated fibrinogen undergoes conformational changes exposing epitopes which are not accessible in soluble fibrinogen and in fibrin still containing FPB [118].

Endothelial cells which are present in a monolayer and are exposed to an overlaid fibrin clot become rapidly disorganised [119]. The contact of endothelial cells with fibrin at their apical surface induces the formation of capillary-like tubular structures via the interaction of fibrin β 15-42 with a receptor on endothelial cells [106]. The interaction of fibrin with endothelial cells via this receptor, probably VE-cadherin [62], may contribute to the mechanism through which fibrin stimulates angiogenesis [120].

Endothelial cells seeded on a fibrin II matrix undergo proliferation [121]. This does not occur on fibrin I matrices or on fibrin matrices prepared from fibrinogen$_{325}$, suggesting that in this process the binding of endothelial cells to the amino terminal region, which is exposed after FPB cleavage, determines the ability to support proliferation. Interaction of endothelial cells with their matrix via integrins is not sufficient to induce proliferation.

Fibrin acts as a physiological secretagogue for endothelial cells

Endothelial cells interact with Bβ 15-42 of fibrin, which induces the secretion of several products. Endothelial cells exposed to fibrin rapidly release vWF from the Weible-Palade bodies. Occupancy of endothelial receptors by unclotted fibrinogen is not sufficient to induce the release. A structural motive present in fibrin interacts with an endothelial cell receptor [117, 122, 123]. The exposure of Bβ 15-42 is a prerequisite for the response, since clotting of fibrinogen with thrombin but not Reptilase supports the release. The Bβ 15-42 interacts directly with endothelial cells, because peptides containing this sequence can inhibit the release response [117]. This release can also be induced by peptides corresponding to Bβ 15-42, albeit less efficient. The required conformation for interaction is probably better stabilised in complete, intact fibrin, or maximal release requires cross-linking on receptors, which can only be mediated by the complete fibrin.

Early in the process of fibrinolysis the carboxyl-terminus of the Aα-chain (which binds $\alpha_v\beta_3$) as well as the Bβ-chain (containing Bβ15-42) are removed. The carboxyl-terminal region of the Aα-chain supports binding of endothelial cells, while the carboxyl-terminus of the Bβ-chain induces proliferation, spreading and vWF release from these cells. Plasmin cleavage of the carboxyl-terminus of the Bβ-chain does not majorly contribute to the mechanical disruption of the fibrin gel, but downregulates the fibrin-induced responses in endothelial cells.

Fibrin also specifically induces a time and concentration dependent release of tPA from endothelial cells [124, 125, 126, 127, 128]. The cleavage of FPB from fibrinogen is also a prerequisite for the induction of tPA release and synthesis. This suggests that this response, as for the secretion of vWF, is mediated by the interaction of endothelial VE-cadherin with Bβ15-42 in fibrin.
In addition, exposure of the endothelium to fibrin leads to a concentration and time dependent decrease in the production of NO and NO synthetase, impairing vasodilatory responses. As a consequence, NO-dependent attenuation of platelet function is impaired [129]. Endothelium exposed to fibrin deposition also releases prostacyclin [125]. Interaction of the endothelial cells with Bβ 15-42 probably is a prerequisite, which suggests a common mechanism with vWF release. It is probably related to the increase in cytoplasmic Ca^{2+} that is induced by the Bβ 15-42 domain of fibrin [130]. This is in contrast with results from Chang et al [17, 131, 132], who state that interaction of endothelial cells with Reptilase formed fibrin induces an increased secretion of prostacyclin.
Several other effects of fibrin have been reported. The exposure of endothelial cells to fibrin *in vivo* and *in vitro* induces the surface-localised expression of tissue factor [133]. Endothelial cells exposed to fibrin secrete chemoattractant proteins such as IL-8 [134, 135,136] and upregulate the surface localised expression of ICAM-1 (intercellular adhesion molecule 1) [136]. Also fibrin rapidly induces a sustained synthesis and secretion of thrombospondin from endothelial cells [126]. It is presently unknown whether these latter effects are induced by Bβ 15-42 domain of fibrin or by other parts of the molecule.

Interaction of endothelial cells with plasma fibrinogen

Endothelial cells lining the inner wall of the vessel constitute a non-thrombogenic surface, which is continuously in contact with high concentrations of fibrinogen present in the circulation. Fibrinogen plays a role in mediating binding of different cell types to endothelial cells, an interaction which plays an important role in inflammation [137, 138, 139]. Inflammation is a component in repair-associated angiogenesis. Dysregulation of the leucocyte-endothelial cell interaction is an early event in the pathogenesis of vascular diseases such as atherosclerosis [119] and delayed wound repair.
On endothelial cells predominantly ICAM-1 and $\alpha_v\beta_3$ are involved in this mechanism, and in a lesser extent also GPIbα [140]. $\alpha_v\beta_3$ not only mediates the adhesion of activated monocytes and leucocytes to endothelial cells, but is also involved in the transmigration of these cells through the endothelium [141].

The vitronectin receptor, $\alpha_v\beta_3$

Binding of fibrinogen to endothelial cells

Fibrinogen binding to endothelial cells is saturable, time and concentration dependent, reversible and specific [1] with binding characteristics resemblig binding of fibrinogen to platelets. The binding characteristics are independent from stimuli which induce fibrinogen binding to platelets, suggesting that fibrinogen can bind to unstimulated cells and that the receptor is consitutively expressed. Initial binding studies have been performed with human umbilical vein endothelial cells which have about 55.000 binding sites per cell with a kDa of 0.23 μM for cells in suspension and 1.5 μM for monolayer cultures. *in vitro* differences in expression of the receptor in different cell types have been reported.
The pattern of recognition depends upon an RGD-recognition specificity, which can be completely inhibited by RGD-containing peptides or by mutation of the RGD-sequence in the ligands [20, 21, 142]. Fibrinogen contains three putative RGD adhesion sites, two sites in the fibrinogen Aα–chain, in the NH_2-terminus at position Aα 95-97 and in the COOH-terminus at position Aα 572-474, and one site in the COOH-terminus of the γ-chain at position 400-411. The interaction can be inhibited by a blocking monoclonal antibody against $\alpha_v\beta_3$ (LM 609©) and by RGD-peptides, suggesting that $\alpha_v\beta_3$ is involved. Studies with blocking monoclonal antibodies directed against either binding site in fibrinogen revealed that endothelial cells only adhere to the COOH-terminus of the Aα-chain [15, 143, 144]. Platelets bind to fibrin and fibrinogen via a homologous but distinct integrin on the COOH-terminus of the γ-chain (γ 400-411) so both cell types do not compete with one another for binding [145].
The limited interaction of endothelial cells with only the COOH-terminus of fibrin(ogen), which is abolished by the attack of plasmin, may represent a regulatory role of the fibrinolysis in endothelial cell binding. Mast cell tryptase, a 31 kDa glycosylated trypsin-like molecule stored in the granules of mast cells, not only renders fibrinogen inclottable by cleaving Bβ 21, but, by cleaving Aα 572, it also abolishes the binding of endothelial cells to fibrinogen [146]. Also dysfibrinogenemia Marburg, lacking Aα 572-574 has an impaired binding of endothelial cells [147].
The capacity of endothelial cells to bind soluble ligands, such as fibrinogen, is influenced by the activation state of the integrin. An activation of $\alpha_v\beta_3$ leads to a higher affinity ligand binding state [16, 148, 149].

Characterisation of the receptor

The fibrinogen binding endothelial cell surface molecule is immunologically related to the platelet glycoprotein GPIIbIIIa [8, 9, 10, 13, 14, 150] which is the fibrinogen binding receptor on platelets. The β-unit is identical to the GPIIIa, while the α-unit is only 34% homologous to the IIa subunit of the

platelet fibrinogen receptor [6, 118]. The receptor is identical to the vitronectin receptor first discovered on melanoma cells [8, 18].

$\alpha_v\beta_3$ present at the luminal side of the endothelial cell is diffuse and granularly localised [11]. This luminal located $\alpha_v\beta_3$ retains binds RGD-mediated fibrinogen and vitronectin, but only if these proteins are clustered on circulating cells or on beads, suggesting they have a role as bridging molecules between the endothelium and vascular cells in the bloodstream. This binding is probably mediated by a specific subpopulation of $\alpha_v\beta_3$, different from the receptors mediating cell adhesion, suggesting that there are two functionally distinct populations of $\alpha_v\beta_3$ on the endothelial cell [151].

The fibronectin receptor, $\alpha_5\beta_1$

Fibrinogen can directly bind to $\alpha_5\beta_1$ on endothelial cells [152], which is the major receptor for fibronectin [153]. The binding site for endothelial $\alpha_5\beta_1$ is localised in the fibrinogen Aα–chain at position 572-574, the same binding site as for $\alpha_v\beta_3$. The binding is mediated by the RGD-mediated binding mechanism of integrin receptors, as discussed above.

The ICAM-1 Immunoglobulin-like receptor

ICAM-1 is a single, transmembrane glycoprotein receptor belonging to the immunoglobulin-like cell adhesion receptor family expressed on different cell types, including endothelial cells and leucocytes [154]. Its expression is induced by inflammatory mediators, such as tumour necrosis factor (TNFα), interleukin-1 (IL-1) and interferon-gamma (IF-γ), shear stress and fibrin. ICAM-1 binds to membrane-bound integrin receptors such as LFA-1 and MAC-1 on leukocytes, CD43, hyaluronan and fibrinogen [155].

The binding of fibrinogen to ICAM-1 on endothelial cells is involved in mediating the binding of inflammatory cells. The first IgG domain of ICAM-1 on endothelial cells [156] binds to γ 119-133 in fibrinogen and the I-domain in the α-unit of MAC-1 on inflammatory cells [157] binds to γ 190-202 in the same fibrinogen [158, 159]. This interaction occurs at physiological concentrations of fibrinogen and is not RGD-mediated [160, 161]. MAC-1 interaction with fibrinogen is dependent upon activation of the receptor [162], but chemoactivated MAC-1 can also interact directly with its counter-receptor ICAM-1 on endothelial cells. The simultaneous interaction of fibrinogen with ICAM-1 on endothelial cells and MAC-1 on leucocytes increases the attachment by 5 times.

Intercellular bridging by fibrinogen is a pathway of leucocyte recruitment to the endothelium independently of the receptor/counter-receptor interaction and it is influenced by the unique topography of ICAM-1 expression on endothelial cells. ICAM-1 is expressed only on the apical, and not on the basolateral side, of endothelial cell protrusions of TNFα–stimulated cells, thereby favouring interaction of MAC-1 bound fibrinogen or β2-interaction. The first step in the adhesion process is the

binding of an activated MAC-1 receptor to fibrinogen, inducing a conformational change in the fibrinogen resulting in the exposure of the binding site for ICAM-1. This is a means of preventing aberrant activation of the adhesion cascade under physiological conditions, first of all by the necessity of an activated MAC-1 and the requirement of conformational change in the fibrinogen [163].
For the binding of fibrinogen on endothelial ICAM-1, an intact cytoskeleton is necessary, suggesting that stress fibres are required for binding [155]. ICAM-1 plays a central role in migration of activated leucocytes to sites of inflammation.
Fibrinogen induces tight adhesion of cells to postcapillary venules under low shear forces, which can be blocked by γ 119-133 and by antibodies against ICAM-1 [164].

Conclusion

Fibrin, which is formed after vessel injury and in areas of tumours, harbours specific features for endothelial cell adhesion and activation. To date, several molecules on endothelial cells have been characterised as fibrin receptors. They comprise the $\alpha_v\beta_3$- and $\alpha_5\beta_1$-integrins, CD44 related chondroitin sulphate proteoglycan and VE-cadherin. The integrins $\alpha_v\beta_3$- and $\alpha_5\beta_1$ bind to the same sequence in the Aα-chain of fibrin, namely Aα 572-574.
The role of $\alpha_v\beta_3$-integrin in angiogenesis has best been studied. Although dispensable in early development, $\alpha_v\beta_3$-integrin can play a role in adult pathological angiogenesis. This is indicated by the ability of $\alpha_v\beta_3$-integrin blocking agents to reduce angiogenesis in several angiogenesis models *in vivo*. Furthermore, $\alpha_v\beta_3$-integrin expression is enhanced in endothelial cells present in angiogenic vessels and during inflammation. It is generally believed that $\alpha_v\beta_3$-integrin can bind to fibrin by interacting with the RGD-sequence in the Aα-chain. However, as complex matrices are involved, it remains to be demonstrated that indeed this interaction plays a pivotal role. Conflicting *in vitro* data are available regarding the role of the $\alpha_v\beta_3$-integrin in the formation of capillary-like tubular structures in a fibrin matrix. In addition to a direct role in cell adhesion, the interaction between integrins and the fibrin matrix also cause cell activation by intracellular signaling. The contribution of $\alpha_5\beta_1$ in endothelial cells adhesion to and invasion into a fibrin matrix still needs further evaluation.
Fibrin not only serves as a passive adhesion matrix, but interaction of endothelial cells with fibrin activates these cells and triggers fibrin-mediated responses, such as induction of cell migration, proliferation and formation of capillary-like tubular structures. These reactions are not only mediated by the integrin-interaction, but also by the interaction between the Bβ 15-42 sequence in fibrin and VE-cadherin. This interaction further induces secretion of several products, which are involved in coagulation, fibrinolysis and attraction of inflammatory cells. This indicates that the fibrin matrix, which is only present during pathological conditions is more than a passive matrix, but can contribute to the subsequent healing process by inducing influx of inflammatory cells and by initiating its own degradation by upregulating fibrinolysis.

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CHAPTER III

Influence of the fibrin structure on the formation and maintenance of capillary-like tubules by human microvascular endothelial cells

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Abstract

Fibrin is a temporary matrix which not only covers a wound, but also provides a structure for invading cells during healing. Changes in the polymerisation conditions before gelation of the clot affect the structure of fibrin and thus might influence the interaction with invading cells. Therefore we tested whether changes in the fibrin structure influence the formation of capillary-like tubular structures by human microvascular endothelial cells (hMVEC) in an *in vitro* angiogenesis model. Opaque ^{125}I-fibrin structures prepared at pH 7.0; fibrin matrices at pH 7.4 and transparent ^{125}I-fibrin structures prepared at pH 7.8 were neutralised (pH 7.4) before seeding hMVEC on top of them in confluent density. Endothelial cells were stimulated with a growth factor (bFGF or $VEGF_{165}$) and a cytokine (TNFα) to induce the u-PA/u-PA-receptor-dependent formation of capillary-like tubular structures. The formation of these structures was quantified by determining the length of the invasive structures by image analysis and by measuring the accompanying ^{125}I-fibrin degradation. Ingrowth of tubular structures proceeded at a faster rate in opaque matrices consisting of thick fibrin fibres as compared to transparent gels with fine fibrin fibres. The more rapid ingrowth of tubular structures in opaque fibrin gels induced by bFGF/TNFα or $VEGF_{165}$/TNFα was accompanied by a larger extent of fibrin degradation. Both processes were inhibited by aprotinin and ε-aminocaproic acid indicating the involvement of plasmin. They were also inhibited by anti-u-PA or anti-u-PA-receptor IgG, but not by anti-t-PA IgG, suggesting the involvement of cell-bound u-PA activity.

However, in the opaque fibrin gels, the tubular structures dissolved upon prolonged incubation due to excessive fibrin degradation. Simulation of hMVEC with bFGF alone did not induce tubular structures, but caused a high degree of t-PA- and plasmin-dependent fibrin lysis and, after several days, a partial detachment of sheets of cells. Gradual inhibition of the excessive fibrin degradation by a series of aprotinin concentrations did not lead to tube formation in bFGF-treated cells. These data indicate that the formation and stability of tubular structures by hMVEC in fibrin is accompanied by controlled fibrinolysis and depends critically not only on cell-bound u-PA-dependent plasminogen activation, but also on the fibrin structure. Because the fibrin structure is largely influenced by the conditions in which fibrin has been polymerised, these conditions may have considerable impact on angiogenesis during wound healing and vascularisation of tumour stroma.

Introduction

Fibrin is a temporary matrix which is formed after wounding or when plasma leaks from blood vessels forming a fibrinous exudate, often seen in areas of inflammation and in tumours [1, 2]. The fibrin matrix not only acts as a barrier preventing further blood loss, but also provides a scaffolding into which new microvessels can infiltrate during wound healing [3, 4]. The structure of fibrin and other matrix proteins associated with it, such as vitronectin, provide new adhesion sites and thus spatial

information for cell-matrix interactions during cell migration and the formation of new tubular structures. This remodelling process requires the balanced formation and dissolution of cell-matrix interaction sites [5]. In addition to the integrins involved in the formation of cell-matrix interaction sites [6-8], cell-bound proteolytic enzymes, such as plasminogen activators (PA) and matrix degrading metalloproteinases, are generally thought to play a role in the invasion of endothelial cells into a matrix structure [9-11].

The ingrowth of endothelial cells in a fibrin matrix was shown to depend critically on u-PA-mediated activation of plasminogen to plasmin, which was localised on the cell surface by interaction with the u-PA receptor [12, 13]. Controlled plasminogen activator activity was essential to form capillary-like tubular structures. Excessive plasminogen activation caused the formation of endothelial cysts in the fibrin matrix [14, 15], while enhancement of PA inhibition limited the extent of capillary tube formation [16]. Because alteration of the fibrin structure leads to changes in the sensitivity of these matrices to plasmin degradation [17] as well to an altered spatial organisation of possible cell-binding sites, we wondered whether the fibrin structure per se influenced the process of angiogenesis. Altered fibrin structures have been reported in various pathological conditions, such as inflammation [18], multiple myeloma [19], factor XIII deficiency [20] and dysfibrinogenemias [21].

In experimental conditions the fibrin structure can be altered by changes in the local pH during fibrin polymerisation, as well as by incorporation of plasma proteins, or proteins secreted by inflammatory cells [22-24]. Because the fibrinolytic sensitivity as well the major cell binding RGD sequence in the Aα-chain differs in human fibrin as compared to the fibrin of several other species [25], we prepared human fibrin matrices at various pH. After neutralising and equilibrating these matrices we evaluated the process of capillary-like tubular structure formation by human microvascular endothelial cells. These studies show that, in addition to cell-bound u-PA and plasmin activity, the fibrin structure per se is an important determinant of the rate of capillary outgrowth as well as of the stability of capillary-like tubular structures.

Materials and methods

Materials

Penicillin/Streptomycin, L-glutamine and medium M199 with or without phenol red, with EBBS, L-glutamine and Hepes were obtained from Biowitthaker (Verviers, Belgium). Trichloroacetic acid (TCA), trypsin 1-300 370 USP/mg from ICN (Costa Mesa, CA) and heat-inactivated newborn calf serum from GIBCO BRL (Paisley, Scotland). Human serum was prepared from pooled fresh blood of 10-20 healthy donors obtained from a local bloodbank. Fibronectin was a gift from Dr J. van Mourik (Central Laboratory of the Blood Transfusion Service, Amsterdam). A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al [26]. Thrombin and heparin

were obtained from Leo Pharmaceutical Products (Weesp, The Netherlands), tissue culture plastics from Costar (Cambridge, MA). Human fibrinogen, batch X 0379-51 containing 3.2 μg plasminogen and 5 μg plasmin per gram fibrinogen from Chromogenix AB (Mölndal, Sweden), and iodinated human fibrinogen (200 μCi/mg fibrinogen) from Amersham (Buckinghamshire, England). $VEGF_{165}$ [27] was a kind gift from Dr. H. Weich, (GBF, Braunsweig, Germany) and TNFα [28], containing $2.45x10^7$ U/mg protein and less than 40 ng lipopolysaccharide per mg protein from Dr. J. Tavernier (Biogent, Gent, Belgium). Recombinant human basic fibroblast growth factor was purchased from Intergen (Purchase, NY) or from Boehringer Mannheim (Mannheim, Germany). Rabbit polyclonal anti-u-PA, anti-t-PA, preimmune IgG and mouse monoclonal anti-u-PA were prepared at our laboratory. Monoclonal anti-u-PA receptor IgG was a gift from Dr. Weidle (Boehringer Mannheim, Penzburg). Aprotinin and recombinant human hirudin were purchased from Pentapharm Ltd (Basel, Switzerland) and ε-aminocaproic acid from Merck (Darmstadt, Germany). Technovit 8100 was obtained from Heraeus Kulzer (Wehrheim, Germany).

Cell culture

Human foreskin microvascular endothelial cells were isolated, cultured and characterised as previously described [29, 30]. Cells were cultured until confluence at 5% CO_2/95% air on fibronectin-coated dishes in M199 supplemented with 2 mM L-glutamine, 20 mM Hepes (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 μg/ml crude endothelial cell growth factor (ECGF), 5 U/ml heparin, 100 IU/ml penicillin and 100 μg/ml streptomycin. Subsequently the endothelial cells were detached with trypsin/EDTA and transferred to new fibronectin-coated dishes at a split ratio of 3:1. Confluent endothelial cells were used from passages 7 to 10.

Preparation of fibrin gels

Human fibrin gels were prepared by the addition of 100 μl of 0.3 U/ml thrombin to 200 μl of 3 mg/ml fibrinogen which had been dialysed against phosphate-buffered saline (PBS) pH 7.4 with the addition of 2 μCi ^{125}I-fibrinogen in a 1 cm^2 well of 48-well plates. The structure of the fibrin clot was modified by varying the pH of the mixture before polymerisation between pH 7.0 and pH 7.8 with NaOH or HCl. After 24h of polymerisation, inactivation of the thrombin, elution of free iodine and adjustment of the pH of the fibrin gels to pH 7.4 was carried out by equilibrating the gels twice for 12h and once for 24h with 0.5 ml M199 containing 10% human serum and 10% newborn calf serum. The structure of fibrin fibres was monitored by turbidity measurement, which is dependent on fibre thickness and gel rigidity [31]. This assay has proven to be an effective measurement of the fibrin fibre thickness and fibrin network structure [32]. The turbidity of fibrin gels polymerised in a 96-well plate was measured at 350 nm by using a Titertek reader.

In vitro angiogenesis model

After preparation of the fibrin matrices confluent endothelial cells were detached from the fibronectin coated dishes with trypsin/EDTA, seeded in a confluent density on the fibrin matrices. After 24h the medium was replaced with medium containing different mediators. Every 48h the medium was changed and collected, over a time period of six days. The formation of tubular structures of endothelial cells by invasion into the underlying matrix was analysed by phase contrast microscopy. Quantification of the length of the structures formed was performed by a computer equipped with Optimas image analysis software and a monochrome CCD camera (MX5) connected to it. Per well three pictures were taken at a low magnification. Because of the turbidity of the opaque fibrin matrices, the tubular structures were traced onto a transparent sheet and their length was measured by a computer analysis program. After fixation the matrices were embedded in Technovit 8100, according to instructions of the manufacturer, and the presence of tubular structures in the three dimensional fibrin matrix was analysed by histological examination of sections perpendicular to the monolayer which were stained with Coomassie Brilliant blue.

Analysis of fibrin degradation

The fibrin degradation products in the collected supernatant media were precipitated at 0° C by the addition of 1 volume of 20% trichloroacetic acid The radioisotope content of the pellet, containing the fibrin degradation products was determined by a γ–counter. Data are expressed as the mean ±SEM.

Results

Structure of fibrin matrices formed at different pH

Fibrin matrices were polymerised in phosphate-buffered saline at pH values ranging from 6.5 to 8.5. Turbidity measurements were performed and the absorbency of the formed matrices was plotted against pH of the polymerisation buffer. Fibrin matrices formed in a more acid environment (pH 6.5 to 7.0) had a high absorbency (opaque fibrin matrices) indicative of a opaque, porous fibrin network composed of thick fibrin fibres, while those polymerised in a more basic environment (pH 7.6 to 8.5) had a low absorbency (transparent fibrin matrices) indicative of a rigid, transparent network consisting of thin fibrin fibres [32]. Small pH variations in a range between 7.0 and 7.8 resulted in dramatic changes in the absorbency and thus in the network structure of the formed matrices. After polymerisation the fibrin matrices were washed and equilibrated three times with serum containing medium (pH 7.4) for 48 hrs; this did not alter the structure of the fibrin matrix as determined by turbidity measurement.

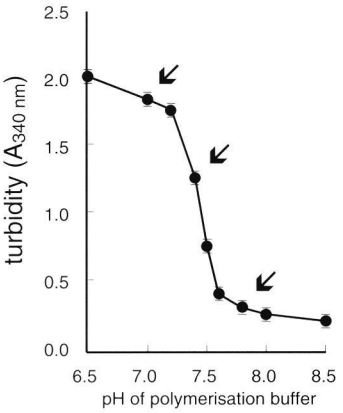
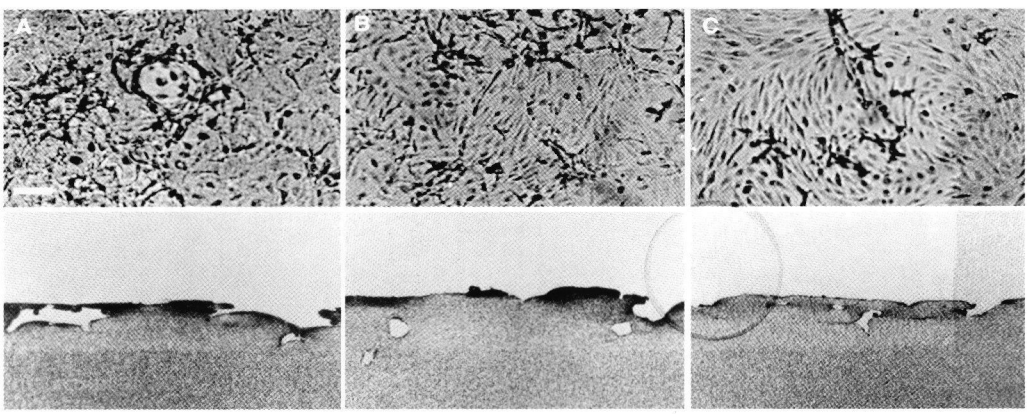


***Figure 1**: The effect of the pH of the polymerisation buffer on the structure of the fibrin matrix: Different fibrin matrices were made by the addition of 100 ml of 0.3 U/ml Thrombin to 200 ml of 3 mg/ml fibrinogen in polymerisation buffers of different pH. The structure of the fibrin was determined by measurement of the turbidity at 350 nm (A 350). Arrows indicate the fibrin matrices which were used in the in vitro angiogenesis assay. Data are expressed as the mean ±SEM of three independent experiments.*

Formation of capillary-like tubular structures in the various fibrin matrices

When human microvascular endothelial cells (hMVEC) grown on a fibrin matrix were exposed to the combination of bFGF and TNFα (bFGF/TNFα) or $VEGF_{165}$ and TNFα ($VEGF_{165}$/TNFα), they invaded the underlying fibrin matrix and formed capillary-like tubular structures, as shown for bFGF/TNFα in figure 2. When these experiments were performed on different fibrin matrices, the extent of tube formation in $^{pH\ 7.0}$fibrin matrices was considerably larger than in $^{pH\ 7.8}$fibrin matrices, although the onset of tube formation was seen at the same time point (usually after two days).

***Figure 2**: Phase-contrast photomicrographs (left panels) and histological cross-sections (right panels) of invasive and tubular structure formation by human microvascular endothelial cells: Three fibrin matrices were used, which were formed at pH 7.0 ($^{pH\ 7.0}$fibrin (a)); pH 7.4 ($^{pH7.4}$fibrin (b)) or at pH 7.8 ($^{pH7.8}$fibrin (c)) and subsequently equilibrated with the culture medium. Endothelial cells were cultured on the surface of those three-dimensional fibrin matrices in M199 supplemented with 10% human serum and 10% newborn calf serum (pH 7.4). Stimulation with 6 ng/ml bFGF and 6 ng/ml TNFα induced the cells to invade the fibrin matrix and form capillary-like tubular structures. Pictures were taken after 4 days. Bar (top panels) represents 10 µm; magnification of right panels is 40-fold.*



Quantification of the length of tubular structures formed by bFGF/TNFα–stimulated cells after 4 days showed that the extent of tubular structure formation at $^{pH\,7.4}$fibrin and at $^{pH\,7.8}$fibrin were 79±3% and 22±4% of those in $^{pH\,7.0}$fibrin respectively (figure 3). Not only quantitative differences but also qualitative differences could be observed. In a $^{pH\,7.0}$fibrin matrix an extensive network of invasive structures was formed, while in a $^{pH\,7.8}$fibrin matrix smaller and more isolated tubular structures were formed (figure 2 & 3).

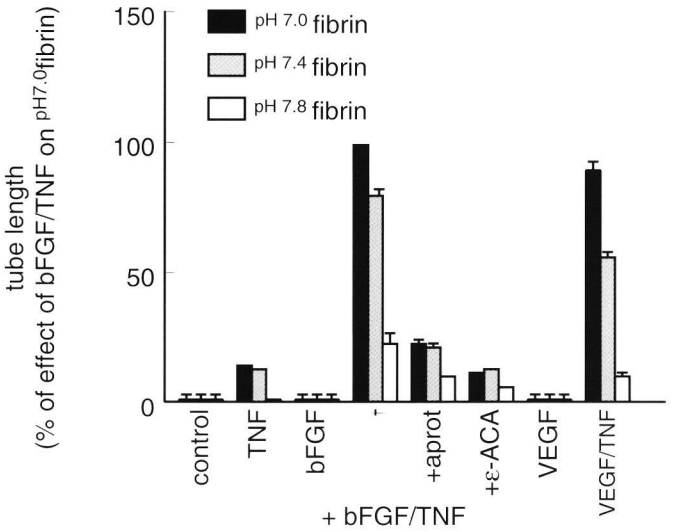


***Figure 3**: Influence of fibrin structure on the formation of invasive and tubular structures by human microvascular endothelial cells: Cells were incubated with 6 ng/ml TNFα, 6 ng/ml bFGF and/or 25 ng/ml VEGF165 for 4 days during which tubular structures were formed. The total length of tubular structures was quantified as described in Materials and Methods and expressed as % of the values obtained after stimulation with bFGF/TNFα on a $^{pH\,7.0}$fibrin matrix. $^{pH\,7.0}$fibrin, $^{pH\,7.4}$fibrin, and $^{pH\,7.8}$fibrin refer to the three different fibrin matrices. Plasmin activity and plasminogen activation were inhibited by aprotinin (100 U/ml) and ε-aminocaproic acid (10 mM), respectively. Data are the mean ±SEM of three independent experiments.*

The formation of tubes was largely prevented by ε–aminocaproic acid or aprotinin, which interfere with plasminogen activation or plasmin activity, respectively. Similar data were obtained with $VEGF_{165}$/TNFα–stimulated hMVEC (figure 3). In agreement with previous findings, the addition of bFGF or $VEGF_{165}$ alone was unable to induce tubular structures [13]. Cross-sectional analysis after 4 days confirmed the presence of tubular structures with a lumen covered with endothelial cells (figure 2B). During a prolonged stimulation of 6 days, the tubular structures in the $^{pH\,7.0}$fibrin matrix were regressed to invaginations in the fibrin matrix. This could be prevented by the addition of a low concentration of aprotinin (0.3 U/ml), suggesting that the disappearance of the structures was the consequence of excessive fibrinolysis (figure 4A-C). The structures formed in a $^{pH\,7.8}$fibrin matrix appeared to be stable during the prolonged stimulation (figure 4D-F).

To determine whether residual thrombin activity or activation of residual prothrombin in serum affected the outgrowth of capillary-like structures [33], the fibrin matrices were preincubated with hirudin (5 U/ml) and the *in vitro* angiogenesis assay was performed in the continuous presence of hirudin. No differences were observed in the extent of tube formation (data not shown).

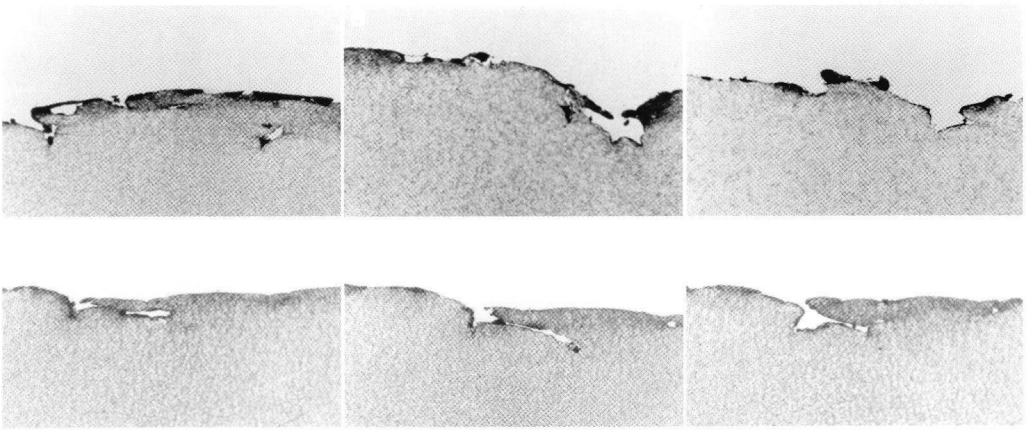


***Figure 4**: Histological cross-sections of endothelial cells cultured on the different fibrin matrices: endothelial cells were stimulated with 6 ng/ml bFGF and 6 ng/ml TNFα to invade the matrix and form tubular-like structures. The structures formed were further assessed by examining cross-sections of different matrices. Tubular structures in $^{pH\,7.0}$fibrin were assessed after 4 (A); 5 (B) and 6 days (C) and in $^{pH\,7.8}$fibrin matrices after 4 (D) and 6 (E and F) days. E And F are consecutive sections through the same tubular structure after 6 days. Magnification is 40-fold.*

Pericellular fibrin degradation accompanies tubular structure formation

To evaluate whether the outgrowth of structures was accompanied by fibrin degradation, the accumulation of lysis products from ^{125}I-labelled fibrin matrices in the conditioned media was quantified. When hMVEC were stimulated with bFGF or $VEGF_{165}$ and TNFα simultaneously, the formation of ^{125}I-fibrin degradation products increased severalfold (figure 5), particularly in conditions with the most extended outgrowth of structures (compare figure 3). This increase in ^{125}I-fibrin degradation products was nearly completely inhibited by the addition of aprotinin or ε–aminocaproic acid (figure 6). Furthermore, for both bFGF/TNFα– and for $VEGF_{165}$/TNFα-stimulated endothelial cells, the outgrowth of tubular structures and degradation of ^{125}I-fibrin matrices were simultaneously inhibited by anti-u-PA IgG but not by anti-t-PA IgG as shown for $^{pH\,7.0}$fibrin matrices (figure 5). Similar results were obtained for $^{pH\,7.4}$fibrin and $^{pH\,7.8}$fibrin matrices.

When u-PA antigen was assayed in the supernatants of bFGF/TNFα-stimulated cells, no significant differences in the u-PA antigen production over a time period of 4 days was found in cells grown on $^{pH\,7.0}$fibrin, $^{pH\,7.4}$fibrin or $^{pH\,7.8}$fibrin matrices (8.8±1.6, 8.1±1.8 and 8.5±1.9 ng/well respectively measured in five different experiments). Also no significant difference in the PAI-1 production was observed during the same incubation period (2216±369, 1749±104 and 2257±573 ng/well respectively), suggesting that the enhanced fibrinolysis was not due to enhanced u-PA production or to decreased PAI-1 synthesis, but related to the properties of the different fibrin matrices.

Furthermore, an anti-u-PA-receptor monoclonal antibody (anti-u-PAR), which blocked cellular u-PA binding, could reduce the formation of tubular structures by 80-95% (figure 5). These data indicate that the u-PA/plasmin system is involved in the outgrowth of structures by the pericellular activation of

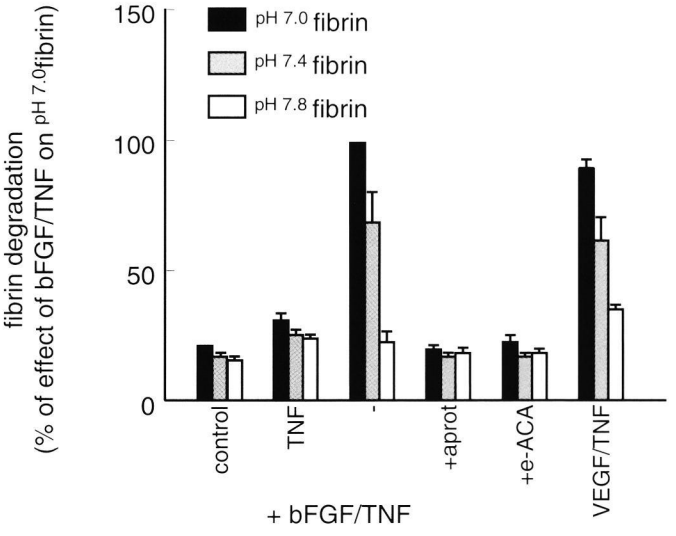


***Figure 5**: Influence of the fibrin structures on fibrin degradation by human microvascular endothelial cells: Cells were incubated with 6 ng/ml TNFα, 6 ng/ml bFGF and/or 25 ng/ml VEGF165 for 4 days during which tubular structures were formed. The total amount of fibrin degradation was quantified as described in Materials and Methods and expressed as % of the values obtained after stimulation with bFGF/TNFα on a $^{pH\,7.0}$fibrin matrix. $^{pH\,7.0}$fibrin, $^{pH\,7.4}$fibrin, and $^{pH\,7.8}$fibrin refer to the three different fibrin matrices. Plasminogen activity and activation were inhibited by aprotinin (100 U/ml) and ε-aminocaproic acid (10 mM). Data are the mean ±SEM of three independent experiments.*

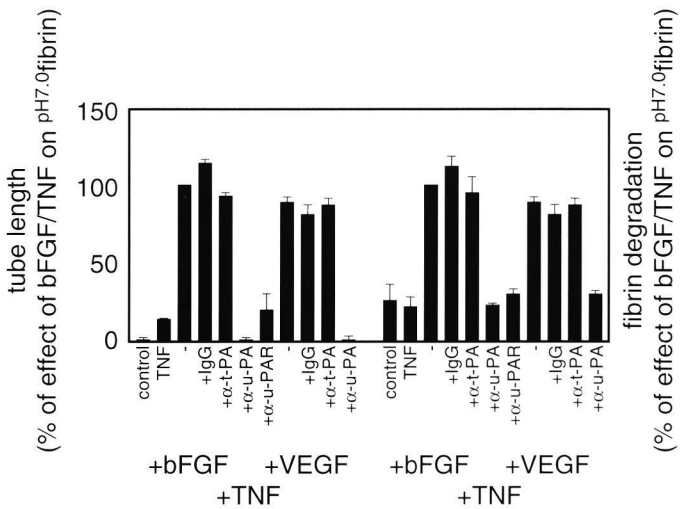


***Figure 6**: Influence of inhibition of plasminogen activators on the formation of tubular structures (left) and fibrin degradation (right) by endothelial cells stimulated with bFGF/TNFα or VEGF165/TNFα. Cells were incubated with 6 ng/ml TNFα, 6 ng/ml bFGF and/or 25 ng/ml VEGF165 for 4 days during which tubular structures were formed. The total length of tubular structures and the amount of fibrin degradation was quantified as described in Materials and Methods and expressed as % of the values obtained after stimulation with bFGF/TNFα on a $^{pH\,7.0}$fibrin matrix. Plasminogen activator activity was inhibited by the addition of 100 µg/ml of neutralising antibodies. Data are the mean ±SEM of three independent experiments.*

plasminogen to plasmin by u-PA, probably bound to the u-PA-receptor. The presence of hirudin during the *in vitro* angiogenesis assay did not influence the extent of fibrin degradation accompanying the formation of tubular structures (data not shown).

bFGF induces excessive fibrinolysis without tube formation

Unexpectedly, when hMVEC were stimulated with bFGF alone, considerable degradation of ^{125}I-fibrin occurred even exceeding the degradation induced by stimulation with bFGF/TNFα (figure 7). In this condition no tubular structures were formed, but sheets of cells detached from the monolayers and

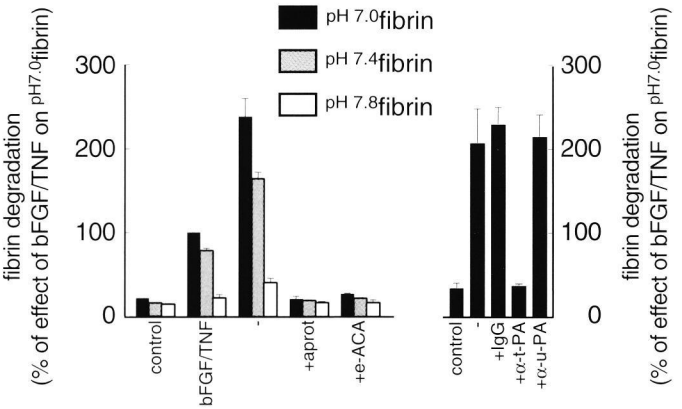


***Figure 7**: Influence of inhibition of the plasminogen activator/plasmin system on 125I-fibrin degradation by cells stimulated by bFGF alone: Cells were incubated with 6 ng/ml bFGF for 4 days. The amount of fibrin degradation was quantified as described in Materials and Methods and expressed as % of the values obtained after stimulation with bFGF/TNFα on a $^{pH7.0}$fibrin matrix. Plasminogen activator activity was inhibited by the addition of 100 µg/ml of neutralising antibodies. Data are the mean ±SEM of three independent experiments.*

gaps were formed in the underlying fibrin matrix. This effect was more prominent on $^{pH7.0}$fibrin than on $^{pH7.8}$fibrin. The lysis of ^{125}I-fibrin induced by stimulation with bFGF was inhibited by the addition of aprotinin, ε-aminocaproic acid (figure 7) and anti-t-PA IgG but not by anti-u-PA IgG (figure 7), suggesting that bFGF-induced t-PA production caused lysis of the fibrin matrix and detachment of

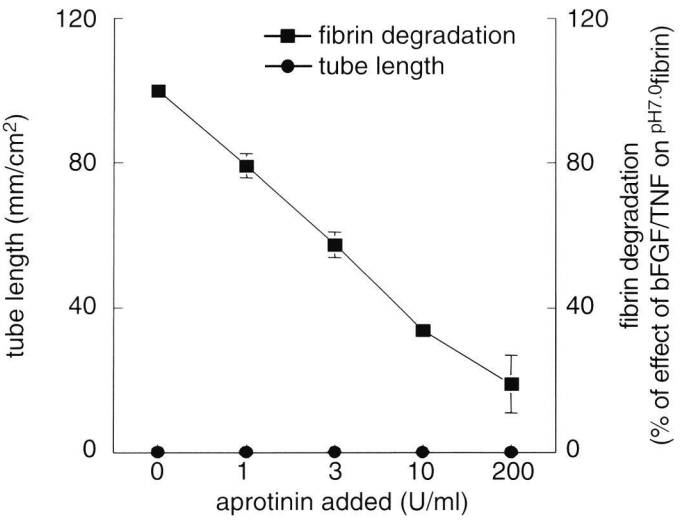


***Figure 8**: Neutralisation of excessive proteolytic activity induced by stimulating endothelial cells with bFGF: human microvascular endothelial cells were seeded on top of a matrix and stimulated with 6 ng/ml bFGF, whereas a gradual decrease in fibrinolytic activity was achieved by titration of aprotinin varying from 0 to 200 U/ml. Fibrin degradation was determined as described in Materials and Methods and expressed as % of effect of bFGF on $^{pH7.0}$fibrin. The formation of capillary-like tubular structures was also measured and expressed as mm/cm^2. Data are the mean ±SEM of two independent experiments.*

hMVEC. Neutralisation of excessive t-PA-induced proteolytic activity by the addition of aprotinin in a concentration ranging from 200 U/ml to 0.03 U/ml maintained the monolayer and controlled the matrix degradation, but did not lead to tubular structures formation (figure 8). Thus the induction of

tubular structures could not be induced by t-PA-dependent fibrinolysis induced by bFGF, but required cell-bound u-PA-dependent plasminogen activation such as that induced by bFGF/TNFα.

Discussion

The present study using an *in vitro* angiogenesis model consisting of human microvascular endothelial cells cultured on top of various human fibrin gels indicates that, in addition to cell-bound plasminogen activator activity, the structural and mechanical properties of fibrin play a regulatory role in the formation of capillary-like tubular structures.

The fibrin matrix, which is formed in pathological conditions, facilitates the invasion of cells and neovascularisation [2,3], and thus contributes to the healing process that follows injury. Because fibrin is specifically associated with pathological conditions, understanding of fibrin-associated angiogenesis can help to identify regulatory processes that can control angiogenesis [34-36], or to stimulate it in areas where it is processing inadequately [37-38]. A number of factors affect the molecular structure of fibrin, and as such may affect the properties of the fibrin matrix that are needed for the formation of a new capillary network [24]. Our finding that opaque and porous fibrin matrices ($^{pH\,7.0}$fibrin) permitted a faster and more extensive outgrowth of tubular structures than did transparent and rigid matrices ($^{pH\,7.8}$fibrin) is in line with the observations of other investigators who showed that the migration of endothelial cells and fibroblasts proceeded faster in the opaque and porous fibrin gels [39,40].

In our experimental condition endothelial cells were only in contact with fibrin at their basolateral side and kept their polarised orientation during the formation of tubular structures [13]. This prevents rapid cell shape changes caused by the apical exposure of endothelial cells to fibrin (ref. 41 and our unpublished observations). In this respect our model differs from the model of Nehls and Hermann [39], who embedded bovine endothelial cells grown on beads in a fibrin gel. In the latter assay the cells invaded faster into an opaque and porous fibrin gel than in a transparent and rigid one, but they were not able to form tubular structures in the opaque gels. In our model, the opaque and porous fibrin structures permitted not only a more rapid ingrowth of human microvascular endothelial cells than did finely dispersed fibrin gels but also a the formation of a more extended tubular network. However, we also observed that these tubular structures could not be maintained during a prolonged incubation of porous fibrin gels. This was accompanied by a considerable degree of lysis of the fibrin gels. These observations agree with previous studies showing that opaque and porous fibrin gels are more sensitive to fibrinolysis [17]. However, it should be noted that not only does the fibrinolytic sensitivity of the fibrin matrices play a role in this process, but the accessibility of important epitopes due to a different fibrin bundle structure formation may also contribute to the different angiogenic responses. This implies not only direct cell binding sites on the fibrin Aα-chain [25] and possibly the Bβ chain [42], but also the association of vitronectin to the fibrin bundles which is likely to occur during incubation in the presence of serum or plasma.

The parallel occurrence of tube formation and fibrin degradation was only found when plasmin was generated by u-PA activity, and not by t-PA activity. For many cells it has been shown that u-PA bound to a cellular u-PA receptor is involved in cell migration and invasion [43-46]. Although the role of u-PA in lateral endothelial migration, such as re-endothelialisation of an arterial inner lining, is uncertain [47,48], invasion of endothelial cells in a three-dimensional fibrin matrix *in vitro* requires cell-bound u-PA activity [12,13,16]. In agreement with these latter studies, we observed that cell-bound u-PA activity was needed for the formation of capillary-like tubular structures in all three fibrin matrices that were used. Although u-PA, which in human endothelial cells is induced by TNFα [49], is a major proteolytic determinant for the invasion of endothelial cells in fibrin, it is likely that other proteases, in particular matrix degrading metalloproteinases (MMPs) are additionally involved in the remodelling of more complex matrices, that also contain interstitial collagens [50]. In this context it is of interest that TNFα induces, in addition to u-PA, the synthesis of a number of MMPs in endothelial cells [51-53]. Furthermore, TNFα can act at other levels. It can enforce the interaction between MVEC and the fibrin matrix by induction of matrix-binding integrins, in particular $\alpha_v\beta_3$ [54, 55], which binds to many matrix proteins including fibrin [56] and vitronectin [57], and it may affect the expression of growth factor receptors.

An unexpected finding of our study was that when endothelial cells were stimulated with bFGF in the absence of TNFα , they degraded the fibrin matrix at a relatively high rate. This lysis depended on plasmin and t-PA activities, but was not accompanied by the formation of tubular structures. The inability of t-PA to induce tubular structures is at variance with the data of Yasunaga et al [58], but was also found in other studies [13]. Previous studies on endothelial cells have shown that stimulation of endothelial cells by bFGF increases t-PA production. Therefore, one may anticipate that enhanced t-PA activity and accompanying plasmin generation is too great to allow the formation of stable tubes. Excessive fibrinolysis preventing tube formation in fibrin matrices was also found with phorbol-ester-stimulated bovine endothelial cells [59]. In that case, the formation of tubular structures could be rescued by the stabilisation of the fibrin matrix by aprotinin [59]. However, although titration of the plasmin inhibitor aprotinin in the fibrin gels caused a gradual decrease in fibrin lysis, it was unable to provide, under any conditions, the environment in which tubular structures were formed by bFGF-stimulated hMVEC. Therefore, we favour the suggestion that only well-balanced u-PA-mediated and cell-bound plasminogen activation contributes to the formation of tubular structures, and that t-PA-enhanced fibrinolysis does not, possibly because it is not confined to the cell surface.

In conclusion our data point to an important role of the fibrin matrix per se in the formation of capillary-like tubular structures, in addition to the role played by cell-bound u-PA activity. In this study, relatively simple fibrin matrices have been studied. Many conditions and components influence the structure of fibrin when they are present during fibrin formation, and proteases from leukocytes can modify the fibrin structure after it has been formed. Therefore, wound healing and recanalisation of

mural thrombi may in part depend on fibrin-modifying factors. Future studies have to reveal whether and how these factors can be influenced.

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CHAPTER IV

Unfractionated and low molecular weight heparin affect fibrin structure and angiogenesis in vitro

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Abstract

Cancer patients treated for venous thromboembolism with low molecular weight heparin (LMWH) have a better survival rate than patients treated with unfractionated heparin (UFH). Since angiogenesis is an important determinant in the progression and metastasis of many solid tumors, the effects of heparins on *in vitro* angiogenesis were investigated. Both UFH and LMWH inhibited to the same the extent bFGF-induced proliferation of human microvascular endothelial cells (hMVEC) as determined by [^{3}H]-thymidine incorporation (36-60% inhibition.) $VEGF_{165}$-induced proliferation was also inhibited, but to a to a lesser extent (19-33% inhibition.)

Turbidity measurements and electron microscopy showed that the presence of LMWH during polymerization of the fibrin matrix led to a more transparent rigid network with thin fibrin bundles, while the presence of UFH resulted in a more opaque malleable network with thick fibrin fibers.

We used a human *in vitro* angiogenesis model, that consisted of hMVEC seeded on top of a fibrin matrix, and stimulated the cells with bFGF/TNFα to induce capillary-like tubular structures. The formation of capillary-like tubular structures was retarded with matrices polymerized in the presence of LMWH (44% and 46% inhibition as compared to a control matrix for 1.5 and 10 U/ml LMWH respectively), while matrices polymerized in the presence of UFH facilitated tubular structure formation (89% and 29% stimulation as compared to a control matrix for 1.5 and 10 U/ml UFH respectively.) Similar results were obtained for cells stimulated with VEGF/ TNFα.

These data determine the inhibitory effect of heparins on proliferation of hMVEC and provide a novel mechanism by which LMWH may affect tumor progression, namely reduced ingrowth of microvascular structures in a fibrinous stroma matrix by rendering it less permissive for invasion.

Introduction

Patients with malignant diseases are at increased risk of venous thromboembolic complications [1]. As a result, many cancer patients are treated with antithrombotic drugs, including heparins. Intravenous dose-adjusted unfractionated heparin (UFH) has been the standard initial treatment, but recent randomized clinical trials have shown that subcutaneous fixed doses of low molecular weight heparin (LMWH) are as safe and effective as UFH [2-4]. Cancer patients who have been treated with LMWH for their venous thromboembolism were found to have a significantly improved three month survival as compared to UFH recipients, whereas incidences of hemorrhages and thromboembolic recurrences were comparable in both treatment groups [5].

Several experimental studies have reported the either stimulatory or inhibitory effects of heparins on tumor growth and metastasis (see [6] for review). These effects may not only reflect their anticoagulant function, but may involve other processes, such as angiogenesis [7]. Animal studies have shown that

LMWH and UFH differentially affect angiogenesis, but the mechanisms by which they act remain unclear [8,9].

Angiogenesis, the formation of new blood vessels, supports the expansion of many solid tumors and facilitates the escape of tumor cells and thus metastasis [10, 11]. Angiogenesis is driven by microvascular endothelial cells (MVEC), which upon activation degrade their basement membrane, migrate into the interstitial matrix, proliferate, and form new capillary-like tubular structures [12]. Tumors release a number of angiogenic growth factors, such as vascular endothelial growth factors (VEGFs) [13], fibroblast growth factors (FGFs) [14] and scatter factor [15]. Induction and maintenance of angiogenesis requires interaction of these growth factors with their respective receptors, which then activate endothelial cells [16, 17], often in concert with other cytokines [18]. Heparan sulfates and heparins modulate the binding of many angiogenic growth factors, and hence may affect endothelial cell responses [19, 20]. Thus far, the effects of heparins on angiogenesis have been attributed to their interaction with angiogenic growth factors. However, other steps in the process of angiogenesis may also be influenced, in particular the interaction of MVEC with the matrix which they invade.

A major component of the initial tumor stroma is fibrin, which is deposited following exudation of plasma from leaky vessels surrounding the tumor [21]. Fibrin provides scaffolding for both invasive cancer cells and endothelial cells, thereby contributing to tumor growth and neovascularization [22, 23]. The structural and mechanical properties of the fibrin matrix play a regulatory role in the formation of capillary-like tubular structures [24, 25]. Modification of the structure of the fibrin network alters its sensitivity towards proteolytic degradation, which affects tube formation [26]. Heparins also affect the structure of the fibrin clot, altering its sensitivity to plasmin degradation [27]. However it is not known to which extent LMWH or UFH affect angiogenesis by altering the structure of the fibrin matrix.

The present study evaluates the effects of UFH and LMWH on growth factor-induced proliferation and the formation of capillary-like tubular structures by human MVEC (hMVEC). Both compounds reduce proliferation of hMVEC to a similar degree. However, the presence of LMWH during the polymerization of the fibrin decreases the formation of tubular endothelial structures, while the presence of UFH enhances its formation. These data provide a novel mechanism by which LMWH may affect tumor progression, namely reduced ingrowth of microvascular structures in a fibrinous stroma matrix by rendering it less permissive for invasion.

Materials and methods

Materials

Penicillin/streptomycin, L-glutamine and medium M199 with or without phenol red, with EBBS, L-glutamine and HEPES were obtained from Biowitthaker (Verviers, Belgium). Trichloroacetic acid (TCA), trypsin 1-300 370 USP/mg were obtained from ICN (Costa Mesa, CA) and heat-inactivated

newborn calf serum from GIBCO BRL (Paisley, Scotland). Human serum was prepared from the pooled fresh blood of 10-20 healthy donors obtained from a local blood bank. Fibronectin was a gift from Dr J. van Mourik (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine brain [28]. Thrombin and unfractionated heparin (UFH) were obtained from Leo Pharmaceutical Products (Weesp, The Netherlands), tissue culture plastics from Costar (Cambridge, MA). Human fibrinogen, batch X 0379-51 containing 3.2 μg plasminogen and 5 μg plasmin per gram fibrinogen was purchased from Chromogenix AB (Mölndal, Sweden) and the low molecular weight heparin (LMWH) Reviparin was from Knoll (Ludwigshaven, Germany). $VEGF_{165}$ was a kind gift from Dr. H. Weich, (GBF, Braunsweig, Germany), and TNFα containing $2.45x10^7$ U/mg protein and less than 40 ng lipopolysaccharide per mg protein from Dr. J. Tavernier (Biogent, Gent, Belgium). Recombinant human basic fibroblast growth factor (bFGF) was purchased from PeproTech (Rocky Hill, NJ) and [^{3}H]-thymidine from Amersham (Buckinghamshire, UK.)

Cell culture

Human umbilical vein endothelial cells (HUVEC) [29] and human foreskin microvascular endothelial cells (hMVEC) were isolated, cultured and characterized as previously described [30,31]. Cells were cultured until confluence in 5% CO_2/95% air atmosphere on fibronectin-coated dishes in M199 supplemented with 2 mM L-glutamine, 20 mM HEPES (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 μg/ml ECGF, 100 IU/ml penicillin and 100 mg/ml streptomycin. The endothelial cells were then detached with trypsin/EDTA and transferred to new fibronectin-coated dishes at a split ratio of 1:3. Confluent endothelial cells were used at passage 9-11 for hMVEC and at passage 2 for HUVEC.

Proliferation assay

Incorporation of [^{3}H]-thymidine in DNA was determined as previously described [18]. Confluent cultures of HUVEC or hMVEC were detached by trypsin/EDTA solution and seeded at a density of 7500 cells per cm^2 on fibronectin-coated 24 well dishes in M199 medium containing 10% NBCS, penicillin/streptomycin, growth factor (2.5 ng/ml bFGF or 12.5 ng/ml $VEGF_{165}$). The inhibition of proliferation was studied by the addition of 0.5 or 10 U/ml UFH or 0.5 or 10 U/ml LMWH. After a preincubation period of 48 hours, a trace amount of [^{3}H]-thymidine (0.5 μCi per 2 cm^2 well in 10 μl) was added and cells were incubated for another 8 hours. The cells then were washed with ice-cold PBS and [^{3}H]-labeled cells were fixed with 0.5 ml 100% methanol, DNA was precipitated with 5% TCA, dissolved in 0.3 M NaOH and counted in a liquid scintillation counter.

Preparation of fibrin matrices

Human fibrin matrices were prepared by the addition of 0.1 U/ml thrombin to 300 µl of 3 mg/ml fibrinogen dialyzed against phosphate-buffered saline (PBS, containing 140 mM NaCl, 13.4 mM $Na_2HPO_4.2H_2O$, 138 mM $NaH_2PO_4.2H_2O$, pH 7.4) in a 1 cm^2 well of 48-well plates. The structure of the fibrin clot was modified by varying the pH of the mixture before polymerization between pH 7.0 and pH 7.8 with NaOH or HCl. After 24 hours of polymerization, inactivation of thrombin and adjustment of the pH of the fibrin gels to pH 7.4 was carried out by equilibrating the gels twice for 12 hours and once for 24 hours with 0.5 ml M199 containing 10% human serum and 10% newborn calf serum.

The influence of heparins on the structure of the fibrin matrix was studied by the addition of 1.5 or 10 U/ml UFH or 1.5 or 10 U/ml of LMWH prior to the polymerization. After 4 hours of polymerization the matrices were washed with culture media.

In a parallel experiment the structure of fibrin fibers was monitored by turbidity measurement with a multichannel spectrophotometer at 340 nm (Titertek multiscan; Flows Labs, McLean, VA) and was plotted against the pH of the polymerization buffer or against the concentration of the added heparin.

For electron microscopy examination of the fibrin network structure, human plasma was clotted on formvar-coated 200 mesh nickel grids by the addition of 1 U/ml of thrombin in the presence or absence of LMWH or UFH. After 30 min, fibrin was washed with distilled water and stained with 1% phosphotungstic acid for 1 min. After repeated washing with water the specimen was dried and the fibrin network formed was analyzed in a Philips 201 electron microscope.

In vitro angiogenesis model

After preparation of the fibrin matrices, confluent endothelial cells were detached from the fibronectin-coated dishes with trypsin/EDTA and seeded in a confluent density on the fibrin matrices. After 24 hours the medium was replaced with medium containing different mediators. Every 48 hours the medium was changed and collected, for a time period of six days. The formation of tubular structures of endothelial cells by invasion into the underlying matrix was analyzed by phase contrast microscopy. Quantification of the length of the structures formed was performed by a computer equipped with Optimas image analysis software connected to a monochrome CCD camera (MX5) [18].

Antigen measurement in conditioned media

u-PA Antigen was measured as previously described [18]. Briefly, 96-well microtiter plates were coated with a mixture of two monoclonal antibodies, UK 2.1 and UK 26.15, recognizing different epitopes of the u-PA antigen. The next day, the plates were blocked with casein and serial dilutions of standard u-

PA (Ukidan; Serono, Aubonne, Switzerland) or culture media were added. Finally, an incubation was performed with HRP-conjugated monoclonal anti-u-PA IgG (LMW 11.1), and then tetramethylbenzidine substrate was added to react. The reaction was stopped with H_2SO_4 after 15 min of incubation. The absorbance was measured with a multichannel spectrophotometer.

PAI-1 antigen was determined by ELISA of the conditioned media collected from cells grown on fibrin and stimulated with different factors in M199 supplemented with 10% human serum and 10% newborn calf serum, according to the instructions of the manufactures (Biopool, Umea, Sweden).

Results

Effect of heparin on the proliferation of hMVEC and HUVEC

Proliferation was measured as the incorporation of [^{3}H]-thymidine in hMVEC stimulated with bFGF (2.5 ng/ml) or $VEGF_{165}$ (12.5 ng/ml). Addition of a low concentration of UFH- or LMWH (0.5 U/ml of UFH or 0.5 U/ml of LMWH) to bFGF-stimulated cells resulted in a significant decrease of

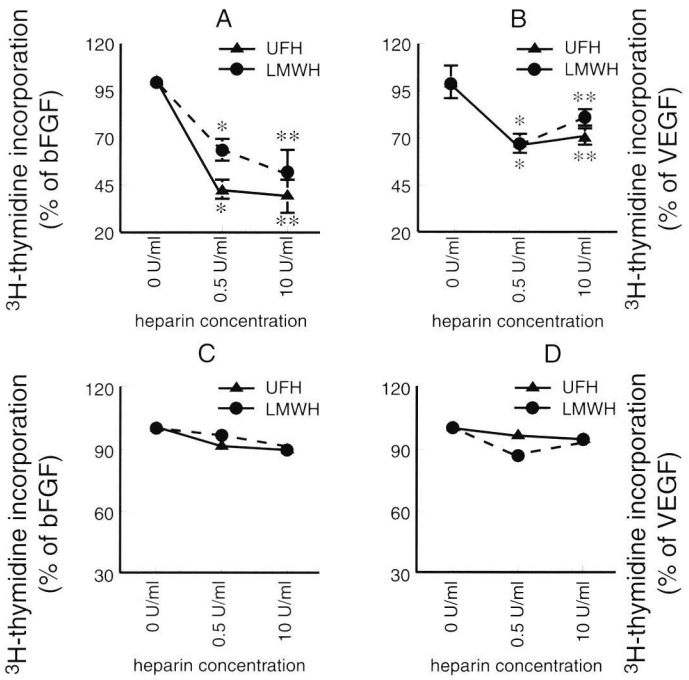


***Figure 1**: Incorporation of [3H]-thymidine in DNA of hMVEC (A, B) or HUVEC (C, D): the inhibition of proliferation was studied by the addition of 0.5 or 10 U/ml UFH or 0.5 or 10 U/ml LMWH. Data are the mean ±SE of three independent experiments for bFGF (A and C) and for VEGF165 (B and D).*
(represents $p < 0.01$ and ** represents $p < 0.05$.)*

[^{3}H]-thymidine incorporation (58±3% and 36±3% inhibition, respectively, as compared to control). The addition of higher concentrations of both heparin preparations resulted in similar or even further inhibition (60±9% and 50±10% inhibition, respectively) (figure 1A). Also $VEGF_{165}$-induced proliferation

was inhibited by the addition of UFH and LMWH, although to a lesser extent (33±3% for 0.5 U/ml UFH and 0.5 U/ml LMWH, and to 28±3% and 19±3% for 10 U/ml respectively) (figure 1B).
Interestingly, this effect of heparin was specific for hMVEC, because UFH or LMWH did not significantly affect the proliferation of HUVEC induced by bFGF (8±2% and 5±8% for 0.5 U/ml UFH and 0.5 U/ml LMWH and to 15±5% for 10 U/ml) (figure 1C) nor $VEGF_{165}$ (2±4% and 9±7% for 0.5 U/ml respectively, and to 3±5% and 4±10% for 10 U/ml, respectively) (figure 1D).

Fibrin matrices polymerized in different conditions

Previously we showed that the structure of fibrin depends on the pH at which fibrin was polymerized [25]. Fibrin matrices polymerized at pH 7.0 or lower had a high turbidity indicative of an opaque, malleable and porous network, while those polymerized in a more basic environment (pH 7.8) had a low absorbency indicative of a transparent, dense and rigid network (figure 2A).

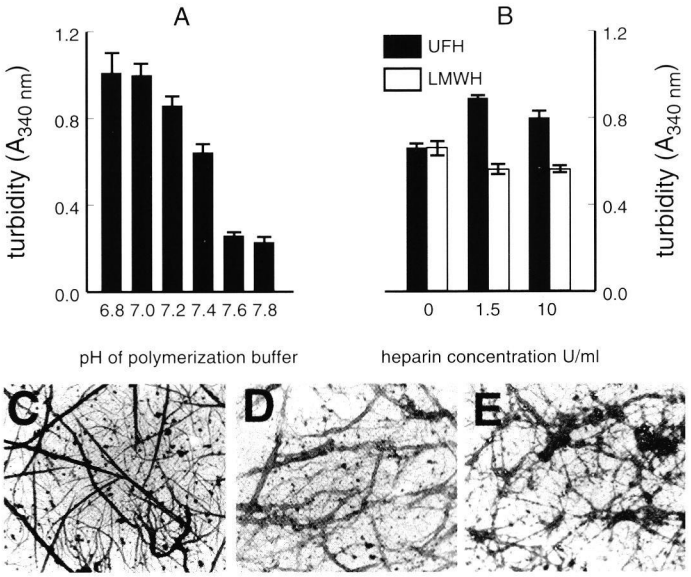


***Figure 2**: The effect of the polymerization conditions on the structure of the formed fibrin matrix.*
***A, B**: Prior to polymerization the pH of the buffer was altered between pH 7.0 and 7.8 (A) or 1.5 or 10 U/ml UFH and 1.5 or 10 U/ml LMWH were added (B). After 4h of polymerization the structure of the fibrin was determined by measurement of the turbidity at 340 nm (A 340). Data are expressed as the mean ±SE of three independent experiments.*

***C, D, E**: Electron micrographs of the fibrin network after polymerization without (C) or in the presence of UFH (D) or LMWH (E).*

The presence of UFH and LMWH during fibrin polymerization also caused an alteration of the turbidity of the matrices formed. UFH induced an increase in turbidity and LMWH a decrease (figure 2B).
Electron microscopic studies on fibrin matrices confirmed that the fibrin network formed in the presence of UFH was composed of thicker fibrin bundles in a more porous network, while bundles in the tighter network formed in the presence of LMWH were thinner and denser (figure 2C-E).

Formation of capillary-like tubular structures in the various fibrin matrices

hMVEC grown on a fibrin matrix and stimulated with the combination of bFGF and TNFα (bFGF/TNFα) or $VEGF_{165}$ and TNFα ($VEGF_{165}$/ TNFα) invaded the underlying fibrin matrix and formed capillary-like tubular structures (figure 4B) [18], as shown for bFGF/TNFα in figure 4A, B.

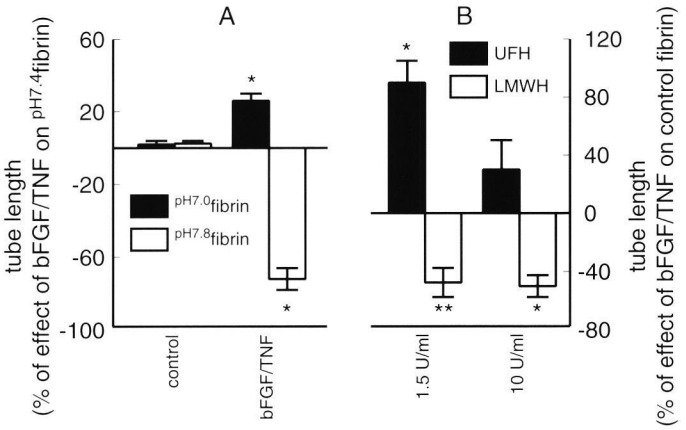


***Figure 3**: Influence of fibrin structure on the formation of capillary-like tubular structures: stimulation of endothelial cells with 5 ng/ml bFGF and 1 ng/ml TNFα for six days induced the cells to invade the fibrin matrix and form capillary-like tubular structures. The total length of tubular structures was quantified and expressed as % of the values obtained after stimulation with bFGF/TNFα on a control fibrin matrix (pH 7.4 (A) and without UFH/LMWH (B)). Data are expressed as mean ±SE of five different experiments performed with duplicate wells. 100% is 98±3 mm/cm². (* represents $p < 0.01$ and ** represents $p < 0.05$).*

The overall length of tubular structures was considerably higher in malleable fibrin matrices produced at pH 7.0 (126±2% of tube formation as compared to matrices polymerized at pH 7.4) than in fibrin

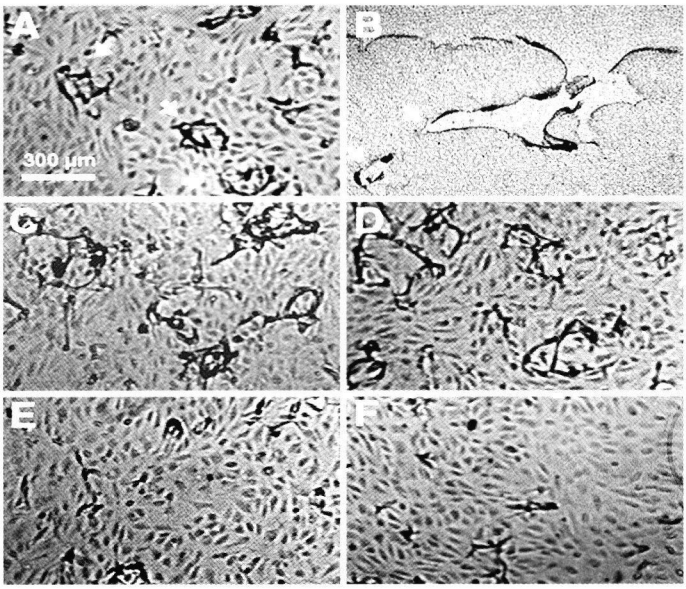


***Figure 4**: Influence of fibrin structure on the formation of capillary-like tubular structures: Stimulation of endothelial cells with 5 ng/ml bFGF and 1 ng/ml TNFα for six days induced the cells to invade the fibrin matrix and form capillary-like tubular structures.*
***A, C-F**: Phase-contrast photomicrographs of tubular structures (A, arrowhead) formed by hMVEC cultured on fibrin matrices formed in the presence of 1.5 or 10 U/ml UFH (C and D), 1.5 or 10 U/ml of LMWH (E and F) and control matrices (A).*
***B**: Histological cross-section: a tubular structure, indicated by an arrowhead, is connected as became evident by serial sections.*

matrices produced at pH 7.8 (30±4% of tube formation as compared to matrices polymerized at pH 7.4), both in bFGF/TNFα (figure 3A), and $VEGF_{165}$/ TNFα- stimulated cells (data not shown). Alteration of the fibrin matrices due to the presence of UFH and LMWH during the polymerization also affected the extent of tube-formation by hMVEC (figure 3B). The total length of the tubes formed after stimulation with bFGF/TNFα of the cells grown on fibrin matrices polymerized in the presence 1.5 U/ml and 10 U/ml UFH was increased with 89±11% and 29±16% respectively, as compared to control matrices (figure 4C-D). However, the presence of similar amounts of LMWH during matrix polymerization caused a decrease in the length of tube formation to 44±10% and 46±7% as compared to control, respectively (figure 4E-F).

Similar results were obtained when hMVEC were stimulated with 25 ng/ml VEGF and 1 ng/ml TNFα. The measured length for control matrices was 62 mm/cm^2, while the measured length for matrices polymerized in the presence of 1.5 U/ml or 10 U/ml UFH was 79.9 mm/cm^2 and 77.6 mm/cm^2, respectively and for matrices polymerized in the presence of 1.5 U/ml or 10 U/ml LMWH was 29.9 mm/cm^2 and 28.9 mm/cm^2, respectively.

The differences in tube formation could not be explained by an altered u-PA activity, because the secretion of u-PA and PAI-1 antigen in all conditions was similar (table 1).

***Table 1**: Measurement of u-PA and PAI-1 antigen in conditioned media. Stimulation of endothelial cells with 5 ng/ml bFGF and 1 ng/ml TNFα induced the cells to invade the fibrin matrix and form capillary-like tubular structures. Every two days the supernatant media were renewed. The total concentrations of u-PA and PAI-1 antigens secreted in the conditioned media were measured by specific ELISA and expressed as the total amount (ng) secreted over a period of six days. 1 U/ml LMWH represents 1 aXa/ml LMWH.*

	PAI-1 ng / 10^5 cells / 6d	u-PA ng / 10^5 cells / 6d
0 U/ml	678±50	21±0.4
1.5 U/ml UFH	694±57	24±0.1
10 U/ml UFH	685±34	23±0.3
1.5 U/ml LMWH	711±20	23±0.1
10 U/ml LMWH	682±20	23±0.4

Discussion

The present study reveals two mechanisms by which UFH and LMWH affect angiogenesis *in vitro*. UFH and LMWH inhibit the proliferation of hMVEC induced by the angiogenic factors bFGF and $VEGF_{165}$ to a similar degree, and differently affect fibrin matrix formation. LMWH causes the formation of more rigid fibrin matrices that inhibit capillary-like tubular structure formation, while the presence of UFH contributes to the formation of a more malleable fibrin matrix and thus facilitates angiogenesis. Angiogenesis is required for the expansion of many solid tumors and facilitates the metastasis of tumor cells to other organs [10]. Factors altering angiogenesis may therefore influence these processes and thereby the prognosis of cancer patients. Various studies have suggested that heparins affect the proliferation of endothelial cells by their effects on angiogenic growth factors, in particular FGFs and

VEGFs [32-34]. Both endothelial heparan sulfates and heparins can promote the interaction of these growth factors with their receptors. One may anticipate that LMWH might inhibit angiogenesis by competing with cellular heparan sulfates for the binding of these growth factors [35]. However no differences between UFH and LMWH on endothelial cell proliferation induced by bFGF or $VEGF_{165}$ were observed. Interestingly, the inhibitory effect of heparin on proliferation was relatively strong in hMVEC, whereas it was non-significant in HUVEC. Because angiogenesis is driven by microvascular endothelial cells, this effect is probably relevant for tumor neovascularization.

The migration and invasion of cells depend on their detachment from and the new attachment of invading cells to their extracellular matrix. The matrix of an initial tumor stroma is predominantly formed by plasma proteins, in particular fibrinogen, released by leaky vessels [22, 36]. Tumor cells induce a state of hyperpermeability in the surrounding vasculature by the release of vascular permeability factors such as VEGF [36]. The fibrinous exudate formed provides an important provisional scaffolding for invasive cells, thereby contributing to tumor growth and neovascularization. *In vitro* angiogenesis studies revealed that the formation of capillary-like tubular structures by endothelial cells in a fibrin matrix depends on local and controled matrix degradation mediated by cell-bound urokinase and plasmin [38-40]. In addition, the structure of fibrin itself plays an important role in the invasion of the matrices by [24, 25] and as is shown here, heparins affect fibrin matrix formation. Differences in the fibrin network structure alter its sensitivity towards plasmin-dependent proteolysis [41, 27] and the display of epitopes involved in endothelial cell-matrix interaction during angiogenesis [42, 43]. Heparins were present during the polymerization of the matrix, but were washed out before their use in the *in vitro* angiogenesis model. It is unlikely that a small amount of heparin remaining significantly affected endothelial proliferation in our *in vitro* angiogenesis model. This suggestion is strengthened by our previous observation that the formation of tubular structures does not critically depend on endothelial proliferation [18, 40]. Finally, the effects of UFH and LMWH on hMVEC proliferation were similar, whereas their effects on capillary-like tube formation paralleled their effects on the fibrin structure. Thus heparins may influence angiogenesis differentially, by their specific effect on the fibrin structure in the fibrinous stroma of a tumor.

These data provide a novel mechanism by which LMWH may affect tumor progression, namely reduced ingrowth of microvascular structures in a fibrinous stroma matrix by rendering it less permissive for invasion.

In conclusion, our data indicate that heparins not only affect the proliferation of endothelial cells, but also affect angiogenesis by altering the structural and mechanical properties of the fibrin network. Whereas the structural alterations of the fibrin matrix by UFH enhanced the invasion of the matrix by capillary-forming endothelial cells, LMWH reduced it. These findings may contribute to the elucidation of the mechanisms by which heparins may affect cancer progression differentially.

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CHAPTER V:

Aberrant fibrin formation and cross-linking of fibrinogen Nieuwegein, a variant with a shortened Aα-chain, alters endothelial capillary tube formation

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Abstract

A congenital dysfibrinogenemia, fibrinogen$_{Nieuwegein}$, was discovered in a young male without any thromboembolic complications. A homozygous insertion of a single nucleotide (C) in codon Aα 453 (Pro) introduced a stop codon at position 454, which resulted in the deletion of the carboxyl-terminal segment Aα 454-610. The ensuing unpaired cysteine at Aα 442 generated fibrinogen-albumin complexes of different molecular weights. The molecular abnormalities of fibrinogen$_{Nieuwegein}$ led to a delayed clotting time and a rigid fibrin network with a low turbidity, probably due to defective lateral association of the fibrin bundles. Electron microscopy confirmed that thin fibrin bundles were organized in a fine network. The use of fibrin$_{Nieuwegein}$ in an *in vitro* angiogenesis model resulted in a strong reduction of tube formation as quantified by image analysis. The ingrowth of human microvascular endothelial cells in this model was shown to be independent of $\alpha_v\beta_3$, indicating that the reduced ingrowth is not due to the absence of the RGD-binding site on Aα 572-574. Rather, the more rigid fibrin structure of fibrin$_{Nieuwegein}$ is the cause, since partial normalization of the fibrin network by changing the pH during polymerization to more acid, resulted in an increase of tube formation. Whereas factor XIIIa, which cross-links primarily γ-chains, further decreased the ingrowth, tissue transglutaminase (TG), which is released in areas of vessel injury, did not. This is in line with the absence of the cross-linking site for TG in the α-chains of fibrinogen$_{Nieuwegein}$. In conclusion, this newly discovered congenital dysfibrinogenemia has a delayed clotting time. It leads to the formation of a rigid fibrin structure, which could not be cross-linked by TG and which is less supportive for ingrowth of endothelial cells.

Introduction

Fibrinogen is a 340-kDa glycoprotein that is present in the blood at a concentration of approximately 3 mg/mL [1]. It is composed of six polypeptide chains, $(\alpha, \beta, \gamma)_2$ which are linked together by disulfide bonds and are organized in a symmetrical dimeric fashion [2]. During blood coagulation thrombin converts fibrinogen to fibrin monomers, which associate into staggered, overlapping, two-stranded fibrils. These two-stranded fibrils laterally associate to form fibrin bundles [3]. The final step in the formation of a three-dimensional fibrin network is the cross-linking of the fibrin subunits by formation of intermolecular bonds. This reaction is catalyzed by factor XIIIa, which in the presence of Ca^{2+} covalently links γ-chains of two adjacent fibrinogen molecules [4, 5], while cell-derived tissue transglutaminase covalently links two α-chains [6]. Apart from its role in hemostasis, the fibrin formed also provides a temporary matrix for cell invasion during the subsequent healing process. Formation of new blood vessels, or angiogenesis, is an important component of tissue-repair after injury [7-8]. Endothelial cells degrade the fibrin matrix locally, migrate into it, proliferate, and form capillary tubes [9]. This process not only requires proteases for cell detachment, invasion and lumen formation, but

also new cell-matrix interactions for reattachment of the invading cells. Different receptors, including integrins, are involved in endothelial cell-matrix interactions during migration and subsequent reattachment [10].

The structure of the fibrin network present in the fibrinous exudate is an important determinant in the extent of capillary tube formation [11-13]. The architecture of the fibrin network and its fibrinolytic sensitivity are determined by the rate of polymerization and its extent of cross-linking [14-17]. The carboxyl-terminal region of the Aα-chain of fibrinogen determines the thickness of bundles formed, and as such the structure of the fibrin network [18-21]. Thin fibrin bundles will associate into a tight and rigid network, while thick bundles associate into a porous and malleable network [22].

Several endothelial cell receptors interact with different domains in the fibrinogen molecule [23-25]. Integrins are heterodimers, composed of an α- and a β- subunit, which bind to the RGD-sequence present in extracellular matrix proteins [26]. The $\alpha_v\beta_3$-integrin is a promiscuous receptor, binding a wide variety of extracellular matrix proteins, as well as fibrinogen [27]. The RGD-sequence at position Aα 572-574 of fibrinogen is involved in binding of the cell-surface localized endothelial $\alpha_v\beta_3$-integrin receptor [28]. The expression of this receptor on endothelial cells is tightly regulated; it is only minimally expressed on resting endothelial cells, but highly upregulated during angiogenesis *in vivo* [29, 30]. Inhibitors of $\alpha_v\beta_3$-integrin [31, 32] can block angiogenesis in various conditions, suggesting an important role for this receptor.

Congenital dysfibrinogenemia, fibrinogens with structural and/or functional defects, have proven not only to be valuable tools to study structure-function relationships in the fibrinogen molecule [33], but also for studying interactions of various cells with fibrinogen and fibrin [34].

In the present study, we characterized a new congenital dysfibrinogenemia, $fibrinogen_{Nieuwegein}$, with a truncated Aα-chain, lacking the binding site for endothelial $\alpha_v\beta_3$-integrin on the Aα-chain as well as the cross-linking site for tissue transglutaminase. The effects of these alterations on endothelial capillary tube formation was studied in an *in vitro* angiogenesis model using human microvascular endothelial cells overlayed on fibrin matrices. Polymerization of $fibrinogen_{Nieuwegein}$ mutant resulted in a transparent rigid network that markedly delayed endothelial capillary-like tube formation *in vitro*.

Materials and methods

Coagulation studies

Blood was collected by venipuncture and anticoagulated with 1/10 volume of 0.11 mol/L trisodium citrate. Platelet-free plasma was prepared by centrifugation for 30 min at 4° C at 2300 x g. The concentration of fibrinogen in plasma was determined by clotting of plasma with Reptilase, dissolution of the washed clot in 0.025 mol/L acetic acid and measurement of the absorbancy at 280 nm, using

an absorption coefficient of $A^{1\%,1cm}$ = 15.0 [35]. Coagulation time was determined by the Clauss thrombin-method [36].

Purification of fibrinogen

Fibrinogen was purified from the plasma using β-alanine as described by Straughn and Wagner [37]. β-Alanine was added to plasma in a final concentration of 0.5 mol/L and kept on ice for 3 min. After removal of the precipitate by centrifugation at 13,000 rpm for 15 min, the concentration of β-alanine in the supernatant was raised to 1.5 mol/L and the mixture was kept for another 30 min on ice. Subsequently, fibrinogen was collected by centrifugation at 13,000 rpm for 15 min. Residual fibrinogen was precipitated from the plasma by raising the β-alanine concentration to 3 mol/L and centrifugation after 30 min incubation on ice. The fibrinogen pellets were dissolved in PBS (phosphate buffered saline, pH 7.4) and dialyzed against PBS. The concentration of the fibrinogen solution was adjusted to 3 mg/mL for subsequent use.

SDS-PAGE of plasma and immunoblot analysis

Fibrin monomers were prepared by clotting plasma with Reptilase. After washing of the clot with PBS, fibrin monomers were dissolved in 0.025 mol/L acetic acid. SDS-PAGE of fibrin monomers after reduction with 7% wt/vol β-mercaptoethanol was performed with 10% wt/vol polyacrylamide gels according to Laemmli [38]. For Western blotting, electrophoresis of plasma or fibrin monomers was performed with or without reduction by β-mercaptoethanol on 4-16% wt/vol polyacrylamide or 5% wt/vol polyacrylamide precast gradient gels (Biorad). The electrophoresis buffer consisted of 0.025 mol/L Tris, 0.19 mol/L glycine, 0.1% wt/vol SDS, pH 8.3, while the sample buffer contained 8 mol/L urea, 0.05 mol/L Tris/HCl, 25 mmol/L EDTA, 2% wt/vol SDS, 0.1% wt/vol bromophenol blue, pH 6.8. After separation on SDS-PAGE gels, proteins were semi-dry blotted onto a nitrocellulose filter. The blots were blocked by incubation with 0.01 mol/L Tris/HCl, pH 8.0, 0.15 mol/L NaCl, 0.1% Tween-20 and 5% skimmed milk overnight at 4° C. Subsequently, the blots were incubated for 2 hours at room temperature in 0.01 mol/L Tris/HCl pH 7.4, 0.15 mol/L NaCl, 0.1% Tween-20 in which the antibodies were diluted. Y18, a monoclonal antibody against the NH_2-terminal part of the Aα-chain of fibrinogen, and G8, a monoclonal antibody against the COOH-terminal part of the Aα-chain of fibrinogen, were conjugated to horse radish peroxidase (HRP) and used in a 1:1000 dilution [39, 40]. A rabbit-anti-human-Fbg coupled to HRP [41] and a rabbit-anti-human albumin coupled to HRP were used in a 1:3000 and 1:1000 dilution, respectively. After 3 times 15 min washes with incubation buffer cross-reacting bands were visualized with BM-blue.

Fibrin structure

Turbidity of the fibrin network

Human fibrin gels were prepared by addition of 1 µL of 10 U/ml thrombin to 100 µL of 3 mg/mL fibrinogen dialyzed against PBS in a 0.33 cm^2 well of 96-well plates. After 4h of polymerization, the turbidity of fibrin gels, which is dependent on fiber thickness and gel rigidity [42], was measured at 340 nm by using a titertek reader.

Electron microscopy of fibrin

Plasma was clotted on a formvar-coated 200-mesh nickel grid by the addition of 1 U/mL of thrombin. After 30 min of clotting, fibrin was washed with distilled water and stained with 1% phosphotungstic acid for 1 min. After repetitive washing with water the specimen was dried and the formed fibrin network was analyzed by electron microscopy.

DNA isolation and DNA sequencing

Genomic DNA was isolated from white blood cells [43] and the DNA encoding the carboxy-terminal part of the Aα-chain from amino acid 391 to 625 was amplified by PCR, using as forward oligonucleotides α6A (TGGGGCACATTTGAAGAGGTGTCA) and as reverse αCD2 (GGAACTTACAGTCGACCACAAAAACAGACC). The 829 bp PCR product of exon V of the fibrinogen Aα-gene was sequenced by Baseclear (Leiden, The Netherlands).

Cell culture

Human foreskin microvascular endothelial cells were isolated, cultured and characterized as previously described [44]. Cells were cultured until confluence at 5% CO_2/95% air on fibronectin-coated dishes in M199 supplemented with 2 mmol/L L-glutamine, 20 mmol/L Hepes (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 µg/mL crude endothelial cell growth factor supplement (ECGFs), 5 U/mL heparin, 100 IU/mL penicillin and 100 µg/mL streptomycin. Subsequently the endothelial cells were detached with trypsin/EDTA and transferred to new fibronectin-coated dishes with a split ratio of 1:3. Confluent endothelial cells were used at passage 11.

In vitro angiogenesis

Preparation of fibrin gels

Human fibrin gels were prepared by addition of 2 µL of 100 µmol/L plasminogen, 2 µL of 100 U/mL thrombin to 100 µL of 3 mg/mL fibrinogen in a 0.33 cm^2 well of 96-well plates. Tissue transglutaminase and factor XIIIa were added in different concentrations together with 5 mmol/L $CaCl_2$. After 4h of polymerization, thrombin was inactivated by equilibrating the gels for 12h with 0.2 mL M199 containing 10% human serum and 10% newborn calf serum. Inhibiting monoclonal antibodies or peptides were added during this incubation period to allow penetration into the fibrin. All experiments were performed with duplicate wells.
Altering the pH prior to polymerization by adding HCl (to pH 7.0) or NaOH (to pH 7.8) changes the structure of the formed fibrin network.

In vitro angiogenesis model

Endothelial cells were detached from the fibronectin-coated dishes with trypsin/EDTA and seeded in a confluent density on the fibrin matrices. After 24 h the medium was replaced with medium containing different test compounds. Every 48 h the medium was changed and collected, for a total of four to six days. The formation of tubular structures of endothelial cells by invasion in the underlying matrix was analyzed by phase contrast microscopy. Quantification of the length of the formed structures was performed by a computer equipped with Optimas image analysis software and a monochrome CCD camera (MX5) connected to it [45].

Immunohistochemical analysis of the formed capillary-like tubular structures.

Fibrin matrices were fixed at 4° C in *p*-formaldehyde PBS, pH 7.4 for 2 hours at 4° C. After fixation the fibrin matrices were washed overnight with 5% sucrose in PBS and embedded in Tissue-Tek and snap-frozen in precooled 2-methyl butane and stored at -80° C. 7 µm-thick sections were made throughout the whole specimen and for immunohistochemical analysis the sections were fixed on the slide by 4% *p*-formaldehyde in PBS. The primary antibody (CD-51, a monoclonal antibody against the α_v-chain was used at a concentration of 1:100) was incubated in dakodiluent for one hour at room temperature, and after washing anti-mouse-Cy3 was added in a 1:100 dilution for one hour. After washing the sections were mounted with Vectashield with DAPI. Since the capillary-like tubes are only composed of endothelial cells, no cell marker was used.

Binding of endothelial cells to fibrin

Fibrin matrices were prepared in a well of a 96-wells plate by adding 50 µL of 3 mg/mL fibrinogen to which 1 U/mL of thrombin was added to induce clotting. After clotting the matrices were equilibrated with M199 with 1% wt/vol HSA with or without the addition of antibodies or peptides for a period of 12 hours. Human microvascular endothelial cells forming confluent monolayers were detached by trypsin/EDTA, sedimented by centrifugation at 250 g for 5 min and resuspended in M199 1% wt/vol HSA at a concentration of 50,000 cells/mL. A volume of 150 µL of the cells in suspension was added to the wells and allowed to attach for 2 hours at 37° C. The wells were washed three times to remove unattached cells, fixed with 2% paraformaldehyde and adhered cell were counted. Attached cells were expressed as the percentage of cells attached to normal fibrin [46].

Statistics

Experiments were performed with duplicate wells and expressed as % of control. For statistical analysis the ANOVA was used followed by a modified t-test according to Bonferroni.

Results

Coagulation parameters of normal plasma and plasma$_{Nieuwegein}$

A 35-year old patient showed a prolonged activated partial thrombin time (aPTT), which was determined prior to biopsy. The clotting time of this plasma, as determined by the Clauss assay, was delayed (normal plasma diluted to 50% in Michaelis buffer clotted within 4.5 sec and the patient plasma within 15 sec). This delayed clotting time was associated with a normal plasma concentration of fibrinogen (3.4 mg/mL). This suggests an abnormality in the fibrinogen molecule, which was identified as fibrinogen$_{Nieuwegein}$. When plasma was coagulated by the addition of thrombin (1 U/mL), the turbidity at 340 nm increased progressively with normal plasma but not with plasma$_{Nieuwegein}$ (figure 1A). Similar turbidity curves were obtained if normal plasma and the patients plasma were coagulated with Reptilase and if purified fibrinogen was used (data not shown).

Determination of the fibrin structure by electron microscopy

Since the measured turbidity is an indicator of the structure of the fibrin network, the low turbidity of coagulated plasma$_{Nieuwegein}$ suggests that the formed fibrin network is tight and rigid, whereas the high turbidity after coagulation of normal plasma is known to reflect a porous and malleable network [42].

To visualize the fibrin network, normal plasma and plasma$_{Nieuwegein}$ were clotted by the addition of 1 U/mL of thrombin and the formed network was analyzed by electron microscopy. The fibrin network formed from fibrinogen$_{Nieuwegein}$ appeared to be tighter than the network formed by normal fibrinogen, and consisted of thin fibrin fibers, instead of the thick fibrin bundles of normal fibrin (figure 1B, C).

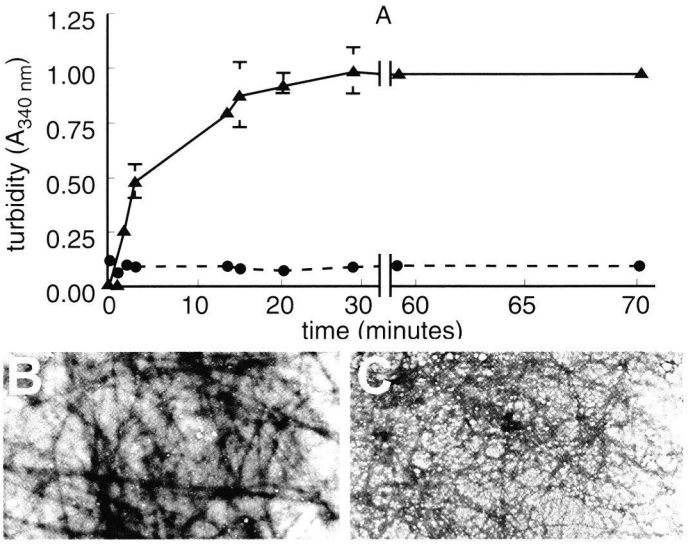


***Figure 1**: Fibrin structure of fibrin$_{Nieuwegein}$. **A**: Plasma was clotted by the addition of 1 U/ml thrombin. Turbidity at 340 nm was measured in time. Triangles represent control plasma and circles plasma$_{Nieuwegein}$. Data are expressed as mean ±SEM of duplicate experiments with triplicate wells.*

***B, C**: Electron microscopic analysis of the formed fibrin structure from normal plasma (B) and plasma$_{Nieuwegein}$ (C).*

SDS-PAGE and immunoblot analysis of fibrin monomers and plasma

SDS-PAGE of fibrin monomers prepared from plasma$_{Nieuwegein}$ (panels 1) and normal plasma (panels 2) was performed under reducing conditions on a 10% wt/vol polyacrylamide gel and stained with Coomassie Brillant blue (figure 2A). With normal fibrin, a band at the Aα-chain position (66.5 kD), a band at the Bβ-chain position (52 kD) and a band at the position of the γ-chain (47 kD) were observed (figure 2A). With fibrin$_{Nieuwegein}$ an additional band below the γ-chain was seen (figure 2A). Immunoblotting with rabbit-anti-human fibrinogen revealed that the band at position 66.5 kD of fibrin$_{Nieuwegein}$ (panel 1) did not crossreact, indicating that this band does not contain fibrinogen (figure 2B). To determine the position of the Aα-chain in fibrin$_{Nieuwegein}$, blots from normal plasma and plasma$_{Nieuwegein}$ were incubated with Y18/HRP, an HRP-linked antibody that is specific for the NH_2-terminus of the Aα-chain (figure 2C) and G8/HRP, an antibody that is specific for the 150 last amino acids in the COOH-terminus of the Aα-chain complexed to HRP (figure 2D). The Aα-chain detected with Y18/HRP in fibrinogen$_{Nieuwegein}$ appeared to be smaller than normal. The absence of cross-reaction with G8/HRP suggests that part of the COOH-terminal part of the Aα-chain is deleted. Further analysis of the band at the position of 66.5 kD with immunoblotting with rabbit-anti-human albumin/HRP revealed that this band contains albumin (figure 2E).

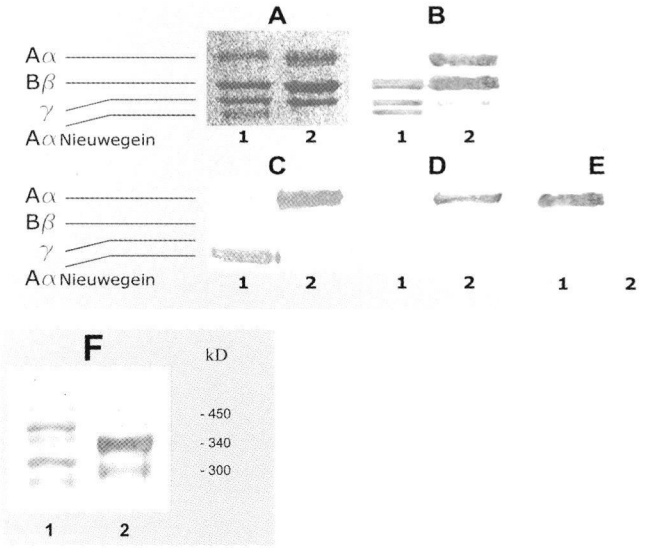


***Figure 2**: Analysis of normal fibrinogen or fibrin monomers (2) and of fibrinogen$_{Nieuwegein}$ or fibrin$_{Nieuwegein}$ monomers (1).*
***A**: Coomassie staining of fibrin monomers run on 10% SDS-PAGE gels after reduction.*
***B-E**: Immunoblots of fibrinogen, run on 10% SDS-PAGE gels after reduction, with rabbit-anti-human fibrinogen/HRP (**B**), G8/HRP (**C**), Mab Y18/HRP (**D**) and rabbit-anti-albumin/HRP (**E**).*
***F**: Analysis of plasma run on a 5% SDS-page gel without reduction and immunoblotted with Mab Y18/HRP.*

To determine the molecular weight of the different forms of fibrinogen, normal plasma and plasma$_{Nieuwegein}$ were analyzed by immunoblotting with Y18/HRP. In normal plasma the different molecular weight forms of fibrinogen could be detected, consistent with precursor fibrinogen, HMW-fibrinogen (340 kD), LMW-fibrinogen (310 kD) and LMW'-fibrinogen (270 kD) [43, 44]. The different molecular forms of fibrinogen$_{Nieuwegein}$ in the plasma have lower molecular weights than those of normal fibrinogen (HMW is approximately 306 kD, LMW approximately 290 kD and an LMW' of 270 kD), due to the presence of an abnormally short Aα-chain. Additional bands were observed for the plasma$_{Nieuwegein}$, which probably represent fibrinogen-albumin complexes, similar as previously described for fibrinogen Marburg [34]. A complex of one or two albumin molecules per HMW- Fibrinogen$_{Nieuwegein}$ and complexes of one albumin molecule per LMW- Fibrinogen$_{Nieuwegein}$ can be formed, leading to complexes with molecular mass of 366 kD, 426 kD and 350 kD respectively.

Amplification and DNA sequence analysis of exon V of the fibrinogen Aα-chain gene

Sequence analysis of base pairs encoding amino acids 391 to 625 in the COOH-terminus in the Aα-chain revealed that in the codon of proline at position 453 a C-nucleotide is inserted, altering the sequence from CCT to CCC. This insertion changes the following codon of Asp at position 454 (GAT) to a stop codon (TGA). A normal sequence for the Aα-chain was not detected in the amplified DNA, indicating that the mutation was homozygous. This insertion results in deletion of amino acids 454 to 610 of the Aα-chain. This is in agreement with the presence of a shorter (±17 kD) Aα-chain in fibrinogen$_{Nieuwegein}$ on 10% wt/vol SDS-PAGE gels in reducing conditions and the presence of

fibrinogen-albumin complexes, due to disulfide formation between the unpaired cysteine at position Aα 442 and a free cysteine present in albumin.

Influence of dysfibrinogen$_{Nieuwegein}$ on *in vitro* angiogenesis

To investigate the effect of the structural defect of fibrinogen$_{Nieuwegein}$ on tube formation, fibrin matrices prepared from this fibrinogen were used in an established *in vitro* angiogenesis model [45]. When human microvascular endothelial cells (hMVEC) seeded on top of a fibrin matrix were stimulated with bFGF and TNFα (bFGF/TNFα), they invaded the underlying fibrin matrix and formed capillary-like tubular structures (figure 3B). Cross-sectional analysis confirmed the presence of tubular structures with a lumen covered with endothelial cells. In agreement with our previous findings [45] no tubular structures were formed in the absence of bFGF/TNFα (figure 3A). When fibrin$_{Nieuwegein}$ was used as a matrix, a marked reduction (75±6%) in tubular structure formation occurred (figure 3C).

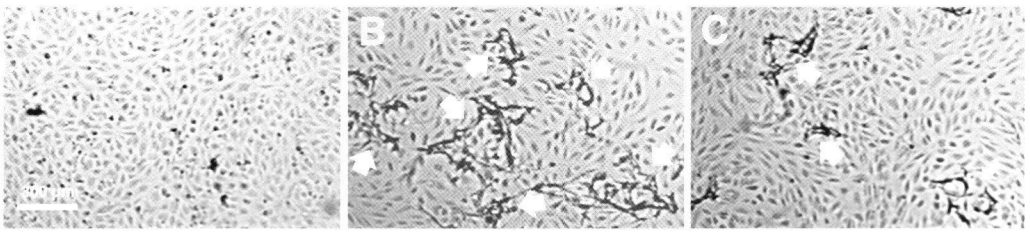


***Figure 3**: Formation of capillary-like tubular structures by hMVEC in a fibrin$_{Nieuwegein}$ matrix. hMVEC were seeded in confluency on the fibrin matrices. Under control conditions they remained as a monolayer on the fibrin (**A**). Stimulated with 5 ng/mL bFGF and 1 ng/mL TNFα they formed invasive capillary-like tubular structures in normal fibrin matrices (**B**). Capillary-like tubular structure formation by bFGF/TNFα-stimulated cells was decreased in fibrinNieuwegein matrices (**C**). After 4 days pictures were taken. Bar represents 300 μM.*

Since the outgrowth of vessels depends on the local degradation of the underlying fibrin matrix by the u-PA/plasmin system, the concentrations of u-PA and PAI-1 antigen [45] were determined in the conditioned media. No effect of the matrix on the accumulation of these proteins was found (data not shown).

Localization of $\alpha_v\beta_3$ on the surface of endothelial cells present in the capillary-like tubular structures in a fibrin matrix

Deletion of the carboxyl terminal part of the Aα-chain in fibrinogen$_{Nieuwegein}$ eliminates the RGD sequence at position Aα 572-574 which constitutes an adhesion site for endothelial $\alpha_v\beta_3$-integrin. To investigate whether this may be the cause of the diminished tube-formation, we first confirmed the

presence of $\alpha_v\beta_3$-integrin on endothelial cells. Immunohistochemical analysis of hMVEC showed an increased appearance of $\alpha_v\beta_3$ on the surface of hMVEC present in capillary-like tubular structures in the fibrin matrix, as well as in cells present on top of the fibrin matrix (figure 4).

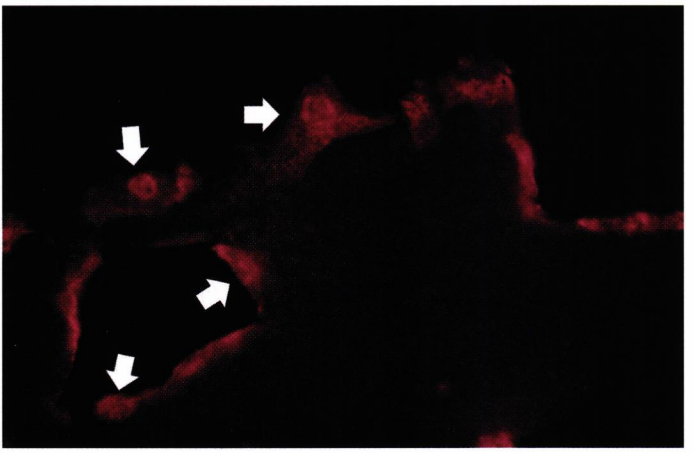


***Figure 4**: Immunostaining for $\alpha_v\beta_3$ receptor on the cell surface of hMVEC stimulated with bFGF/TNFα. After an incubation period of 5 days with bFGF/TNFα (the fibrin matrices were sectioned and endothelial cells were stained for $\alpha_v\beta_3$-integrins. Arrowheads point to endothelial cells present in the monolayer on top of the matrix and to cells in the capillary-like tubular structure in the fibrin.*

Role of RGD-sequences in fibrin and endothelial $\alpha_v\beta_3$ during cell attachment to and tube formation in a fibrin matrix

A possible interaction between the RGD-sequences in fibrin and the endothelial $\alpha_v\beta_3$-receptor during cell-adhesion and tube formation was evaluated using cyclic-RGD peptides (c-RGD, 10 μg/mL) and an inhibiting moAb against $\alpha_v\beta_3$ (LM609, 10 μg/mL). This antibody completely blocks the adhesion of hMVEC to a vitronectin matrix [46].

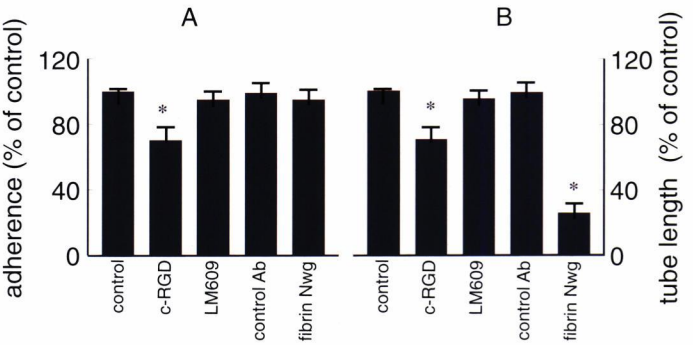


***Figure 5**: Effect of c-RGD (10 μg/mL) and anti-$\alpha_v\beta_3$-antibody (LM609, 10 μg/mL) on the adherence to and invasion in fibrin matrices by hMVEC. **A**: Cells were allowed to adhere to fibrin in the presence c-RGD (10 μg/mL), anti-FITC (10 μg/mL) and LM609 (10 μg/mL). Cells were also allowed to adhere to a matrix prepared from fibrinogen$_{Nieuwegein}$. After 2 hours the adhered cells were counted and expressed as % of control (no addition). Results from six cultures in three independent experiments are given and expressed as mean ±SD. **B**: hMVEC on top of the fibrin matrix were stimulated to invade by 5 ng/mL bFGF and 1 ng/mL TNFα alone or in the presence of c-RGD (10 μg/mL), anti-FITC (10 μg/mL) or LM609 (10 μg/mL). Cells were also allowed to invade in a matrix prepared from fibrinogen$_{Nieuwegein}$. After six days the formed tube length was measured and expressed as % of control (no addition). Data are the mean ± SD of six cultures in three independent experiments (* represents $P < 0.05$).*

The attachment of endothelial cells to an underlying fibrin matrix was slightly reduced by the presence of c-RGD (20±9%, n=3), and unaffected by the presence of LM609 (95±4% of control values, n=3) (figure 5A). Binding of endothelial cells to $fibrin_{Nieuwegein}$ was not impaired either. These data indicate that $\alpha_v\beta_3$-integrins on endothelial cells are not involved in initial attachment of hMVEC to fibrin.
During the process of capillary-like tubular structure formation in the underlying fibrin matrix the presence of c-RGD reduced the extent of outgrowth by 20±9% (n=3), whereas LM609 did not alter outgrowth (95±3% as compared to control, n=3) (figure 5B).

Influence of the fibrin structure on *in vitro* angiogenesis

The effect of the abnormal polymerization of $fibrinogen_{Nieuwegein}$ on capillary-like structure formation was further analyzed in two ways. Firstly, because the fibrinogen molecules in $plasma_{Nieuwegein}$ can form complexes with albumin, either one or two per molecule, albumin was added to the polymerizing fibrinogen in a 1 to 1 (1:1) or 2 to 1 (2:1) molar ratio to investigate the effect of albumin on the formation of capillary-like tubular structures. The extent of capillary-like tubular structure formation was only slightly affected in the highest concentration of albumin tested. This suggests that the presence of albumin per se in $fibrinogen_{Nieuwegein}$ does not significantly influence tube formation (figure 6). These results however do not exclude the possibility that covalent binding of albumin to $fibrinogen_{Nieuwegein}$ affects tube formation.

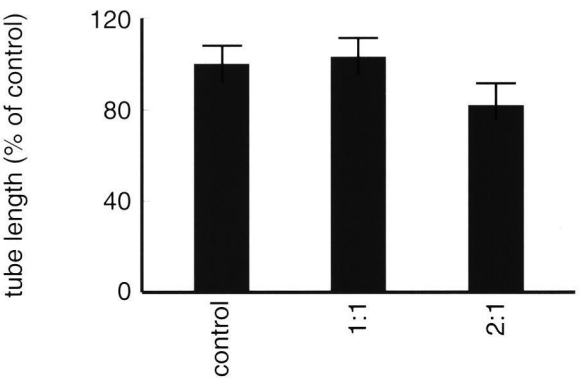


***Figure 6**: Fibrin matrices from normal fibrinogen were polymerized in the presence of albumin. After 4 days capillary-like tubular structures were measured and expressed as % of control. Control represents a fibrin matrix with no albumin, 1:1 represents albumin added in 1 to 1 molar ratio to polymerizing fibrinogen and 2:1 represents albumin added in 2 to 1 molar ratio to polymerizing fibrinogen. Data are mean ± SD of four cultures in two independent experiments.*

Secondly, as shown in figure 7A, the pH of the polymerization buffer influences the structure of the fibrin matrices [12]. An increase of pH during the polymerization of normal fibrin from pH 7.0 to pH 7.8 results in a decrease of turbidity. Analysis of tube formation reveals a parallel decrease in the tube length formed. Similarly, an increase in turbidity of fibrin matrices prepared from $fibrinogen_{Nieuwegein}$ was observed when the pH of the buffer was decreased to pH 7.0 (figure 7A), with a parallel increase

(53±19%) in tube formation (figure 7B). Taken together these findings suggest that the configuration of the fibrin network is an important determinant of the outgrowth of tubes in a fibrin matrix.

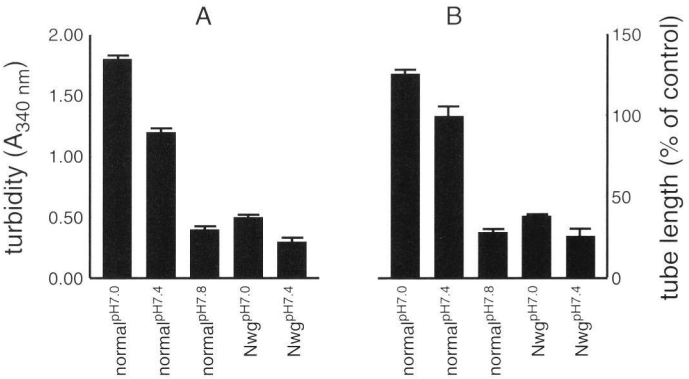


***Figure 7**: Fibrin matrices from normal fibrinogen and fibrinogen$_{Nieuwegein}$ were polymerized at pH 7.0, pH 7.4 and pH 7.8. **A**: Turbidity at 340 nm was measured after 2 hours of polymerization. **B**: hMVEC were seeded on top of these fibrin matrices and stimulated with 5 ng/mL bFGF and 1 ng/mL TNFα to form invasive capillary-like tubular structures. Capillary-like tubular structure formation was quantified after 4 days and expressed as % of control (normal fibrin at pH 7.4). Data are mean ±SD of six cultures in three independent experiments.*

Effect of cross-linking of fibrin on the extent and maintenance of tube formation

Not only the bundle structure is an important factor in the architecture of the fibrin matrix, but also the extent of cross-linking. Tissue transglutaminase, which is released during wounding of a vessel, covalently links two Aα-chains in adjacent fibrin molecules, while factor XIIIa, which is present in the circulation, covalently links two γ-chains.

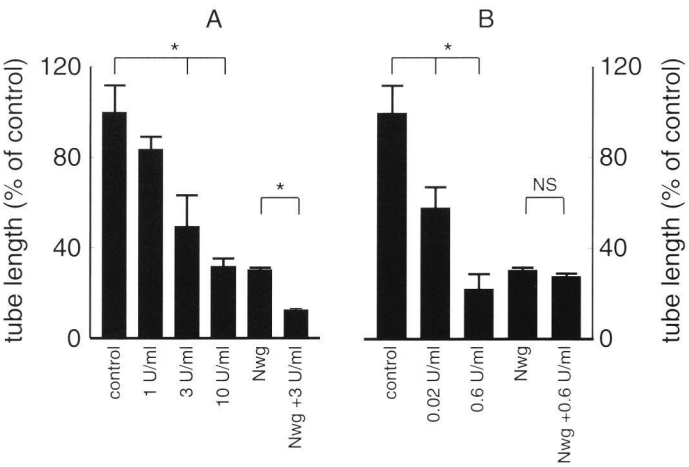


***Figure 8**: Effect of factor XIIIa and tissue transglutaminase on the formation of capillary-like tubular structures in fibrin. Fibrin matrices prepared from normal fibrinogen and fibrinogen$_{Nieuwegein}$ were cross-linked by the addition of different concentrations of factor XIIIa (**A**) and tissue transglutaminase (**B**). hMVEC were seeded on top of the fibrin and stimulated with 5 ng/mL bFGF and 1 ng/mL TNFα to form invasive capillary-like tubular structures. The total length of capillary-like tubular structures was quantified after 4 days and expressed as % of control (normal fibrin). Data are mean ±SD of 8 cultures in four independent experiments (*represents $p < 0.05$).*

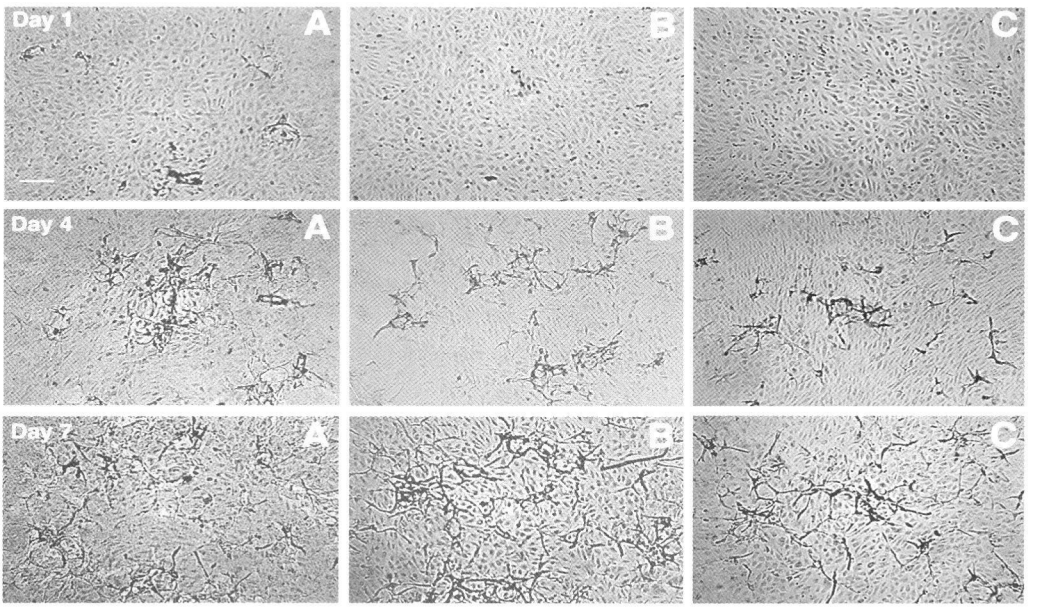
Cross-linking of normal fibrin matrices with factor XIIIa resulted in a decrease of the total length of the tubes formed (figure 8A). Factor XIIIa, added during the polymerization of normal fibrin in a concentration of 1 U/mL, did not significantly affect the extent of tube formation (84±8% as

compared to control), while higher concentrations of 3 U/mL and 10 U/mL factor XIIIa inhibited tube formation by 50±13% and 68±3%, respectively (figure 8D). Cross-linking of the fibrin network formed from $fibrinogen_{Nieuwegein}$ with 3 U/mL factor XIIIa resulted in a proportional decrease (42±1%, n=5) of the length of tubes formed.

Addition of tissue transglutaminase during the polymerization of normal fibrin reduced the length of the formed tubes by 42±9% and 78±5% for 0.02 U/mL and 0.6 U/mL tissue transglutamase, respectively (figure 8B). Addition of 0.6 U/mL tissue transglutaminase to polymerizing $fibrin_{Nieuwegein}$ did not affect the extent of tubes formed (figure 8B).

Interestingly, cross-linking of the normal fibrin matrix with transglutaminase retards the initiation of capillary-like tubular structure formation (figure 9B, C) and as such the increase in formed tube length after 4 days. During a prolonged period of 7 days, the tubular structures in the non cross-linked fibrin matrices were regressed to invaginations in the fibrin matrix, as revealed by examinations of histological cross-sections (figure 9A). The structures formed in cross-linked matrices however appeared to be stable during the prolonged stimulation (figure 9B, C). These data suggest that cross-linking of the fibrin matrix by TG not only affects the initiation and extent of tubular structure formation but also the maintenance of the structures formed.

***Figure 9**: Effect of tissue transglutaminase on the initiation and stability of capillary-like tubular structures in fibrin. Fibrin matrices were cross-linked by the addition of different concentrations of tissue transglutaminase. hMVEC were seeded on top of the fibrin and stimulated with 5 ng/mL bFGF and 1 ng/mL TNFα to form invasive capillary-like tubular structures. At day 1, day 4 and day 7 pictures were taken of control (A), 0.02 U/mL transglutaminase (B) and 0.6 U/mL transglutaminase (C). Bar represents 200 μm. Inserts at day 4 and day 7 are histological cross-sections of the corresponding fibrin matrices.*



Discussion

Congenital dysfibrinogenemia$_{Nieuwegein}$, with an abnormally short Aα-chain results in the formation of an altered fibrin network and delays the formation of capillary-like tubular structures in an *in vitro* angiogenesis model.

The presence of an abnormally short Aα-chain in fibrinogen$_{Nieuwegein}$ was the result of the appearance of a stop codon at Aα 454, due to which the molecular mass of HMW- and LMW- fibrinogen is smaller than the molecular mass of normally occurring HMW- and LMW-fibrinogen molecules [47, 48]. The lack of amino acids at position Aα 454-610 resulted in the appearance of a free SH-group in the cysteine at position Aα 442, which normally forms an intrachain disulfide bridge with the SH-group in the cysteine at Aα 472 [49]. As described previously for other congenital dysfibrinogenemias containing an unpaired cysteine, complexes with free SH of other molecules can be formed [32]. Three different fibrinogen-albumin complexes can arise, as previously described for Fibrinogen Marburg [34], two complexes of one or two albumin molecules per HMW-fibrinogen and a complex of one albumin molecule per LMW- fibrinogen. Complexes with free SH-group in other fibrinogen also can occur leading to fibrinogen/fibrinogen complexes with different high molecular mass [50].

A decrease in turbidity of the formed fibrin$_{Nieuwegein}$ network was found. It has been long known that the turbidity of a fibrin network is a good indicator for its structure [42]. A low turbidity (transparent network) is an indicator for a tight network, consisting of thin rigid fibrin bundles, whereas a high turbidity is a feature of a malleable network, consisting of thick fibrin bundles. The presence of thin fibrin bundles in the rigid network formed from plasma$_{Nieuwegein}$ was confirmed by electron microscopy. The Aα-chain of fibrinogen plays an important role in the lateral association of the fibrin monomers and delay of this association leads to the formation of a tight network, which consists of thin fibrin bundles [14, 15]. The presence of cross-linked albumin to cysteine Aα 442 in fibrinogen$_{Nieuwegein}$ delays the polymerization rate, due to which a tight fibrin network is formed [18, 19].

The effect of the structural and functional defect of fibrinogen$_{Nieuwegein}$ on the formation of capillary-like tubular structures in an *in vitro* angiogenesis model was analyzed. hMVEC were stimulated to form capillary-like tubular structures in the underlying fibrin matrix by the co-operative action of bFGF and TNFα [45]. This process depends on local degradation of the fibrin matrix and detachment of cell-matrix contacts and subsequent renewal of cell-matrix contacts needed for invasion in the underlying matrix and to form a network of tube-like structures. A dramatic decrease in the length of the formed tubules was observed on a fibrin$_{Nieuwegein}$ matrix.

The deleted Aα-segment in fibrinogen$_{Nieuwegein}$ contains the Arg-Gly-Asp sequence at position Aα 572-574, which interacts with the $\alpha_v\beta_3$-integrin. An important role of $\alpha_v\beta_3$-integrin during the process of angiogenesis *in vivo* and during fibrin-endothelial cell interaction *in vitro* has been suggested [30-32]. The absence of the $\alpha_v\beta_3$-integrin binding sequence in fibrinogen$_{Nieuwegein}$ and inhibition of the active form of the $\alpha_v\beta_3$-integrin by LM609 on endothelial cells did not affect endothelial cell binding to the fibrin,

although $\alpha_v\beta_3$-integrin was clearly present on hMVEC in our experimental conditions. In contrast to the expectation [28], the interaction of $\alpha_v\beta_3$–integrin with the RGD at position Aα 572-574 is apparently not necessary for initial attachment of endothelial cells to fibrin. Other receptors, including other RGD-mediated integrins, on endothelial cells are more important for initial endothelial cell-fibrin interactions during attachment [24, 25]. Blocking of the $\alpha_v\beta_3$-integrin receptor by LM609, an inhibiting monoclonal antibody against $\alpha_v\beta_3$, in our *in vitro* model also did not block the formation of capillary-like tubular structures. Our data indicate that the reduced formation of capillary-like tubular structures in fibrin$_{Nieuwegein}$ is not caused by the absence the RGD-binding site at position Aα 572–574.

During the formation of capillary-like tubular structures the architecture of the formed fibrin matrix plays a critical role [11-13]. An opaque fibrin matrix, consisting of a malleable fibrin network, facilitated the formation of capillary-like tubular structures more than a transparent matrix, with a rigid fibrin network. Turbid fibrin matrices prepared from intact fibrinogen have a rapid and extensive ingrowth of capillary-like tubular structures. The process of tube formation in a transparent fibrin matrix prepared from intact fibrinogen developed similarly as the formation in a fibrin$_{Nieuwegein}$ matrix, suggesting that the initiation and extent of tube formation is dependent upon the conformation of the network. In fibrinogen$_{Nieuwegein}$ the presence of cross-linked albumin bound to the cysteine at Aα 442 leads to steric hindering of the lateral association of the fibrin monomers. This results in the formation of a rigid network. A rigid fibrin network is less sensitive to plasmin mediated fibrinolysis than a malleable fibrin network [51], and as such less permissive for rapid ingrowth of endothelial cells [12]. The formation of capillary-like tubular structures was not influenced by the presence of non-cross-linked albumin in the fibrin matrices.

Fibrin matrices prepared from intact fibrinogen without additional cross-linking support rapid ingrowth of tubular structures, but these structures could not be maintained during prolonged incubation. Not only the initiation and progression of capillary-like tubular structures formation, but also the stability and maintenance of the structures formed are influenced by the extent of cross-linking of the fibrin. Cross-linking results in a decrease of fibrinolytic sensitivity of the fibrin matrices [52], due to which endothelial cells more slowly can degrade the matrix. During migration of cells the stabilization of the temporary matrix plays an important role, increased stability of provisional fibrin matrix promotes wound healing *in vivo* [53]. The cross-linking site of tissue transglutaminase in the Aα-chain is deleted in fibrinogen$_{Nieuwegein}$, resulting in an impairment of cross-linking. But, the formation of a tight fibrin network by fibrin$_{Nieuwegein}$ already supports tube formation during a prolonged incubation period, overcoming the defect of cross-linking by transglutaminase.

From these results we can conclude that fibrinogen$_{Nieuwegein}$ has an abnormal short Aα–chain, leading to the absence of the RGD-sequence at position Aα 572-574 and to the formation of a rigid fibrin network partly by the presence of cross-linked albumin at the unpaired cysteine at position Aα 442. When tested in an *in vitro* angiogenesis model the formation of capillary-like tubular structures was delayed. This is probably not due to the absence of the $\alpha_v\beta_3$-integrin binding site but to the formation

of a tight fibrin matrix. The tight fibrin network of fibrinogen$_{Nieuwegein}$ leads to the formation of a stabile provisional matrix, which retards the ingrowth of cells, but favours the stability of angiogenic microvessels once they are formed.

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CHAPTER VI

Impaired capillary-like tubular structure formation in a fibrin matrix by depletion of plasminogen

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Abstract

Adult angiogenesis, associated with pathological conditions, is usually accompanied by the formation of a fibrinous exudate. The formation of capillary structures in a fibrinous matrix *in vivo* can be mimicked *in vitro* by a model, composed of human microvascular endothelial cells (hMVEC) seeded on top of a fibrin matrix and stimulated with bFGF/TNFα to form capillary-like tubular structures. Previous studies showed that local degradation of the matrix by cell-bound urokinase/plasmin activity is required for tube formation. We investigated whether capillary-like tubular structure formation also occurred in experimental conditions avoid of plasmin activity. To that end, the plasmin inhibitor aprotinin was used or the fibrin matrix and the culture medium were depleted from plasminogen. Depletion of plasminogen and the addition of the plasmin inhibitor aprotinin resulted in a decrease of tube formation by 60%. In the absence of plasmin activity the initiation of tube formation occurred, but progression to fully developed tubular structures with a lumen was impaired. Immunohistochemical analysis showed the presence of several MMPs, namely MMP-1, MMP-2 and MMP-3 associated with endothelial cells present in the capillary-like tubular structures. The formation of initial structures in the absence of plasmin activity was inhibited by the metaloproteinase inhibitor BB94. These data indicate that the u-PA/plasmin and MMP-system cooperate in the formation of capillary-like tubular structures in a fibrin matrix.

Introduction

Tissue injury causes the disruption of blood vessels and the extravasation of plasma fibrinogen, which results in the formation of a fibrin clot or fibrinous exudate. This temporary matrix not only prevents further blood loss, but also serves as a scaffolding to support cell migration during wound healing [1]. The invasion of new blood vessels during the formation of granulation tissue is driven by microvascular endothelial cells (MVEC), which upon activation locally degrade their basement membrane, migrate into the supporting matrix, proliferate, and form new capillary-like tubular structures [2]. For this process migrating endothelial cells use and produce at least two protease systems, namely the plasminogen activator/plasmin system and matrix metalloproteinases (MMPs).

The temporary fibrin matrix can be degraded by plasmin, which is activated from its zymogen by two types of plasminogen activators, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) [3]. Although plasmin is an important proteolytic determinant for the invasion of endothelial cells in fibrin [4, 5], it is suggested that both u-PA/plasmin and MMP systems are involved in the degradation of the extracellular matrix [6-14]. This is also supported by the fact that endothelial cells at the leading edge of a new blood vessel concomittantly express components of both protease systems [15, 16] and that their expression is regulated by the same growth factors and

cytokines [17-21]. Furthermore, different components in the extracellular matrix, including fibrin [11, 22, 23], are common substrates for as well plasmin as MMPs [24-26].

MMPs are secreted as inactive zymogens and *in vitro* studies have shown that the u-PA/plasmin sytem directly or indirectly activates a number of pro-MMPs, such as pro-MMP-1, pro-MMP-9, pro-MMP-10 and pro-MMP-13 [27-30]. A complex interrealtionship between both protease systems is further supported by the fact that MMPs can also control plasminogen activation by u-PA/u-PAR at the cell surface [31, 32], and that some MMPs, such as MMP-3 and MMP-12, can form angiostatin, an important inhibitor of angiogenesis, from plasminogen [33-35].

Recently, Hiroaka et al [11] reported that capillary-like tubular structures were formed when tissue segments of plasminogen knockout mice were embedded in a fibrin matrix. This suggests that angiogenesis *in vitro* in a fibrin matrix can occur plasmin-independently [11]. Indeed, these authors reported that this process was driven by MT-MMP activity [11].

The formation of new capillaries in a temporary matrix *in vivo* can be mimicked *in vitro* by a model composed of human MVEC (hMVEC) seeded on top of a three dimensional matrix and stimulated by bFGF/TNFα to form capillary-like tubular structures [36]. bFGF and TNFα cooperatively induce the surface localization of u-PA receptor (u-PAR) and the secretion of urokinase (u-PA), leading to pericellular activation of plasminogen to plasmin by cell-surface localized u-PA. TNFα can, in concert with a growth factor (aFGF), induce the secretion of several components of the MMP-system such as MMP-1, MMP-2 and MMP-8 in MVEC [37, 38].

To investigate whether the MMP-system might play a plasmin-independent role in the formation of capillary-like tubular structure, we investigated the effect of plasminogen depletion on the process of tube formation. In addition we demonstrated the presence of several MMPs in the tubular structures and have shown that MMP activity can act in concert with cell bound u-PA/plasmin activities.

Materials and methods

Materials

Penicillin/streptomycin, L-glutamine and medium M199 with or without phenol red, with EBBS, L-glutamine and HEPES were obtained from Biowitthaker (Verviers, Belgium). Trypsin 1-300 370 USP/mg from ICN (Costa Mesa, CA) and heat-inactivated newborn calf serum from GIBCO BRL (Paisley, Scotland). Human serum was prepared from pooled fresh blood of 10-20 healthy donors obtained from a local bloodbank. Fibronectin was a gift from Dr J. van Mourik (Central Laboratory of the Blood Transfusion Service, Amsterdam). A crude preparation of endothelial cell growth factor was prepared from bovine brain [36]. Thrombin and heparin (UFH) were obtained from Leo Pharmaceutical Products (Weesp, The Netherlands), tissue culture plastics from Costar (Cambridge, MA). Human fibrinogen, free of plasminogen, was purchased from Kordia (Leiden, The Netherlands).

TNFα, containing $2.45x10^7$ U/mg protein and less than 40 ng lipopolysaccharide per mg protein from Dr. J. Tavernier (Biogent, Gent, Belgium). Recombinant human basic fibroblast growth factor (bFGF) was purchased from PeproTech (Rocky Hill, NJ). Plasminogen was a kind gift of Dr. D. Rijken. Polyclonal antibodies against MMP-1, MMP-2 and MMP-3 were from Biotrends. Tissue-Tek at Sakura (Zoeterwoude, The Netherlands), Streptavidin Cy-3 at Sigma Chemicals (St Louis, MO). Lowacryl K11M kit was from TAAB laboratories (Aldermaston, UK). Vectashield and biotin labeled anti-rabbit IgG were from Vector Laboratories (Burlingame, CA, USA). Aprotinin was purchased from Pentapharm Ltd (Basel, Switzerland) and ε-aminocaproic acid from Merck (Darmstadt, Germany). Collagen was from Collagen Corporation (Palo Alto, CA), pyrogen-free human serum albumin from CLB (Amsterdam, The Netherlands), and BB94 was a kind gift from Dr E.A. Bone (British Biotech Ltd, Oxford, UK)

In vitro angiogenesis model

Cell culture

Human foreskin microvascular endothelial cells (hMVEC) were isolated, cultured and characterised as previously described [39, 40]. Cells were cultured until confluence at 5% CO_2/95% air on fibronectin-coated dishes in M199 supplemented with 2 mM L-glutamine, 20 mM Hepes (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 μg/ml crude endothelial cell growth factor (ECGF), 100 IU/ml penicillin and 100 mg/ml streptomycin. The endothelial cells were then detached with trypsin/EDTA and transferred to new fibronectin-coated dishes at a split ratio of 1:3. Confluent endothelial cells were used at passage 9-11.

Preparation of the three dimensional matrices.

Three dimensional matrices were prepared by addition of 300 μl in a 1 cm^2 well of 48-well plates of a mixture of 0.1 U/ml thrombin and 2 mg/ml fibrinogen. After polymerisation the matrices were equilibrated with 0.5 ml M199 containing 10% human serum and 10% newborn calf serum to inactivate residual thrombin. Human and new born calf serum were depleted of plasminogen by incubating 2 volumes of serum with 1 volume of lysine-sepharose for 2 hours at 4° C, after which the lysine sepharose was removed by centrifugation. This process was repeated three times. In conditions in which plasminogen was required 1 μM of Lys-plasminogen was added.
After preparation of the matrices, confluent endothelial cells ($0.7x10^5$ cells/cm^2) were washed three times 5 minutes with M199 containing 100 mM ε-aminocaproic acid (εACA) and 1% human serum albumin (HSA), where after they were detached from the fibronectin coated dishes with trypsin/EDTA. The cell suspension was washed twice with 100 mM εACA in M199 before they were

seeded in the culture media on the matrices in a confluent density. After 24 hours the medium was replaced with medium containing different mediators. Every 48 hours the medium was changed and collected, this for a time period of four to six days. The formation of tubular structures of endothelial cells by invasion into the underlying matrix was analysed by phase contrast microscopy. The total length of the structures formed was measured in six randomly chosen microscopic fields (7.3 mm^2/field) by a computer equipped with Optimas image analysis software connected to a monochrome CCD camera (MX5) and was expressed as mm/cm^2 [36].

Preparation of the specimens.

The matrices were fixed at 4° C in *p*-formaldehyde in phosphate buffered saline (PBS, pH 7.4) for 2 hours at 4° C. After fixation the matrices were washed overnight with 5% sucrose in PBS and embedded in Tissue-Tek and snap-frozen in precooled 2-methyl butane and stored at -80° C. 7 µm-thick sections were made throughout the whole specimen and stained with the appropriate antisera as described below.

Histology

For immunohistochemical analysis of the hMVEC invaded in the matrices, the 10 µm thick sections were fixed on the slide by 4% *p*-formaldehyde in phosphate buffered saline (PBS). The primary antibody (rabbit-anti human MMP-1, MMP-2 and MMP-3, at a concentration of 1:100) was incubated PBS containing 1% FCS for one hour at room temperature, after which it was biotin-labeled by biotinylated goat anti-rabbit IgG. As secondary antibody Streptavidin-Cy3 was added in a 1:100 dilution for one hour and after washing the sections were mounted with vectashield with DAPI. Since the capillary-like tubes are only composed of endothelial cells, no cell marker was used.

Statistics

Experiments were performed with duplicate wells and expressed as % of control. For statistical analysis the ANOVA was used followed by a modified t-test according to Bonferroni.

Results

Plasminogen depletion from the culture conditions impairs capillary-like tubular structure formation in a fibrin matrix

hMVEC seeded on top of a fibrin matrix and cultured without addition of a growth factor or a cytokine remained as a quiescent monolayer on top of the matrix (figure 1A). Stimulation of the hMVEC with the combination of 5 ng/ml bFGF and 1 ng/ml TNFα (bFGF/TNFα) induced invasion and formation of structures in the underlying matrix (figure 1B). Cross-sectional analysis after 6 days confirmed the presence of tubular structures consisting of endothelial cells surrounding a lumen in the underlying fibrin matrix (figure 1C) [36, 41, 42].

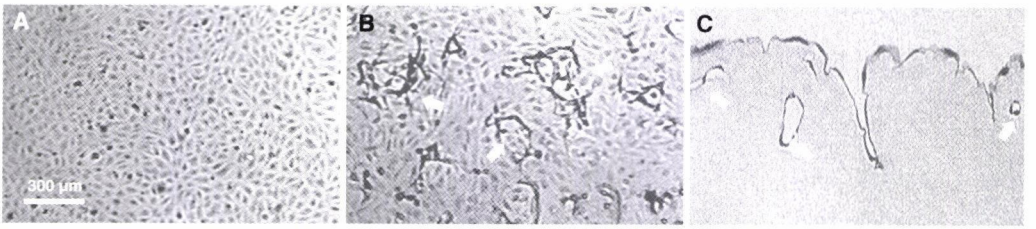


***Figure 1**: Formation of capillary-like tubular structures by hMVEC. hMVEC were cultured on top of a three-dimensional fibrin matrix in M199 with 10% human serum and 10% NBCS and were not stimulated (A) or stimulated with the addition of 5 ng/ml bFGF and 1 ng/ml TNFα (B). After 4 days of culture non-phase photomicrographs were taken. Histological analysis of cross-sections of capillary-like tubular structures (C). Bar represents 300 µm.*

Inhibition of plasmin activity by the addition of aprotinin (100 U/ml) reduced the formation of tubular structures by 59±16% (figure 2). A similar decrease in structure formation (72±6%) was observed when plasminogen was depleted from the fibrin matrix and the culture medium (figure 2).

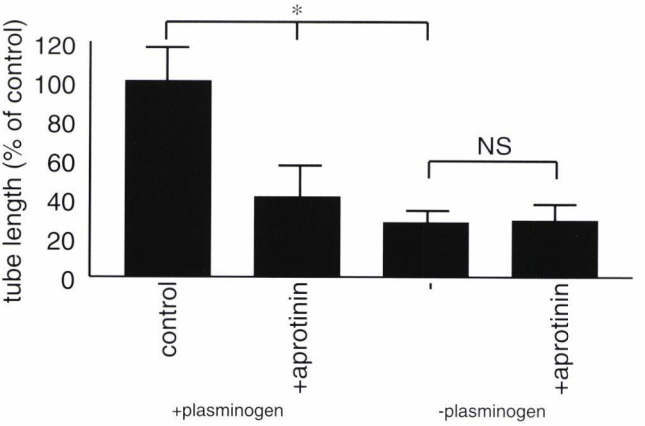


***Figure 2**: Plasminogen depletion impairs tube formation in a fibrin matrix. hMVEC were cultured on the surface of a three-dimensional fibrin matrix in M199 with 10% human serum and 10% NBCS (with or without plasminogen) and were stimulated with 5 ng/ml bFGF and 1 ng/ml TNFα, with or without the addition of aprotinin (100 U/ml). After 4 days of culturing, total tube length was measured as described and expressed as % of tube length formed by bFGF/TNFα-stimulated hMVEC cultured in the presence of plasminogen (control). The data are expressed as % of control and represent mean ±SD of six cultures in three independent experiments.*

The formation of these latter structures could not be further inhibited by the addition of aprotinin (100 U/ml), suggesting that the formation of these structures did not depend on residual plasmin activity (figure 2).

The residual structures formed in both conditions did not resemble the normally formed capillary-like tubular structures, but looked like dense, elongated cord-like structures consisting of sprouting cells (figure 3A, B). Closer analysis by cross-sectional histological examination revealed that the structures did not resemble normal capillary-like tubular structures, but were composed superficially invaded elongated cells, which did not form any substantial lumen (figure 3C, D).

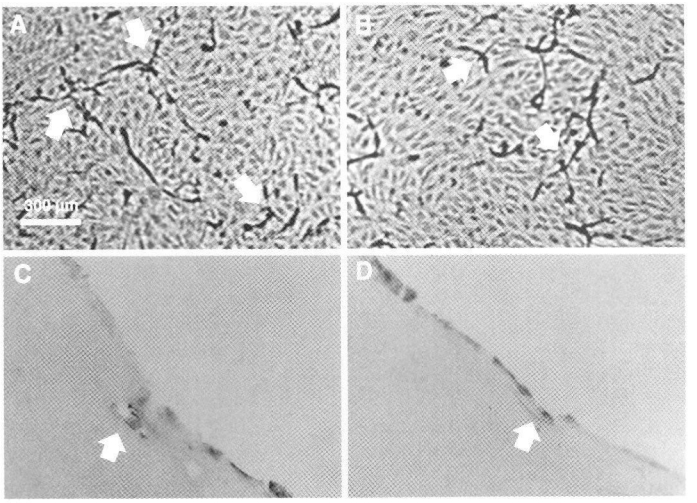


***Figure 3**: Histological analysis of the structures formed in the presence of the plasmin inhibitor aprotinin or in plasminogen deficient conditions. Analysis of the structures formed by endothelial cells seeded on the fibrin matrices and stimulated with 5 ng/ml bFGF and 1 ng/ml TNFα in the presence of plasminogen and aprotinin (100 U/ml) (A, C) or in plasminogen deficient conditions (B, D). After 4 days of culture, pictures were taken and the fibrin matrices were fixed and embedded and cross-sections were analysed (C, D). Bar represents 300 µM.*

Localization of MMP -1, MMP-2 and MMP-3 in capillary-like tubular structures formed by hMVEC in a fibrin matrix

To identify whether alternative proteases, such as MMPs, are expressed by the hMVEC invading the underlying fibrin matrix, the localization of MMP-1, MMP-2 and MMP-3 was analysed. When hMVEC were stimulated with bFGF/TNFα for 4 days, tubular structures were clearly visible underneath the endothelial monolayer. The presence of MMP-1 (figure 4A), MMP-2 (figure 4B), MMP-3 (figure 4C) was confirmed by immunostaining with different polyclonal antibodies. MMP-3 expression was clearly visible in the cytoplasm surrounding the cell nuclei (figure 4C), while MMP-1 (figure 4A) was mainly localised in the basolateral side of the cells and in the surrounding fibrin matrix. MMP-2 staining was localized diffusely around the cell, as well at the basolateral as at the apical side of the cell (figure 4B).

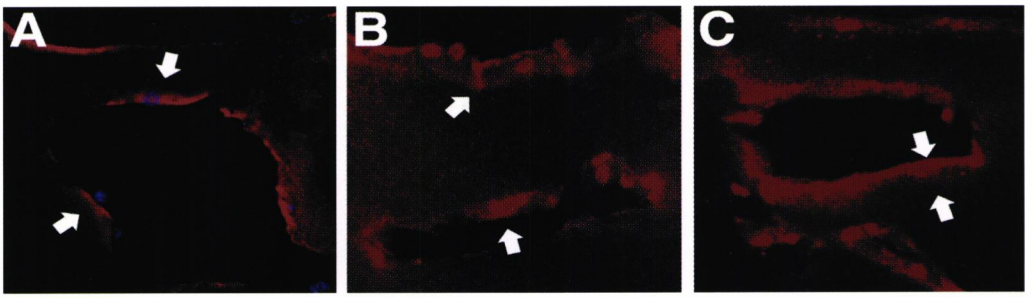


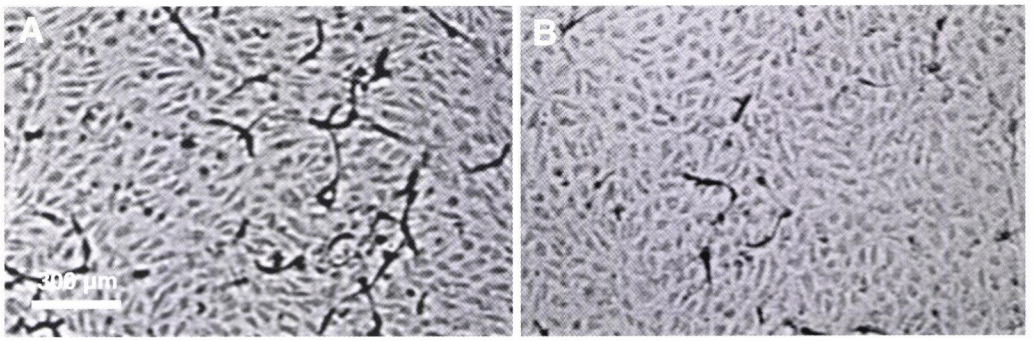
***Figure 4**: Localisation of MMPs in endothelial cells present in the capillary-like tubular structures in a fibrin matrix. hMVEC were cultured on top of a three-dimensional fibrin matrix in M199 with 10% human serum and 10% NBCS and were stimulated with 5 ng/ml bFGF and 1 ng/ml TNFα. After 4 days of culture, the fibrin matrices were fixed and embedded as described and 10 μm cross-sections perpendicular to the matrix surface were cut. Immunohistochemistry was performed as described. Immunostaining for MMP-1 (A), for MMP-2 (B) and for MMP-3 (C). Arrowheads indicate areas of positive staining.*

Inhibition of structure formation in plasminogen deficient conditions by the MMP-inhibitor BB94

To investigate whether the structure formation, which occurred in plasminogen-deficient conditions, required MMP-activity, a general MMP-inhibitor (10 μg/ml BB94, or Batimastat) was added to the culture media (figure 5). Structure formation was largely, but not completely (48±21%) inhibited by the addition of BB94, suggesting that MMP activity is involved in the formation of these elongated lumen less structures (figure 5).

Comparily, if both plasmin and MMP activities were inhibited in conditions in which plasminogen was present, an almost complete inhibition of tube formation occurred, while aprotinin by itself only led to an inhibition of 60% (figure 5C).

***Figure 5A**: hMVEC were seeded on top of a fibrin matrix and were stimulated with bFGF and TNFα in plasminogen deficient conditions. **B**: The addition of BB-94 resulted in a decrease of tube formation. After 4 days pictures were taken. Bar represents 300 μM.*



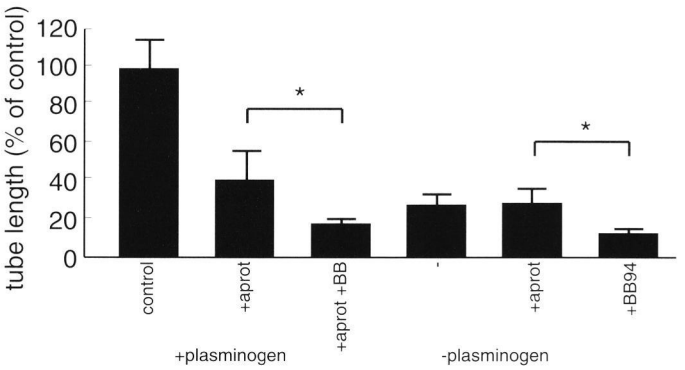


***Figure 5C**: Tube length was measured. Data are expressed as % of control and represent mean ±SD of six cultures in three independent experiments.*

Discussion

Human microvascular endothelial cells, seeded on top of a fibrin matrix, and stimulated with a growth factor and a cytokine invaded the underlying matrix and formed capillary-like tubular structures consisting of endothelial cells surrounding a lumen [36, 41, 42]. The depletion of plasminogen and inhibition of plasmin activity did not impair the initiation but the progression and formation of capillary-like tubular structures. The length and the characteristics of the structures formed were impaired. The structures formed did not resemble the normally formed tubular structures, which consisted of endothelial cells surrounding a lumen in the underlying fibrin matrix, but looked like solid cord-like structures. These structures were composed of aligned endothelial cells which were superficially invaded in the fibrin matrix and which were unable to form any substantial lumen. Analogous cord-like lumen less structures have been reported by Pepper et al., when insufficient fibrin lysis was the result of upregulated protease inhibition by TGF-β [43]. This suggests that degradation of the fibrin matrix by plasmin is critical for the formation of capillary-like tubular structures with a patent lumen.

In the conditions of deficient plasmin activity hMVEC have a residual MMP activity. This MMP activity only contributed to the initiation of structure formation, but did not lead to the progression and formation of normal tubular structures. This was probably due to insufficient proteolytical degradation of the fibrin matrix and as such to patent lumen formation. These data are in concert with previous reports that showed that full formation of capillary-like tubular structures depends on the plasminogen activator/plasmin system in a fibrin matrix [4, 36]. Interestingly, Hiraoka et al showd that MT1-MMP secreted by human umbilical vein endothelial cells (HUVEC) acts as a pericellular fibrinolysin, which directs their invasion into a plasminogen-free fibrin matrix. However, in contrast to HUVEC, hMVEC did not activate MMP-2 upon stimulation by TNFα, which suggests that these cells express little MT1-MMP [37]. This is reflected by the inability of the cells to activate MMP-2 upon stimulation by TNFα, which contrasts to the ability of HUVEC [37]. Differences in expression and secretion of MMPs between macrovascular and microvascular endothelial cells have previously been reported [44]. The

absence of MT1-MMP expression by MVEC might explain why these cells do not form fully developed capillary-like tubular structures in plasminogen deficient conditions.

Our study is confined to the formation of capillary-like tubular structures in a fibrin matrix *in vitro*. *In vivo* it has been shown that endothelial cells present in the leading tip of new blood vessels express u-PA, u-PAR and MMPs as well as their inhibitors [15, 16]. Using our *in vitro* angiogensis model, the expression of MMPs by hMVEC present in the capillary-like tubular structures in a fibrin matrix was analysed. In a previous report we have shown that endothelial cells present in the capillary-like tubular structures express u-PA and u-PAR [42]. Here we report that these hMVEC also express MMPs, such as MMP-1, MMP-2 and MMP-3. Other MMPs may also be involved, but have not yet been studied. When the outgrowth of capillary-like tubular structures was reduced by inhibition of plasmin or plasminogen deficiency, the contribution of metalloproteinase activity became visible. Thus, the presence of components of both systems in capillary-like tubular structures suggests that both protease systems are involved in the process of tube formation *in vitro*. Future studies have to elucidate whether these protease systems both act on fibrinolysis or represent different contributions such as fibrinolysis by the PA/plasmin system and degradation of the basement membrane by the MMPs.

It should be noted that our experiments have been conducted in matrices of purified fibrin. In a fibrinous exudate *in vivo* the fibrin network is enriched with other plasma proteins, such as vitronectin. Furthermore, it is intermingled with collagen fibers from the interstitium. The role of the plasminogen activator/plasmin may be overemphasized as compared to the MMP system by the use of purified fibrin matrices. Future *in vivo* angiogenesis studies, and *in vitro* studies with use of more complex matrices containing fibrin, collagen and other proteins will be helpful to elucidate the role of the PA/plasmin and MMP system and their mutual interaction.

Acknowledgements

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CHAPTER VII

The inactivation of single-chain urokinase-type plasminogen activator by thrombin on cultured human endothelial cells

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Abstract

Single-chain urokinase-type plasminogen activator (scu-PA) is cleaved by thrombin, resulting in an inactive molecule called thrombin-cleaved two-chain urokinase-type plasminogen activator (tcu-PA/T). It has been reported that binding to soluble urokinase receptor protects scu-PA from inactivation by thrombin, but there is no knowledge about cell-mediated inactivation of scu-PA. We have studied whether scu-PA bound to cultured human umbilical vein endothelial cells (HUVEC) could be inactivated by thrombin. High molecular weight scu-PA was bound to HUVEC and incubated with increasing amounts of thrombin for 30 min at 37° C. Cell-bound urokinase-type plasminogen activator (u-PA) was released and levels of scu-PA, tcu-PA/T and active two-chain u-PA were measured using sensitive bioimmunoassays. Cell-bound high molecular weight scu-PA was efficiently inactivated by thrombin. 50% inactivation of scu-PA occurred at about 0.2 nM thrombin. Only low binding of low molecular weight scu-PA to HUVEC was observed. In the presence of monoclonal anti-urokinase receptor IgG, at least 50% of the binding of scu-PA to HUVEC was inhibited. The relative amount of tcu-PA/T that was generated by thrombin was not affected by the monoclonal antibody. These results indicated that scu-PA bound to HUVEC via the urokinase receptor can be inactivated by thrombin. The efficient inactivation of cell-bound scu-PA suggests that a cofactor for thrombin may be involved, like thrombomodulin or glycosaminoglycans. It is concluded that scu-PA bound to the urokinase receptor on a cell surface can be inactivated by thrombin, which may have profound effects on u-PA-mediated local fibrinolysis and extracellular proteolysis during processes in which thrombin is also involved.

Introduction

Urokinase-type plasminogen activator (u-PA) plays an important role in extracellular proteolysis and fibrinolysis by converting plasminogen into plasmin [1]. This serine protease circulates as a single-chain precursor form (scu-PA), which can be activated into two-chain u-PA (tcu-PA) after cleavage at Lys 158 – Ile 159 by plasmin or other activators [1]. Cleavage of scu-PA can also result in inactivation. Thrombin cleaves scu-PA at Arg 156 - Phe 157, two residues prior to the activation site, in this way generating an inactive tcu-PA form called thrombin-cleaved tcu-PA (tcu-PA/T) [2]. The inactivation of scu-PA has been postulated as a mechanism for protecting a fresh blood clot from early fibrinolysis [3, 4]. This process does indeed take place *in vivo*, as we have demonstrated the presence of tcu-PA/T in human body fluids under pathological conditions which involve the production of large amounts of thrombin [5, 6].

The inactivation of scu-PA by thrombin is accelerated by thrombomodulin, both in a purified system and in a plasma milieu [7-9]. Thrombomodulin is a membrane protein on endothelials cells which forms a 1:1 complex with thrombin, in this way promoting both the anticoagulant and antifibrinolytic

properties of thrombin (for review see [10, 11]). Wilhelm *et al.* have shown that binding of scu-PA to soluble urokinase receptor protects scu-PA from inactivation by thrombin [12]. However, this protective effect is abolished in the presence of soluble thrombomodulin [12]. These data imply that the urokinase receptor and thrombomodulin may play a significant role in regulating the inactivation of scu-PA by thrombin. Binding of scu-PA to the urokinase receptor promotes the activation of plasminogen either by augmentation of the intrinsic activity of scu-PA or via reciprocal activation of scu-PA and plasminogen [13-16]. Inactivation of receptor-bound scu-PA by thrombin or the thrombin/thrombomodulin complex thus may affect processes that are mediated by plasminogen activation via receptor-bound u-PA, such as local fibrinolysis, cell migration, tumor invasion, tissue remodelling and angiogenesis [17-19]. However, there is no knowledge about thrombin-mediated inactivation of scu-PA on a cell surface. In the present study, we examined the inactivation of scu-PA bound to cultured human endothelial cells by thrombin.

Materials and methods

Penicillin/streptomycin, L-glutamine and medium M199 were obtained from Biowitthaker (Verviers, Belgium). Heat-inactivated newborn calf serum was obtained from Gibco/BRL (Paisley, UK). Human serum was prepared from pooled fresh blood of 10-20 healthy donors obtained from a local bloodbank. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag *et al.* [20]. Heparin was obtained from Leo Pharmaceutical Products (Weesp, The Netherlands). Gelatin was obtained from Merck (Darmstadt, Germany) and tissue culture plastics from Costar (Cambridge, MA, USA).

High molecular weight (HMW) single-chain urokinase-type plasminogen activator (scu-PA) from a transformed human kidney cell line was a gift from Dr. F. Hammerschmidt (Sandoz, Vienna, Austria) [21]. Low molecular weight (LMW) scu-PA purified from human embryonic kidney cell cultures was a gift from Dr. J. Henkin (Abbott Laboratories, Abbott Park, Ill, USA) [7]. Human thrombin (specific activity of 2.9 NIH U/μg, as indicated by the manufacturer) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hirudin was obtained from Pentapharm (supplied by Kordia, Leiden, The Netherlands). Monoclonal anti-human urokinase receptor IgG (Moab H-2) was a gift from Dr. Weidle (Boehringer Mannheim, Penzburg) [18, 22]. An inhibitory monoclonal IgG directed against epidermal growth factor-like domain 5 of human thrombomodulin was obtained from American Diagnostica (Greenwich, CT, USA). Monoclonal anti-human mannose receptor IgG (15-2) was a gift from M. Barrett-Bergshoeff of this institute [23].

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated, cultured and characterized as described previously [24, 25]. Cells were cultured in a 12-wells gelatin-coated (1%) culture plate until confluence at 5% CO_2/95% air in medium M199 supplemented with 2 mM L-glutamine, 20 mM HEPES (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 µg/ml crude endothelial cell growth factor, 5 U/ml heparin, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37° C. Confluent cells were used from passage 2.

Inactivation of HMW scu-PA or LMW scu-PA by thrombin on HUVEC

Confluent HUVEC cultured in 3.8 cm^2 wells of a 12-wells culture plate were incubated for 2 h at 37° C with serum-free medium. Prior to the incubation with scu-PA, endogenous ligand bound to the urokinase receptor or other binding sites was removed by washing the cells with 50 mM glycine/HCl, pH 3.0, 100 mM NaCl for 3 min at 37° C, followed by immediate neutralization with M199 containing 1% BSA (buffer A). Cells were then incubated with 1 µg/ml HMW scu-PA or LMW scu-PA in buffer A for 30 min at 37° C in a final volume of 400 µl. Unbound scu-PA was removed and increasing amounts of thrombin (0 - 10 nM) in buffer A were added for 30 min at 37° C in a final volume of 400 µl. After incubation thrombin was removed and cells were washed once with buffer A supplemented with 1 ATU/ml hirudin to inhibit thrombin. Cell-bound u-PA was released by adding 405 µl 50 mM glycine/HCl, pH 3.0, 100 mM NaCl, 0.1% BSA for 20 min at 37° C, and supernatants were collected. The supernatants were neutralized by adding 135 µl 150 mM Tris, 0.4% BSA, 0.04% Tween 80 containing 4 ATU/ml hirudin and centrifuged. Levels of tcu-PA, scu-PA and tcu-PA/T were assessed in 25 µl supernatant using bioimmunoassays for tcu-PA, scu-PA and tcu-PA/T (see below) [5, 26, 27]. Total u-PA antigen was determined in 20 µl supernatant using a urokinase ELISA [28]. As a control, the experiment with HMW scu-PA and LMW scu-PA was also performed in blank wells without HUVEC.

Effect of monoclonal anti-urokinase receptor IgG (Moab H-2) on the inactivation of HMW scu-PA by thrombin on HUVEC

HUVEC were incubated with 1 µg/ml HMW scu-PA in the absence and presence of 2.5 and 10 µg/ml monoclonal anti-urokinase receptor IgG (Moab H-2) in buffer A for 30 min at 37° C, as described above. Unbound scu-PA and Moab H-2 were removed and bound scu-PA was inactivated by 0.1 nM thrombin in buffer A for 30 min at 37° C. Cell-bound urokinase was released and levels of tcu-PA, scu-PA and tcu-PA/T were assessed in 25 µl supernatant using the bioimmunoassays, as described above.

Effect of monoclonal anti-thrombomodulin IgG on the inactivation of HMW scu-PA by thrombin on HUVEC

HUVEC were incubated with 1 μg/ml HMW scu-PA in buffer A for 30 min at 37° C, as described above. Unbound scu-PA was removed and bound scu-PA was inactivated by 0.1 and 1 nM thrombin in buffer A in the absence and presence of 2.5 and 25 μg/ml monoclonal anti-thrombomodulin IgG for 30 min at 37° C. As a control antibody, 25 μg/ml monoclonal anti-mannose receptor IgG was used. Cell-bound urokinase was released and levels of tcu-PA, scu-PA and tcu-PA/T were assessed in 25 μl supernatant using the bioimmunoassays, as described above.

Bioimmunoassay (BIA) for tcu-PA, scu-PA and tcu-PA/T

The inactivation of scu-PA on HUVEC was measured using a BIA for tcu-PA and scu-PA, and a BIA for tcu-PA/T as described before [5, 26, 27]. In these assays, the (potential) activity of the various u-PA forms is measured. Briefly, 25 μl supernatant in duplicate was incubated in 96-well plates coated with rabbit polyclonal anti-u-PA antibody. After washing, the immobilized material was activated in parallel wells with buffer (BIA for tcu-PA), 1 nM plasmin (BIA for scu-PA) or 20 nM cathepsin C (BIA for tcu-PA/T). Urokinase activity was determined using Glu-plasminogen and the plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide. The levels of tcu-PA and scu-PA were calculated from a calibration curve obtained by serial dilutions of purified scu-PA, and the level of tcu-PA/T was calculated from a calibration curve obtained by serial dilutions of purified tcu-PA/T.

Statistics

Correlations between levels of u-PA measured in the BIA and levels of u-PA measured in the ELISA were tested using the Pearson correlation coefficient method.

Results

Inactivation of scu-PA by thrombin on HUVEC

The total amount of cell-bound HMW u-PA was calculated as the sum of the levels of tcu-PA, scu-PA and tcu-PA/T as measured in the BIAs. The recovery of total cell-bound HMW u-PA was found to decrease with increasing amounts of thrombin, while no effect was observed on the binding of HMW u-PA to blank wells (table 1). The same phenomenon was found when total u-PA antigen was measured by ELISA (data not shown). In addition, a positive correlation was observed between the levels of the total amount of u-PA recovered in the BIAs and the levels of total u-PA antigen recovered in the

Thrombin (nM)	total HMW u-PA bound to HUVEC (pg)	total HMW u-PA bound to blank wells (pg)	total LMW u-PA bound to HUVEC (pg)	total LMW u-PA bound to blank wells (pg)
0	4357 ± 791	628	241 ± 31	112
0.1	3747 ± 824	619	249 ± 35	113
1	2940 ± 623	586	251 ± 27	112
10	2391 ± 763	611g	238 ± 30	110

***Table 1**: Effect of thrombin on the recovery of cell-bound u-PA in the BIAs. HMW scu-PA or LMW scu-PA (1 µg/ml) were bound to HUVEC for 30 min at 37° C in four (n=4) and three experiments (n=3), respectively. As a control, HMW scu-PA (n=2) and LMW scu-PA (n=1) were also bound to blank wells. Cells were subsequently incubated with increasing amounts of thrombin for 30 min at 37° C. Cell-bound u-PA was released and levels of tcu-PA, scu-PA and tcu-PA/T in the supernatants (total volume of 540 µl) were determined using bioimmunoassays (see Materials and Methods.) The total amount of bound u-PA (mean ± SD, n=4 for HMW scu-PA bound to HUVEC; mean, n=2 for HMW scu-PA bound to blank wells; mean ± SD, n=3 for LMW scu-PA bound to HUVEC; n=1 for LMW scu-PA bound to blank wells) was expressed as the sum of the amounts of tcu-PA, scu-PA and tcu-PA/T.*

ELISA ($r = 0.903$, $p < 0.001$), indicating a simultaneous decrease of both u-PA activity and u-PA antigen (data not shown). The effect of thrombin on the recovery of cell-bound u-PA may be explained by thrombin-mediated release of u-PA bound to HUVEC, resulting in a decreased amount of cell-bound u-PA. To correct for this phenomenon, levels of tcu-PA, scu-PA and tcu-PA/T were expressed as a percentage of the total amount of u-PA bound to HUVEC per thrombin concentration (figure 1). Both the absolute levels of scu-PA (data not shown) and the levels of scu-PA expressed as a percentage of total cell-bound u-PA were found to decrease with increasing amounts of thrombin (figure 1). Simultaneously, tcu-PA/T was generated which was reflected both by an increase of absolute levels of tcu-PA/T (data not shown) and an increase of tcu-PA/T expressed as a percentage of total cell-bound u-PA (figure 1). About 0.2 nM thrombin was needed to induce 50% inactivation of scu-PA. The levels of tcu-PA were very low and were not affected by thrombin, suggesting that scu-PA was hardly activated during the incubation.

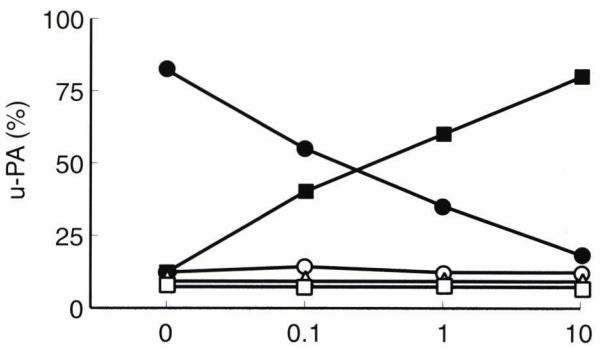


***Figure 1**: Inactivation of HMW scu-PA by thrombin on HUVEC. HMW scu-PA (1 µg/ml) was bound to HUVEC (A) in four experiments (n=4) or to blank wells (B) in two experiments (n=2) for 30 min at 37° C and subsequently incubated with increasing amounts of thrombin for 30 min at 37° C. Levels of cell-bound tcu-PA (▲), scu-PA (●) and tcu-PA/T (■) were measured in bioimmunoassays (see Materials and Methods) and were expressed as a percentage (mean ±SD, n=4) of the total amount of cell-bound u-PA per thrombin concentration (see table 1.) Levels of tcu-PA (△), scu-PA (○) and tcu-PA/T (□) bound to the blank wells were expressed as a percentage (mean, n=2) of the total amount of bound u-PA per thrombin concentration (see table 1.)*

About 10% binding of HMW u-PA to the blank wells was found as compared to binding to HUVEC in the absence of thrombin (table 1). scu-PA bound to the blank wells was also found to be inactivated by thrombin, but to a lesser extent than scu-PA bound to HUVEC (figure 1). In the blank wells, 50% inactivation was not even reached at the highest concentration of thrombin used (10 nM).

In the absence of thrombin, about 5% binding of LMW scu-PA to HUVEC was found as compared to HMW scu-PA (table 1). Thrombin did not affect the recovery of bound LMW u-PA in the BIAs. The binding of LMW scu-PA to HUVEC was about twofold higher than the binding of LMW scu-PA to blank wells (table 1).

These findings implied that in this experimental set-up about 90% of the binding of HMW scu-PA to HUVEC was specific and mediated via the N-terminal part of scu-PA. In addition, scu-PA bound to HUVEC via cellular binding sites was efficiently inactivated by thrombin.

Role of the urokinase receptor in the inactivation of scu-PA by thrombin on HUVEC

To examine specifically whether scu-PA bound to the urokinase receptor could be inactivated by thrombin, we studied in a second experiment the effect of monoclonal anti-urokinase receptor IgG (Moab H-2) on the inactivation of HMW scu-PA by thrombin. The binding of HMW scu-PA to HUVEC was inhibited by Moab H-2 in a concentration-dependent manner (figure 2A). At 10 μg/ml monoclonal antibody, 50% inhibition of scu-PA binding was found. This finding indicated that in this experimental set-up at least 50% of HMW scu-PA is bound to HUVEC via the urokinase receptor. Since cell-bound HMW scu-PA could be completely inactivated by 10 nM thrombin (figure 1), the urokinase receptor must be at least partially involved in this process. The involvement of the urokinase receptor in the inactivation of cell-bound scu-PA by thrombin was confirmed by the finding that the relative amount of generated tcu-PA/T was not affected by Moab H-2 (figure 2B). If only scu-PA bound to HUVEC via other binding sites than the urokinase receptor had been inactivated by

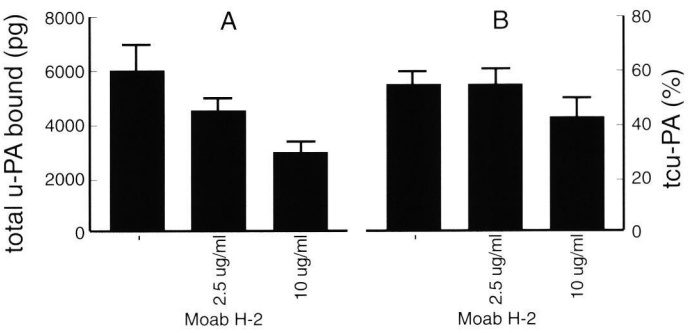


***Figure 2**: Effect of monoclonal anti-urokinase receptor IgG (Moab H-2) on the binding of HMW scu-PA to HUVEC and on the inactivation of scu-PA by thrombin on HUVEC. HMW scu-PA (1 μg/ml) was bound to HUVEC in the absence (open bars) and presence of 2.5 μg/ml (hatched bars) or 10 μg/ml (solid bars) Moab H-2 for 30 min at 37° C in three experiments (n=3.) Subsequently, cell-bound scu-PA was incubated with 0.1 nM thrombin for 30 min at 37° C. Levels of cell-bound tcu-PA, scu-PA and tcu-PA/T were measured in bioimmunoassays (see Materials and Methods.) The total amount of bound u-PA (mean ± SD, n=3) was expressed as the sum of the amounts of tcu-PA, scu-PA and tcu-PA/T (A) and generated tcu-PA/T was expressed as a percentage of the total amount of bound u-PA (B).*

thrombin, then an increase in the relative amount of generated tcu-PA/T could have been expected in the presence of Moab H-2.

Role of thrombomodulin in the inactivation of scu-PA by thrombin on HUVEC

Monoclonal anti-thrombomodulin IgG and the control antibody monoclonal anti-mannose receptor IgG did not affect the total amount of HMW u-PA that was recovered in the BIA and had no significant effect on the inactivation of scu-PA by thrombin at the concentrations used (data not shown).

Discussion

In this study, thrombin-mediated inactivation of scu-PA bound to HUVEC was examined. The results demonstrated that binding of scu-PA to HUVEC does not protect scu-PA from inhibition by thrombin. Cell-bound scu-PA was almost completely inactivated into tcu-PA/T by 10 nM thrombin. In this experimental set-up, at least 50% of the binding of scu-PA to HUVEC appeared to be mediated by the urokinase receptor. The involvement of other binding sites besides the urokinase receptor, like protease nexins and glycosaminoglycans cannot be excluded [29-32]. From our results it is concluded that binding of scu-PA to HUVEC via the urokinase receptor offers no protection against inactivation, as has been claimed for the binding of scu-PA to soluble urokinase receptor by Wilhelm *et al.* [12]. An explanation may be that due to the binding of thrombin to an endothelial cofactor, the protective effect of the urokinase receptor is abolished. In the study of Wilhelm *et al.*, thrombin bound to its cofactor thrombomodulin was capable of inactivating urokinase receptor-bound scu-PA [12].

Cell-bound scu-PA was very efficiently inactivated by thrombin. About 0.2 nM thrombin was needed to induce 50% inactivation of scu-PA, which is far less than observed in buffer or in a plasma milieu [7, 9]. In the absence of thrombomodulin, 50% inactivation of scu-PA in buffer is induced by 5-15 nM thrombin, while more than 300 nM is needed in a plasma milieu because of the presence of thrombin inhibitors [7, 9]. In the presence of thrombomodulin, about 0.2-1.0 nM thrombin is needed to induce 50% inactivation in buffer, and about 80-90 nM is needed in a plasma milieu [7, 9]. These data suggest that the inactivation of cell-bound scu-PA by thrombin may be potentiated by a cofactor. Thrombomodulin is an endothelial cofactor for thrombin and potentiates the activation of protein C and thrombin activatable fibrinolysis inhibitor by thrombin at the endothelial cell surface [11, 33-35]. In this study, the inactivation of cell-bound scu-PA by thrombin was not affected by 25 μg/ml (170 nM) monoclonal anti-thrombomodulin IgG, suggesting that the role of thromobomodulin is of minor importance. However, the amount of monoclonal antibody used in this study may not have been sufficient to prevent the binding of thrombin to thrombomodulin. Molinari *et al.* have demonstrated

that endogenous thrombomodulin enhances the inactivation of scu-PA by thrombin in a perfused rabbit heart model [8]. In addition to thrombomodulin, other components on the endothelial surface may serve as a cofactor for thrombin. It is known that heparin and related glycosaminoglycans like heparin sulphate and chondroitin sulphate potentiate the inactivation of scu-PA by thrombin [8, 36]. Heparin-like molecules are present on endothelial cells and are able to bind both thrombin and u-PA [31, 32, 37-39]. Further research is needed to examine the role of thrombomodulin or other cofactors like glycosaminoglycans in the inactivation of cell-bound scu-PA by thrombin.

A strong and rapid decreasing effect of thrombin on the recovery of cell-bound u-PA was observed. This observation may be explained by the release of the urokinase receptor from HUVEC by thrombin [40]. Cleavage of the ligand-binding domains of the urokinase receptor by tcu-PA as described by Hoyer-Hansen *et al* [41] is not likely, since no activation of scu-PA into active tcu-PA was observed.

The inactivation of cell-bound scu-PA seems not to be restricted to a specific cell type. In a preliminary experiment, we found that scu-PA bound to cultured human smooth muscle cells is also inactivated by thrombin (unpublished observations). Recently, it was demonstrated that scu-PA associated to human platelets via an unidentified urokinase binding protein is inactivated by thrombin into tcu-PA/T. This reaction is followed by re-activation of tcu-PA/T due to thrombin-mediated release of a lysosomal enzyme by platelets [42]. This phenomenon may be of importance for the regulation of fibrinolysis. Besides in fibrinolysis, thrombin-mediated inactivation of cell-bound scu-PA may have profound effects on processes in which both u-PA and thrombin are involved, like angiogenesis, atherosclerosis and restenosis.

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CHAPTER VIII

General conclusions

General conclusions

In this chapter some specific findings described in this thesis are discussed in a broader context. The major finding of our studies is that the architecture and composition of the fibrin matrix determines the initiation, progression and stability of capillary-like tubular structure formation in fibrin. This finding is based on *in vitro* studies, in which the structure of the fibrin matrix was modulated by physical conditions during coagulation, by biological agents, in particular heparins, and by a structural defect in fibrinogen Nieuwegein. It is important that these studies will be followed by *in vivo* studies, in which the fibrinous exudate can be studied in the context of many parameters that contribute to angiogenic processes. Because a fibrinous exudate will be sensitive to lysis, enforcement of the fibrinous matrix by an inert and supportive matrix is probably needed. A sponge, which can be transplanted subcutaneously, would be a good model for such studies [1]. Alternatively, wound chambers [2] may be used, as they allow not only to inspect a spontaneously formed fibrinous exudate, but also the neovascularisation of modified fibrin layers that have been placed on top of a skin wound.
Three aspects have received special attention in this thesis: (a) the involvement of proteases; (b) the role of integrins; (c) the structure and extent of cross-linking of the fibrin network.

Involvement of proteases

Formation of capillary-like tubular structures involves cell movement into the temporary fibrin matrix. This requires an active proteolytic system that can cleave a path for cell migration and invasion [3, 4]. The existing endothelial cell-matrix interactions, which anchors the cell to the extracellular matrix, are disrupted. At the leading edge of the moving cell expression and activation of matrix degrading enzymes contribute to degradation of the extracellular matrix [5, 6]. This degradation facilitates the invasion process by creating a path for cellular movement [7].
In our *in vitro* angiogenesis model, invasion of endothelial cells in the underlying fibrin matrix is mainly accomplished by the PA/plasmin system. The cell-bound plasmin activity not only disrupts the cell-matrix interactions, but also degrades the fibrin to create space for cell invasion. The parallel occurrence of tube formation and fibrin degradation is only found when plasmin is generated by u-PA/u-PAR activity, and not by t-PA activity. Binding of u-PA and plasminogen to their receptors results in a localised activation of u-PA and plasminogen. This confines the protease activity to the immediate vincinity of the cell [8-11]. The plasmin activity, which is generated by t-PA, is not confined to the pericellular area. t-PA-mediated plasminogen activation occurs throughout a large area of the fibrin matrix, and since the fibrin/plasmin complex is protected from inactivation by α_2-antiplasim, it causes uncontrolled lysis of the entire matrix structure [12].
Normal capillary-like morphogenesis depends on a precise protease-antiprotease equilibium. Physiological protease inhibitors play an important regulatory role in controlling proteolytic activity

[4, 13]. Increased u-PA expression by endothelioma cells results in uncontrolled fibrin degradation [14]. Similarly, when u-PA activity is uncontrolled by deficiency in PAI-1, angiogenesis can be impaired in certain conditions [15]. This results in degradation of matrix proteins, that represent the scaffolding structure for the migrating cells. As a consequence cells can no longer adhere and pull themselves through the matrix, and undergo apoptosis [16, 17].

Plasminogen deficiency *in vivo* usually does not impair angiogenesis, which suggests that other protease systems, probably MMPs, can compensate during development and in several pathological conditions [18]. Our *in vitro* studies indicate that u-PA/plasmin activity is a prerequisite for tube formation. The model is composed of purified fibrin [19], and therefore the role of u-PA /plasmin may be limited to situations in which endothelial cells encounter and must degrade fibrin to form new capillary sprouts. *in vivo* more complex temporary matrices are formed [20]. They are formed from fibrin which is entangled in the interstitial collagen and in which plasma proteins such as vitronectin are entrapped. It is likely that matrix degrading metalloproteinases (MMPs) play an important role in the digestion of several of these physiological matrices [21]. In particular, membrane-bound MT-MMPs in combination with other MMPs provide a means of cell-bound proteolytic activity with a broad substrate spectrum, including proteins sensitive for the u-PA/plasmin system [22-24].

The formation of fully developed capillary-like tubular structures *in vitro* depends upon plasmin activity. Depletion of plasminogen or inhibition of plasmin activity did not prevent the initiation of superficially invading structures, but caused complete inhibition of further cell invasion and the formation of capillary-like tubular structures. For the formation of a patent lumen although plasmin activity needs to be present. Although MMPs are capable of degrading fibrin [25-27], they can not result in the formation normal tubes in the absence of plasminogen. The fact that in plasminogen deficient conditions the residual MMP activity could lead to initiation of tube formation suggests a role for MMPs in angiogenesis. This role is also emphasised by the fact that the same factors induce the expression of both protease systems and that both systems are upregulated in the leading tip of a neovessel [4, 13, 28-32]. Future studies have to elucidate whether these protease systems both act on degradation of the fibrinous exudate, or each have aspecific role, i.e. degradation of the temporary fibrin matrix by the PA/plasmin system and degradation of the basement membrane by the MMPs. For therapeutical applications it should be taken in account that selection of the type of protease inhibition to achieve inhibition of angiogenesis depends on the balance of both protease systems, and that in many conditions a complete inhibition of angiogenesis requires interference with both protease systems.

Role of integrins

Not only proteases play a role during the migration of endothelial cells. The process of angiogenesis involves multple inteactions between endothelial cells and proteins present in the extracellular matrix

[33, 34]. The adherence, invasion and migration of endothelial cells during angiogenesis is regulated by one or more members of the integrin family [17, 35-37]. The temporary fibrinous exudate provides a template of different adhesion sites onto which endothelial cells can adhere during migration. During angiogenesis *in vivo* $\alpha_v\beta_3$-integrin plays an important role. In several angiogenesis models *in vivo*, inhibition of $\alpha_v\beta_3$-interaction with the extracellular matrix results in the inhibition of capillary network formation [17, 38]. It is suggested that endothelial $\alpha_v\beta_3$-integrin interacts with and RGD-sequence in the Aα-chain of fibrin(ogen) [39, 40]. In our *in vitro* model, consisting of pure fibrin and endothelial cells an important role for $\alpha_v\beta_3$ seems to be absent. Blocking of the $\alpha_v\beta_3$-integrin receptor and the absence of a binding site in a congenital dysfibrinogenemia, at position Aα 572-574, did not impair formation of capillary-like tubular structures. The interaction between endothelial $\alpha_v\beta_3$-integrin and the RGD-sequence at position Aα 572-574 is not necessary for attachment and migration, this in contrast to what expected. This discrepancy between the *in vivo* data and our *in vitro* findings can be explained in several ways. Either endothelial cells in contact with fibrin preferentially use another, yet unclear, receptor. Other receptors, even RGD-mediated integrins, on endothelial cells could be more important for initial endothelial cell-fibrin interactions during attachment [41, 42].

The network structure of fibrin

The process of vessel formation and the resulting stabilisation of the newly formed vessel is a complex event that depends on the interaction of cells with the extracellular matrix. Our studies revealed that the formation of capillary-like tubular structures by endothelial cells in a fibrin matrix depends on the structure of the formed network. Many conditions and components influence the structure of fibrin when they are present during fibrin formation [43]. We found that opaque and porous fibrin matrices permit a faster and more extensive outgrowth of tubular structures than transparent and rigid matrices. Opaque, malleable matrices are more sensitive for degradation by plasmin than transparent, rigid matrices [44]. Not only the fibrinolytic sensitivity of the fibrin matrices plays a role, but the accessibility of important epitopes for endothelial cell adhesion might be different in different fibrin bundle structures [45]. Malleable matrices support faster ingrowth of tubular structures, but the formed vessels are not very stabile and after a prolonged incubation period the capillary network collapses. This emphasises the impact of the fibrin-modifying conditions during fibrin formation on the angiogenenic process and as such on wound healing. The quality, rate and stability of vessels formed after wounding or during recanalisation of mural thrombi may in part depend on the microenvironment. Recently *in vivo* studies have demonstrated that a stable fibrin matrix favours wound healing. It has been suggested that stabilisation of the temporary matrix supports better migration of cells and ,as such, results in a better wound healing *in vivo* [46]. Therapeutic interventions can be easily undertaken, if the fibrin-modifying conditions, which favour angiogenesis, are better understood.

It should be taken in account that drugs, such as heparin, influence the fibrin structure. Clinical studies have shown that cancer patients treated with unfractionated heparin (UFH) have a shorter survival than patients treated with low-molecular-weight heparin (LMWH) [47]. In this context we investigated the effect of LMW and UFH on the structure of the fibrin network and as such on angiogenesis. We found that heparin favours the formation of a malleable fibrin network, while LMWH favours the formation of a rigid network [48]. As such, the presence of UFH during fibrin polymerisation favours a faster formation of capillary-like tubular structures, while LMW inhibits the formation. It should be taken in account that permissive matrices do not contribute to the stability of the vessels formed. The angiogenesis, which occurs in areas of tumours, is uncontrolled and as such detrimental to the organism [49]. UFH may contribute to this unorganised angiogenesis, and may help to enhance side effects, such as additional vascular leakage from these unorganised vessels.

A permissive matrix not only supports migration of endothelial cells, also tumour cells will invade easier in malleable matrix. Nehls et al have recently shown that hyaluronan, which is secreted by tumours, alters the fibrin structure to more malleable [50]. As such tumours contribute to the formation of a permissive temporary matrix which supports rapid tumour migration.

When heparins are given to patients with diseases associated with angiogenesis, it should be taken in account that they may influence the structure of the fibrin and as such the angiogenic process. There is a need for systematic evaluation of such effects *in vivo*.

Effect of fibrin stabilisation

The fibrinolytic sensitivity of fibrin matrices can also be influenced by cross-linking. Factor XIIIa, present in the circulation, primarily cross-links γ-chains and tissue transglutaminase (TG), which is released after endothelial injury, cross-links α-chains. The impact of cross-linking of an intravascular thrombus on fibrinolysis has been long known. Extensive cross-linking by XIIIa strengthens the clot, due to which it is less susceptible to plasmin degradation [51]. The role of stabilisation of a fibrinous matrix in neovascularisation of granulation tissue however is a recent field of investigation. Recently, it has been shown that addition of TG to wounds leads to a better healing, probably due to a stabilisation of the fibrin matrix [46].

Our data indicate that the initiation, progression and maintenance of capillary-like tubular structures are influenced by the extent of cross-linking of the fibrin by TG. Cross-linking results in a decrease of fibrinolytic sensitivity of the fibrin matrices [52], due to which endothelial cells more slowly can degrade the matrix. This results in the perisitance of the matrix until wound healing is terminated.

The stabilisation of the matrix by TG could regulate assembly of the granulation tissue and the neovascularization process, that is essential for an effective healing response. Migration of endothelial cells into fibrin forms an indispensable part of the healing process. During migration, the stability of the provisional fibrin matrix is of utmost importance, and TG's ability to stabilise the matrix that resists

degradation may be vital for orchestrating tissue repair. The important role of tissue transglutaminase during wound healing is further supported by the fact that endothelial cells invading a fibrin clot express TG [46].

Taken together, these data indicate that the stability of the fibrin matrix plays an important mediator of angiogenesis by regulating important events in vascular assembly.

Perspective

The recognition that the fibrin matrix structure plays an important role in the formation and stability of capillary-like structures contributes to unravelling the basic mechanisms of endothelial cell-matrix interactions, which play an important role in angiogenesis. Since angiogenesis in the adult is thought to play role in a number of diseases, insights in these mechanisms have the potential to contribute to the development of therapeutic applications. Modulation of endothelial cell-matrix interactions may provide a means to stimulate angiogenesis in poorly vascularised areas, as well as to inhibit angiogenesis in diseases, where it contributes to the progression of the disease. Because the extracellular matrix is different in wounds, bone and various tumours, it may provide a means for selective interference of the angiogenesis process in tumours without interference in the healing of wounds or bone fractures. Future developments have to show whether this is indeed the case and can be implemented in a therapeutic context.

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Curriculum Vitae

De auteur van dit proefschrift werd in Leuven (België) geboren op 2 februari 1968. Na het voltooien van de studie latijn-wetenschappen in het Paridaens-instituut te Leuven werd aan de Katholieke Universiteit te Leuven de studie geneeskunde in 1986 begonnen. Nadat deze in juni 1993 met onderscheiding afgerond werd, is de auteur als wetenschappelijk medewerker werkzaam geweest in CELGEN aan de Katholieke Universiteit te Leuven.

Vanaf april 1995 tot december 1999 werd in het Gaubius Laboratorium, TNO Preventie en Gezondheid, binnen de afdeling Vaat-en Bindweefsel onderzoek onder leiding van Prof. Dr. V.W.M. van Hinsbergh promotieonderzoek verricht, zoals in dit proefschrift beschreven is.

Nawoord

Dit proefschrift is tot stand gekomen met de hulp van velen. Op deze manier zou ik alle collega's, familie-leden en vrienden willen bedanken. In de eerste plaats wil ik mijn collega's in het Gaubius lab bedanken voor alle hulp. Also I would like to thank all the people in the Vascular Biology lab for their help with the immunofluorescence and electron microscopy.

Samenvatting

Voor de groei en het herstel van weefsel is de toevoer van voedingsstoffen en zuurstof en de afvoer van afvalstoffen van essentieel belang. Aanvoerende bloedvaten voorzien in deze behoeften van groeiende tumoren en herstellende wonden. Het vaatbed breidt zich samen met groeiend en herstellend weefsel uit. Inzicht in de mechanisme van nieuwvorming van bloedvaten is van essentieel belang om het proces van tumor groei en metastasering te begrijpen en te kunnen verhinderen. Tevens kunnen deze inzichten helpen om een manier te vinden om slecht helende wonden te voorkomen. De nieuwe vaatjes worden gevormd in een tijdelijke, dragende ondergrond, oftewel fibrine.

Fibrine is normaliter niet aanwezig in het lichaam. De tijdelijke fibrine plug wordt gevormd uit fibrinogeen. Fibrinogeen is een eiwit dat in een bepaalde concentratie aanwezig is in het bloed. Als er een verwonding optreedt dan komt het stollingsysteem op gang. Het eindproduct van dit systeem is trombine. Trombine zorgt ervoor dat fibrinogeen geactiveerd wordt waardoor deze een netwerkje of een stolseltje gaat vormen. Dit stolseltje, of fibrine zorgt ervoor dat de bloeding gestelpt wordt, maar moet op den duur ook weer opgeruimd worden en vervangen worden door het weefsel dat er eerst was. De eerste fase van dit opruimwerk bestaat uit de vorming van aan- en afvoerend bloedvaatjes, oftewel angiogenese.

Vanuit de omringende bloedvaatjes begint dit angiogenese proces. Dit proces wordt gestuurd door de cellen aan de binnenkant van de bloedvatwand, de endotheel cellen. Het groeiende - vragende - weefsel, dat extra bloedvat voorzieningen nodig heeft, roept endotheel cellen van de binnenkant van nabijgelegen vaatjes op. Deze cellen krijgen het vermogen om zich te verplaatsen en te vermenigvuldigen. Doordat ze deze mogelijkheden krijgen zijn ze in staat om vanuit hun moeder bloedvaatje een nieuw bloedvaatje naar het vragende weefsel te vormen.

Bij dit proces trekken de zich nieuwvormende bloedvaatjes door het fibrine stolseltje. Om dit te kunnen doen moeten de celletjes zich eerst losmaken van hun plaats, de fibrine afbreken om een baan te vormen en zich vervolgens een stapje verder weer vast te zetten, om dan weer opnieuw zich los te maken en weer verder te trekken.

De afbraak van fibrine moet in de buurt van de cel en op een gecontroleerde manier gebeuren. Als de cellen niet genoeg fibrine afbreken, dan worden er erg nauwe vaatjes gevormd. Als er te veel fibrine afgebroken wordt, dan kunnen er geen vaatjes gevormd worden omdat de cellen dan hun tijdelijke ondergrond vernietigd hebben.

Om de fibrine op een gelokaliseerde manier af te kunnen breken hebben de geactiveerde cellen een perfect uitgebalanceerd mechanisme. Op het voortstuwende deel dragen de cellen aan de oppervlakte een receptor. Dit is een eiwit dat fungeert als een haakje of ankertje waaraan een specifiek eiwit zich kan vastmaken. Aan de receptor zal u-PA zich binden. u-PA is een eiwit dat in staat is andere eiwitten te activeren. Een van deze eiwitten, oftewel plasminogeen, bevindt zich ook aan de oppervlakte van het voortstuwende deel van de cel. Plasminogeen wordt dan door u-PA tot plasmine geactiveerd. Het is

plasmine dat op zijn beurt in staat is om de cellen los te maken en om fibrine af te breken. Dit proces wordt nauwkeurig onder controle gehouden door remmers, die de activiteit van elk van de enzymen uitbalanceert. Antilichamen tegen u-PA en tegen de u-PA receptor en remmers van de plasmine activiteit zijn in staat om dit proces te blokkeren.

Naast het u-PA/plasmine systeem beschikken de cellen over een ander mechanisme om zich te verplaatsen. Dit systeem wordt het MMP-systeem genoemd. MMP staat voor matrix metalloproteinase, enzymen die de matrix kunnen afbreken en tot een specifieke familie behoren. De bijdrage van dit systeem in de verplaatsing van endotheel cellen in een tijdelijke fibrine ondergrond is echter van minder belang dan de bijdrage die het u-PA/plasmine systeem levert. Maar als de vorming van nieuwe bloedvaatjes volledig geremd moet worden, moet met dit systeem ook rekening gehouden worden.

Niet alleen de fibrine afbrekende capaciteit die de cellen gekregen hebben spelen een rol bij de verplaatsing van de cellen, maar ook de eigenschappen van de tijdelijke fibrine ondergrond. Als fibrinogeen omgezet wordt in fibrine dan wordt de structuur van dit fibrine netwerk bepaald door de omstandigheden. Sommige omstandigheden zorgen ervoor dat een fibrine netwerk gevormd wordt dat bestaat uit dikke bundels die zich organiseren in een grofmazig netwerk met grote tussenruimten. In andere omstandigheden wordt dan weer preferentieel een netwerk gevormd met dunne bundeltjes in een fijnmazig netwerk. De opbouw van het netwerk bepaalt hoe goed het later afgebroken zal worden. Het grofmazige netwerk wordt beter en sneller afgebroken dan het fijnmazige netwerk. Dat heeft tot gevolg dat de cellen zich sneller verplaatsen in het grofmazige netwerk. Maar aangezien de tijdelijke ondergrond sneller afgebroken wordt is hij minder stabiel en blijft hij niet tot op het einde stevig genoeg om de nieuwgevormde vaatjes te dragen. Op de lange duur zal dit vaatje dus inklappen en ten onder gaan, wat niet bevorderlijk is voor de toevoer naar het vragende weefsel.

Niet alleen de omstandigheden in het lichaam tijdens de vorming van de fibrine ondergrond bepalen hoe de structuur is, ook ziekten en medicijnen kunnen de structuur veranderen. Dit kan soms onverwacht het proces van vaatvorming beinvloeden.

Verplaatsende cellen maken niet alleen hun ankers met de matrix los, maar bij de verplaatsing moeten ze ook steeds nieuwe ankerplaaten maken. Cellen beschikken over een heel arsenaal adhesie receptoren die kunnen binden aan fibrine. Een adhesie receptor is een soort sleuteltje dat op het oppervlak van de cel zit en dat past in een slotje in de ondergrond. Door deze specifieke interactie kunnen cellen zich vastmaken aan de ondergrond en kan de ondergrond ook informatie aan de cel doorspelen. Tot nu toe is het echter nog niet helemaal duidelijk welke receptoren endotheel cellen gebruiken om zich vast te zetten en te bewegen over een fibrine ondergrond. Een literatuur studie werd gedaan om een overzicht te krijgen welke interacties er plaats vinden. De belangrijkste receptor die naar voren gekomen is, is de vitronectine receptor of $\alpha_v\beta_3$-integrine, die een interactie aangaat met een slotje op fibrine.

Van een patient werd een nieuw afwijkend fibrinogeen dat dit slotje voor de avb3-integrine mist gekarakteriseerd. De afwezigheid van dit slotje en ook het toevoegen van een antilichaam tegen dit integrine kon de binding van cellen aan fibrine en de vorming van nieuwe bloedvaatjes in de fibrine

matrix niet verhinderen. Verder onderzoek naar andere receptoren die wel van primair belang zijn in dit proces zal moeten aantonen welke receptor een sleutelrol speelt.

Publications

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