Vasculogenesis and Angiogenesis: Molecular and Cellular Controls

Part 2: Interactions between Cell and Extracellular Environment

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Summary

Angiogenesis, defined as a new blood vessel formation from a pre-existing vessel, is initiated by angiogenic growth factors and their receptors that induce endothelial cell migration and proliferation. Extracellular proteolysis is essential for deassembly and reassembly of endothelial cells to their environmental matrix. The aim of this review is to update data on the role of the coagulation and fibrinolysis system, metalloproteinases and adhesion molecules during this step of angiogenesis.

The first part of this review examined molecular and cellular control of vasculogenesis and angiogenesis, describing in detail the temporal sequence of interactions between angiogenic growth factors and their receptors and among growth factors. After recalling the different stages of angiogenesis, the second part of the review will describe the events leading to deassembly of endothelial cells starting from their basal membrane, an essential prerequisite for their migration, and the events leading to reassembly of endothelial cells (EC), smooth muscle cells (SMC) and the extracellular matrix (ECM) thereby forming a new vessel.

During embryogenesis, cells aggregate into cooperative groupings called tissues. The endothelium is made up of a single layer of en-

dothelial cells. Like the whole epithelium, its cells are juxtaposed and joined. The EC are closely related to the ECM of the underlying connective tissue. This ECM assumes a particular aspect at the basal pole of the cells forming the basal lamina (or basal membrane). The intercellular and cell-matrix interactions take place thanks to membrane molecules known as adhesion molecules. Adhesion is ensured by two biochemically identical molecules (homophilic adhesion) or two different molecules (heterophilic adhesion).

The cells of a growing vessel recognize each other with the help of homophilic adhesion molecules which ensure tissue cohesion. These molecules belong to the cadherin family, the superfamily of immunoglobulins and the selectin family and take part in vessel reassembly during angiogenesis. In addition, these cells interact with the basal lamina lying just beneath the EC layer. The ECM is made up of a complex three-dimensional network of proteins, polysaccharides and signal molecules. In contact with the endothelium, the matrix forms a basal lamina whose chemical composition differs from classic ECM, namely by the prevalence of collagen type. The basal lamina is responsible for regulating cell function. The network formed by the ECM traps growth factors so that they are released on matrix degradation. Endothelial cell interactions with their

matrix environment aim to maintain the stability of the newly formed vessel and are ensured by heterophilic adhesion molecules known as integrins. Nevertheless, integrins also play a role in new vessel formation by tying the EC and thereby allowing their progression through the ECM towards chemotactic growth factors. This displacement is made possible by the rupture of contacts with the EC environment thanks to the metalloproteinases and some elements of the coagulation and fibrinolysis system (figure 1).

Nevertheless, if angiogenesis were constantly activated, it would result in an anarchic and undefined proliferation of new vessels as seen in cancer for example. In addition, anti-angiogenic molecules exist physiologically allowing the angiogenesis process to be stopped once the new vessel has been formed.

The Different Stages of Angiogenesis

Formation of a new blood vessel from a pre-existing vessel, or angiogenesis, is conditioned by compulsory remodelling (figure 2). First and foremost, the extension of a new vessel is characterised by deassembly of the vessel from its environment (the other endothelial cells, pericytes or SMC and the extracellular matrix), a proliferation stage, a migration stage and a re-assembly stage making the newly formed vessel stable.

The Activation Stage Includes:

- triggering the angiogenesis process following stimulus by so-called angiogenic molecules or growth factors
- enhanced cell permeability and the formation of extracellular fibrin deposits
- deassembly of the vessel wall with enzymatic degradation and dissolution of the ECM architecture
- degradation of the basal lamina
- migration of the EC into the perivascular space towards angiogenic stimulation which attracts the EC and is hence known as a chemotactic factor, followed by invasion of the ECM
- proliferation of endothelial cells enabled by the loss of inhibited cell-to-cell contact
- formation of the capillary lumen by EC coalescence.

The Resolution Stage Involves:

- inhibition of EC proliferation
- cell migration arrest

- reconstitution of the basal lamina
- maturation of junctional complexes
- assembly of the vessel wall
- progressive recruitment and differentiation of SMC and pericytes as the endothelial tube elongates
- organization of the three-dimensional architecture of the vascular tree.

Factors Allowing Cell Progression through the ECM

Presentation of the Coagulation and Fibrinolysis System, Integrins and Metalloproteinases

Coagulation and Fibrinolysis

Stimulation by Vascular Endothelial Growth Factor (VEGF) by increasing vascular permeability allows the passage of fibrinogen from the circulating compartment towards the extravascular region. Fibrinogen triggers the coagulation cascade modulated by fibrinolysis to prevent the formation of thrombi and vascular occlusion. The tissue factor of exposed EC initiates extrinsic coagulation, leading to the surface of circulating platelets activated to form a complex transforming prothrombin into thrombin. Thrombin converts the fibrinogen into monomers of fibrin thereby contributing to clot stability. In addition to its vessel protection properties, thrombin also has angiogenic properties.

Regulation of coagulation, or fibrinolysis, is ensured by two key serine proteases which dissolve the deposits of fibrin and the fibrin clot but also play a more direct role in angiogenesis by breaking down ECM proteins. These two proteases are tPA (tissue-type Plasminogen Activator) and uPA (urokinase-type Plasminogen Activator) which transform plasminogen into the active form responsible for dissolution of the fibrin clot. uPA is secreted in the form of an inactive precursor, pro-uPA, which binds to its receptor, uPAR, by glycosylphosphatidylinositols anchored in its membrane. uPA triggers a cascade of proteinases at the cell surface by activating certain metalloproteinases which break down the ECM thereby favouring cell migration. The uPA RNAm is regulated by growth factors, cytokines, and steroids. The role of tPA in angiogenesis is not as well documented as that of uPA and will not be tackled in this review.

Plasminogen Activator inhibitor-1 (PAI-1) is

Figure 1 Schematic diagram of the different molecules involved in the deassembly and reassembly of the vessel from its matrix environment during angiogenesis (redrawn from Mattot et al., 1998).

the main inhibitor of uPA and tPA and together with a2-antiplasmin regulates plasmin production. In addition, PAI-1 determines the rate of uPA binding to uPAR by favouring rapid endocytosis of the uPA-uPAR-PAI-1 complex. By blocking the interactions between vitronectin, a ligand of integrins, uPAR and integrins, PAI-1 favours the detachment of the EC from its matrix environment thereby facilitating its migration.

Metalloproteinases

Metalloproteinases (MMP) belong to the zinc finger protein family whose activation depends on the presence of calcium. There are two types of MMP: secreted MMP and membrane-anchored MMP or MT-MMP (membrane-type MMP). They contain four domains:

a signal peptide, a propeptide domain which is split when the enzyme is activated, a catalytic domain and a C-terminal domain binding the substrate. Under basal conditions, MMP are produced in the form of an inactive proenzyme subsequently activated by withdrawal of the propeptide, thereby revealing the site in the active zinc finger. These molecules are proteolytic enzymes allowing the EC to degrade its basal lamina then its matrix environment before migrating. The MMP include collagenases (MMP-1, MMP-8, MMP-13), stromelysins (MMP-3, MMP-10, MMP-11), other MMP (MMP-7 and MMP-12) and lastly the gelatinases (MMP-2 or gelatinase A, MMP-9 or gelatinase B). The gelatinases seem to be implicated in the angiogenesis process. Secreted MMP are activated in the extracellular compartment or on con-

Figure 2 Description of remodelling triggered by angiogenesis (redrawn from Suh, 2000).

tact with the plasma membrane for MMP-2. MT-MMP contain more than one transmembrane domain responsible for anchoring the enzyme to the plasma membrane and are activated within the cell.

For example, MT1-MMP associated with the plasma membrane allows targeting of ECM degradation in its immediate vicinity and activates the precursor of another MMP, MMP-2. The MMP are specifically inhibited by TIMPs (tissue inhibitors of MMPs). They are regulated at all the levels previously described from pro-MMP to activated MMP¹¹. They are not constitutively expressed but regulated by cytokines, cell-to-cell interactions and cell-matrix interactions¹⁶. Gelatinase A activation is well known: TIMP-2 binds to the N-terminal domain of MT1-MMP, whereas progelatinase A binds to TIMP-2 via its C-terminal domain. Another MT1-MMP molecule found nearby binds to progelatinase A and splits the propeptide domain giving rise to a partially active form which becomes fully active by autocatalysis. The

thrombin-thrombomodulin-protein C complex leading to activated protein C can also split the peptide domain and gelatinase A (figure 3)¹¹.

Integrins

Twenty-two different types of integrins are currently known. They are membrane proteins made up of the α and β chains of the same family which heterodimerize in various combinations of different affinity.

Their function is to allow adhesion of the cell expressing them to the ECM or to nearby cells. By their extracellular segment, integrins specifically bind to different ligands like fibronectin, vitronectin, laminin, collagen, von Willebrand factor and fibrinogen. Some integrins recognize an RGD sequence (arginine-glycine-aspartate) conveyed by ECM, whereas others specifically recognize other sequences. The integrin cytoplasmic segment is related to proteins which bind to cytoskeletal elements (actin microfilaments) (figure 4). Integrins are therefore responsible for the heterophilic binding which

Figure 3 Schematic drawing of the main activation pathways of gelatinase A (MMP-2) (redrawn from Nguyen et al., 2001).

ensures the cohesion or integration of cells and their matrix environment.

Integrin activation entails the interaction of cells with their substrate and intracellular signal transduction. These intracellular signals trigger the events regulating myriad cell functions including cell survival, proliferation and migration¹. This results in elevation of calcium and intracellular pH, synthesis of inositol lipids and phosphorylation of tyrosines kinases such as focal adhesion kinases (FAK), Src kinases, adaptive proteins such as Shc, p130CAS and CrkII, resulting in stimulation downstream of the Ras/Mitogen-activated protein (MAP) kinase pathway.

The integrins identified at endothelial level are integrins $\alpha_6\beta_1$, $\alpha_5\beta_1$, $\alpha_2\beta_1$ and $\alpha_v\beta_3$ which preferentially bind to laminin, fibronectin, collagens and vitronectin respectively (Table). SMC express more specifically integrins β_1 and α_v , whereas $\alpha_v\beta_3$, which recognizes ligands with an RGD sequence, is particularly important during angiogenesis.

Role of these Molecules in Angiogenesis

The hypercoagulating activity and raised thrombin rates seen in cancer are associated with the angiogenic effect of thrombin. In a review based on their own work, Maragoudakis and Tsopanoglou⁹ summarize the different arguments in favour of thrombin's role in angiogenesis, irrespective of the coagulation cascade:

1) Thrombin has an angiogenic effect observed in chick chorio-allantoic membrane (CAM) models, an avian embryo adnexa (extraembryonic tissue) supporting vessel growth and resulting from fusion of the chorion and allantois;

2) In cultured endothelial cell models of human umbilical vein, thrombin triggers 50% of the attachment of endothelial cells to the matrix support of the culture medium. This effect is dose dependent and completely reversible;

3) Thrombin is responsible for activation of progelatinase A and the enhanced secretion of MMP-2 in vascular endothelial cells, the metalloproteinases making up the main proteolytic

system essential to tissue remodelling and cell migration;

4) Thrombin favours vectorial secretion of extracellular matrix proteins by endothelial cells: three hour endothelial cell exposure to thrombin results in an increased basolateral deposition of extracellular matrix proteins (collagen I, fibronectin);

5) Lastly, thrombin enhances the mitogenic effect of endothelial cells induced by VEGF, and measured by an increase in DNA basically during the early hours of exposure to thrombin and by transient positive regulation (8-16 hours) of receptor genes VEGFR2 (Flk-1/KDR) and VEGFR1 (Flt-1). This effect is dose dependent and specific to VEGF (for example, FGF-R1 RNAi does not increase). These events occur via PKC and MAP kinase activation.

There is evidence of other bonds between thrombin and the molecules implicated in angiogenesis. When the angiogenic system is activated, e.g. in cancer in which high concentrations of thrombin are present, thrombin binds to the thrombomodulin at the endothelial surface and activates protein C. Activated protein C is then able to activate MMP-2 which had been inactive thus far (pro-MMP2) and which involves rupture of the basal membrane. Thrombin has a short-lasting effect and it is then rapidly incorporated into blood clots and inactivated by heparin, antithrombin III, the inhibitor of protein C. MMP-9 probably takes part in this stage of basal membrane degradation which precedes EC migration. The gelatinases are activated by the TIMP. In addition, the EC find themselves in contact with ECM collagen type I as the basal membrane has been broken down. Collagen type I is itself able to positively regulate MT1-MMP and activate gelatinase A and this activation continues

until the new basal membrane has been formed (figure 5)¹¹.

The fibrinolysis system which intervenes at the same time to prevent thrombus formation also plays a direct role in angiogenesis. Plasmin's function is to dissolve fibrin clots, but it also activates metalloproteinases activating the proMMP form into intermediate MMP and active MMP. Its form is stabilized by binding to vitronectin, an ECM protein.

The quiescent endothelium does not express uPA, uPAR or PAI-1. Instead, uPA and uPAR are expressed by the endothelium during angiogenesis whereas PAI-1 is expressed by both endothelial and stromal cells. Paracrine induction of PAI-1 is thought to combat excess pericellular proteolysis¹⁴. uPA plays a major role in angiogenesis not only in breaking down the ECM, but also by activating TGF β or favouring the release of bFGF from the ECM. In addition, uPA is pro-angiogenic in the CAM model due to increased bFGF. When its secretion is stimulated by growth factors, it has a chemotactic and mitogenic effect on vascular cells. uPA binding to uPAR via its growth-like factor domain locates and activates protease but itself starts the signal transduction leading to migration/invasion and proliferation¹⁹.

PAI-1 is strongly expressed in many models of tumoral angiogenesis. Absence of PAI-1 in the host at the time of tumor cell grafting severely impairs its invasion and neovascularization in the host. This phenomenon is reversed on administration of PAI-1 in mice knocked out for the gene coding for PAI-1. This may seem paradoxical since PAI-1 decreases plasmin, which is itself pro-angiogenic. The role of interactions between PAI-1, the PA system and vitronectin, composing the ECM has been established in KO PAI-1^{-/-} mice. PAI-1 serves not only to maintain matrix structure by counteracting the excess proteolysis required for EC migration and formation of the endothelial tube, but its multiple interactions guide EC motility through the ECM. Devy⁶ showed that PAI-1 can be both pro- and anti-angiogenic in relation to the concentration present.

Although the plasminogen/plasmin system is implicated in extracellular proteolysis during angiogenesis, knockout mice for uPA, uPAR, PAI-1, plasminogen or vitronectin have normal embryonic development, growth and fertility, suggesting that each of these factors taken

Table The different integrins implicated in angiogenesis and their ligands

$\alpha_1\beta_1$, $\alpha_2\beta_2$	$\alpha_v\beta_3$, $\alpha_v\beta_5$	$\alpha_5\beta_1$
Collagens	Fibronectin	Fibronectin
Laminins	Vitronectin	
	Fibrinogen	
	Osteopontin	
	von Willebrand's factor	
	Thrombospondin

Figure 4 General schematic diagram of the integrin system (redrawn from Catala, 2000).

alone is not essential to angiogenesis - at least during development¹⁴.

On culture of EC, there is an increased expression of MMP-1, -2 and -9. If VEGF is added to the culture medium of human dermal microvascular EC, there is an increase in MT1-MMP and MMP-2 with a decreased expression of TIMP-1 and -2. Under these conditions, changes in MMP expression are less important than those observed for proteins of the PA system¹⁴. These processes are also at work in

pathological conditions as tumoral angiogenesis is decreased in mice knocked out for MMP-2 in a model of melanoma cells of the B16BL6 line¹⁴. KO mice for MMP-9 have a decrease of bone growth cartilage, whereas KO mice for MT1-MMP die between the third and 16th week due to decreased vascular invasion of calcified cartilage.

$\alpha v\beta_3$ is the integrin most implicated in angiogenesis. $\alpha v\beta_3$ binds to MMP-2 in a non-RGD dependent fashion and serves to localise the ac-

Figure 5 Schematic drawing of the interactions between thrombin, gelatinase A (MMP-2) and collagen type I (redrawn from Nguyen et al., 2001)

tive form of metalloproteinase at the surface of angiogenic vessels. This allows the angiogenic cell to break down the ECM during its invasion. After degradation of the basal membrane, the EC enter into contact with collagen type I which is the main protein of the stroma and contains the RGD sites otherwise inaccessible to $\alpha v\beta_3$. In breaking down the collagens, MMP exposes the RGD sites which can then bind to integrin.

This expression of RGD sites after degradation of the ECM will facilitate the invasion and progression of EC through the matrix: via their integrins, the EC move through the ECM. This mechanism is arrested by formation of a new basal lamina avoiding contact between the collagen type I and EC⁷.

αv -/- mutant mice die both in utero and some hours after birth due to brain and bowel vessel abnormalities associated with haemorrhage, whereas β_3 -/- mice present no vascular malformation, suggesting that the function of αv in these two organs may develop by formation of another heterodimer⁷. Fibronectin and integrin $\alpha_5\beta_1$, its main receptor, are both essential to angiogenesis since $\alpha_5\beta_1$ -/- mice die in utero from severe vascular system impairment.

Integrin $\alpha v\beta_3$ expression was found to be enhanced in EC only during angiogenesis and not in quiescent vessels in the chick chorio-allantoic membrane model. In this model, angiogenesis is induced by bFGF and TNF α at ten days and blocked by monoclonal antibodies directed against $\alpha v\beta_3$ whereas these antibodies do not affect vessels already formed². The ligand mediating angiogenesis and binding to $\alpha v\beta_3$ seems to be vitronectin. The binding of $\alpha v\beta_3$ antagonists like peptides containing RGD sequences or specific anti-integrin antibodies leads to a high apoptotic endothelial cell load from angiogenesis vessels. This suggests that $\alpha v\beta_3$ is essential to the survival and maturation of EC during angiogenesis. Interestingly, anti- $\alpha v\beta_3$ antibodies trigger not only inhibition of vascular proliferation, e.g. during scarring or retinal neovascularization, but also in tumoral and developmental cells¹⁸.

Integrin activation pathways seem to be specific to angiogenic stimulation: $\alpha v\beta_3$ is implicated in angiogenesis induced by b-FGF and $\alpha v\beta_5$ is implicated in angiogenesis induced by VEGF and PKC (Protein Kinase C)¹⁸.

Hence, in these cell invasion processes, integrins, MMP, the PA (Plasminogen Activator)

system and PAI-1, associated with the ECM, are tightly bound and cooperate closely:

- uPAR interacts with integrins $\beta 1$ and $\beta 2$ of circulating endothelial cells
- MMP-2 and -9 colocalise with $\beta 1$ integrins
- $\alpha v\beta 3$ integrin binds directly to a MMP and vitronectin
- uPA and PAI-1 balance the high affinity binding between vitronectin and uPAR
- the cell adhesion depending on vitronectin and migration implicating the αv -integrins or uPAR are blocked by PAI-1 irrespective of its role as a protease inhibitor¹⁵.

Factors Allowing Recognition of Cells and their Reassembly

The EC are interconnected by tight junctions and adhesion junctions. Adhesion junctions are made up of calcium-dependant transmembrane adhesion molecules called cadherins. The extracellular domain of vascular endothelial cadherin (VE-cadherin) initiates cell adherence, whereas the cytoplasmic domain interacts with cytoskeletal proteins such as β catenin, plakoglobin and protein p120, and also helps to strengthen the intercellular junction⁸. Hence, the expression of VE-cadherin on the EC surface allows these cells to identify each other as belonging to the same tissue and to form a coherent organ. Carmeliet⁴, generating mice in which the cytoplasmic domain that binds β catenin was truncated, showed that this domain was essential for EC survival. EC which differentiate normally in mutants assemble in vascular plexi but are unable to undergo the remodelling and maturation of the vascular tree observed in normal animals. Yolk-sac EC detach from each other and disperse in the cavity. Mutant animals die at E9.5. The authors showed that truncation of VE-cadherin triggers EC apoptosis and impairs transmission of the signal of endothelial survival ensured by Akt kinase and Bcl2.

Anti-angiogenic Factors

Some inhibitors have been identified in non vascularized tissues or in cellular supernatants of non transforming cells near a tumoral extension. Endogenous inhibitors have also been

recognised together with inhibitors resulting from the breakdown of pro-angiogenic molecules.

The endogenous inhibitors include TIMP, natural MMP inhibitors, thrombospondin-1 and -2, and anti-angiogenic cytokines (IL4, IL10, IL12) which counter-balance the pro-angiogenic cytokines (TNF α , TGF, IL8, LPS, IL1 β , MCP-1)¹⁷. The inhibitors generated by ECM breakdown products or proteins implicated in angiogenesis include lytic fragments of the C-terminal tip of MMP2, PEX. By accumulating in neovascularization tissues, these fragments are thought to block the binding of MMP2 to $\alpha v\beta 3$ and hence block angiogenesis in a CAM model³. Angiostatin is a fragment of plasminogen¹³, and endostatin, a fragment of collagen XVIII¹³. Angiostatin, endostatin, maspin, PEDF and the truncated form of anti-thrombin III belong to the serpins and have anti-angiogenic properties. Hence, breakdown of the ECM and cleavage of certain pro-angiogenic molecules results in a sort of negative retrocontrol of angiogenesis.

Conclusions

In response to an angiogenic stimulus triggered by a growth factor, already existing microvessel endothelial cells break down their basal membranes using proteolytic enzymes, namely the metalloproteinases. Endothelial cells are then able to migrate towards this chemotactic stimulus and follow their progression by breaking down the extracellular matrix. The cells then proliferate, identify each other and reassemble to form a vascular lumen then reconstitute their basal membranes and pericellular matrix environment concomitant with smooth muscle cell proliferation and migration to form a new vessel. In ischaemic diseases, stimulating the activation of angiogenic growth factors seems to be the strategy of choice. In cancer, associated with abnormal cell proliferation in addition to an undefined proliferation of vessels, it seems logical to develop molecules able to curb angiogenesis either by using antibodies against growth factors or favouring physiological inhibitors. An exact identification of these mechanisms will pave the way for new therapeutic strategies.

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