The Proteolytic Activation of Vascular Endothelial Growth Factor-C

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Summary

The enzymatic cleavage of the protein backbone (proteolysis) is integral to many biological processes, e.g. for the breakdown of proteins in the digestive system. Specific proteolytic cleavages are also used to turn on or off the activity of proteins. For example, the lymphangiogenic vascular endothelial growth factor-C (VEGF-C) is synthesized as a precursor molecule that must be converted to a mature form by the enzymatic removal of C- and N-terminal propeptides before it can bind and activate its receptors. The constitutive C-terminal cleavage is mediated by proprotein convertases such as furin. The subsequent activating cleavage can be mediated by at least four different proteases: by plasmin, ADAMTS3, prostate-specific antigen (PSA) and cathepsin D. Processing by different proteases results in distinct forms of "mature" VEGF-C, that differ in their affinity and their receptor activation potential. This processing is tightly regulated by the CCBE1 protein. CCBE1 regulates the activating cleavage of VEGF-C by ADAMTS3 and PSA, but not by plasmin. During embryonic development of the lymphatic system, VEGF-C is activated primarily by the ADAMTS3 protease. In contrast, it is believed that plasmin is responsible for wound healing lymphangiogenesis and PSA for tumor-associated pathological lymphangiogenesis. Cathepsin D has also been implicated in tumor lymphangiogenesis. In addition, cathepsin D in saliva might activate latent VEGF-C upon wound licking, thereby accelerating wound healing. The molecular details of proteolytic activation of VEGF-C are only recently extensively explored, and we likely do not know yet all activating proteases. It appears that the activity of VEGF-C is regulated for different specific functions by different proteinases. Although VEGF-C clearly plays a pivotal role for tumor progression and metastasis in experimental animal studies, the relevance of most correlative studies on the role of VEGF-C in human cancers is quite limited until now, also due to the lack of methods to differentiate between inactive and active forms.

Key Words: VEGF-C, lymphangiogenesis, proteinases, proteolysis

Die proteolytische Aktivierung des Vaskulären Endothelzellwachstumsfaktors-C

Zusammenfassung

Enzymatische Schnitte der Polypeptidkette von Proteinen sind Bestandteil vieler biologischer Prozesse, so z.B. bei der Zerlegung von Proteinen während der Verdauung. Gezielte enzymatische Schnitte werden auch benutzt, um die Aktivität bestimmter Proteine ein- oder auszuschalten. So z.B wird der lymphangiogene Vaskuläre Endothelzellwachstumsfaktor-C (VEGF-C) als Vorläufermolekül synthetisiert, welches durch die enzymatische Entfernung von Teilen des Proteins in eine aktive Form umgewandelt werden muss, bevor es seine Rezeptoren binden und aktivieren kann. Dabei werden C- und N-terminale Propeptide von VEGF-C entfernt. Die konstitutive C-terminale Spaltung wird durch Proproteinkonvertasen wie Furin vermittelt. Die darauffolgende, aktivierende Spaltung kann durch mindestens vier verschiedene Proteasen vermittelt werden: Plasmin, ADAMTS3, prostata-spezifisches Antigen (PSA) und Cathepsin D. Resultierend aus der Prozessierung durch unterschiedliche Proteasen entstehen unterschiedliche, "reife" VEGF-C-Formen, die sich in ihrer Affinität und ihrem Rezeptor-Aktivierungspotential unterscheiden. Das CCBE1-Protein reguliert die Prozessierung von VEGF-C durch ADAMTS3 und PSA, aber nicht die durch Plasmin. Während des physiologischen Wachstums des Lymphgefäßsystems in der Embryonalentwicklung wird VEGF-C hauptsächlich durch die ADAMTS3-Protease aktiviert. Im Unterschied dazu wird vermutet, dass Plasmin für die Wundheilung und PSA für die mit Tumorwachstum assoziierte, pathologische Lymphangiogenese verantwortlich ist. Cathepsin D wurde ebenfalls mit der Tumor-Lymphangiogenese in Verbindung gebracht; darüberhinaus könnte das im Speichel enthaltene Cathepsin D, durch Wundlecken, latentes VEGF-C aktivieren und dadurch die Wundheilung beschleunigen. Die molekularen Details der proteolytischen Aktivierung von VEGF-C werden erst seit kurzer Zeit intensiv erforscht und wahrscheinlich sind noch nicht alle aktivierenden Proteasen bekannt. Jedoch scheint es, dass die Aktivität von VEGF-C für verschiedene spezifische Funktionen von unterschiedlichen Proteinasen reguliert wird. Obwohl VEGF-C in experimentellen Tierversuchen eine zentrale Stellung in der Tumorprogression und -metastasierung einnimmt, ist die Aussagekraft der bisherigen korrelativen Studien zur Rolle von VEGF-C bei Tumorerkrankungen des Menschen begrenzt. Dieser Umstand ist nicht zuletzt in den fehlenden Möglichkeiten begründet, zwischen der inaktiven und den aktiven Formen zu unterscheiden.

Schlüsselwörter: VEGF-C, Lymphangiogenese, Proteinasen, Proteolyse

List of Abbreviations	
ADAM	A Disintegrin- and Metalloproteinase
ADAMTS3	A Disintegrin- and Metalloproteinase with Thrombospondin Motifs-3
CatD	Cathepsin D
CCBE1	Collagen- and Calcium-Binding EGF domain-containg protein 1
ELISA	Enzyme-Linked Immunosorbent Assay
ECM	Extracellular Matrix
HS	Hennekam Syndrome
HSPG	Heparansulfate Proteoglykan
KLK3	Kallikrein-related Peptidase 3 (synonymous with PSA)
MMP-3	Matrix-Metalloproteinase-3
PSA	Prostata-Specific Antigen
TGF-β	Transforming Growth Factor-β
VEGF-A	Vascular Endothelial Growth Factor-A, often simply referred to as "VEGF"
VEGF-C, -D	Vascular Endothelial Growth Factor-C, -D
VEGFR	VEGF Receptor
VHD	VEGF Homology Domain

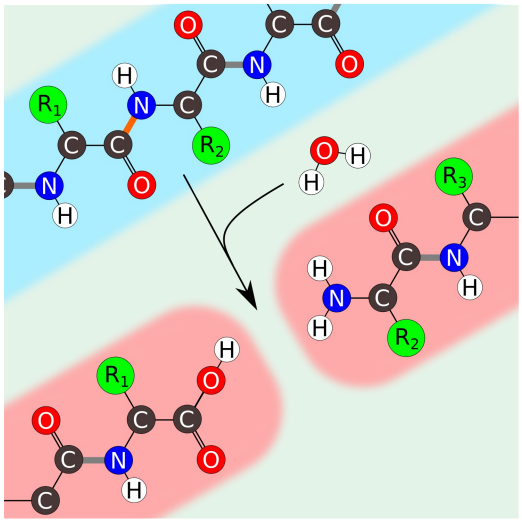

Figure 1

Proteolysis. *The hydrolytic cleavage of a peptide bond (orange) of a protein (blue background) into two fragments (red background). The peptide bonds of the protein backbone are shown as thick lines. The amino acid side chains are symbolized as green circles. Without enzymatic catalysis by proteinases this chemical reaction is extremely slow.*

Proteinases (protein cleaving enzymes)

Proteinases (or proteases) are enzymes that cleave proteins by hydrolysing the peptide bonds of the protein backbone (Figure 1). They occur inside (intracellular) and outside (extracellular) of cells, and are essential for a multitude of cell and body functions. For example, proteinases process antigens in the course of an immune reaction for antigen presentation, they break down damaged or unnecessary proteins (e.g. in lysosomes) and they digest food proteins in the gastrointestinal tract. In the stomach, for example, pepsin is generated from the precursor pepsinogen by autoproteolysis at a low pH, and in the intestine, trypsin is generated from the precursor trypsinogen by autocatalysis.

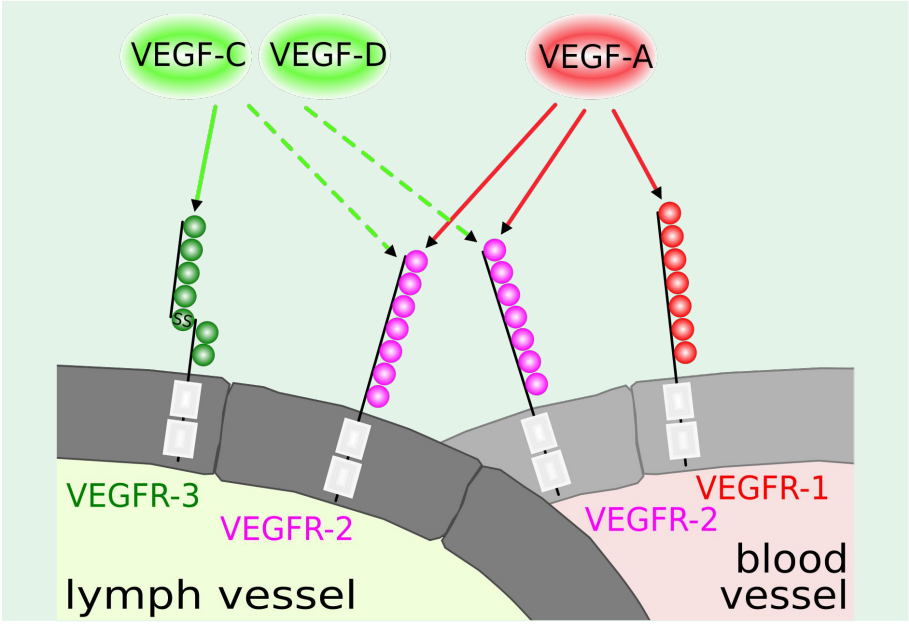

Figure 2

The growth factors VEGF-A, VEGF-C and VEGF-D and their receptors*. The growth and function of blood and lymphatic vessels is controlled by Vascular Endothelial Growth Factors (VEGFs). VEGF-A is the quintessential growth factor for blood vessels, while VEGF-C is the quintessential growth factor for lymphatic vessels. VEGF-A is recognised by VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). VEGF-C and VEGF-D are recognised by VEGF receptor-3 (VEGFR-3) and, under certain circumstances, also by VEGFR-2. VEGFR-1 is largely specific for endothelial cells of blood vessels and VEGFR-3 for endothelial cells of lymphatic vessels. In contrast, VEGFR-2 is found on both vessel types. If, for example, active VEGF-C or VEGF-D binds to VEGFR-3 on the lymphatic endothelial cell surface, the signal is transduced into the cell nucleus, where it provokes a proliferative and migratory response, thus initiating vessel growth.*

Activation of proteins

Many proteins are produced as inactive precursors and are activated by proteolytic cleavage when their function is required. The proteinases themselves are also produced as inactive pro-proteinases and must be activated by proteolytic removal of their propeptides. This is of uppermost importance, since the uncontrolled activity of proteinases would otherwise destroy cells and decompose the extracellular matrix (ECM).

The perhaps best known proteolytically controlled processes include blood coagulation, the limitation of blood clotting and its reversal, i.e. the dissolution of blood clots [1,2]. Many bood clotting factors are proteinases, which in turn activate other proteinases, etc. ("proteolytic cascade") to catalyze the proteolytic conversion of soluble fibrinogen into polymerizing fibrin in the final step of the blot clotting cascade.

Activation of growth factors

Many growth factors and also some cytokines are produced as inactive precursors, which only become active through proteolytic cleavage ("processing"). Among the better known growth factors that are activated through proteolytic cleavage are e.g. the Transforming Growth Factor-β (TGF-β)[3], but also the lymphangiogenic growth factors VEGF-C and VEGF-D. Many studies have analyzed the mechanisms and the regulation of VEGF receptor activation by VEGFs [4], whereas relatively little is known about the upstream processes of mobilisation and activation of VEGFs.

The VEGF family

The biology of the growth factors VEGF-C and VEGF-D has been described in detail in a previous review

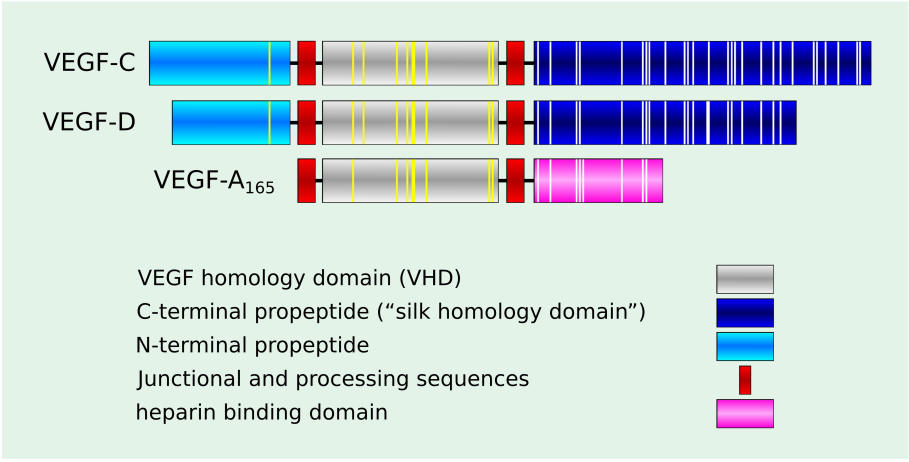

Figure 3

Schematic representation of the domain organisation of VEGF growth factors using VEGF-C/D and VEGF-A as examples. *The VEGF growth factors consist of the central VEGF homology domain (in grey) and optional accessory domains (in blue and magenta). The proteolytic cuts usually take place between the domains (in red). The characteristic cysteine patterns of the VEGF family and the C-terminal propeptide are represented by yellow and white lines, respectively.*

[5]. For this reason, only a short introduction follows, in which the relevant properties and characteristics of VEGF-C and VEGF-D are explained. VEGF-C and VEGF-D belong to the VEGF family (see also Figure 2 for a graphical short overview of VEGF-A, VEGF-C, VEGF-D and their receptors).

Characteristic for the members of the VEGF family is the VEGF Homology Domain (VHD) as the central and dominant structural element. This domain is almost 100 amino acids long and has a characteristic arrangement of eight cysteine (C) amino acid residues ($CX_{22}CPXCVX_3RCXGCCX_6CX_{33\text{-}35}CXC$), which form disulfide bridges among themselves and thereby give the VEGFs a very stable core. This core also forms the receptor binding epitope and thus determines to which of the three VEGF receptors (-1, -2 and -3) a VEGF binds. In addition to this core, most VEGFs have other domains that are either upstream (N-terminal) or downstream (C-terminal) from the VHD (Figure 3). These additional domains give VEGFs the ability to interact with other binding partners.

For example, different isoforms of VEGF-A have C-terminal heparin binding domains of varying strength, with which they bind heparan sulfate proteogycans. This variation in binding strength causes a more or less prominent immobilization on cell surfaces and the extracellular matrix (ECM), which in turn results in distinct activity profiles of the isoforms. Proteases such as plasmin can convert the longer ECM-bound VEGF-A isoforms into shorter, more diffusible isoforms [6,7]. Cleavage by different matrix metalloproteinases (MMPs), especially MMP-3, converts e.g. the main isoform $VEGF\text{-}A_{165}$ into a shorter, non-heparan sulfate-binding isoform [8].

The C-terminal domain of VEGF-C

Similar to VEGF-A, VEGF-C and VEGF-D are also immobilized on cell surfaces and the ECM via their C-terminal domain [9]. In contrast to all other VEGF family members, the C-terminal domain of VEGF-C and VEGF-D blocks the growth factor activity [10]. Most likely, this domain sterically hinders access to the receptor binding site. This assumption would also explain why the C-terminal domain of VEGF-C is almost twice as large as its VHD. The origin of the protein sequence of the C-terminal domain is mysterious, since no homologous sequences seem to exist in the genomes of vertebrates. Homologous proteins are, however, found in the salivary secretions of some silkworm mosquito larvae, e.g. Chironomus tentans [11]. For this reason, this domain has also been called *silk homology domain*, although its amino acid sequence is unrelated to the classical silk proteins.

Hypoxia regulates angiogenesis, but how is lymphangiogenesis regulated?

VEGF-A, which is mainly responsible for the formation of blood vessels, is tightly regulated at the transcriptional level. If the oxygen supply to a

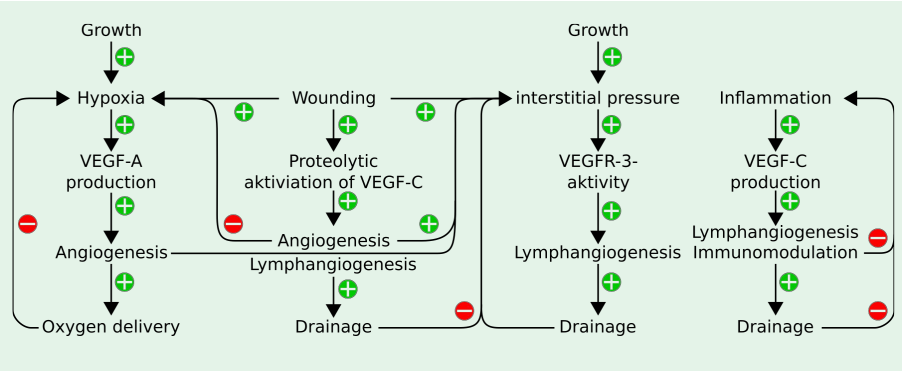

Figure 4

Schematic representation of known control loops in angiogenesis and lymphangiogenesis.

The production of VEGF-A and VEGF-C is usually self-limiting due to negative feedback. As soon as a sufficient oxygen supply has been established or the tissue pressure normalized, the signaling for blood vessel or lymphatic growth is reduced.

tissue is insufficient (hypoxia), the production of VEGF-A is switched on, which in turn leads to blood vessel growth and normalisation of the oxygen pressure [12]. In contrast, VEGF-C production hardly improves tissue oxygenation, but it does improve tissue drainage and immune cell trafficking (see Figure 4). Presumably for this reason and in contrast to VEGF-A, the production of VEGF-C is controlled by proinflammatory signals and not or only insignificantly by hypoxia [13-15]. In addition, VEGF-C can contribute to the limitation of inflammatory reactions by increased drainage [16,17] and immunomodulation [18]. VEGF-C also plays a vital role for the lymph vessels of the small intestinal villi (lacteals). These in fat absorption specialised vessels require for their maintanance the permanent stimulation by VEGF-C [19], which is produced by macrophages in response to the microbial intestinal flora [20].

Increased interstitial tissue pressure amplifies the growth of lymph vessels via the pressure-dependent signal transduction of VEGF receptor-3 (*mechanotransduction*) mediated by β1 integrin and integrin-linked kinase (ILK), thereby normalising tissue pressure [21,22]. Whether tissue pressure also has an influence on VEGF-C production or activation is not known yet.

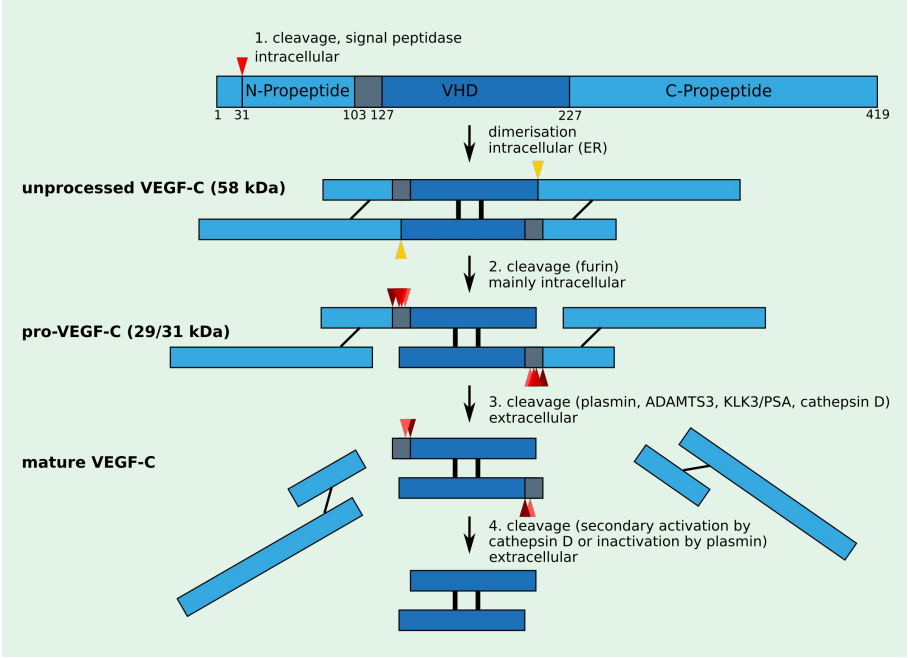

Figure 5

Schematic representation of the proteolytic activation of VEGF-C. *VEGF-C is synthesized as a precursor with a size of 58 kDa. This unprocessed form (also called "prepro-VEGF-C") is more than twice as large as the mature VEGF-C and, after the signal peptide has been cleaved off during transport into the endoplasmic reticulum, is converted into pro-VEGF-C in the trans-Golgi network by the proprotein convertases PC5, PC7 and especially furin. This occurs by cleaving the polypeptide chain C-terminally to the VHD (marked by a yellow triangle). If furin is blocked, unprocessed VEGF-C is not converted into pro-VEGF-C [23]. Pro-VEGF-C can bind but not activate VEGFR-3 and therefore acts as a competitive inhibitor of active VEGF-C, which has been shown both in vitro and in vivo [24]. Only a further proteolytic cut N-terminally of the VHD (marked by red triangles) converts pro-VEGF-C into the biologically active form. Mature VEGF-C has by far the highest affinity for the binding and activation of VEGFR -2 and -3 [10]. Unprocessed VEGF-C is hardly detectable in the cell culture supernatant and probably occurs physiologically only inside the cell [10].*

During embryonic development VEGF-C is activated by ADAMTS3

Mutations in the Collagen- and Calcium-Binding EGF domain-containg protein 1 (CCBE1) gene are responsible for the systemic lymphatic dysplasia in Hennekam Syndrome Type I [25]. CCBE1 regulates the proteinase ADAMTS3, which is the primary proteinase that activates VEGF-C during embryonic growth [26,27]. The ADAMTS proteinases are cell surface or ECM-localized multidomain enzymes closely related to the ADAM proteinases. In contrast to the membrane-bound ADAM proteinases, the ADAMTS proteinases are secreted and contain one or more repeats of the thrombospondin type 1 motif. Some functions of this protein family, such as procollagen processing or proteoglycan cleavage, have been linked to the regulation of angiogenesis [28]. Due to its structure, ADAMTS3 belongs together with ADAMTS2 and ADAMTS14 to the procollagenase group [29] and also cleaves, at least in vitro, procollagen N-propeptide [30].

If the function of ADAMTS2 is mutationally impaired, proteolytic collagen maturation is disturbed and a connective tissue defect is the consequence (Ehlers-Danlos syndrome, dermatosparaxis type) [31]. In contrast, patients without or with compromised ADAMTS3 genes show no deficits in collagen synthesis, but distinct defects in the development of the lymphatic system [32,33].

Although the biosynthesis of VEGF-D is very similar to that of VEGF-C (shown schematically in Figure 5) [34], ADAMTS3 cannot activate VEGF-D [26,35]. And, notably, none of the other procollagenases of the ADAMTS family (ADAMTS2 and ADAMTS14) do activate VEGF-C [26].

Activation of VEGF-C in wound healing by plasmin and cathepsin D

The restoration of oxygen supply and immune function through blood and lymph vessels are paramount for wound healing. An acceleration of wound healing by VEGF-C was first observed in animal experiments in 2004 [36,37]. When platelets are activated, VEGF-C is released from the α-

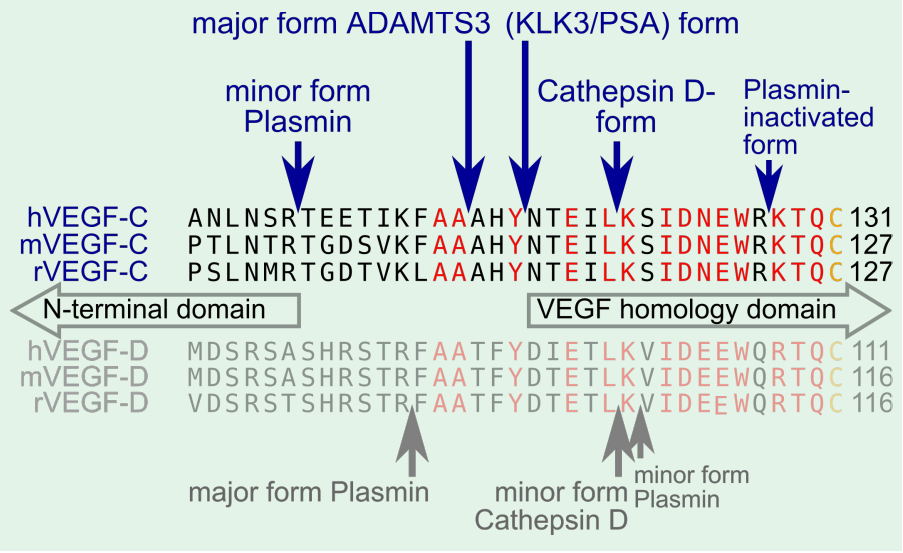

Figure 6

Schematic representation of the proteolytic processing sites in the amino acid sequences of VEGF-C and VEGF-D. *The activation of VEGF-C and VEGF-D is achieved by proteolytic cleavage of the protein between the N-terminal domain and the VEGF homology domain. The further C-terminally (in the figure to the right) the cleavage of VEGF-C occurs, the lower the biological activity of the resulting VEGF-C form [42]. The cleavage of VEGF-C by plasmin within the VEGF homology domain leads to a complete inactivation of VEGF-C [26]. Alternatively, VEGF-C can also be inactivated through sequesteration by soluble isoforms of its receptors [44]. Figure 6 was modified under the Creative Commons license from [42].*

granules [38]. Plasmin, which is later involved in the dissolution of the temporary fibrin matrix [39], probably activates both platelet-derived VEGF-C and latent VEGF-C (pro-VEGF-C which was embedded in the extracellular matrix [40, 41]).

Cathepsin D is another activator of VEGF-C [42]. Accelerated wound healing by saliva [43] can perhaps be partly attributed to the activation of VEGF-C by Cathepsin D, which is found in saliva. However, many other enzymes are released during wound healing which have a fairly broad substrate specificity, e.g. MMP-3, and which may contribute to the activation of VEGF-C as well as to the release/activation of ECM-sequestered (inactive) VEGF-A.

Activation of VEGF-C by prostate specific antigen (PSA, KLK3)

Somewhat surprisingly, but not entirely unanticipated [45], it turned out that PSA (prostate specific antigen), which is controversially used in prostate cancer screening, can activate VEGF-C [42]. Despite being the most frequently used blood test for early cancer detection, it is less well known that PSA is a proteinase whose main biological task is to liquefy the gel-like consistency of the male ejaculate, which allows the sperm cells to swim [46].

VEGF-A had been detected in seminal fluid more than 20 years ago [47,48], and was later shown to have a positive effect on the motility of spermatozoa [49]. However, only recently it was recognized that also VEGF-C is present in the male ejaculate and that the activation of this seminal VEGF-C occurs concurrently with the liquefaction of the ejaculate by PSA [42]. Whether seminal VEGF-C is an epiphenomenon or has any function for reproduction has not yet been clarified. VEGF-C is certainly required for the implantation of the embryo into the endometrium, where it acts on the blood vessels [33]. However, VEGF-C could also play a role in the implantation-associated immune modulation [50] or it might - as already described for VEGF-A [49] - have a direct chemotactic or chemokinetic effect on spermatozoa.

The key position of CCBE1 as cofactor of activation

When it is important to react quickly to changing demands, regulation at the

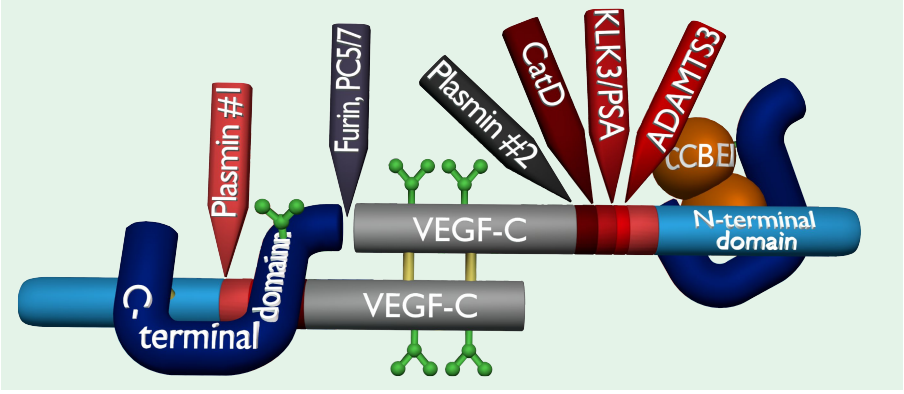

Figure 7

Schematic representation of the hypothetical mechanism of action of CCBE1. *The C-terminal domain of pro-VEGF-C (dark blue) blocks the access of enzymes to proteolytically sensitive sequences (shown on the left half of the dimer). CCBE1 causes a conformational change in VEGF-C and thus exposes the proteolytic target site (shown on the right half of the dimer). The CCBE1 effect on VEGF-C activation has been demonstrated for ADAMTS3 and KLK3/PSA , and it is suspected for Cathepsin D. After the activation of VEGF-C, e.g. at the primary interface of plasmin (#1), Cathepsin D can shorten the protein with an additional cut ("secondary activation"). The shorter the N-terminal end of active VEGF-C, the weaker it binds to and activates its receptors. With a proteolytic cut at the secondary plasmin cleavage site (#2), VEGF-C loses all activity towards VEGFR-2 and VEGFR-3.*

gene expresion level introduces a delay due to the upstream processess of transcription and translation. The production and on-demand activation of inactive ("latent") VEGF-C bypasses this delay. A similar form of storage and activation is known e.g. from TGF-β [51]. The heparin-binding isoforms of VEGF-A are also reversibly inactivated by binding to extracellular proteins and can be reactivated if required, e.g. by plasmin-mediated proteolytic cleavage [6]. A summary of all previously published VEGF-C activating enzymes and the exact positions of the cleavage sites is shown in Figure 6.

The CCBE1 protein regulates the VEGF-C-activating function of the ADAMTS3 proteinase. CCBE1 consists of two domains: the N-terminal domain, which is formed by three EGF-like repreats, and the C-terminal domain. which consists of two collagen motifs. Both domains are able to accelerate the activation of VEGF-C by ADAMTS3 independently. The N-terminal domain of CCBE1 is responsible for the colocalization of VEGF-C and ADAMTS3 with CCBE1 to form the activation complex, and the C-terminal domain accelerates the catalytic cleavage of VEGF-C by ADAMTS3 [40]. Presumably, CCBE1 removes the masking of the proteolytic target site of VEGF-C, which is normally blocked by its own C-terminal domain (Figure 7). The different activation paths of VEGF-C with regard to the localisation of the activation complex are explained in Figure 8.

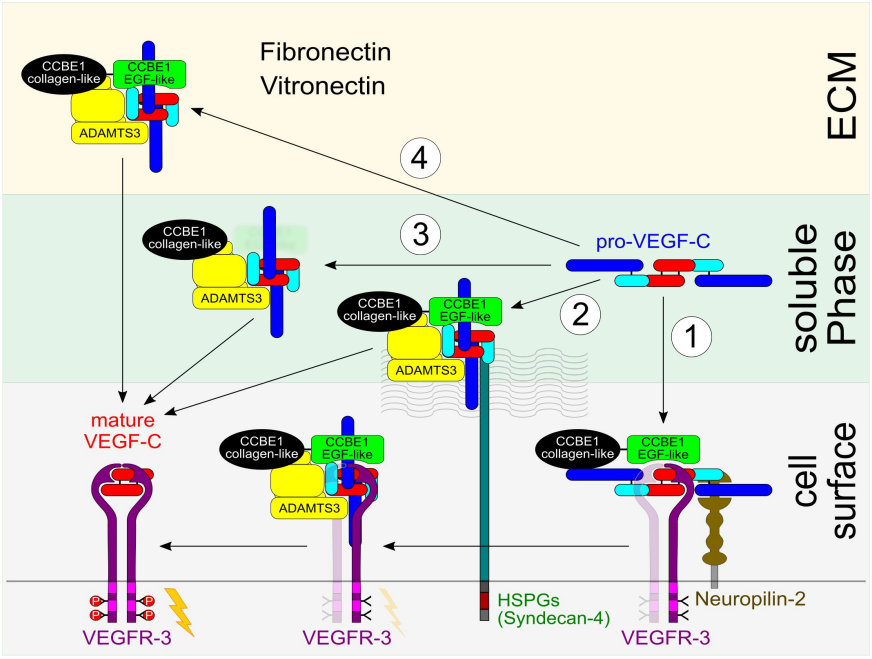

Figure 8

The different activation paths of VEGF-C. *The proteolytic cleavage of pro-VEGF-C by ADAMTS3 activates and mobilizes VEGF-C. The activation of VEGF-C can take place in four different settings:*
1. activation of VEGFR-3-bound pro-VEGF-C [26]
2. activation of HSPG-bound VEGF-C [9]
3. activation of VEGF-C in the soluble phase [35]
4. activation of ECM-bound VEGF-C [40].
VEGFR-3-bound but inactive VEGF-C can start signaling immediately after proteolytic activation (activation mode 1), whereas HSPG-bound VEGF-C must first dissociate from the HSPG and translocate to VEGFR-3 (activation mode 2). The activation of VEGF-C can also take place in the soluble phase (activation mode 3). Immunohistochemically, however, the vast majority of pro-VEGF-C, CCBE1 and ADAMTS3 are found bound to the extracellular matrix (ECM, activation mode 4) or on cell surfaces (activation modes 1 and 2). CCBE1 fulfills two independent functions for VEGF-C activation: the C-terminal domain accelerates the proteolytic cleavage, while the N-terminal domain recruits pro-VEGF-C to efficiently form the trimeric activation complex. Figure 8 was modified under the Creative Commons license from [40].

Hennekam Syndrome (HS) is a rare congenital disease with a generalized lymphedema as its main feature. At first, mutations in the CCBE1 gene were identified as the cause, but meanwhile, mutations in three different

Proteinase	Cleavage site	Remarks	Activates the following receptors	Reference(s)
Plasmin (primary cleavage site)	Arg102↓Thr103	minor form, probably responsible for the activation of VEGF-C in wound healing	VEGFR-2 VEGFR-3	[26,41]
ADAMTS3	Ala111↓Ala112	main form, for lymphangiogenesis during embryonal development, CCBE1- regulated	VEGFR-2 VEGFR-3	[26,32,33,40]
KLK3/PSA	Tyr114↓Asn115	activates VEGF-C in seminal fluid, CCBE1- regulated	VEGFR-2 VEGFR-3	[42]
Cathepsin D	Leu119↓Lys120	found in seminal fluid and saliva	preferentially VEGFR-3	[42]
Plasmin (secondary cleavage site)	Arg127↓Lys128	inactive form		[26]

Table 1

Four proteinases are known in the literature as VEGF-C activators. *Plasmin occupies a special position because it inactivates VEGF-C during prolonged exposure by cutting at a secondary site.*

genes are known to trigger HS. The function of two of these genes (CCBE1 and ADAMTS3) within the VEGF-C signal transduction pathway is known. It is assumed that the third gene (FAT4) also has an important function within the VEGF-C signal transduction pathway.

Activation of VEGF-C in tumours

VEGF-C and its activation are indispensable for the development of the lymphatic system [33,52], and in the adult organism, at least some lymphatic networks need a constant supply of VEGF-C for their maintenance [19]. To prevent lymphatic dysfunction, the amount of active VEGF-C must be precisely regulated. A degregulation with severe consequences can e.g. be triggered by tumors.

The relationship between VEGF-A-mediated blood vessel formation and tumour growth has been well studied and is also specifically blocked in anti-angiogenic tumour therapy, e.g. by the antibody drug bevacizumab (Avastin) [53]. It has always been assumed that the majority of tumours never become clinically relevant because they do not acquire the ability to stimulate blood vessel growth [54]. Without switching on VEGF-A production and without the resulting vascularisation ("angiogenic switch"), these tumours can never grow larger than a few millimetres because they lack sufficient oxygen and nutrients [55,56].

However, tumours can produce not only VEGF-A but also VEGF-C. The effects of VEGF-C on tumour growth occur at several levels:

1. VEGF-C can activate VEGFR-2 and thus replace VEGF-A as an angiogenic factor [57].

2. VEGF-C can stimulate VEGFR-3, which is found particularly on newly sprouting blood vessels in the tumour vasculature [58].

3. Tumor cells themselves can express VEGF receptors and be stimulated in an autocrine or paracrine fashion by VEGF-C [59].

4. VEGF-C can stimulate lymph vessel growth and thus promote metastasis [60-62].

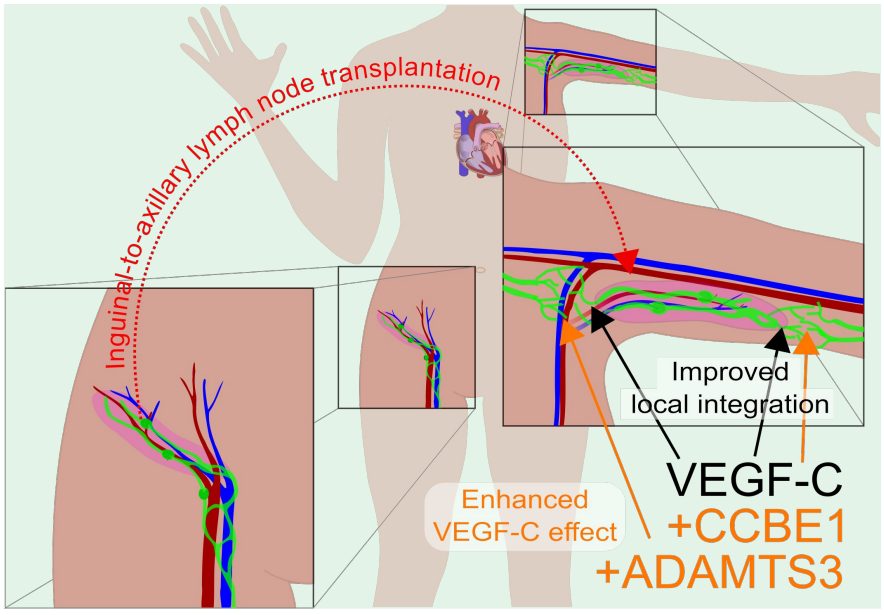

Figure 9

Gene therapy with AdVEGF-C. *An increasingly popular therapy for breast cancer-associated lymphedema is autologous lymph node transplantation [88,89]. In pre-clinical studies, the treatment success (integration of the transplanted lymph node into the local lymphatic network) could be improved by the simultaneous administration of VEGF-C [90]. With this strategy, Lymfactin® has successfully completed Phase I clinical trials and is now in Phase II. As a further development of Lymfactin®, a simultaneous administration of VEGF-C with the VEGF-C-activating ADAMTS3 and/or CCBE1 is being discussed.*

Unlike for the blockade of VEGF-A, there is no approved drug therapy for the blockade of VEGF-C. This lack might result from the fact that proteolytic activation produces many different forms of VEGF-C. Effective blocking would likely need to block all forms of VEGF-C in addition to all forms of VEGF-D, as VEGF-D can provide similar signals for tumour growth as VEGF-C [63].

Which proteinases activate VEGF-C in tumour diseases has not yet been experimentally investigated, but cathepsin D and PSA are likely to play a role for at least certain tumour types. The expression of cathepsin D has long been correlated with tumour metastasis [64]. Although, in contrast to cathepsin D, the correlation between PSA and tumour development has been studied much more intensively, various studies have come to different conclusions regarding a tumour-promoting function of PSA [65-69]. Some authors postulate that PSA promotes early tumour growth but inhibits its development in later stages [70]. In any case, with the activation of VEGF-C by cathepsin D and PSA, possible mechanistic links have been identified, which allows to experimentally address and answer these and similar questions.

Pro-VEGF-C or active VEGF-C?

The vast majority of studies on the role of VEGF-C in tumor growth describe the correlation of VEGF-C levels with disease progression. However, none of these studies distinguishes between active, mature VEGF-C and inactive pro-VEGF-C. This can be attributed to the fact that pro-VEGF-C has

only been known to be inactive since 2014 and that no commercially available test does distinguish between the two forms. For RNA-based expression analyses (e.g. Gene-Chip®, RNA-Seq) such differentiation is essentially impossible, since all VEGF-C forms are translated from the same mRNA transcript of the *VEGFC* gene. A differentiation of the different VEGF-C forms could be achieved with an antibody-based test (ELISA, Western blot), but such a test has not been developed yet. Moreover, the majority of commercially available antibodies against VEGF-C are not even capable of detecting VEGF-C with the necessary sensitivity [42]. It is therefore not surprising that the research data are confusing.

Meanwhile, the number of clinical studies that correlate VEGF-C expression of tumours with the course of the disease has exceeded three hundred (Pubmed query: https://mjlab.fi/pubmed1). Some studies have found a link between VEGF-C levels and disease progression [71,72], while others could not demonstrate such a link [73]. In any case, controlled animal experiments mostly confirm the instrumental role of VEGF-C for tumour metastasis [60,74,75], and molecular biological mechanisms have also been identified for the relationship [76].

Activation of VEGF-C for prolymphangiogenic therapies

Although lymphedema can be treated, the aim of research remains a causal therapy, because lymph drainage and bandaging only help to control the symptoms of the underlying lymphatic insufficiency. With Bestatin (Ubenimex) and Lymfactin®, the first trials for drug-based lymphedema therapies have been started in the recent years. However, the Bestatin studies of the US company Eiger BioPharmaceuticals have been discontinued in autumn 2018 after the second phase since neither primary nor secondary objectives had been achieved [77]. In contrast, the phase 2 studies of Lymfactin®, sponsored by the Finnish pharmaceutical start-up Herantis, have just been expanded [78]. The two drugs are based on different mechanisms of action. Following the observation that the analgesic ketoprofen relieves lymphedema symptoms in a mouse model [79], the ketoprofen-like but more specific Bestatin was selected for clinical trials [80]. Ketoprofen and Bestatin are non-steroidal anti-inflammatory drugs, and not much detail is known about their influence on the lymphatic system. In contrast, Lymfactin® is a genetically engineered biopharmaceutical that is based on the body's own VEGF-C production after administration of a recombinant adenoviral vector (AdVEGF-C, see Figure 9), whose mechanism of action is well researched [81-83]. Depending on the area of application, the availability of endogenous proteinases and CCBE1 for the activation of VEGF-C for Lymfactin®/AdVEGF-C could, however, be a limiting factor. In animal experiments, e.g. muscle tissue reacted to VEGF-C-providing gene therapy only with moderate lymphangiogenesis. Only when VEGF-C was co-administered with CCBE1 the lymphangiogenic response became strong [26].

Because the lymphatic system is important not only for drainage but also for immunity, it is not surprising that VEGF-C has been identified as a pharmacological target for several diseases affecting the immune system. These include chronic inflammatory bowel disease [84], psoriasis [85] and rheumatoid arthritis [17], but also neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease [86]. Also intruiging, albeit controversial, is a report about the successful therapy of myocardial infarction in an animal model with a single dose of VEGF-C [87].

It should be noted that these prolymphangiogenic applications pursue an objective that is contrary to that of tumour therapy. In lymphedema and immune diseases, the typical goal is to increase expression and activation of VEGF-C, whereas in tumour therapy, the goal is to block VEGF-C expression or activation. Balancing these opposing goals might prove a complex task, particularly in the case of edema which occurs as a result of surgical cancer treatment.

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