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The lymphangiogenic growth factors VEGF-C and VEGF-D

Part 1: Fundamentals and embryonic development

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Summary

VEGF-C and VEGF-D are the two central signaling molecules that stimulate the development and the growth of lymphatic system. Both belong to the VEGF protein family which plays important roles in the growth of blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis). In mammals the VEGF family comprises five members: VEGF, PIGF, VEGF-B, VEGF-C and VEGF-D. The family was named after its first discovered member VEGF ("Vascular Endothelial Growth Factor"). VEGF-C and VEGF-D form functionally and structurally a subgroup within this family. They differ from the other VEGFs by their peculiar biosynthesis: they are produced as inactive precursors and need to be activated by proteolytic removal of their long N- and C-terminal propeptides. Unlike the other VEGFs, VEGF-C and VEGF-D are direct stimulators of lymphatic growth. They exert their lymphangiogenic function via VEGF receptor-3, which is expressed in the adult organism almost exclusively on lymphatic endothelial cells. In this review we give an overview of the VEGF protein family and their receptors with the emphasis on the lymphangiogenic VEGF-C and VEGF-D, and we discuss their biosynthesis and their role in embryonic lymphangiogenesis.

Keywords: VEGF-C, VEGF-D, growth factors, lymphangiogenesis

Die lymphangiogenen Wachstumsfaktoren VEGF-C und VEGF-D

Teil 1: Grundlagen und Embryonalentwicklung

Zusammenfassung

VEGF-C und VEGF-D sind die zwei zentralen Signalmoleküle, die für die Entwicklung und das Wachstum des Lymphgefässsystems verantwortlich sind. Beide gehören zur VEGF-Proteinfamilie, deren Mitglieder hauptsächlich im Wachstum von Blutgefäßen (Angiogenese) und Lymphgefäßen (Lymphangiogenese) ihre Funktionen haben. Die VEGF-Familie umfasst in Säugetieren fünf Mitglieder: VEGF, PIGF, VEGF-B, VEGF-C und VEGF-D. Benannt wurde diese Familie nach ihrem zuerst entdeckten Mitglied VEGF ("Vascular Endothelial Growth Factor"). VEGF-C und VEGF-D bilden funktionell und strukturell eine Untergruppe innerhalb der VEGF-Familie. Sie unterscheiden sich von den anderen VEGFs durch ihre besondere Biosynthese: sie werden als inaktive Vorgängerproteine produziert, für deren Aktivierung ihre langen N- und C-terminalen Propeptide enzymatisch abgespalten werden müssen. Im Gegensatz zu den anderen VEGFs sind VEGF-C und VEGF-D direkte Stimulatoren für das Wachstum lymphatischer Gefäße. Ihre lymphangiogene Wirkung erfasst VEGF-C und VEGF-D über den VEGF-Rezeptor-3 (VEGFR-3), der im erwachsenen Organismus fast nur auf den Endothelzellen der Lymphgefäße zu finden ist. In diesem Artikel geben wir einen Überblick über die VEGF-Proteinfamilie und deren Rezeptoren mit dem Schwerpunkt auf den lymphangiogenen Mitglie-
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**Figure 2.**

**Schematic representation of the VEGF growth factors and VEGF receptors.** The VEGF receptors are transmembrane receptors with an extracellular domain consisting of seven immunoglobulin (Ig) homology domains and an intracellular split-kinase domain. The outer three Ig homology domains (shown in different color) are sufficient to interact with the respective ligands. The VEGFs consist of two individual polypeptide chains, which are connected by disulfide bridges. Typically, two identical protein chains connect to form a VEGF molecule (homodimer), but also two different protein chains can connect to form a so-called heterodimer (e.g. PlGF and VEGF-A). Similarly, two different VEGF receptors can ligate to form heterodimers and such a ligation can be mediated by a ligand; for example, VEGF-C can ligate VEGF receptor-2 and VEGF receptor-3 resulting in a VEGFR-2/3 heterodimer. Such receptor heterodimers may have special functions [64, 86]. Among the VEGF receptors, VEGF receptor-3 is exceptional, as it is the only one whose extracellular domain is proteolytically cleaved [87].

**Figure 1.**

**Model of the activation of tyrosine kinase receptors by a bivalent ligand.** Simultaneous binding of a ligand (e.g. VEGF-C, shown in blue) to the extracellular domains of two receptor molecules (e.g. VEGFR-3, shown in gray) positions the intracellular catalytic domains (shown in red) in such a way that they can phosphorylate each other. This changes the three-dimensional structure of the intracellular receptor part and exposes affinities to other intracellular signaling molecules (second messengers) that are activated by docking to the phosphorylated tyrosine residues. Such activations propagate in a cascade fashion until the signal enters the cell nucleus where it modifies the transcriptional activity of target genes. The intracellular signaling of VEGF receptors is reviewed in detail by Olsson et al. [5].

**The VEGF growth factors**

Mammals have five different VEGFs: VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF (placental growth factor).

**VEGF-A.** VEGF-A was the first VEGF growth factor that was discovered and it is often simply referred to as VEGF. In the early literature it is also known as vascular permeability factor (VPF) because of its property to increase the permeability of blood vessels [8]. Its main function is in the stimulation of blood vessel growth (angiogenesis). VEGF-A has two different receptors on endothelial cells: VEGF receptor-1 (VEGFR-1) and VEGFR-2.

**VEGF-B and PlGF.** In contrast to VEGF-A, VEGF-B and PlGF are only able to interact with VEGFR-1. Almost all the important functions of VEGF-A are mediated via the signal transduction of VEGFR-2; and accordingly, VEGF-B and PlGF are only weakly angiogenic. VEGFR-1, in contrast to VEGFR-2, has even an inhibitory function by binding the potently angiogenic VEGF-A, but not triggering a similarly strong reaction as VEGFR-2 does [9-13]. VEGF-B and PlGF, however, seem to have specific functions for angiogenesis of the heart muscle [14, 15], for certain pathological processes [16] and fatty acid metabolism [17].

**VEGF-C and VEGF-D.** VEGF-C and VEGF-D can both interact with VEGFR-2 and VEGFR-3. Their main task is the stimulation of the growth of lymphatic vessels (lymphangiogenesis). VEGF-C was discovered in 1996 as a binding partner of VEGF receptor-3, for which no ligand had been found yet [18]. A little later, murine VEGF-C was described independently, but the authors named the molecule VRP [VEGF-related protein; 19]. Soon thereafter, the...
specific lymphangiogenic properties of VEGF-C were demonstrated in various animal models [20, 21]. Because in some of these models, the angiogenic properties of VEGF-C became apparent [21-23], a VEGF-C mutant was developed that interacts exclusively with VEGF receptor-3 and thus no longer has angiogenic potency [the so-called C156S VEGF-C mutant; 24]. With this mutant, the lymphangiogenic function of VEGF-C could be separated from the angiogenic function and independently studied.

VEGF-D is the second specific lymphangiogenic growth factor. It was identified and described independently by three different research teams, once under the acronym FIGF [c-fos-induced growth factor; 25] and twice as VEGF-D [26, 27].

VEGF-E and VEGF-F. In addition to these five mammalian VEGFs there are VEGF-E and VEGF-F. VEGF-E is the collective name for proteins closely related to the VEGFs that were detected in the genome of certain pathogenic viruses and that are involved in the disease etiology [28-32]. The collective term VEGF-F denotes homologous proteins that have been identified as accessory components of snake venoms [33-37]. Their function is probably to increase the permeability of blood vessels in order to potentiate the effects of the primary venom components.

The structure of VEGF molecules

VEGF family members are molecules that feature a central domain homologous to VEGF. This domain is referred to as VEGF homology domain (VHD, shown in red in Figure 3). This homology can be identified at all levels of the protein structure (from the amino acid sequence up to the three-dimensional structure of the protein). The VHD contains the receptor binding domain. In addition, most VEGFs feature accessory domains that further determine the specific properties of individual VEGFs, e.g. the affinity of VEGF-A to the co-receptors Neuropilin-1 and -2 [38], or of VEGF-C to the co-receptor Neuropilin-2 [39, 40].

All of the VEGF family members are composed of two polypeptide chains (dimeric proteins). During the biosynthesis of the polypeptide chains they align in an antiparallel fashion with a hydrophobic contact area facing each other, and become covalently linked with two disulfide bridges. The resulting shape resembles roughly a flattened ellipsoid. At each end of this ellipsoid, there is one epitope that can bind a matching VEGF receptor. Both epitopes are composites of parts from both polypeptide chains. This composite nature of the receptor binding epitopes explains why monomeric VEGF (VEGF with only one polypeptide chain) is biologically inactive: it cannot ligate two receptors [41].

Alternative splicing

Like almost all secreted proteins, the VEGFs are glycoproteins. Most of them are produced in different forms. The diversity is produced either by alternative splicing or by posttranslational modification of the protein (e.g. by proteolytic cleavage). Through alternative splicing of the VEGF-A mRNA, a variety of different VEGF-A isoforms are produced that differ primarily in their affinity for heparan sulfate proteoglycans (HSPGs), which occur primarily on cell surfaces and in the extracellular matrix [see Figure 3; 12, 41]. The interaction with HSPGs immobilizes the so-called "heparin-binding" VEGF-A isoforms. Thus a concentration gradient can form, which can be used by blood vessels for orientation and directed growth [42, 43].

The activation of VEGF-C and VEGF-D

Splice isoforms have been also described for VEGF-C and VEGF-D, but their functions are unknown [19]. VEGF-C and VEGF-D get their diversity...
mainly by enzymatic (proteolytic) cleavage of their precursor molecules (see Figure 4).

The affinity of the VEGF-C precursor molecules for the VEGF receptor-3 is modest and its affinity is even lower for VEGF receptor-2. With increasing processing, the affinities for both receptors increase, and fully processed mature VEGF-C and VEGF-D have in addition to their lymphangiogenic also strong angiogenic potencies [44-47]. How the activation of VEGF-C and VEGF-D is controlled in vivo, is not precisely known. It is believed that the availability and activity of specific proteases is central to this control: The activation of VEGF-C and VEGF-D by specific proteases would therefore be one of the crucial factors determining whether VEGF-C and VEGF-D act as lymphangiogenic or angiogenic effectors. In addition to regulating the activity of VEGF-C, the C-terminal propeptides of VEGF-C and VEGF-D have other functions: similar to the heparin-binding domain of VEGF-A, they endow the molecules with heparin affinity [48]. It is also interesting that the C-terminal propeptide contains a repetitive arrangement of cysteine residues, which is otherwise almost exclusively known from the salivary proteins of silk weaving mosquito larvae of the genus Chironomus; hence the name “Silk homology domain” for the C-terminal propeptide [18, 49]. However, the whys and hows of this similarity are unknown.

**Vasculogenesis or angiogenesis?**

Two different mechanisms can lead to the formation of new blood vessels: vasculogenesis and angiogenesis. Vasculogenesis is the differentiation of progenitor cells (angioblasts or lymphangioblasts) into endothelial cells and the formation of a primitive vascular network while angiogenesis refers to the growth of new blood vessels from pre-existing vessels. Vasculogenesis plays mainly a role during the early development of the vascular system, whereas angiogenesis is the main mechanism for vascular growth during the later phases of embryonic development and in the adult organism.

In mammals, the lymphatic system forms by angiogenic processes emanating from the large veins [50, 51]. The initially disputed fact that also vasculogenesis can contribute to the development of the lymphatic system was shown e.g. in birds [52] and frogs [53]. To form new vessels, endothelial cells have to execute a complex program: they need to switch from their resting state back into active cell cycling. One of the main triggers of cell cycle re-entry for blood vessel endothelial cells is the lack of oxygen (hypoxia), which necessarily results from avascular growth. Molecular oxygen sensors activate a genetic switch that activates the angiogenesis program.
The triggers for the expansion of the lymphatic system are less well known, but the interstitial pressure seems to play an important role for embryonic lymphangiogenesis and the inflammatory status for pathologic lymphangiogenesis.

**Differences between the blood and lymphatic system**

The pressure within the blood vessel system leads to leakage of blood plasma, which thereby becomes tissue fluid. The main function of the lymphatic vessels is to return this excess tissue fluid back into the blood circulation. After uptake by the lymphatics, the tissue fluid becomes lymph fluid. On its way back into the blood vessel system, the lymph passes through the lymph nodes, which are base stations of the immune system. In the lymph nodes, immune cells which specifically recognize antigens, mature and multiply. Another function of the lymphatic vessels is limited to the colon: the uptake and transport of dietary fats with long chain fatty acids and fat-soluble vitamins.

Blood vessels and lymphatic vessels are constructed differently. Blood endothelial cells are connected to each other via tight junctions and adherens junctions and have a continuous basement membrane on their tissue-facing (basolateral) side. In contrast, lymphatic endothelial cells are only loosely connected by overlaps, and their basement membrane is incomplete. They are connected to the underlying tissue by elastic fibers (anchoring filaments). These fibers mechanically open the overlaps at elevated tissue pressure and thus ensure the flow of tissue fluid into the lymphatic vascular lumen.

Blood and lymphatic endothelium also differ in the expression of various markers: both blood and lymphatic endothelial cells express the general endothelial marker PECAM-1 (platelet endothelial cell adhesion molecule 1), but they express a different set of VEGF receptors: blood endothelial cells express VEGFR-1 and -2, while lymphatic endothelial cells express VEGFR-2 and -3. Fenestrated endothelium [61], high endothelial venules [HEVs; 62, 63] and the blood vessels of tumors are exceptions in that they do express the lymphatic marker VEGFR-3.

The mechanisms of the directional growth are similar for blood vessels and nerve cell axons: a specialized cell on the tip of the vascular sprout (tip cell) determines the direction of growth of subsequent cells (stalk cells) by extending filopodia that sense growth factor concentration gradients.

**VEGF-C and VEGF-D in embryonic development**

VEGF-C and VEGF-D have two receptors: VEGFR-2 and VEGFR-3. VEGFR-2 is the primary receptor on endothelial cells of blood vessels (BECs) and stimulates their growth, while VEGFR-3 exerts the same function on lymphatic endothelial cells (LEC). Accordingly, VEGF-C and VEGF-D can act both as angiogenic and lymphangiogenic growth factors. VEGFR-3 was discovered before VEGF-C and VEGF-D and therefore, VEGF-3 was for some time an “orphan receptor”, i.e. a receptor without known binding partners (ligands). However, soon after the discovery of VEGFR-3, the specific expression pattern of VEGFR-3 suggested, that its function was closely related to the lymphatic system. In the early stages of embryonic development all endothelial cells express VEGFR-3, but with advancing age, its expression becomes more and more restricted to lymphatic endothelial cells.

Genetically engineered mice that do not express VEGFR-3, die between the 9th and 10th day of embryonic development due to generalized edema. This confirmed the essential role of VEGFR-3 in the development of the cardiovascular system, since at this time, the development of the lymphatic system has not started yet.

Mice that not express the VEGFR-3 ligand VEGF-C die about three days later due to generalized edema. In these mice, the lymphatic system does not develop.

Interestingly, neither the development of the blood vascular system nor of the lymphatic vascular system is af-
fected by the lack of the second lymphangiogenic growth factor VEGF-D [72]. The absence of both VEGF-R-3 ligands (VEGF-C and VEGF-D) during embryonic development does not lead to the same severe disorders in the development of the vascular system as the absence of VEGF receptor-3 [73]. Therefore, there might be yet unknown ligands for VEGF receptor-3. Alternatively, VEGF-R-3 might be activated independently of a ligand [74, 75].

A molecule that synergizes with VEGF-C and which is required for the development of the lymphatic system and lymphangiogenesis in general is CCBE1 (collagen and calcium binding EGF domains 1 protein). The blockade of lymphatic development appears in CCBE1-deficient and VEGF-C-deficient mice around the same time and is phenotypically very similar. However, it is unclear what exact role CCBE1 plays for the lymphatic system [76-78]. Mutations in the human CCBE1 gene can be responsible for Hennekam syndrome, a rare genetic disease whose main symptoms include lymphedema and lymphangiectasia of the intestine [79].

### Differences between mice and humans

Because we owe a considerable part of our knowledge about the molecular mechanisms of lymphangiogenesis to laboratory mice, it is necessary to mention two important differences between mice and humans with respect to the VEGF-C/VEGF-D/VEGF receptor-3 signaling pathway (depicted in Figure 5): While mature human VEGF-D can activate the angiogenic receptor VEGFR-2, this is not the case for mouse VEGF-D [80]. It is therefore believed that VEGF-D could have a different function for mice and humans. Furthermore, there are two splice variants of human VEGF receptor-3 [a short and a long isoform; 81], whereas in mice, only one isoform can be detected. The appearance of two VEGFR-3 splice variants can be attributed to a retroviral insertion into the FLT4 gene [82], and the two isoforms differ in the signals, which are generated upon stimulation with VEGF-C [83, 84].

The second part of this review will focus on the roles that VEGF-C and VEGF-D play in various diseases that involve the lymphatic system. For some of these diseases mouse models exist. However, due to the above mentioned differences between mice and humans, one should be careful when extrapolating from preclinical studies in animal models to clinical trials with human patients.

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