# Vascular Endothelial Growth Factors VEGF-B and VEGF-C

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Vascular endothelial growth factor, which was identified almost 10 years ago, has so far been considered as the only growth factor relatively specific for endothelial cells. VEGF is an important regulator of endothelial cell proliferation, migration, and permeability during embryonic vasculogenesis and in physiological and pathological angiogenesis [reviewed in (1–3)]. The pivotal role of VEGF in embryogenesis is emphasized by the unprecedented result that the inactivation of even a single VEGF allele results in embryonic lethality (4,5). VEGF acts through its two known high-affinity receptors Flt1, vascular endothelial growth factor receptor 1 (VEGFR-1) and KDR/Flk1, or VEGFR-2 (6–10). A third receptor tyrosine kinase homologous with VEGFR-1 and VEGFR-2, designated Flt4, was cloned as an orphan receptor by two research groups and was shown not to bind VEGF (11–14). Additional VEGF receptors of unknown nature also exist on endothelial and tumor cells (15,16).

The second member of the VEGF family of growth factors, placenta growth factor (PlGF), is 53% identical with VEGF within its platelet-derived growth factorlike region and binds only VEGFR-1 (17–19). Both VEGF and PlGF are dimeric glycoproteins related in structure to the platelet-derived growth factors A and B (PDGF-A and PDGF-B). This relation is based on the presence of several conserved amino acid residues including 8 equally spaced cysteines. Compared with VEGF, the mitogenic or permeability-enhancing activities of PlGF are weak; however, PlGF is able to potentiate the action of VEGF in vivo and in vitro (19). PlGF–VEGF heterodimers occur in vivo and have intermediate potency in mitogenic stimulation of endothelial cells (35).

Two novel endothelial cell-specific growth factors, structurally related to VEGF and PlGF, were recently discovered. These factors, designated as vascular endothelial growth factor B (VEGF-B) or VEGF-related factor (20,21) and vascular endothelial growth factor C (VEGF-C) or VEGF-related protein (22,23) expand the known VEGF family and demonstrate the complexity of regulation of endothelial functions. This review summarizes the initial studies on VEGF-B and VEGF-C.

## VEGF-B/VRF

The human and mouse VEGF-B cDNA clones were isolated from human fibrosarcoma and erythroleukemia tumor cell cDNA libraries and from an adult mouse heart cDNA library, respectively, by using a serendipitously found partial mouse cDNA clone as a probe (20,24). Independently, another group found the same gene when attempting to identify candidate genes for multiple endocrine neoplasia type 1 (MEN1). The product of this alternatively spliced gene was designated as VRF (21).

The two currently known isoforms of VEGF-B are generated by alternative splicing of mRNA from the VEGF-B gene, spanning about 4 kb of DNA. The human and murine VEGF-B genes are composed of 7 exons, and their exon–intron organization resembles that of VEGF and PlGF genes (21,24,25). The mature VEGF-B proteins (devoid of signal sequence) have 167 (VEGF-$B_{167}$) and 186 (VEGF-$B_{186}$) amino acid residues, respectively. VEGF-$B_{186}$ is generated by using an alternative splice acceptor site in exon 6, resulting in an insertion of 101 bp between nucleotides 410 and 411 in the coding sequence of VEGF-B167. This insertion introduces a frame shift and a stop codon at the position corresponding to nucleotides 521–523 of the coding region of VEGF-$B_{167}$ cDNA (Fig. 1). Thus, the two VEGF-B isoforms have an identical NH2-terminal domain of 115 aa and different COOH-terminal domains. Although the C-terminus of VEGF-$B_{167}$ is highly basic, that of VEGF-$B_{186}$ is rich in alanine, proline, serine, and threonine amino acid residues and has no significant similarity with amino acid sequences of known proteins (21,24). Unlike other growth factors of the VEGF-family, both isoforms of human and mouse VEGF-B lack the consensus sequence for N-linked glycosylation (NXT/S); instead, $VEGF_{186}$ is O-glycosylated (24).

VEGF-$B_{167}$ remains cell associated with secretion, but it is released into the culture medium with treatment of the producing cells with heparin or high salt. The cell (or matrix) association of VEGF-$B_{167}$ likely occurs via its basic region, as observed for the highly basic splice variants of VEGF. This notion is supported by the fact that VEGF-$B_{186}$, lacking the highly basic region, is freely secreted from cells and is not bound to cell-surface or pericellular heparan sulfate proteoglycans (20,24).

The apparent molecular masses of the secreted VEGF-$B_{167}$ and VEGF-$B_{186}$ polypeptides are 21 kDa

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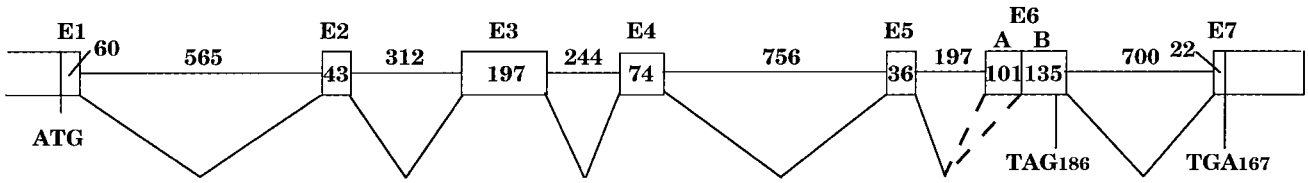


Fig. 1. Schematic structure of the human VEGF-B gene (Reproduced from Olofsson et al, 1996 with permission from the publisher, the American Society for Biochemistry and Molecular Biology). The sizes of introns and exons are indicated in base pairs. The alternative splicing of exon 6 and the resulting translational termination codons are also shown.

and 32 kDa, respectively, in reducing conditions and approximately twice this in nonreducing conditions, indicating that both forms are produced as disulfide-linked homodimers. Both VEGF-$B_{167}$ and VEGF-$B_{186}$ also can form disulfide-linked heterodimers with VEGF. In the absence of heparin, the VEGF-$B_{167}$ · $VEGF_{165}$ heterodimers remain cell associated (20,24). Because homodimers of $VEGF_{165}$ are efficiently secreted into the medium, VEGF-$B_{167}$ appears to determine the release of the heterodimers from cell surface.

Heterodimerization of VEGF and VEGF-$B_{167}$ might therefore control the bioavailability of VEGF (20). The receptor for VEGF-B has not been published, and it is currently unknown whether the VEGF-B polypeptides perform their in vivo function as homodimers, as heterodimers with VEGF, or both. VEGF-B-containing conditioned medium was able to stimulate thymidine incorporation into DNA in human umbilical vein endothelial cells and bovine capillary endothelial (BCE) cells, suggesting that VEGF-B is angiogenic (20).

Both isoforms of VEGF-B have an almost identical pattern of expression, being predominantly expressed in embryonal and adult muscle tissues (myocardium and skeletal muscle), and are coexpressed with VEGF in many tissues, most prominently in the heart (24,26). Mouse VRF expression was detected in 8-day-old embryos in structures most likely corresponding to the neural tube. On embryonic day 14, VRF is expressed in most tissues of the embryo, although most prominently in heart, spinal cord, and cerebral cortex. On day 17, most of the in situ hybridization signal is concentrated in the heart, brown fat, and spinal cord (26).

The VEGF-B gene was localized to chromosome 11q13, proximal to the cyclin D1 gene, which is amplified in a number of human carcinomas (21,27). However, the amplification of cyclin D1 was not accompanied by amplification of VEGF-B in several mammary carcinoma cell lines studied (27).

## VEGF-C/VRP

VEGF-C was initially identified (22) as a factor stimulating tyrosine phosphorylation of an orphan receptor Flt4, which is structurally homologous to VEGFR-1 and VEGFR-2. VEGF-C was then purified from medium conditioned by the PC-3 prostatic adenocarcinoma cells by using receptor-affinity chromatography on Flt4 extracellular domain coupled to Sepharose. A 5′ fragment of VEGF-C cDNA was amplified by serial polymerase chain reaction (PCR) using degenerate primers, and the entire cDNA was cloned from a cDNA library prepared from PC-3 cells using the labeled PCR-amplified 5′ VEGF-C cDNA fragment as a probe (22). Independently, another group identified an expressed sequence tag (EST) homologous with VEGF in the database and isolated the cDNA encoding VEGF-C by using the EST probe in screening of the library. The protein encoded by this cDNA was designated as VRP (23).

The open reading frame of the VEGF-C cDNA encodes a protein of 419 amino acid residues, with a predicted molecular mass of 46.9 kDa. The VEGF-C preproprotein consists of an N-terminal signal peptide, followed by an N-terminal propeptide, the VEGF-homology domain, and a C-terminal propeptide (Fig. 2). The C-terminal propeptide contains four tandemly repeated cysteine-rich motifs that are characteristic of a protein component of silk produced by the larval salivary glands of the midge, *Chironomus tentans.* Homologous portions of VEGF-C are about 30% identical with those of VEGF165, 27% with those of VEGF-B167, 25% with those of PlGF-1, and 22–24% with those of PDGF-A and PDGF-B. VEGF-C contains three putative N-glycosylation sites in the VEGF homology domain, and it is efficiently secreted as a disulfide bonded homodimer. Most of the secreted VEGF-C is proteolytically processed from the precursor polypeptide. VEGF-C binds Flt4, which we renamed as VEGFR-3 and VEGFR-2, and stimulates their tyrosine phosphorylation (22,36). An angiogenic activity of VEGF-C was suggested by its ability to stimulate migration of BCE cells in collagen gel (22). VRP promotes the growth of human lung endothelial cells, although its effect was about 100-fold less potent than the effect of VEGF (23).

Two VEGF-C mRNAs of 2.4 and 2.0 kb are detected in Northern blotting of RNA from many embryonal and adult tissues. In adult humans, the VEGF-C mRNA is expressed most prominently in heart, placenta, ovary, small intestine, and the thyroid gland. Tumor cells express almost exclusively the 2.4-kb mRNA form, suggesting that it corresponds to the described VEGF-C cDNA clone obtained from the PC-3 tumor cell line (22). The identity of the 2.0-kb VEGF-C mRNA remains to be determined. Two VRP cDNA clones containing 152-bp and 557-bp deletions when compared with the full-length VRP cDNA clone have been described. Due to the shift of the reading frame, which occurs 15 amino acid residues downstream of the deletion, the predicted proteins encoded by the two deleted cDNAs contain either none or only part of the core cysteine region similar to VEGF. These deleted VRP forms are presumably generated by alternative splicing (23). The VEGF-C gene was localized to chromosome 4q34, close to the human aspartylglucosaminidase gene (27).

In addition, the mouse VEGF-C cDNA has been cloned and encodes a protein of 415 amino acid resi-

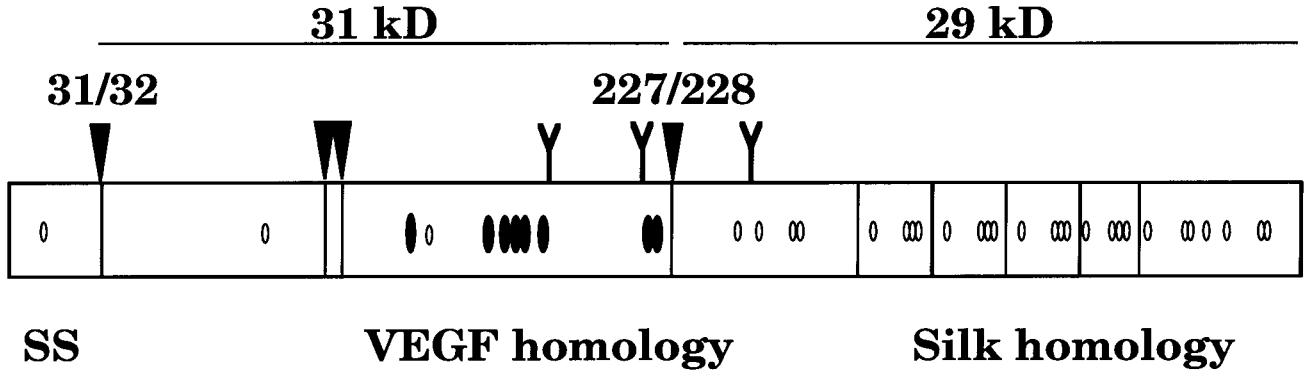


Fig. 2. Schematic structure of VEGF-C (22,23,36). The Cys residues are indicated as ovals, and the Cys residues conserved throughout the VEGF/PDGF family are indicated as large filled ovals. Three potential sites of N-linked glycosylation (y) and two cleavage sites (arrowheads) with flanking amino acid residue numbers and major polypeptide sizes. SS: Signal Sequence are also marked in the figure.

dues, which is 85% identical with the human VEGF-C and similarly processed (28). In in situ hybridization, mouse VEGF-C mRNA was detected in 8.5-day embryos in the cephalic mesenchyme, along the somites, in the tail region, and extraembryonally in the allantois. In 12.5-day embryos, VEGF-C mRNA was particularly prominent in regions where the lymphatic vessels undergo sprouting from embryonic veins, such as perimetanephric, axillary, and jugular areas. The signal also was detected between the vertebral corpuscles, in the lung mesenchyme, in the neck region, and in the developing forehead. The developing mesenterium, which is rich in lymphatic vessels, also showed strong VEGF-C expression (28). The distribution of the VEGFR-3 mRNA follows a somewhat similar temporal and spatial pattern (28,29). This observation suggests a paracrine mode of ligand–receptor interaction, with VEGF-C expressed in mesenchymal cells adjacent to the VEGFR-3-positive endothelia.

The partially adjacent VEGFR-3 and VEGF-C expression patterns suggest that VEGF-C functions in the formation of the venous and lymphatic vascular systems during embryogenesis. Constitutive expression of VEGF-C in adult tissues further suggests that this growth factor also is involved in the maintenance of functions of, for example, differentiated lymphatic endothelium where VEGFR-3 is expressed (22,28,29).

VEGF-C expression was first detected by Northern blotting and hybridization analysis in day 7 p.c. embryos (28). This finding was striking, considering the first appearance of VEGFR-3 mRNA on day 8.5 of gestation (29), which suggests a possible role for VEGF-C during earlier stages of embryonal development. Such a function might be exercised through the ability of VEGF-C to function as a ligand for VEGFR-2, which is expressed in presumptive progenitors of yolk sac blood islands as early as 7 day p.c. Interestingly, VEGFR-2 is essential for the development of both haematopoietic and endothelial cells (30,31). The question as to whether VEGF-C also is a factor essential for development of hemangioblasts is still unanswered. This function of VEGF-C might account for the difference between the phenotypes of the VEGF- and VEGFR-2- deficient mice, namely the delayed endothelial cell differentiation in VEGF−/− mice and the aborted hematopoietic and endothelial cell development in the VEGFR-2−/− mice. However, the VEGF-C mRNA detected by Northern blotting on day 7 p.c. may be derived from placenta/fetal membranes, where VEGF-C was also expressed according to in situ hybridization data.

An additional growth factor of the PDGF/VEGF family, designated as c-fos-induced growth factor (FIGF), was isolated using differential mRNA screening of normal and fos−/− fibroblasts (32). Although FIGF apparently showed autocrine mitogenic and morphogenic effects on fibroblasts, its prominent similarity to VEGF-C suggests that it also may possess angiogenic activity.

A brief characterization of the polypeptides of the VEGF family is presented in Table 1.

## PERSPECTIVES

The discovery of two novel members of the VEGF family increases our understanding of the complexity of the regulatory signals for endothelial cells and promotes new areas of research in vascular biology. Many of the already established experimental models and approaches used in VEGF studies might obviously be applied to studies of VEGF-B and VEGF-C. However, not only endothelial functions should be taken into consideration here because recent results show that VEGF might induce certain biological effects via the targeting of nonendothelial cells (33,34).

The main questions about the biological roles of VEGF-B and VEGF-C have not been answered. In this regard, different transgenic approaches including gene targeting are of great importance. Studies on VEGF-C might bring invaluable information concerning the function of lymphatic vessels. To our knowledge, VEGFR-3 is the only known receptor (tyrosine kinase) specific for the lymphatic endothelium.

Important issues also concern the analysis of possible regulation of VEGF-B and VEGF-C expression by hypoxia and other factors known to regulate VEGF expression and the function of the splicing forms of VEGF-B and VEGF-C. Such alternatively spliced forms might possess different functions in vivo, for example, due to the differences in their receptor specificity/affinity, bioavailability, stability, and ability to form heterodim-

TABLE 1. Comparison of the human VEGF family members[1]

| | $VEGF_{165}$ | PIGF-1 | PIGF-2 | $VEGF\text{-}B_{167}$ | $VEGF\text{-}B_{186}$ | VEGF-C | FIGF/VEGF-D[3] |
|---|---|---|---|---|---|---|---|
| Total length (22) | 191 | 149 | 170 | 188 | 207 | 419 | 358/354 |
| Predicted molecular weight (kDa) | 22.3 | 16.7 | 19.3 | 21.3 | 21.6 | 46.9 | 40.9 |
| %Identity with $VEGF_{165}$[2] | 100 | 40.4 | 40.1 | 42.7 | 33.0 | 31.4 | 26.7 |
| Number of Cys residues | 16 | 10 | 11 | 16 | 8 | 38 | 29 |
| Number of N-glycosylation sites | 1 | 2 | 2 | 0 | 0 | 3 | 3 |
| Heparin binding | + | − | + | + | ? | − | ? |
| Receptor(s) | VEGFR-1 VEGFR-2 | VEGFR-1 | VEGFR-1 | ? | ? | VEGFR-2 VEGFR-3 | ? |
| Heterodimers | PlGF-1 PlGF-2 | $VEGF_{165}$ | $VEGF_{165}$ | $VEGF_{165}$ | $VEGF_{165}$ | ? | ? |

[1]The data are presented for the human factors, except for FIGF (mouse).
[2]In appropriate domain.
[3]Mark Achen, personal communication.
aa: amino acid residues.

ers with other members of the VEGF family. The discovery of VEGF-B and VEGF-C highlights the structural similarities of VEGF family polypeptides and simplifies the search for novel homologous molecules.

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