Current Biology of VEGF-B and VEGF-C

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Endothelial growth factors and their receptors may provide important therapeutic tools for the treatment of pathological conditions characterised by defective or aberrant angiogenesis. Vascular endothelial growth factor (VEGF) is pivotal for vasculogenesis and for angiogenesis in normal and pathological conditions. VEGF-B and VEGF-C provide this gene family with additional functions; for example, VEGF-C also regulates lymphangiogenesis.

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Abbreviations

IL	interleukin
PDGF	plateltet-derived growth factor
PlGF	placenta growth factor
TNF-α	tumour necrosis factor α
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor

Introduction

The inner lining of blood and lymphatic vessels, as well as the endocardium, consists of endothelial cells. The blood vasculature forms by two processes: by vasculogenesis, the *de novo* formation of endothelial channels from differentiating angioblasts; and by angiogenesis, the sprouting or splitting of capillaries from pre-existing vessels (reviewed in [1]). Polypeptide growth factors and their receptors are major components of the regulatory machinery that governs these processes. Two receptor tyrosine kinase families, the vascular endothelial growth factor (VEGF) receptors (VEGFR-1/Flt-1, VEGFR-2/KDR and VEGFR-3/Flt4) and the angiopoietin receptors (Tie-1 and Tie-2/Tek) are the key players, being largely specific for endothelial cells. Other receptor families, such as the Eph family, also provide major contributions to vessel differentiation [2•, 3]. Targeted gene disruptions in mice have verified their central importance in vessel growth, remodelling and maturation ([4-8, 9••], reviewed in [10]).

Although the adult vasculature is normally quiescent, it can become activated to form new capillaries, for example, in wound healing and tumourigenesis. There is convincing evidence that tumours are angiogenesis dependent [11]. In the prevascular phase a tumour's volume rarely exceeds a few cubic millimetres and vessel density in invasive cancers (e.g. in prostate cancer) positively correlates with metastatic potential and prognosis [12]. During the so-called angiogenic switch in tumourigenesis, the balance between angiogenesis inhibitors (e.g. endostatin and thrombospondin-1) and angiogenesis inducers (e.g. VEGF) is shifted and rapid vessel ingrowth follows, supporting tumour expansion [11]. By default endothelial cell turnover rates are low in resting vessels, whereas they are high in tumour vasculature. Angiogenesis is suggested to be a rate-limiting step in tumour development and angiogenesis inhibitors are thus attractive drugs for anticancer therapy. There are several benefits of directing drugs to the endothelium, including its general accessibility through the blood circulation and the absence of drug-resistance in normal diploid and genetically stable endothelial cells, as opposed to the frequent development of resistance to cytotoxic therapy in genetically heterogeneous and unstable cancer cells [13, 14].

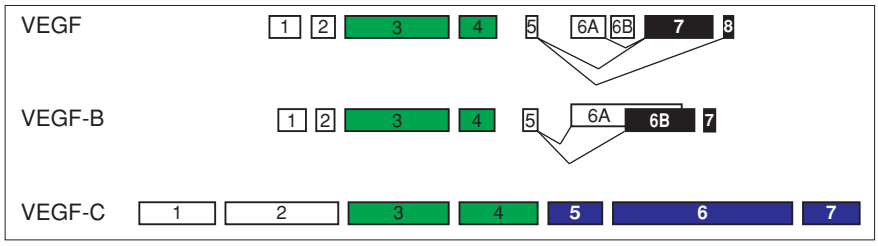
VEGF is a hypoxia-inducible endothelial cell mitogen. It stimulates endothelial cell migration, vessel permeability [15], and promotes survival of the newly formed vessels [reviewed in 16]. VEGF is crucial for embryonic development as targeted inactivation of even a single VEGF allele results in embryonic lethality [17, 18], and it is also required for survival in early postnatal life when the endothelium is still proliferating [19•]. Although VEGF is highly specific for endothelial cells, it has become increasingly clear, that it also elicits responses in non-endothelial cell types. For example, it is chemotactic for monocytes [20, 21] and can inhibit the maturation of dendritic cells [22]. VEGF receptors are as well expressed in certain non-endothelial cell types in the testis and epididymis where overexpression of VEGF caused spermatogenic arrest, epithelial hyperplasia and infertility [23•]. VEGF is also thought to be a key regulator of bone formation via its effects on the osteoblasts and osteoclasts of growth plates [24, 25]. The different splice variants of VEGF seem to differ in their function: in contrast to $VEGF_{165}$, $VEGF_{121}$ is unable to bind to the non-tyrosine kinase receptor neuropilin-1[26], and in new-born gene targeted mice $VEGF_{120}$ cannot compensate for the loss of the longer isoforms, leading to ischemic cardiomyopathy and death [27•].

The family of VEGF-related molecules has recently grown and contains presently five mammalian members: VEGF; placenta growth factor (PlGF); VEGF-B; VEGF-C; and VEGF-D. The viral homologues, collectively called VEGF-E, are encoded by different strains of the Orf virus [28].

VEGF-B, a protein that comes in two flavours

Two mRNA splice variants are generated from the VEGF-B gene, which is located on human chromosome 11q13 [29-31]. The gene contains seven exons. The coding sequence of

Figure 1



Comparisonof exon organisation and major splice isoforms of VEGF, VEGF-B and VEGF-C. The VEGF homology domain containing the conserved cysteine residues of the PDGF-subtype cystine knot is shown in green, the heparin binding domain in black, and the 'silk' homolgy domain in blue.

the first five exons is incorporated into both splice forms. Alternative splicing results in the use of different, but overlapping reading frames in exon 6 (Figure 1). Consequently the two isoforms of the polypeptide share the same 115 amino-terminal amino acid residues, but have distinct carboxy termini [31]. After the 21 amino acid signal sequence has been cleaved off, the two polypeptides are 167 (VEGF-B_{167}) and 186 (VEGF-B_{186}) amino acids in length [30-32]. The apparent molecular masses of the secreted homodimers of VEGF-B_{167} and VEGF-B_{186} are 42 kDa and 60 kDa, respectively.

The amino acid sequences of VEGF-B_{167} and $VEGF_{165}$ are ~44% identical and their intermolecular disulfide bridging patterns are similar. The two subunits are joined by disulfide bridges between the second and fourth cysteine residues of the platelet-derived growth factor (PDGF) subtype cystine knot consensus sequence [33•]. Exon 6B of VEGF-B_{167} is homologous to exon 7 of $VEGF_{165}$; both encode protein sequences rich in basic amino acid residues, which after secretion bind the growth factor to cell-surface heparan sulfate proteoglycans [31]. In contrast, the carboxy-terminal domain of VEGF-B_{186} is hydrophobic and contains many serine, threonine and proline residues. VEGF-B_{167} and VEGF-B_{186} also differ in their glycosylation pattern: whereas VEGF-B_{167} is not glycosylated, VEGF-B_{186} contains O-linked glycans [31]. Furthermore, VEGF-B_{186} is proteolytically processed at Arg127, giving rise to a 34 kDa dimer [33•, 34•].

VEGF-C defines a subfamily within the VEGF family

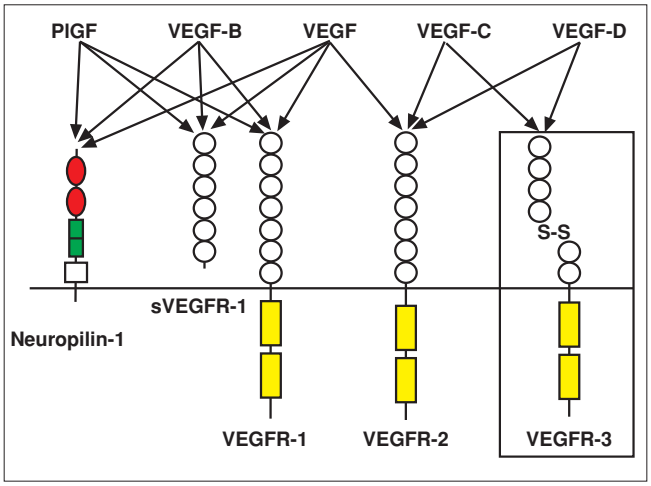
Within the VEGF family of growth factors, VEGF-C and its closest relative, VEGF-D, constitute a subgroup, which is characterised by the presence of unique amini- and carboxy-terminal extensions flanking the VEGF-homology domain [35-37, 38•, 39]. The carboxy-terminal domain contains a repetitive pattern of cysteine residues, Cys-X_{10}-Cys-X-Cys-X-Cys, resembling a motif characteristic of the Balbiani ring 3 protein, a secretory protein and a component of silk produced in larval salivary glands of the midge *Chironomus tentans*. The central core (the VEGF homology domain) exhibits ~30% identity to VEGF [35] and is encoded by exons 3 and 4 of the seven exons [40] (Figure 1), which is a feature conserved in other members of the VEGF family [31, 41, 42]. The VEGF homology domains of VEGF-C and VEGF-D are 61% identical [38]. The human VEGF-C gene has been localised to chromosome 4q34 [29]. VEGF-C is synthesised as a precursor protein, which undergoes subsequent proteolytic processing reminiscent of the PDGF-A and -B chain processing, suggesting an evolutionary relationship [35, 43, 44]. The carboxy-terminal domain is cleaved upon secretion, but remains bound to the amino-terminal domain by disulfide bonds giving rise to a disulphide linked tetramer composed of 29 and 31 kDa polypeptides. Proteolytic processing of the amino-terminal propeptide releases the mature form, which consists of two 21 kDa polypeptide chains corresponding to the VEGF homology domain [43]. The 29/31 kDa form seems to be the most prevalent form of VEGF-C in various biological systems [36, 43].

Dissimilar regulation of VEGF-B and VEGF-C

The promoters of the genes of VEGF family typically lack a TATA-box and so transcription is initiated at more heterogeneous sites [40, 42, 45, 46]. As for the VEGF-B promoter, the VEGF-C promoter sequences also lack putative binding sites for hypoxia-regulated factors [40] and consequently neither VEGF-B nor VEGF-C mRNA levels are regulated by hypoxia [47]. The VEGF-B promoter contains binding sites for the Egr-1 transcription factor, but lacks AP-1 sites that are present in the VEGF promoter [45]. Several growth factors, including PDGF, epidermal growth factor (EGF), transforming growth factor (TGF)-β and cytokines TNF-α and IL-1 (α and β), as well as the diacylglycerol analogue phorbol myristate acetete (PMA) increased the steady state levels of VEGF-C, but not VEGF-B mRNA in human lung fibroblasts [47, 48]. The VEGF-C mRNA induction by IL-1 and TNF-α might be mediated by the transcription factor NF-κB binding sites in the VEGF-C promoter [40]. In general, VEGF-C mRNA levels are downregulated by steroid hormones [49, 50]. In contrast, the VEGF-B mRNA levels seem more or less invariable and show only tissue-type-specific regulation.

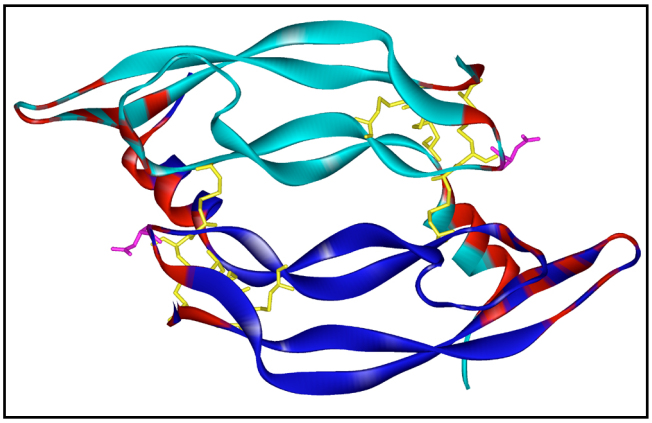
VEGF-B is expressed early during fetal development and is widely distributed, being prominently expressed in the cardiac myocytes, in skeletal muscle and smooth muscle cells of large vessels [51, 52]. Interestingly, VEGF-B is also expressed in the perichondrium of developing bone [52] and in the nervous system, especially in the cerebral cortex [53]. In adult mice, VEGF-B mRNA is abundant in heart and kid-

Figure 2



The vascular endothelial growth factors and their receptors. VEGFR-3 (boxed) is largely restricted to lymphatic endothelium. The different structural elements of the receptors are illustrated as follows: open circle, immunoglobulin domain; yellow box, tyrosine kinase domain; green box, domain homologous to coagulation factors V and VIII; red oval, CUB domain; open box, MAM domain. S-S, disulfide bridge; sVEGFR-1, soluble form of VEGFR-1.

Figure 3



The structure of VEGF was determined by X-ray crystallography. Similar structure was deduced both when crystallised alone and in a complex with VEGFR-1/Flt-1 [59, 86]. Two of the eight cysteins covalently link two VEGF monomers and the remaining six form a cystine knot (shown in yellow). When bound to VEGFR-1/Flt-1, the residues shown in red contact the receptor, predominantly in a hydrophobic fashion, Asp-63 (shown in purple) is the only side chain involved in direct charge-mediated interaction with the receptor. Based on data from mutagenesis studies the binding site for VEGFR-2/KDR is thought to be very similar. When compared to a model of VEGF-C, most divergence is seen in the receptor interface, the flat central part formed by two four-stranded b sheets appears largely unchanged.

ney, where it overlaps with strong VEGF expression [54]. When comparing the isoforms, VEGF-B_{167} mRNA seems to be the most prevalent, representing up to 90% of the transcripts in adult mouse tissues (X. Li, personal communication).

VEGF-C mRNA can be detected in the cephalic mesenchyme (connective tissue of the head region) and along the somites at embryonic day E8.5. At E12.5, VEGF-C expression is prominent in the mesenchyme, around the developing metanephrons and in the jugular area, in regions where the lymphatic vessels are developing in association with venous sac-like structures [55]. This pattern is conserved between species; in quail and chick embryos VEGF-C was observed in regions that would become rich in lymphatic endothelium later in avian development [56•]. In adult mice, the expression of VEGF-C decreases but its mRNA can still be found in the lung, heart, liver and kidney [55, 57]. Both VEGF-B and VEGF-C are suggested to act in a paracrine fashion in normal tissues via their receptors VEGFR-1 and VEGFR-3, respectively [52, 55].

VEGF-B is a ligand for VEGFR-1 and neuropilin-1

Figure 2 summarises the interactions of known VEGFs with their receptors. VEGF-B, like PlGF, is a selective ligand for VEGFR-1 [33•]. The first three immunoglobulin domains of this receptor are sufficient for VEGF-B binding, which is in agreement with the finding that VEGF (known to bind in this region to VEGFR-1) competes with VEGF-B for VEGFR-1 binding. Alanine-scanning mutagenesis of VEGF had suggested that the interaction with VEGFR-1 occurs mainly via the charged residues Asp63, Glu64 and Glu67, whereas binding to VEGFR-2 is primarily mediated by Arg82, Lys84 and His86 [58]. Subsequently, however, the co-crystallisation of VEGF with the second immunoglobulin domain of VEGFR-2 showed that many hydrophobic residues participate in the formation of the binding interface, the only direct polar interaction being the one between Asp63 of VEGF and Arg224 of VEGFR-1 [59] (see Figure 3). In addition, the three conserved acidic residues Asp63, Glu64 and Glu67 are all conserved in a viral VEGF-E (strain NZ2), which does not bind VEGFR-1. Nevertheless, when the homologous residues in VEGF-B (Asp63, Asp64 and Glu67) were replaced by alanine residues, VEGF-B binding to VEGFR-1 was clearly decreased [33•].

Recently, neuropilin-1, a receptor for semaphorins/collapsins involved in axonal guidance, was shown to act as an isoform-specific co-receptor for $VEGF_{165}$ and PlGF-2 [26, 60]. In addition to its neuronal expression, neuropilin-1 is also present in the developing embryo in endothelial cells of capillaries and blood vessels and in mesenchymal cells surrounding the blood vessels, as well as in certain other non-neuronal tissues, including the endocardial cells of the embryonic heart [26, 61]. The importance of neuropilin-1 in the circulatory system was verified when homozygous knockout embryos died of cardiovascular failure at E10.5-12.5. Additionally, overexpression of neuropilin-1 under the β-actin promoter was lethal due to severe anomalies of both the nervous and cardiovascular systems [61, 62]. Amino acid residues encoded by exon 7 of $VEGF_{165}$ mediate its

Table 1

Comparison of the biochemical and functional properties of the VEGF family members.

	VEGF	PlGF	VEGF-B	VEGF-C	VEGF-C
Isotype length*	121, 145, 165, 189, 206	131 (PlGF-1), 152 (PlGF-2)	167, 186	388	333
% Identity with VEGF	100	46	45	30	31
Heparin interaction	121 - 145 + 165 + 189 + 206 +	131 - 152 +	167 + 186 -	ND	ND
Heterodimers	PlGF-1 PlGF-2	$VEGF_{121}$ $VEGF_{165}$	$VEGF_{165}$	ND	ND
Receptors	VEGFR-1 VEGFR-2 NP-1‡	VEGFR-1, NP-1#	VEGFR-1, NP-1§	VEGFR-2,† VEGFR-3	VEGFR-2,† VEFR-3
Activity	Angiogenesis, survival	Migration of EC and monocytes	ND	Lymphangiogenesis, angiogenesis	Proliferation of ECs

*Number of amino acids (without signal peptide). †Only the mature forms of VEGF-C and VEGF-D interact with VEGFR-2. ‡Only $VEGF_{165}$. #Only PlGF-2. §$VEGF\text{-}B_{167}$ and the processed form of $VEGF\text{-}B_{186}$. EC, endothelial cell; ND, not determined; NP-1, neuropilin-1

interaction with neuropilin-1, which enhances the ability to bind VEGFR-2 and to induce chemotaxis [26]. $VEGF\text{-}B_{167}$ also interacts with neuropilin-1 [34•]; the interaction is mediated by the exon 6B encoded domain, which contains a sequence homologous to the neuropilin binding peptide of $VEGF_{165}$ and mediates heparin binding of $VEGF\text{-}B_{167}$. Surprisingly, the non-heparin binding isoform $VEGF\text{-}B_{186}$ also bound neuropilin-1, but only in its proteolytically cleaved form [34•]. The neuropilin-binding epitope in $VEGF\text{-}B_{186}$ was mapped to the first 12 amino acid residues following the core region, that is common to both $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$. The proteolytic cleavage in $VEGF\text{-}B_{186}$ thus unmasks an epitope for neuropilin-1 binding. Thus both VEGF-B and VEGF-C confim the idea, that all VEGF family members contain, in addition to the VEGF-like core, a distinct domain with unique characteristics, which confers binding specificity. In case of VEGF this 'unique' domain can fold independently from the VEGF homolgy domain and its structure could be determined by NMR spectroscopy [63].

VEGF-C signals via VEGFR-2 and VEGFR-3

Although both the full-length and the mature forms of VEGF-C bind VEGFR-3 [35], only the mature VEGF-C can bind to and activate VEGFR-2 [43]. VEGF-C shares receptor specificity with its closest homologue VEGF-D, which is also processed in a similar fashion [38•]. The receptor binding affinity of recombinant mature VEGF-C to VEGFR-3 is approximately threefold higher than for VEGFR-2 [43]. The receptors become readily phosphorylated upon VEGF-C binding and induce downstream signalling; for example, VEGFR-3 activation leads to phosphorylation of Shc and activation of extracellular signal-regulated kinase (ERK)1 and ERK2 [64], which are associated with mitogenesis.

When the first of the two residues Cys156 and Cys165, which in the other members of the PDGF/VEGF family are involved in interchain disulfide bonding, was replaced by a serine residue in the mature form of VEGF-C, the mutant was unable to bind to or to activate VEGFR-2 [65•]. This Cys156 mutant of VEGF-C also failed to induce vascular permeability, thus illustrating that VEGFR-3 is not involved in this process [65•].

Biological activities of VEGF-B

VEGF-B and VEGF have only partially overlapping receptor specificities and as indicated by the lethality of VEGF knock out in embryos, no other growth factor could compensate for the loss of even a single VEGF allele [17, 18]. Analysis of VEGF-B function has been hampered by difficulties in obtaining active recombinant VEGF-B protein, which have been solved only recently. VEGF-B might modulate VEGF signalling by forming heterodimers with VEGF [31, 51]. The VEGF-B knock out mice are viable and fertile with no obvious morphological changes, implying that VEGF-B does not play a major role in vascular development (K. Aase, U. Eriksson, unpublished data). Nevertheless, these mice show a subtle cardiac phenotype.

Targeted gene inactivation of the VEGF-B receptor VEGFR-1 leads to embryonic death around E 8.5 [4]. The VEGFR-1 deficient mice fail to form organised vessels and appear to have an overgrowth of endothelial cells. The phenotype has been explained by an increased mesenchymal→hemangioblast transition in the VEGFR-1 knockout mice, the formation of disorganised vascular channels being

a secondary phenotype [66]. The role of VEGFR-1, however, remains enigmatic: homozygous mice expressing VEGFR-1 without the tyrosine kinase domain but having an intact extracellular domain and membrane spanning region are normal, except that their macrophages show reduced migration *in vitro* in response to VEGF and PlGF [67]. Furthermore, overexpression of a VEGFR-2 devoid of the cytoplasmic kinase domain acts as a dominant negative receptor inhibiting tumour growth in nude mice [68], and an excess of the soluble form of VEGFR-1 (sVEGFR-1 in Figure 1) effectively inhibits tumour growth and metastasis [69]. These data suggest that a ligand binding form of VEGFR-1 defective of the cytoplasmic signal-transducing functions can substitute for the full-length receptor and that VEGFR-1 and VEGFR-2 do not form functionally critical heterodimers *in vivo*. If VEGFR-1 simply acts as a 'sink' for VEGF, it would be interesting to find out whether a soluble VEGFR-1 protein can restore the defective vasculature in embryoid bodies differentiated from the VEGFR-1 (-/-) embryonic stemm cells.

Dual role of VEGF-C: angiogenesis and lymphangiogenesis

VEGF-C, unlike VEGF, is a potent inducer of lymphangiogenesis. Transgenic mice overexpressing VEGF-C under the keratin 14 promoter, which directs transgene expression to the basal keratinocytes of the skin epidermis, had a selective hyperplasia of the superficial lymphatic vasculature [70]. In contrast, the phenotype of transgenic mice with $VEGF_{164}$ overexpressed from same promoter demonstrated its specificity for blood vessels [71]. Exogenously added recombinant mature VEGF-C induced proliferation of lymphatic endothelial cells and development of new lymphatic sinuses in differentiated avian chorioallantoic membrane (CAM) [72].

VEGF-C, however, also has VEGF-like properties. It effects on endothelial cells include stimulation of blood vascular endothelial cell proliferation and migration *in vitro* [35, 36], and *in vivo* VEGF-C increases vascular permeability [43]. Furthermore, using recombinant human VEGF-C or a VEGF-C-encoding plasmid, angiogenesis was promoted in a rabbit ischemic hindlimb model [73]. In accordance with this finding, VEGF-C induces neovascularisation in the mouse cornea micropocket assay and in the immature CAM assay [74•]. Mice deficient of VEGFR-3 died from cardiovascular failure at E9.5 [9••]. Vasculogenesis and angiogenesis were not perturbed, but the remodelling and maturation of large vessels were severely impaired. VEGFR-3 may thus have an essential role even before the formation of the lymphatic system, indicating that its ligands would also act at several developmental time points.

VEGF and VEGF-C might be redundant in vasculogenesis in the early embryo. Differences between the phenotypes of the VEGF-deficient mice [17, 18] and the VEGFR-2/Flk-1-deficient mice [75] indicate that the lack of VEGFR-2 affects an earlier step in development (i. e. the differentiation of the 'hemangioblasts' to endothelial and hematopoietic cells). This suggests the existence of a VEGFR-2 ligand that would partially compensate the absence of VEGF. VEGFR-2 positive cells isolated from the posterior mesoderm of early chicken embryos at gastrulation stage could give rise *in vitro* to endothelial or hematopoietic colonies and their endothelial cell differentiation required exogenously added VEGF [76]. Interestingly, a similar effect was obtained with somewhat higher concentrations of recombinant mature VEGF-C [56•]. The triggering of endothelial cell differentiation was presumably mediated by VEGFR-2, as these precursor cells did not express VEGFR-3 [56•].

Tumour angiogenesis

Recent evidence indicates that both VEGF-B and VEGF-C are expressed in tumour tissues. VEGF-B expression is upregulated in ovarian carcinoma relative to normal ovarian surface epithelium [77] and VEGF-B is commonly present in both benign and malignant human tumours (e.g. in breast carcinoma, melanoma and fibrosarcoma) [78], as well as in a variety of cultured tumour cell lines [47].

VEGF-C mRNA was detected in approximately half of the tumour samples studied [78], and notably, all lymphomas contained VEGF-C mRNA, possibly reflecting the expression of VEGF-C in the corresponding normal cells. VEGF-C might also be involved in lymph node metastasis as upregulated expression of VEGF-C was detected in prostatic carcinoma and the VEGF-C expression was correlated with tumour dissemination into the lymph nodes [79]. VEGF-C and its receptor VEGFR-3, however, are also associated with angiogenesis in breast cancer [80]. The carcinoma cells in at several of the studied tumours expressed the VEGF-C protein and, surprisingly, VEGFR-3 became upregulated on the angiogenic capillaries.

Conclusions

Table 1 summarises certain biochemical and functional properties of the known VEGFs. Whereas VEGF-C appears to be a potent inducer of both angiogenesis and lymphangiogenesis, the function of VEGF-B remains to be established. Because of the selective nature of VEGF-B and VEGF-C, they could be used to target either the vascular endothelium or the lymphatic endothelium. Therapeutical modulation of growth factor signalling in pathologic conditions represents the major challenge in the angiogenesis field. Thus, tumour growth, metastasis and diabetic retinopathy could be prevented by inhibition of angiogenesis, whereas pro-angiogenic stimuli could help patients with myocardial or peripheral ischemia. Rapid progress is being made to control vascular responses to arterial injury, such as balloon angioplasty and in limb ischemia based on gene transfer or local delivery of the VEGF protein [81-84]. In this context, additional VEGF-like molecules, such as VEGF-B and VEGF-C, might provide novel approaches to target angiogenesis, either on their own or in combination with VEGF or

perhaps the angiopoietins [85].

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- •• of outstanding interest

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