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Extracellular matrix in angiogenesis: dynamic structures with translational potential

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Abstract: The vascular network is an integral component of most organs. Beyond assuring an adequate supply of oxygen and nutrients for normal tissue function, vascular structures provide also a critical interface in the balance of tissue homoeostasis and immune functions. Therefore, understanding the biology of the vascular system is a challenging and important objective because it is vital to many physiological and pathological processes. Unravelling mechanisms of blood vessel expansion and remodelling would offer therapeutic options to ameliorate disorders that are currently leading causes of mortality and morbidity, including cardiovascular diseases, cancer, chronic inflammatory disorders, diabetic retinopathy, tissue defects caused by trauma or chronic skin ulcers. This article will review cellular and molecular mechanisms controlling angiogenesis in the light of recent reports and data from our own laboratories. We will focus on the interaction of growth factors with extracellular matrix (ECM) components during the formation of vascular structures in health and disease. Finally, this article will provide a rationale for targeting the ECM–morphogen interplay for therapeutic angiogenesis.

Key words: angiogenesis – extracellular matrix – tissue repair

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Molecular control of blood vessel growth

New blood vessel formation is a vital component for tissue homoeostasis and remodelling. Vessel growth is a complex cascade of biological events, which is characterized by several features: in most organs, the expansion of new blood vessels occurs through both angiogenesis (sprouting of capillaries from pre-existing blood vessels) and vasculogenesis (*de novo* formation of blood vessels by mobilization of bone marrow-derived endothelial progenitor cells) (1). Furthermore, vascular growth is a multistep process that requires a dynamic, temporally and spatially regulated interaction between endothelial cells, multiple soluble growth factors and a complex network of diverse extracellular matrix (ECM) components. Finally, endothelial cell communication with other cell types, either through direct cell–cell contacts or soluble mediators is essential for effective blood vessel formation. During the past decades, several excellent experimental model systems have been developed and now are available for the study of blood vessel growth and regression during development, health, and disease. Among those, formation of new vascular structures during tissue repair after injury represents a paradigm to examine blood vessel formation under physiological conditions. Cancer and intraocular vascular disorders represent the most intensively studied cases of uncontrolled blood vessel growth.

Role of growth factors and cytokines

In recent years, a complex network of cytokines has been identified and is critical for vessel formation, and extensive insight has been gained into the cytokines transcriptional regulation, processing, binding to cell-surface receptors and signalling pathways. Diverse model systems, including Zebrafish, Xenopus, C. elegans and genetically engineered mouse models, provided substantial evidence that functional blood vessel formation requires a dynamic interaction among endothelial cells, non-endothelial cells, diverse pro- and antiangiogenic growth factors and ECM components (2,3). Owing to the complex network of numerous mediators controlling angiogenesis, a high functional redundancy in the molecular actors in this process is presumed, and severe deficiencies in new blood vessel formation might only be apparent in a specific biological context. For example, on the basis of cell culture and *in vivo* studies, fibroblast growth factors 1 and 2 (FGF-1, FGF-2) were postulated to be pivotal mediators in angiogenesis. However, it was surprising that the overall phenotype and impaired wound angiogenesis in skin defects in FGF-1 and FGF-2 double-knockout mice was rather mild (4). Not until the discovery of the angiopoietins and their receptors Tie-1 and Tie-2, as well as the vascular endothelial growth factors (VEGFs) and their receptors, was the identification of growth factor receptor systems with a more specific and essential function in the vascular system achieved. Most of the knockout and transgenic phenotypes of these molecules are embryonic lethal because of defects in the vascular system (5). Among these, VEGF-A and its receptors appear of particular importance in physiology and disease processes; the VEGF family is the angiogenic ligand–receptor system that over the past decades has attracted the highest interest and potential use in pharmacotherapy in the clinic (6,7). For example, since 2008, antibody-mediated blocking of VEGF activity in combination with chemotherapy has been approved for treating patients with diverse cancer entities (8). Furthermore, since 2006, aptamer-mediated blocking of VEGF pathways was approved by the FDA for the treatment of neovascular age-related macular degeneration (9). These basic biological results and drug approvals described

previously indicate that, from a clinical standpoint, the inhibition of vascular growth by blocking VEGF signalling is highly effective. This contrasts with the lack of evidence for the efficient therapeutic use of VEGF proteins to induce the growth of functional vascular structures in clinical conditions. Although today substantial knowledge exists on the role of VEGF ligands and their receptors, and preclinical models indicate effective restoration of tissue integrity and function by external application of VEGF, the evidence for the efficient therapeutic use of VEGF protein to induce the growth of functional vascular structures in clinical studies is still lacking (8). Although various reasons on different levels might account for this limited success, these circumstances illustrate that it is still unresolved how VEGF ligands act in concert with their environmental components to induce the formation of functional vascular networks. Over the past years, compelling evidence has arisen supporting a pivotal role for the ECM for proper VEGF function, raising novel implications for proangiogenic therapeutic concepts (10,11).

VEGF proteins and their receptors

The VEGF family consists of seven members, VEGF-A through VEGF-F, and placenta growth factor (PlGF), which occur in several different splice variants and processed forms (5,6). These ligands share a common structure of eight characteristically spaced cysteine residues in a VEGF homology domain. VEGF members have different physical and biological properties and bind in an overlapping pattern to three receptor tyrosine kinases, known as VEGFR-1, VEGFR-2, and VEGFR-3, as well as to co-receptors including neuropilins (Nrp) and heparan sulphate proteoglycans (HSPG) (5,6). Through these interactions, VEGF ligands and their receptors provide a complex network of potent mediators controlling the expansion and remodelling of the vascular system.

Vascular endothelial growth factor-A was the first member of the VEGF family to be identified (12,13). Since its discovery, VEGF-A has been one of the most studied angiogenic growth factors and is thought to be of singular importance in vascular biology. VEGF-A levels are regulated through transcriptional control and mRNA stability. Moreover, by differential mRNA splicing, the single human VEGF-A gene gives rise to at least eight isoforms (VEGF121, 145, 148, 162, 165, 183, 189, and 206; the same splice variants exist in the mouse, only in slightly different positions), the relative abundance of which varies among different tissues (6,14); the 165-amino acid isoform is the major gene product found in human tissues. The domains encoded by exons 1–5 of the VEGF-A gene are present in all VEGF splice variants and contain information required for the recognition of the tyrosine kinase VEGF receptors 1 (VEGFR-1/Flt-1) and 2 (VEGFR-2/KDR/Flk-1) (15). The isoforms are distinguished by the presence or absence of the peptides encoded by exons 6 and 7 of the VEGF-A gene that code for two independent heparin-binding domains (HBDs). Substantial evidence indicates that differences in the expression of the HBDs are critically involved in the diverse biochemical and functional properties of the VEGF-A splice forms, including binding to cell surfaces and ECM (16,17), receptor binding characteristics (18), endothelial cell adhesion and survival (19) and vascular branch formation (20). VEGF-A transcription is regulated by numerous external factors including growth factors, proinflammatory cytokines, hormones and cellular stress (21). One of the best-characterized factor of VEGF-A synthesis is hypoxia.

In addition to mRNA splicing, post-translational processing of VEGF molecules contributes to the generation of additional VEGF variants that differ in receptor specificity, affinity and biological activity. There is increasing evidence that proteolytic processing of VEGF members plays a crucial role in their post-translational modification and regulation of function (22–25). This observation suggests that the proteolytic microenvironment in which vessel formation is taking place should be considered as a critical determinant controlling VEGF-ligand-mediated activities. Initial evidence supporting the hypothesis that VEGF-A proteolytic processing regulates its activity was derived from studies analysing the activity of the VEGF189 isoform (23). Whereas native VEGF189 binds to VEGFR-1 but not VEGFR-2, maturation of native VEGF189 by urokinase (uPA) within the exon 6-encoded sequence resulted in its VEGFR-2 binding and exerted a mitogenic effect on endothelial cells. Whether VEGF189, in particular its uPA-mediated activation, plays a role *in vivo* has not been analysed so far. In addition, plasmin has been identified as a critical factor regulating the activity of VEGF family members. For example, we (S.E.) and other investigators demonstrated the sensitivity of VEGF165 protein to serine proteases, in particular plasmin (22,26,27). Plasmin–VEGF-A165 interaction attenuates its overall angiogenic potency. In contrast, processing of VEGF-C and VEGF-D by plasmin results in their activation (24,25). Both growth factors are secreted as full-length inactive forms consisting of amino- and carboxyl-terminal propeptides and a central VEGF homology domain. Proteolytic cleavage removes the propeptides to generate mature forms that bind receptors with much greater affinity than full-length forms. The biological significance of plasmin-mediated activation of VEGF-C and VEGF-D *in vivo* has yet to be determined. Whereas VEGF-A acts primarily on the vascular endothelium, the role of VEGF-C and VEGF-D has been implicated in lymphangiogenesis.

A distinct feature of VEGF-A is that it has been shown to regulate multiple events during vascular growth including vascular permeability, migration and proliferation of pre-existing endothelial cells and the recruitment of marrow-derived endothelial progenitor and perivascular cells to the local site of vascular remodelling (28–30). By now, substantial evidence in various preclinical animal and disease models corroborates a critical role for VEGF-A during the repair of soft tissues, including the skin (30,31). Recently, these studies have been complemented by cell type–specific ablation of the VEGF-A gene in the epidermis or myeloid cells. VEGF-A gene deletion in either cell compartment leads to delayed wound closure kinetics (32,33). Furthermore, impaired tissue repair is a major side effect of anti-VEGF tumor therapies, emphasizing VEGF's critical role in the healing response also in humans (34). Together, these data provide substantial evidence that VEGF-A activity is a critical regulator of soft tissue repair. For a more comprehensive overview on the biology of other VEGF family members and their receptors, we refer to several excellent reviews (5,6).

Importance of extracellular matrix components

The ECM is a fundamental component of the microenvironment of cells and forms a complex three-dimensional network assembled from multiple components, including collagens, non-collagenous glycoproteins, elastin, proteoglycans and matricellular proteins in an organ-specific manner. Traditionally, the ECM was considered as an inert, space-filling material between cells that

Table 1. Functions of ECM in vascular biology

Functions as adhesive substrate
Controls cell function and gene expression
Provides structural integrity
Sequesters and stores cytokines
Generates growth factor gradients
Mediates the release of growth factors in the presence of cell-mediated forces or proteolytic degradation
Spatio-temporal regulation of factor release
Presents growth factor to their receptors
Facilitates growth factor receptor and integrin crosstalk
Sensing and transduction of mechanical forces

ECM, extracellular matrix.

provides mechanical support and integrity to organs. However, in recent years, it has become clear that the matrix provides a dynamic and bioactive structure that fundamentally controls cell behaviour through chemical and mechanical signals (35). Also, endothelial cell function and vascular growth are critically dependent on the interactions with the surrounding ECM. The identification of mutant gene defects in human disease, together with the systematic analysis of ECM functions in genetically modified mice (Table 1), has given exciting insights into the diverse biological activities of specific ECM components in vascular biology (3,36). The ECM controls endothelial cell activities by diverse mechanisms ranging from cell anchorage, integrin-mediated activation and signalling to binding, release and activation of soluble growth factors and alteration in the supramolecular matrix architecture (Table 2).

The role of basement membranes

With regard to cell attachment, blood endothelial cells are anchored in the basement membrane, a dense polymeric sheet that is crucial for the proper functioning of blood vessels. Indeed, experimental models yielded substantial evidence that basement membranes provide the essential information for the organization and orientation of endothelial cells. It is known that the removal of individual components of the basement membrane can lead to leakiness of blood vessels (Table 1). The major constituents of this polymer structure are laminins, nidogens, collagen IV, the heparin sulphate proteoglycan perlecan, as well as other macromolecules (37). In addition, basement membranes often contain collagens XV and XVIII, SPARC and fibronectin (FN) (38). During neoangiogenesis, endothelial cells must degrade these basement membrane components and the surrounding ECM with the help of proteases, mainly metalloproteases, serine proteases and cysteine proteases (39). Interestingly, proteolytic processing of basement membrane components during vascular remodelling leads in some ECM macromolecules to cleavage of their NC1 domain. These ECM subdomains have been called matrikines, including the fragments generated from the NC1 domain of collagen IV $\alpha1/\alpha2/\alpha3$ chains (termed arresten, canstatin, tumstatin, respectively), collagen XV (restin) and collagen XVIII (endostatin) (40,41). *In vitro* and *in vivo* studies revealed the potent antiangiogenic effects of matrikines that are mediated by the modulation of integrin-dependent signalling pathways (36). Therefore, pro- and antiangiogenic signals are mediated by the basement membrane.

The role of integrins

Most of the endothelial cell–matrix contact occurs through the major ECM receptor system, namely the integrins. Integrins have

Table 2. Overview of ECM loss of function in mice and vascular phenotype

ECM component	Vascular phenotype (loss of function)	Reference
Fibronectin	Embryonic lethal (E9.5), severe vascular defects	88,89
Inactivation of RGD site in Fibronectin	Embryonic lethal (E10.5), placental and cardiovascular defects	90
Collagen I	Embryonic lethal (E12-14), aortic rapture, tissue integrity defects	91,92
Collagen III	Perinatal death, blood vessel fragility	93
Collagen IV	Heterozygous mice cerebral haemorrhages, perinatal death	94
Collagen XV	Viable, cardiovascular defects	95
Collagen XVIII	Abnormal ocular blood vessels	96
Elastin	Perinatal death, arterial occlusion caused by excessive proliferation of vascular SMC	97
Emilin-1	Viable, mild aortic alterations	98
Fibrillin-1	Perinatal death, aortic rupture	99
Fibulin-1	Perinatal death, haemorrhages, vascular defects	100
Fibulin-4	Perinatal death, vascular defects	101
Fibulin-5	Viable, vascular abnormalities	102,103
Nidogen-1	Viable, disruption of brain capillaries	104
Laminin $\alpha4$ chain	Viable, impaired microvessel maturation	105,106
Laminin $\alpha5$ chain	Placental vessel defect	107
Perlecan	Vascular damage at sites of mechanical stress	108
Thrombospondin-1	Increased vascular density in skin, cornea; excessive vascularization and impaired wound healing	109–111
Thrombospondin-2	Increased vascular density in multiple tissues; increased wound angiogenesis and healing	111,112
CCN1	Embryonic lethal, placental and embryonic vascular defects; increased endothelial cell apoptosis	113
CCN2	Embryonic lethal and vascular defects in the growth plate	114

ECM, extracellular matrix.

a cell type–specific and context-dependent expression. At least eight members of the integrin family such as collagen-binding integrins ($\alpha1\beta1$, $\alpha2\beta1$), laminin-binding integrins ($\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$), and RGD-binding integrins ($\alpha5\beta1$, $\alpha v\beta3$, $\alpha v\beta5$) play important roles in endothelial cell biology (42). However, $\alpha v\beta3$ as well as $\alpha v\beta5$ integrins are probably the most dramatically upregulated integrins on endothelial cells during angiogenesis (43). For example, whereas the integrin $\alpha v\beta3$ is not expressed on quiescent blood vessels (44), it has been linked to endothelial cell migration, invasion and survival during tissue remodelling (45). In these studies, it has also become clear that integrins do not simply provide anchorage to the ECM, but rather are integral structures for endothelial cell activation and signal transduction. In recent years, numerous cytoplasmic components have been identified that become activated upon binding of activated integrins to their ligand (46). Along these lines, multimolecular complexes have been characterized that assemble onto the cytoplasmic tails of activated integrins to engage and organize the cytoskeleton and activate signalling pathways that ultimately lead to changes in gene expression. These studies have significantly advanced the understanding of cell–matrix interactions. Of note, most of these studies have not been performed in endothelial cells, so that further research is needed to understand whether the current concept of integrin activation is also valid for the vascular system.

Integrin ligation has also been shown to be required for cellular responses to most angiogenic growth factors (47), suggesting that the local matrix environment orchestrates endothelial cell responses, mediated by soluble factors. For example, Soldi et al.

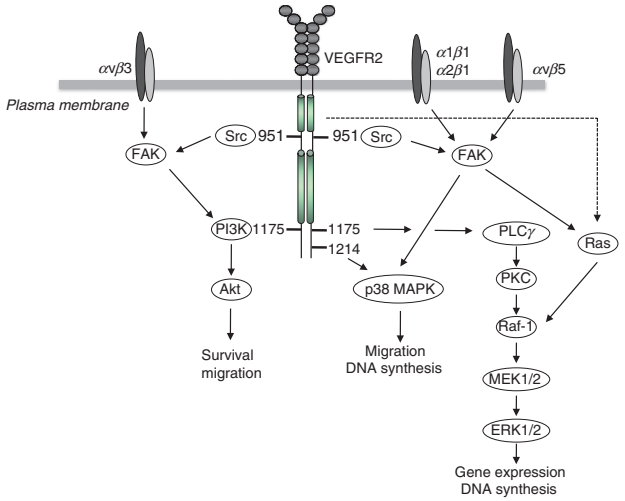

Figure 1. VEGFR-2–integrin crosstalk and biological outcomes: VEGFR-2 activation leads to the activation of PLCγ, which binds to phosphorylated Tyr1175 (Tyr1173 in the mouse) and mediates the activation of the MAPK/ERK1/2 cascade and proliferation of endothelial cells (83); Tyr1175/1173 phosphorylation has also been linked to VEGF-induced PI3K activation (84); AKT is activated downstream of PI3K and mediates the survival of endothelial cells (85); VEGFR-2 stimulates Ras that has been linked to an angiogenic phenotype (86); Ras-independent induction of the Raf/MEK/MAPK pathway, through PLCγ-activated PKC, has been shown in primary liver sinusoidal endothelial cells (87). Increased tyrosine phosphorylation of FAK, mediated in part through Src, is a point of convergence for VEGFR-2 and integrin-mediated survival, migration and proliferation signalling; exposure of endothelial cells to matrix-bound VEGF-A elicits prolonged activation of Tyr1214 and extended activation kinetics of p38 MAPK, these events requiring the association of VEGFR-2 with β1 integrins (56). FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; PI3K, phosphatidylinositol 3'kinase; PKC, protein kinase C; PLCγ, phospholipase C-γ.

(48) showed that tyrosine-phosphorylated VEGFR-2 co-immunoprecipitated with the β3 integrin subunit, but not with β1 or β5, from human umbilical vein endothelial (HUVE) cells stimulated with VEGF-A165. VEGFR-2 phosphorylation and mitogenicity induced by VEGF-A165 were enhanced in cells plated on the $\alpha v\beta 3$ ligand, vitronectin, compared with cells plated on the major $\alpha 5\beta 1$ ligand, FN, or the major $\alpha 2\beta 1$ ligand, collagen (Fig. 1). Consistently, blocking β3-integrin activation resulted in attenuated biological effects triggered by VEGF-A165. Furthermore, evidence was provided that $\alpha v\beta 3$ and $\alpha v\beta 5$, together with focal adhesion kinase (FAK), play distinct roles in the activation of the Ras-ERK cascade, leading to endothelial cell survival during angiogenesis in response to bFGF and VEGF, respectively (49) (Fig. 1). Collectively, these studies provided evidence for the importance of the integrin–growth factor crosstalk in endothelial cells and for the bidirectional flow of information between the vascular extracellular and intracellular compartments. Although a more detailed review on this topic is beyond the scope of this article, it should be mentioned that paradoxically genetic ablation of the genes encoding $\alpha v\beta 3$ and $\alpha v\beta 5$ failed to block angiogenesis and in some cases even enhanced it (50). Furthermore, pharmaceutical inhibitors of $\alpha v\beta 3$ and $\alpha v\beta 5$ that entered clinical trials to inhibit tumor angiogenesis have been unsuccessful so far (51). Therefore, a more complete understanding of how integrins control blood vessel growth is required.

Impact of growth factor–matrix interactions

Several angiogenesis-regulating growth factors such as FGF, VEGF and transforming growth factor-β1 (TGF-β1) or their activity-modifying molecules bind to ECM components. In particular, HSPGs have been identified as growth factor binding sites (52,53). Growth factor–ECM interactions can significantly control tissue homoeostasis and cell function. One of the best clinically understood examples is provided by the pathophysiology underlying vascular alterations in Marfan Syndrome, a connective tissue disorder caused by fibrillin-1 mutations. Over the past years, it became clear that in addition to impairing mechanical tissue integrity, the fibrillin-1 mutation perturbs activation and signalling of TGF-β1 and thereby contributes significantly to the vascular abnormalities (54). This discovery has led to novel pharmacotherapeutic approaches in Marfan Syndrome using TGF-β1 blocking reagents.

Notably, binding of growth factors to the ECM has also been implicated to be of vital importance to establish concentration gradients of these factors, a process known to be of fundamental importance for the regulating patterning developmental events such as vascular or axonal growth. The HBD of VEGF-A isoforms mediates binding to the ECM and is thought to regulate VEGF concentration gradients. For example, transgenic mouse studies provided the compelling evidence that for the development of a functional vascular network, only the presence of the heparin-binding VEGF-A165 isoform is absolutely required (17,20). Fifty percentage of transgenic mice that exclusively expressed the short VEGF-A120 isoform died perinatally because of impaired myocardial angiogenesis and ischaemic cardiomyopathy (17). Furthermore, vascular structures in such mice revealed increased calibre and reduced branching. These findings indicated that the heparin-binding VEGF-A isoforms provide essential stimulatory cues to initiate vascular branching. Consistent with this notion was the finding that transgenic mice expressing only VEGF-A188 also display abnormalities in vessel branching, however in the opposite direction from those mice expressing only VEGF-A120, with an excess of thin and disorganized branches (20). Furthermore, these mice also showed diverse defects in the skeletal system, resulting in stunted growth and reduced survival. Remarkably, only mice expressing exclusively the VEGF-A165 isoform revealed a normal development.

The molecular mechanisms by which the multiple VEGF isoforms with variable affinity for ECM proteins control vascular morphogenesis remain unclear. Gerhardt et al. (10) showed that during vessel sprouting in the postnatal mouse retina, endothelial tip cell induction and migration depend on a concentration gradient of VEGF-A, whereas proliferation of tube-forming stalk cells is regulated by its final concentration (Fig. 2a). Thus, vessel patterning during retinal angiogenesis depends on the balance between two different qualities of the extracellular VEGF-A distribution, which regulate distinct cellular responses in defined populations of endothelial cells. In a subsequent study by Hellström, it was demonstrated that endothelial tip and stalk cells not only differ in their morphology but also in their gene expression and signalling pathways (11). Together, their findings suggested that delta-like 4-Notch1 signalling between the endothelial cells within the angiogenic sprout serves to restrict tip cell formation in response to VEGF-A (Fig. 2a), thereby establishing the adequate ratio between tip and stalk cells required for correct sprouting and branching patterns. In a recent study, Gerhardt et al. provided further evidence for the mechanisms directing tip cell formation. They could

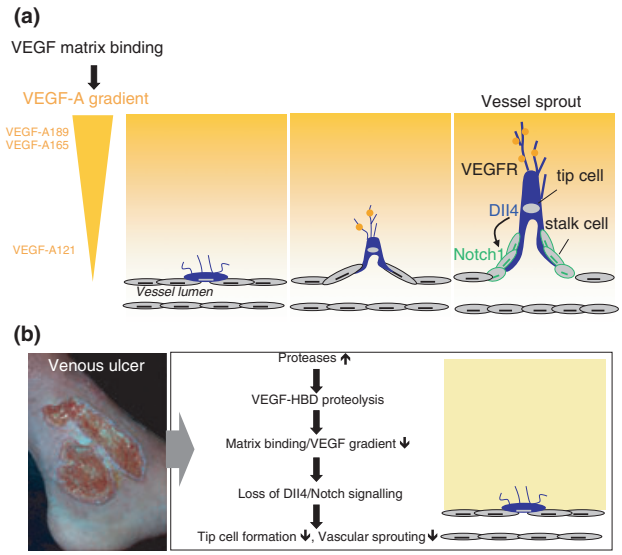

Figure 2. Spatial distribution of VEGF-A regulates vascular sprouting: VEGF concentration gradient determines vascular sprouting: (a) Model of vascular sprouting studied in mouse retina: sequential steps from left to right illustrate the induction of a tip cell (blue) by VEGF-A concentration gradient (orange), and proliferating stalk cells (green); VEGF-A concentration gradient leads to directed filopodia extension/migration and Dll4 expression in VEGFR-1/-2-expressing tip cells; Dll4/Notch signalling in proliferating stalk cells limits tip cell formation. (b) Unbalanced proteolytic activity and disturbed angiogenesis is a hallmark of venous ulcers: plasmin-mediated cleavage of the heparin-binding domain in VEGF-A165 leads to reduced matrix binding and gradient formation of VEGF, resulting in disturbed Dll4/Notch signalling and dysregulated polarization of tip cells and disturbed angiogenesis.

show that endothelial cells compete for the tip cell position through relative levels of VEGFR-1 and VEFGR-2 (55). Currently, it is not known whether the concept of tip–stalk cell communication is also relevant in other organ systems or the human system during vascular remodelling, and continuing studies are required.

A recent study by Chen et al. (56) suggested that in the context of matrix components, activation of VEGFR-2 is determined by matrix-bound or soluble VEGF-A isoforms. These *in vitro* findings showed that in the presence of collagen, matrix-bound VEGF induced the prolonged phosphorylation of tyrosine 1214, resulting in the sustained activation of p38 MAPK (Fig. 1). It was speculated that the progressive recruitment of integrins as endothelial cells invade the ECM facilitates clustering of VEGFR-2, leading to binding between integrins and VEGFR-2 and ultimately resulting in sustained receptor activation.

The role of cell–cell communication

Endothelial cell communication with other cell types, either through direct cell–cell contacts or soluble mediators, is essential for effective blood vessel formation. Interactions between endothelial cells and vascular mural cells (perivascular cells and/or vascular smooth muscle cells) have come into focus as central processes in the regulation of vascular formation, stabilization, remodelling and function (57). Failure of the interactions between the two cell types, as seen in different genetic mouse models, results in severe and often lethal cardiovascular defects (58,59). Abnormal interactions between the two cells types are also implicated in a number of human pathological situations, including tumor angiogenesis, diabetic microangiopathy and stroke. A role for vascular mural cells in dermal wound healing was predicted several decades ago, but so far the precise functional relevance of pericytes for normal and pathological skin development is largely unknown. A recent study identified perivascular cells as a critical source for laminin-332 synthesis, suggesting their unexpected role in epidermal–dermal junction formation (60).

Recent reports point to a critical and novel link between macrophages and angiogenesis. While there is substantial evidence that macrophages promote angiogenesis by releasing proangiogenic factors, such as VEGF (61,62), little is known regarding their potential relevance in direct macrophage–endothelial cell contacts during vascular growth. *In vitro* studies reported that, under certain circumstances, macrophages can differentiate into endothelial cells (63). However, evidence for the physiological relevance of this process during new vascular growth is still lacking. A recent study described the unexpected finding that macrophages act as critical regulators for tip cell fusion and vascular anastomosis (64). Mouse models deficient in macrophages revealed an attenuated vascular network in the brain, which was associated with reduced macrophage–tip cell contacts. The potential physiological relevance of macrophages for anastomosis in other organ systems, as well as how these findings might translate into proangiogenic therapies are interesting questions and await further investigation.

Angiogenesis in health and disease: what can we learn for the therapeutic induction of vascular growth?

Restoration of blood supply in organs that suffer ischaemia because of the sequelae of underlying metabolic diseases or trauma remains a challenging and unresolved task in medicine. Treatment modalities that normalize endothelial cell function and promote tissue vascularization might provide a central strategy to normalize and accelerate tissue regeneration and function following metabolic or mechanical damage. In the past decade, a number of preclinical and clinical studies have been performed to analyse the effect of biological agents, bioactive devices, or environmental conditions to stimulate the formation of new blood vessels in vulnerable tissues and organs. As one of the most challenging areas of modern biotechnology, growth factors have been intensively investigated for therapeutic angiogenesis. Two main strategies are being developed for therapeutic angiogenesis using growth factors, recombinant growth factor proteins and gene therapy (65). So far, most clinical trials of angiogenic therapy have been performed for peripheral arterial disease and ischaemic heart disease and to a lesser extent for chronic non-healing skin wounds. Growth factors that had been tested for therapeutic vascular growth include members of the fibroblast growth factor family (FGF-1, FGF-2, FGF-4), VEGF protein family, angiopoietins, hepatocyte growth factor, TGF-β1 and PDGF-BB (66,67). Although these factors have been successful in therapeutic angiogenesis in diverse preclinical models, thus far their use in clinical trails has not delivered the expected results (65). In addition, clinical trials also pointed out serious side effects of angiogenesis therapy and emphasize the need for additional research to better understand specific functions of angiogenic mediators under consideration and how to modify these mediators to eliminate deleterious side effects (68). Therefore, significant progress is still needed in proangiogenic pharmacotherapy.

Among the growth factors successfully employed in the therapeutic treatment of chronic skin ulcers, granulocyte/macrophage

colony-stimulating factor (GM-CSF) also acts by improving vascularization (69). GM-CSF is known to regulate bone marrow-derived precursor cell recruitment and endothelial cell differentiation (70,71). Enhanced local VEGF-A transcription, potentially by macrophages, pointed to the proangiogenic effects mediated by GM-CSF. This finding indicated that the use of cytokines indirectly stimulating VEGF-A expression at physiological levels might represent a choice for inducing therapeutic angiogenesis possibly overcoming the side effects often reported with direct angiogenic growth factor administration.

Based on comprehensive evidence from many other laboratories as well as our own laboratories, we suspect that the interplay of proangiogenic growth factors with the architecture and components of the ECM is vital for the functional and sustained growth of vascular structures. More research is required to better understand the dynamics and optimal balance between binding and diffusibility of proangiogenic morphogens. So far, this fundamental aspect of growth factor biology has been relatively poorly respected when developing novel regenerative approaches. Furthermore, experimental and clinical findings indicate that the requirements for the perfect proangiogenic microenvironment might be variable among different organs and particular disease conditions. For example, in proinflammatory disease conditions, the local activity of diverse tissue proteases might be increased; therefore, not only matrix components are degraded but also growth factors themselves become a target of unbalanced proteases, so that the equilibrium of matrix-bound and diffusible growth factor may be disturbed. We revealed evidence for the biological significance of VEGF-A165 proteolytic processing in chronic non-healing human skin ulcers.

We (S. E.) and co-workers as well as other investigators demonstrated the sensitivity of VEGF165 protein to serine proteases, in particular plasmin (22,26,27). Plasmin digestion of VEGF165 yields two fragments: an amino-terminal homodimer (VEGF1-110) containing VEGF receptor binding determinants and a carboxyl-terminal polypeptide comprising the HBD (VEGF111-165) (22,26,27). Whereas the heparin-binding affinity of the intact VEGF165 protein and the VEGF111-165 cleavage product was nearly equivalent, no heparin binding was observed for the VEGF1-110 cleavage product (22), indicating that the heparin-binding function of VEGF165 is completely mediated by the carboxyl-terminal domain. Loss of the carboxyl-terminal polypeptide through plasmin digestion significantly attenuated VEGF165 mitogenic activity on HUVE cells (22,27), supporting the crucial significance of the HBD for VEGF-A function. Current knowledge about the prevalence and biological significance of proteolytic digestion of VEGF-A, particularly in the *in vivo* situation, is scarce.

We (S. E.) and colleagues provided evidence for the biological significance of VEGF165 proteolytic processing in skin wound healing. Comprehensive morphological and functional data indicate that endothelial cell function and angiogenesis is significantly disturbed in human chronic non-healing wounds associated with vascular disease, diabetes mellitus and ageing (26). A typical feature of chronic non-healing wounds, particularly recalcitrant venous ulcers, is an unbalanced and increased activity of proteases, which results from a prolonged and increased inflammatory response at the wound site, as well as from a high bacterial burden. Indeed, we and others identified increased levels of matrixmetalloproteinases and serine proteinases, including plasmin in wound lysates from non-healing versus healing wounds (72). Therefore, we investigated a potential causative link between uncontrolled proteolytic activity, impaired angiogenesis and decreased VEGF-A activity in chronic non-healing wounds. Our data demonstrated that in contrast to healing wounds, VEGF-A165 protein is a target of proteolytic processing in chronic non-healing wounds (Fig. 2b) (26,27). Plasmin cleavage of VEGF-A165 lead to the loss of its HBD with subsequent attenuation of its biological activity.

Furthermore, Lee et al. analysed the biological impact of MMP processing of VEGF-A in *in vitro* and *in vivo* tumor models. It was shown that VEGF-A165 is also a target of various MMPs present in tumor progression (73). MMP cleavage appeared to occur in sequential steps, finally resulting in cleavage of the heparin-binding motif. Carcinoma cells were transfected with different VEGF-A variants and injected subcutaneously into a mouse model. Interestingly, tumors that expressed the MMP-cleaved VEGF fragment VEGF1-113 grew poorly and showed capillary dilation of existing vessels. In contrast, tumors expressing the MMP-resistant VEGF-A variant displayed faster growth kinetics, and the vasculature was characterized by increased capillary density with multiple and frequent branch points.

Overall, these studies provide substantial evidence that VEGF-A165 proteolysis occurs *in vivo* and might add an additional level of control for VEGF-A165-mediated processes. Different structural–functional properties might account for the altered VEGF activities of processed VEGF-A. Presented studies suggest that in particular loss of the HBD results in VEGF-A fragments with altered functions. Rendering the VEGF-A molecule resistant to proteolytic cleavage might increase the portion of matrix-bound VEGF-A165 molecules that are more efficient in supporting a functional angiogenic response, when compared with soluble VEGF-A.

Translational challenges: designing morphogens and biomaterials for pharmacotherapy of blood vessel growth

Based on comprehensive studies from many groups, it appears evident that when thinking about therapeutic strategies to restore the function of diseased or damaged organs, it is unlikely that solely the replacement of a single tissue component, meaning exclusively growth factors, ECM or cells, will be a successful approach. Rather, a better understanding of how these different components act together to form a functional organ is needed. Furthermore, increasing evidence suggests that successful pharmacotherapeutic tissue repair and regeneration approaches requires careful consideration of the particular needs of the damaged and diseased tissue, so that therapeutics can be modified and adapted accordingly. Finding a proper equilibrium between binding and diffusion of proangiogenic morphogens in time and space has emerged as paradigm to induce functional blood vessel growth. To enable pursuit of this line of thinking, it will be important to understand in more detail the interplay and dynamics between proangiogenic and ECM components and to learn how this interaction can be controlled to promote therapeutic vascular growth. Studying growth factor and ECM interactions has become an emerging area of research, and different molecular strategies are under current investigation to translate experimental findings in therapeutic approaches.

Stabilization of the 'heparin-binding domain' of growth factors

Structure–function analysis of numerous growth factors has revealed specific domains that often interact with HSPG in the ECM. Typically, these growth factors consist of a stretch of basic amino acids, which is named a HBD and that mediates binding to the ECM via electrostatic interactions. We reasoned that by stabilizing the HBD by engineering proteolytic resistance to prevent cleavage of this domain, growth factor binding to the ECM can be modulated and ultimately impact its functional properties. This strategy might be of particular benefit during pharmacological interventions in proinflammatory conditions with high proteolytic activity. Therefore, we generated a plasmin-resistant VEGF-A165 mutant, characterized by increased integrity of the HBD in the context of plasmin activity. We hypothesized that plasmin resistance of VEGF-A165 results in retained ECM binding and ultimately a superior proangiogenic potency in the microenvironment of a chronic skin ulcer (in which proteases are over-represented compared with normal skin) compared with the wild-type molecule. To test this hypothesis, we investigated the stability and activity of locally applied VEGF165 wild-type or a VEGF165 mutant resistant to plasmin proteolysis (VEGF165^{A111P}) in a genetic mouse model of diabetes-impaired healing (*db/db* mouse) (31). The stability of the mutant VEGF165 was substantially increased in wound tissue lysates of diabetic mice in comparison with VEGF165 wild-type protein, indicating a prolonged activity of the plasmin-resistant VEGF165 mutant. Resistance of VEGF165 to plasmin cleavage resulted in a superior angiogenic response in wounds of diabetic mice, characterized by the increased stability of vascular structures, increased recruitment of perivascular cells and delayed and reduced endothelial cell apoptosis, ultimately leading to amelioration of the diabetes-delayed healing kinetics. Different structural–functional properties of the VEGF-A165 mutant versus the wild-type protein could account for the superior angiogenic potency of the mutant and require further investigation. These include prolonged half-life in the proinflammatory microenvironment of diabetic wounds, enhanced signalling through co-receptors including neuropilins, enhanced activity owing to improved ECM binding and facilitated integrin–VEGFR crosstalk and signalling, and generation of VEGF-A concentration gradients (Fig. 3). These mechanisms may act separately and/or in concert to enhance and prolong the activity of the plasmin-resistant VEGF-A165 molecule in the *db/db* wound environment. Therefore, beyond providing a tool to obtain mechanistic insight into basic cell biological principles, the plasmin-resistant VEGF-165 mutant might also represent a molecule with clinical potential.

Synthetic covalent linkage of growth factors and peptide domains to the extracellular matrix

As a second approach to engineering growth factor dynamics in matrices, synthetic covalent linkage of growth factors to the ECM might provide enhanced matrix–growth factor interaction that ultimately is beneficial for tissue generation and vascular growth. Both biologically derived and synthetic materials have been explored for this approach. For this approach, we (J. H.) and colleagues explored fibrin as therapeutic matrix to induce effective functional blood vessels, developing a novel methodology for the modification of fibrin. In these studies, we have shown that peptides or proteins fused with the factor XIIIa substrate sequence NQEQVSPL, derived

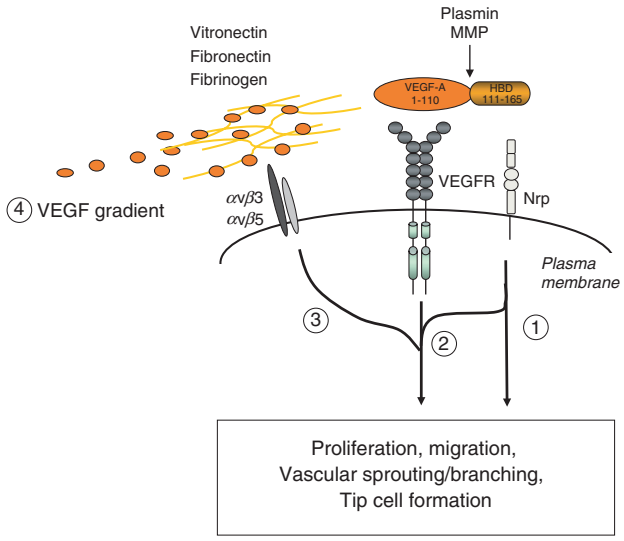

Figure 3. The heparin-binding domain of VEGF proteins convey critical signals in angiogenesis. 1. The heparin-binding domain (HBD) of VEGF-A165 is the epitope for the neuropilin receptor (Nrp) and potentially directly or via interaction with VEGFR-1/-2 controls endothelial cells function; 2. Nrp potentiates the activity of VEGFR-2; 3. HBD mediates the binding of VEGF-A165 to the extracellular matrix and thereby facilitates the crosstalk between VEGFR-2 and integrins; 4. HBD generates a concentration gradient of VEGF-A165 that mediates vascular sprouting. Proteases (plasmin, MMPs) control HBD-mediated angiogenesis by cleavage.

from the N-terminal domain of α2-plasmin inhibitor (α2-PI1-8), can be covalently attached to fibrin through the action of factor XIIIa during coagulation (74). When the short VEGF-A121 isoform was fused to the α2-PI1-8 domain [α2PI(1-8)-VEGF121 fusion protein], *in vitro* studies revealed that the molecule was efficiently bound to the fibrin matrix during coagulation (75,76). When applied on the embryonic chicken chorioallantoic membrane and in adult mice, it was demonstrated that cell-demanded liberation of VEGF121 from fibrin implants induces local and controlled blood vessel growth. Analysis at several levels from endothelial cell morphology and endothelial interactions with periendothelial cells, to vessel branching and network organization, demonstrated that the α2PI(1-8)-VEGF121 fusion protein bound to fibrin induces vessel formation more potently than native VEGF121 and that those vessels possess more normal morphologies at the light microscopic and ultrastructural level. Permeability studies in mice validated that vessels induced by α2PI(1-8)-VEGF121 do not leak (76). Overall, the studies indicate that the quality of the angiogenic response to covalently bound VEGF-A121 to a fibrin matrix is superior compared with diffusible VEGF-A121. In a subsequent approach, this system was further developed in that a new VEGF variant α2PI-Pla-(1-8)-VEGF121 was generated, which couples to fibrin via a plasmin-sensitive sequence (Pla) (73). *In vitro*-generated VEGF release profiles demonstrated that α2PI-Pla-(1-8)-VEGF121 was released fourfold more quickly than a2PI(1-8)-VEGF121, both being retained compared with native VEGF-A121. Both matrix-bound VEGF forms were more effective in inducing endothelial cell proliferation and progenitor cell maturation (77). Therefore, cell-demanded release of engineered VEGF-A variants from fibrin implants may present a therapeutically safe and practical modality to induce local angiogenesis.

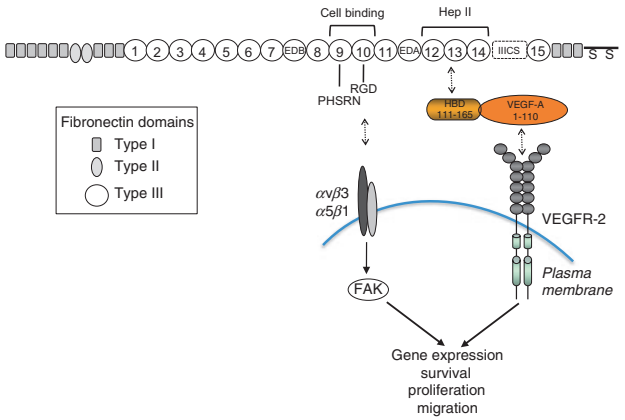

Figure 4. Schematic diagram of the domain structure of fibronectin and function in angiogenesis. Growth factors, including VEGF-A165, bind with high affinity to the heparin-binding domain (Hep II); synergism mediated by simultaneous stimulation of VEGFR-2 and integrins.

Most recently, this technology has been used to integrate recombinant FN fragments into a fibrin scaffold. FN is a key multifunctional adhesion protein found in blood and the interstitial ECM. Structure–function analysis have shown that specific domains and cell-binding sites permit FN to interact with a remarkably wide range of cell types, ECM components and cytokines (Fig. 4). Growth factor binding is mediated particularly by a domain consisting of the 12th to 14th type III repeats of FN (FN III 12-14). It was shown that FN III12-14 fragments covalently bound within a fibrin matrix resulted in significantly enhanced binding of diverse growth factors to the fibrin matrix, ultimately resulting in enhanced smooth muscle cell sprouting *in vitro* (78). This study provides proof for the concept that by protein engineering, physiological matrix–growth factor interactions can be modulated with functional impact on cell behaviour. Overall, synthetic covalent linkage of growth factor cues into fibrin matrices has been shown to be a practical approach in tissue regeneration and vascular growth. The physiological relevance of this technology for therapeutic tissue regeneration is under current investigation. This approach might hold great potential in clinical applications.

Design of novel matrix materials

In general, ECM components from natural sources (e.g. purified fibrin from blood or collagen from skin) are considered advantageous for the use of ECM scaffolds in tissue engineering because of their inherent properties of biological recognition and remodelling. However, synthetic materials have also been extensively explored and are used as three-dimensional extracellular microenvironment to mimic the regulatory characteristics of natural extracellular matrices (79,80). Although progress in biomaterial research has been rapid, lack of efficient vascularization is still a major limitation in regenerative therapies. Therefore, approaches have to be developed to guide and facilitate recruitment of progenitor and/or endothelial cells into material scaffolds to promote the sprouting of capillaries and the formation of functional vascular structures. However, endothelial cell function and vessel formation is complex and sensitive to many diverse stimuli including biochemical signals such as growth factors and ECM components, as well as biophysical properties including matrix stiffness, pore size and proteolytic susceptibility (79). Therefore, experimental approaches that allow the investigation of the systematic and independent variation of biomolecular and mechanical features on blood vessel growth are required. In this regard, biomaterials research will not only serve to advance clinical approaches for tissue regeneration but also provide better understanding of fundamental processes of cell invasion, growth and differentiation. The development of the so-called *hybrid materials* appears to offer a suitable approach to create substrates with a defined functionality, biocompatibility and adaptability regarding composition and structure. Poly(ethylene-glycol) (PEG) is a commonly used synthetic component of these hybrid systems because of its excellent biocompatibility and chemical properties (81). In addition, bioactive components have been integrated into the PEG-based hydrogel matrices, including heparin, cyclic RGD adhesion peptides and growth factors. Recent culture experiments demonstrated the interplay of growth factor presentation, adhesive characteristics and elasticity of these gel matrices to regulate endothelial cell behaviour and indicate clinical potential (82). Future studies have to prove the physiological relevance of these materials for tissue growth and regeneration.

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Conflict of interest

The authors state no conflict of interest.

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