Angiogenesis in a fibrinous matrix

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Angiogenesis, also called neovascularization, is the formation of vessels from pre-existing ones. Angiogenesis is a tightly controlled process that is essential for normal physiological events such as wound healing, but is also indispensable for tumor growth and metastases. Endothelial cells form the inner surface of the blood vessel that, upon activation by for instance growth factors, cytokines or inflammatory cells, penetrate their basal membrane and subsequently migrate and invade into the extracellular matrix (ECM), surrounding the blood vessel and proliferate to form neovessels. During wound healing, fibrin initially forms the provisional matrix and once endothelial cells express the proper integrin receptors, they invade this temporary matrix and start to synthesize a permanent matrix. Although the role of fibrinolysis during angiogenesis in wound healing has been challenged, a role for the plasminogen activators uPA and tPA as well as for plasmin, has been demonstrated. These proteases are also modulators of the angiogenic process as their activation affect the formation of neovessels. TAFI has also been described as a modulator of fibrinolysis in angiogenesis as it may act as a direct inhibitor of plasmin generation. Although the role of TAFI in cutaneous wound healing has been shown, it still remains to be explained why TAFI-deficient mice display normal angiogenesis.

Integrins are cell adhesion molecules that support the binding of endothelial cells to ECM components such as fibrin, fibronectin and vitronectin. In particular the $\alpha v\beta 3$- and $\alpha 5\beta 1$-integrins received special attention, because they are expressed on angiogenic endothelial cells. Cross talk between integrins as well as compensatory responses by other integrins display the complex mechanism of action and currently the exact role of angiogenesis is a matter of controversy. Nevertheless, pre-clinical trials with anti-integrin molecules were promising and have led to ongoing clinical trials. Besides their role in cell adhesion, integrins also serve as transmembrane receptors that link the inner cell surface with the outside of the cell. In this way integrins modulate cell signaling pathways that lead to cytoskeleton reorganization and eventually cell motility, that is required for angiogenesis.

The work described in this thesis comprises the different aspects of angiogenesis that in general can be divided into 3 parts. The first part describes the role of fibrin as the most important ECM component during wound healing. The second part describes modulators of the angiogenic process that include plasminogen as well as TAFI, but also integrins as their expression and their subsequent activation also affect angiogenesis. In the third part a closer look at cell signaling pathways that are involved in angiogenesis has been taken, in particular the ones that affect cytoskeleton reorganization.

In **chapter 1** the current knowledge of angiogenesis is reviewed and the different aspects that affect this process are described in more detail. This includes the role of ECM

components, proteolysis and growth factors, but also the regulation of angiogenesis by modulators such as TAFI and integrins as well as cell signaling molecules that affect the regulation of the cytoskeleton.

In **chapter 2** the role of fibrin during wound healing has been further reviewed and provides an overview of the important steps from fibrinogen leakage into the wound, the formation of a provisional matrix, the stabilization of the platelet plug and the formation of the fibrin matrix. Subsequent proteolysis and remodeling are described for the process of angiogenesis during wound healing, in which the interactions between fibrin and endothelial cells play an essential role. In summary, the structural composition of fibrin and the binding of fibrin to cells and proteins highly determine the wound healing process.

Activation of plasminogen has been proposed to play an essential role in proteolytic degradation of extracellular matrices in tissue remodeling events. In **chapter 3** we studied the contribution of plasminogen to proteolytic degradation of extracellular matrices, using subcutaneously inserted sponges and compared plasminogen deficient mice to their wild-type counterparts. Immunohistochemical analyses of the granulation tissue revealed a significantly reduced influx of cells into the provisional matrix and a decreased vascularity in plasminogen deficient mice compared to their wild-type counterparts. Not only we have demonstrated that the fibrinolytic system contributes to effective angiogenesis, but also that in a fibrinous matrix in plasminogen deficient mice, angiogenesis and tissue remodeling can still proceed, although at a slower rate.

In **chapter 4** the role of plasminogen during cell migration and angiogenesis was further evaluated by the addition of TAFI and CPB during *in vitro* tube formation, which is largely plasminogen dependent. Supplementation of the matrix with TAFI or CPB resulted in a reduced formation of tubular structures. Pre-treatment of cells with CBP in combination with CPB supplementation to the matrix resulted in an additional reduction of tube formation, comparable to the inhibition with aprotinin. This may suggests a complete interference of the uPA/plasmin system.

Capillary-like tube formation in the *in vitro* model using a fibrinous matrix, largely depends on the uPA/plasminogen system, but also requires the role of RGD-binding integrins. In **chapter 5**, we demonstrated that tube formation was minimally affected by the addition of either the $\alpha v\beta 3$-integrin inhibiting MoAb LM609 or the $\alpha 5$-integrin inhibiting MoAb IIA1. Remarkably, when $\alpha v\beta 3$- and $\alpha 5\beta 1$-integrins were inhibited simultaneously, tube formation was significantly reduced. It was accompanied by a reduction of uPA

antigen accumulation and less release of fibrin degradation products. In addition, αvβ5-integrin blocking antibodies further enhanced the inhibition by LM609 and IIA1, but did not reduce tube formation when the blocking antibodies were added alone. Inhibition of neovascularization in the murine fibrinous exudate by the αvβ3- and α5β1-integrin inhibiting peptide GRGDSP, demonstrated a reduction in angiogenesis, whereas the αv-inhibiting cRGD did not significantly affect neovascularization. These data demonstrate that blocking of tube formation in a fibrinous exudate requires the simultaneous inhibition of αvβ3- and α5β1-integrins and may bear impact on attempts influencing angiogenesis in a fibrinous environment.

In **chapter 6** we examined the differential gene expression of tubule forming and non-tubule forming endothelial cells on a fibrinous matrix. After 7 days of stimulation with VEGF, bFGF and TNF-α, the culture consisted of monolayer endothelial cells and capillary-like tube-forming endothelial cells. A method was developed to separate the two populations of endothelial cells from each other, keeping the cellular integrity intact to ensure mRNA extraction and cDNA production. Array analysis was performed and differential gene expression was verified by realtime PCR as well as by laser-capture microdissected cross-sections. These data revealed that CDC42GAP, an inhibitor of active-sate small Rho GTPases, was one of the genes that were downregulated in tubule forming endothelial cells compared to the monolayer counterparts. Overexpression of CDC42GAP in endothelial cells reduced the formation of tubule structures, while the suppression of this GTPase by siRNA slightly enhanced this process. Therefore, CDC42GAP was identified as a counter-regulator for tube formation.

Chapter 7 contains a general discussion of our work in relation to the recent developments in angiogenesis research work. The contribution of both the fibrin structure as well as plasminogen to the wound healing process is further discussed. Plasminogen, TAFI and integrins are all modulators that influence the angiogenesis process and for each of these modulators, their contribution to the formation of tubular structures in a fibrinous matrix, was discussed. Furthermore, an attempt was made to provide insight in the contribution of the GAP gene family members to angiogenesis and the role of CDC42GAP in particular.

Together these data provide further insight in the mechanisms involved in the formation of new microvascular structures in a fibrinous matrix. They may be helpful in improving the modulation of angiogenesis in pathological conditions in which it should be inhibited or enforced.