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Molecular Basis of Vascular Anomalies

Miikka Vikkula,* Laurence M. Boon, John B. Mulliken, and Bjorn R. Olsen

Vascular anomalies comprise a heterogeneous group of disorders that are divided into tumors (hemangiomas) and malformations. Recent advances in biomedical research provide insights into the molecular basis of these disorders and a deeper understanding of vascular morphogenesis. In the future, this emerging knowledge will contribute to novel ways to treat vascular anomalies and to regulate pathologic angiogenesis. (Trends Cardiovasc Med 1998;8:281–292) © *1998, Elsevier Science Inc.*

The earliest development of vascular channels can be identified as differentiation of mesenchymal cells into endothelial cells (ECs). This in situ formation of vascular tubes (vasculogenesis) leads to the formation of a primary capillary plexus (Risau et al. 1988). The plexus undergoes remodeling and sprouting (angiogenesis) within almost all tissues of the mammalian body. The endothelial differentiation is accompanied by the recruitment of smooth muscle cell (SMC) precursors from mesenchymal and neural crest cells. These precursor cells migrate toward the endothelial tubes and differentiate to become the smooth muscle cell layers of blood vessel walls (Nakamura 1988, Kirby and Waldo 1995). Further changes in size and mural structure lead to the formation of arteries, capillaries, veins, and lymphatics, each with their own characteristics.

Recent studies are shedding some light on the cascade of developmental processes leading to the formation of the embryonic vascular network. Studies in transgenic mice have demonstrated a crucial role for vascular endothelial growth factor (VEGF) and for angiopoietin-1 (Ang-1) and -2 (Ang-2) and their receptors (Carmeliet et al. 1996a, Fong et al. 1995, Maisonpierre et al. 1997, Shalaby et al. 1995, Suri et al. 1996). These signaling molecules and their receptors are also implicated in human vascular anomalies believed to be caused by localized errors in vascular morphogenesis.

We discuss what is known about the molecular basis of anomalies that are primarily vascular. For late-appearing vascular lesions associated with other primary conditions (such as "angiokeratomas" in lysosomal storage diseases, kaposi sarcoma in immunodeficient conditions, and hemangioblastomas in von Hippel-Lindau disease; as well as telangiectasias in DNA repair disorders, such as ataxia-telangiectasia and xeroderma pigmentosum) the reader is referred to recent reviews (Mitsuyasu 1993, Maher and Kaelin 1997, Greenblatt 1998, Woods 1998).

• Classification of Vascular Anomalies

Vascular anomalies occur mainly in the skin. In the literature, almost all cutaneous vascular anomalies have, at some point, been called hemangiomas. The term *hemangioma* should, however, be restricted to a rapidly growing vascular tumor of infancy (Figure 1). This benign tumor is usually not present at birth, appears during the first 2 weeks of life, and undergoes a natural regression within 5 to 10 years (Mulliken and Glowacki 1982, Mulliken and Young 1988). Hemangiomas are the most common tumors of infancy. They do not occur in adolescents or adults. The other major category of vascular anomalies are malformations, lesions comprised of dysplastic vessels lined by quiescent endothelium. Vascular malformations are usually, but not always, obvious at birth. They never regress, they grow proportionately with the patient, and sometimes they expand. Vascular malformations can be subcategorized according to channel morphology and rheology as either slow-flow or fast-flow. This "biologic" classification of vascular anomalies into tumors and malformations, proposed in 1982 (Mulliken and Glowacki), has been accepted by the International Society for the Study of Vascular Anom-

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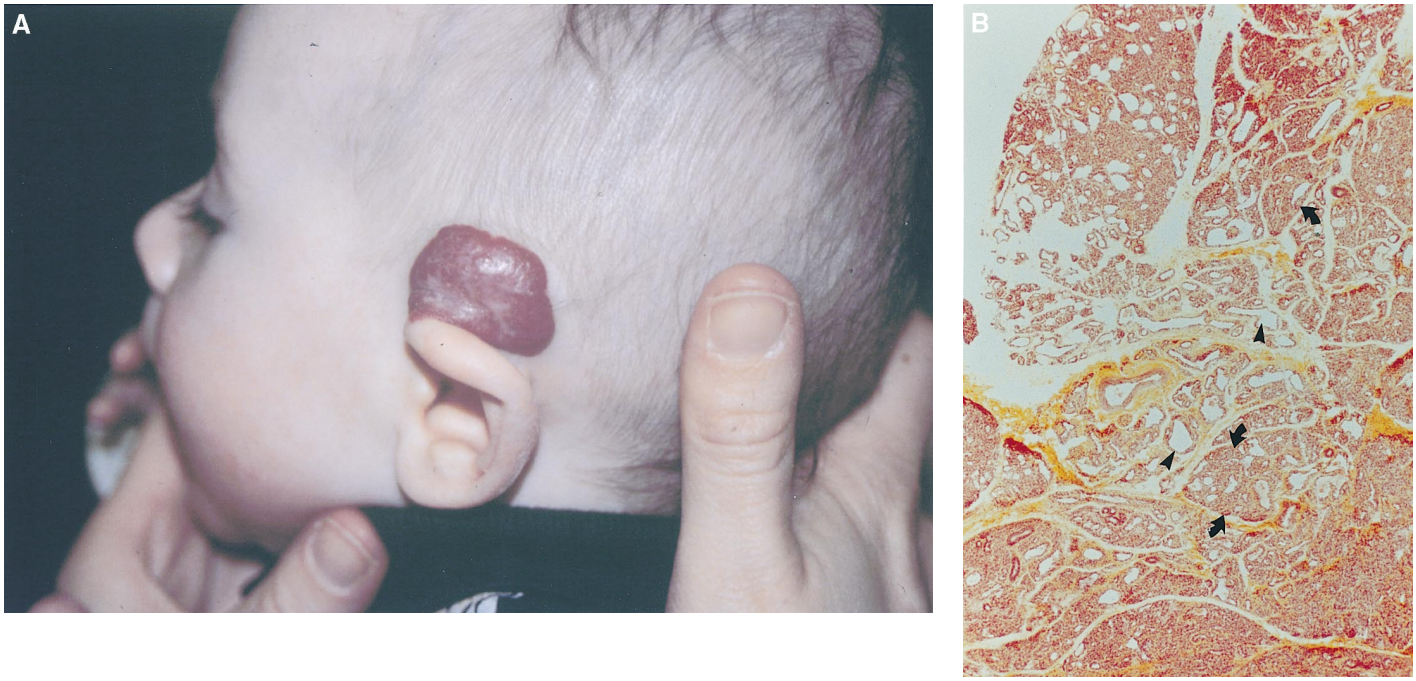

Figure 1. (**A**) A 10-month-old girl with a typical proliferating phase hemangioma (red, raised and warm on palpation) in the periauricular region. (**B**) Histology of a proliferating hemangioma from a 3-month-old boy. Note lobules packed with endothelial cells (*arrows*) and several large draining veins (*arrowheads*) (hematoxylin-eosin-safranine × 2.5).

alies (Rome 1996) and is used in this presentation.

• Vascular Tumors (Hemangiomas)

Hemangiomas are composed of plump, rapidly dividing endothelial cells (see Figure 1). The clinical phases of hemangioma's life cycle can be demonstrated by immunohistochemical markers (Takahashi et al. 1994). Upregulation of angiogenesis occurs, as shown by expression of proliferating cell nuclear antigen (PCNA) and two angiogenic peptides, VEGF and basic fibroblast growth factor (bFGF). Type IV collagenase is also present during the proliferative phase (defined as the first year of life), suggesting that collagen breakdown is necessary for the growing capillaries. E-selectin, an endothelial cell–specific leukocyte adhesion molecule, is also upregulated in the *proliferative phase* (Kräling et al. 1996), as is monocyte chemoattractant protein-1 (MCP-1) (Isik et al. 1996). By 1 year of the child's life, hemangioma's growth slows, as the tumor enters the involuting phase (1–7 years). During this period, mast cells and other monocytes and fibroblasts are seen. There is progressive deposition of perivascular and interlobular fibrous tissue. Apoptosis begins prior to 1 year and reaches its apogee at 2 years (Razon et al. 1998). Involution is also characterized by the autocrine induction of tissue inhibitor of metalloproteinase (TIMP-1), a suppressor of new blood vessel formation (Takahashi et al. 1994). After 5 to 7 years, hemangioma's life cycle is ended, the tumor is in its involuted phase; only a few tiny vessels remain, lined by flat/mature endothelium and surrounded by fibrofatty tissue. Multilaminated basement membranes, an ultrastructural hallmark of the proliferative phase hemangioma, persist in the involuted phase (Mulliken and Young 1988).

There are rare instances in which a fully grown hemangioma is present at birth; this is called congenital hemangioma. Curiously, these tumors often undergo a rapid involution within the first year of life (Boon et al. 1996b). The trigger for hemangiogenesis must have occurred in utero; the earliest reported congenital hemangioma was noted at 14 weeks of gestation (Bronshtein et al. 1992).

Endangering or life-threatening hemangiomas are treated with corticosteroids, as the first-line drug; if there is no response, interferon alfa can be effective (Ezekowitz et al. 1992, Boon et al. 1996a). Measurements of urinary bFGF can be used to monitor pharmacologic therapy (Folkman et al. 1997). Interferon alfa (2a or 2b) may be an effective antiangiogenic agent because it reduces bFGF expression (Singh et al. 1995).

Life-threatening thrombocytopenia is a rare complication of infantile vascular tumors. Since the first description in 1940, this condition has been known as Kasabach-Merritt syndrome. This phenomenon has been believed to be associated with giant hemangioma. However, recent studies have confirmed that Kasabach-Merritt coagulopathy occurs with another, more malignant, type of infantile vascular tumor, known as kaposiform hemangioendothelioma (KHE) (Enjolras et al. 1997, Sarkar et al. 1997). Spontaneous murine hemangioendotheliomas, vascular endothelial tumors that mimic KHE, are lethal (Hoak et al. 1971). Angiostatin, an angiogenesis inhibitor, has been successfully administered to treat these murine vascular tumors (Lannutti et al. 1997). Thus, antiangiogenic therapy (at least with angiostatin) may provide an efficient way to treat human KHE.

The triggers that initiate the common infantile hemangioma, the rare congenital hemangioma, and KHE are unknown. There is no evidence for Mendelian inheritance of hemangioma, but occasionally siblings are affected (Cheung et al. 1997). The trigger seems to be a local event, not a hereditary predisposition. Could it be stimulation of "dor-

mant angioblasts"—primitive cells that gave rise to embryonic vessels? There is no convincing evidence for an infectious etiology (Dupin et al. 1998). Polyoma middle T antigen is known to induce vascular tumors in experimental animals (incorrectly called hemangioma). However, the natural evolution of these tumors (they often undergo malignant transformation and grow throughout life) is very different from that of human hemangiomas (Williams et al. 1989). Could hemangioma begin with a local loss (or gain) of genetic material or information (somatic mutation) producing altered cells and autocrine growth? Perhaps spontaneous regression is a secondary phenomenon, a response by neighboring cells to the tumor. Mice expressing Fes/Fps protein-tyrosine kinase transgene with an activating mutation have multiple hemangioma-like tumors at specific sites; however, these tumors never regress, and the mice die of internal hemorrhage (Greer et al. 1994). Identification of the molecular trigger for hemangiomas is of great interest both clinically and biologically. Because the triggering event presumably involves a potent stimulator of angiogenesis, identification of the molecules involved may provide information that could be used for controlling these common infantile tumors and angiogenesis.

• Vascular Malformations

Vascular malformations differ, both clinically and histologically, from hemangiomas. They are usually present at birth, grow proportionately with the patient, and persist throughout life. Spontaneous regression does not occur, although very rarely deflation can occur with lymphatic malformation. Most vascular malformations are sporadic. However, there are rare families evidencing Mendelian inheritance, making genetic analysis possible. Vascular malformations are subdivided into simple forms (arterial, venous, capillary, or lymphatic malformation) and combined forms (such as arteriovenous and capillary-venous-lymphatic malformation) (Mulliken and Young 1988).

Venous Malformation

Venous malformations (VMs) are the most common referrals to centers for vascular anomalies, accounting for up to 50% of patients. There are, however, no data for their prevalence in the general population. They usually occur sporadically, although several families with dominant inheritance have been reported (Boon et al. 1994, Vikkula et al. 1996; McKusick 1998 [OMIM #600195, venous malformations, multiple cutaneous and mucosal]).

VM has a bluish-purple color, is often raised, and can be emptied by compression. VMs are most often located in the skin and/or mucosal membranes, but visceral lesions occur (see Figure 2 for a typical VM). An interesting family with mucocutaneous venous malformations associated with gastrointestinal bleeding was reported in 1958 by Bean (OMIM #112200). The disorder was inherited as an autosomal dominant trait, known as Bean or Blue Rubber Bleb Nevus syndrome. The individual lesions have similar clinical characteristics to cutaneous venous malformation but with associated gastrointestinal lesions; it could be an allelic VM phenotype. Glomangiomas are another form of venous-like cutaneous anomalies also known to be inherited in an autosomal dominant pattern (Rudolph 1993).

What are venous malformations? How do they form and why do they enlarge? To answer these questions, we used immunohistochemistry to visualize the two most important cellular compo-

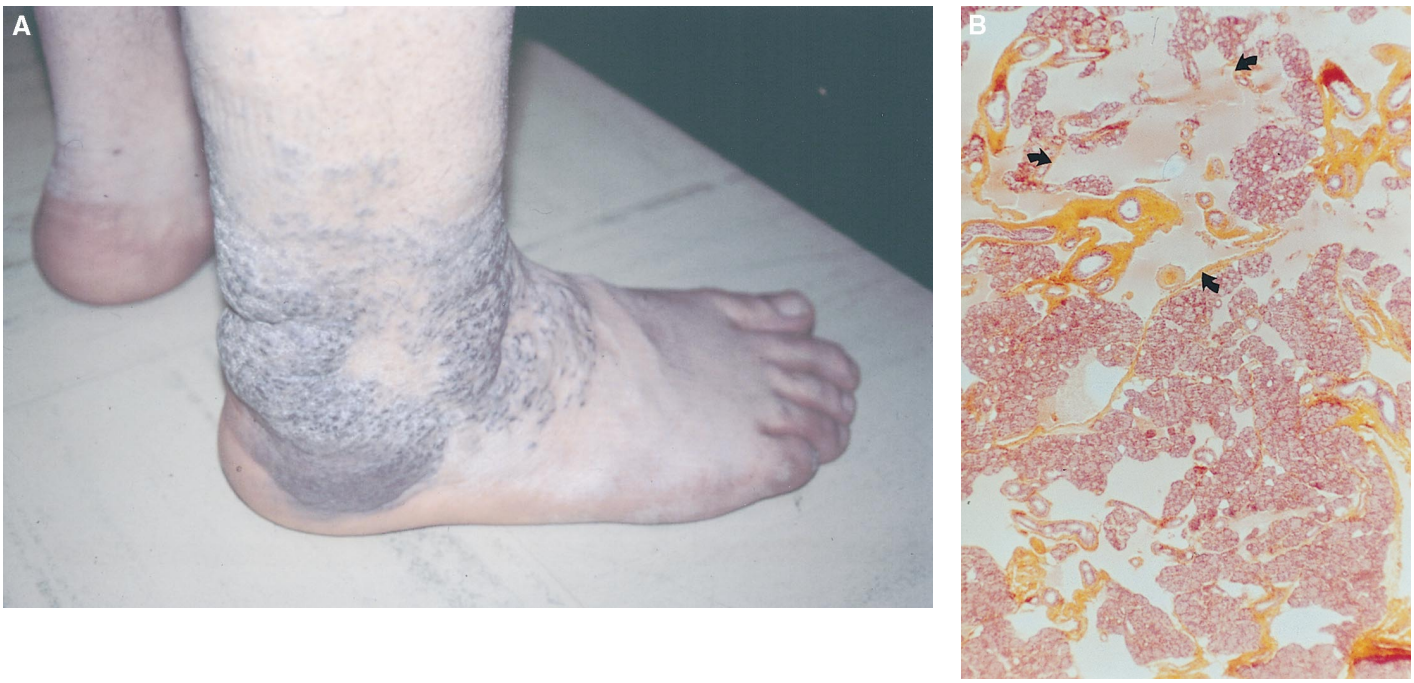

Figure 2. (A) A 30-year-old man with an extensive venous malformation (VM) of the right lower extremity. It is purple-bluish in color, soft and compressible with thrombi on palpation. **(B)** VM from the salivary gland of an 11-year-old male is composed of large, ectatic channels with thin walls (*arrows*) (hematoxylin-eosin-safranine × 2.5).

nents of blood vessel walls, the endothelial and smooth muscle cells. Using antibodies against von Willebrand factor and smooth muscle α-actin, we found that VMs are composed of a number of saccular channels, lined by flat endothelium, with a deficient SMC layer (Vikkula et al. 1996). Antibodies against E-selectin (expressed by proliferating endothelial cells) and against the universal proliferation marker Ki-67 have demonstrated that the endothelium in VMs is not proliferative (Kräling et al. 1996). Thus, it is likely that the observed deficiency of smooth muscle in the walls is a result of diminished recruitment of SMCs rather than an increase in the proliferation of ECs. This could be due to lack of differentiation, migration, and/or proliferation of SMCs or could be the result of increased apoptosis.

We used reverse genetics to unravel the molecular cause of this anomaly in two separate families with autosomal dominantly inherited VMs. By random mapping, we localized the region of the defective gene to chromosome 9 and subsequently pinpointed the causative mutation (Boon et al. 1994, Vikkula et al. 1996). The mutated gene encodes the receptor tyrosine kinase TIE-2. This receptor has been shown to be expressed specifically in endothelial cells (Dumont et al. 1995). Furthermore, homozygous Tie-2 knock-out mice die in utero because of defects in angiogenesis and remodeling (Dumont et al. 1994, Sato et al. 1995).

Although the normal function of the TIE-2 signaling pathway is unknown, two ligands, angiopoietin-1 (Ang-1) and -2 (Ang-2), expressed by mesenchymal cells that surround developing blood vessels, have been identified (Davis et al. 1996, Maisonpierre et al. 1997). Ang-1 binds to TIE-2 and increases receptor autophosphorylation. It does not appear to act as a growth factor that induces endothelial proliferation; yet it causes sprouting angiogenesis in vitro (Davis et al. 1996, Koblizek et al. 1998). The normal function of the TIE-2 pathway may be to regulate signaling between ECs and SMCs. This hypothesis is supported by the mutation we identified in our two families with inherited VMs. The arginine-to-tryptophan substitution in the intracellular kinase domain of TIE-2 caused a 6- to 10-fold increase in the phosphorylating activity of the receptor as measured in vitro (Vikkula et al. 1996). However, the characteristic histological finding in these VMs was a relative deficiency of SMCs. This could be explained by an uncoupling of the proliferation of ECs and concomitant recruitment of SMCs. This explanation is consistent with the defects seen in Ang-1 knock-out mice, that is, abnormal remodeling of the primary capillary plexus resulting in persistent immature vessels with large lumina (Suri et al. 1996). Studies of transgenic mice that overexpress Ang-2 have shown similar results (Maisonpierre et al. 1997). This TIE-2 ligand binds to the receptor without inducing receptor autophosphorylation, and, as it can compete for binding to TIE-2 with Ang-1, it may block the activating effects of Ang-1. In transgenic mice overexpressing Ang-2, similar defects, as with the loss of Ang-1, were seen (Maisonpierre et al. 1997).

Because the human molecular alteration associated with relative deficiency of SMCs is a mutation of the TIE-2 receptor expressed in ECs, perhaps there is another signaling molecule that carries information from ECs to SMCs or their precursors. This could be platelet-derived growth factor (PDGF)-BB or-AA, transforming growth factor-β (TGF-β), heparin-binding epidermal growth factor (HB-EGF), or another, perhaps unidentified, molecule. Experiments with murine knock-outs have provided insights into the in vivo function of some of these factors. Knock-out mice of the best candidate, PDGF-B, have shown that, although PDGF-B is crucial for the recruitment of microvascular pericytes, it does not seem to be as important in veins (Lindahl et al. 1997). TGF-β1$^{-/-}$ and TGF-βr2$^{-/-}$ mice exhibit defects in yolk sac vasculogenesis. These mice die in utero probably owing to inadequate differentiation of endothelial cells (Dickson et al. 1995, Oshima et al. 1996). However, the TGF-β ligand-receptor system still could be important for the development of intraembryonic vasculature at later stages. This is supported by EC-SMC coculture experiments showing that TGF-β is needed for SMC differentiation (Hirschi et al. 1998). Tissue factor knock-out mice have disorganized yolk sac vasculature (large vitelline vessels replaced by an irregular plexus) and lack of smooth muscle α-actin positive pericytes. Nevertheless, the importance of tissue factor for intraembryonic vascular morphogenesis remains unclear (Bugge et al. 1996, Carmeliet et al. 1996b, Toomey et al. 1996). Reduction of smooth muscle α-actin positive pericytes and SMCs occurs in LKLF$^{-/-}$ mice. These animals have a problem of vascular maturation in the aorta and veins in the late embryonic stage (Kuo et al. 1997). The LKLF transcription factor, expressed in ECs but not in SMCs, is therefore directly or indirectly linked to the recruitment of SMCs/pericytes. Although the histologic characteristics are strikingly similar to the observed phenotype in VMs with TIE-2 mutation, the expression levels of Tie-2, PDGF-B, HB-EGF, and TGF-β are normal in ECs of LKLF$^{-/-}$ mice, arguing against function via these pathways (Kuo et al. 1997).

The basic molecular defect in VMs is not necessarily caused by feed-back signaling via a growth factor from endothelium to smooth muscle. Production of extracellular matrix (ECM), by both ECs and SMCs, and attachment of cells to this ECM are important for vascular development and maintenance. Type VIII collagen is associated with SMC migration (Sibinga et al. 1997); fibronectin is essential for proper vascular development (George et al. 1993); and type XVIII collagen, via proteolytic cleavage, releases a potent inhibitor of angiogenesis, endostatin (O'Reilly et al. 1997). Mutations in blood vessel matrix components have been identified in several vascular disorders, such as Marfan syndrome, familial aneurysms, and Ehlers-Danlos syndrome (Superti-Furga et al. 1988, Kontusaari et al. 1990, Collod-Béroud et al. 1997). Moreover, attachment to ECM via $\alpha_v\beta_3$ integrin is needed for EC survival (Brooks et al. 1994). As endothelium and smooth muscle produce vascular ECM, growth factor signaling pathways are likely to be involved in controlling the composition of ECM. For example, TGF-β has been shown to stimulate matrix deposition in intimal hyperplasia (Wolf et al. 1994). Tie-2$^{-/-}$ mice, as well as the Ang-1$^{-/-}$ mice and Ang-2 overexpressing mice, exhibit detachment between endocardium and myocardium (Dumont et al. 1994, Suri et al. 1996, Maisonpierre et al. 1997). Thus, it is possible that normal function of the TIE-2 pathway is related to ECM production and/or EC-ECM attachment.

There are other possible mechanisms involved in the formation of VMs, as these anomalies are multifocal and not all vessels are affected. Are there environmental factors such as infection, microtrauma, or hormonal influences, or perhaps a somatic mutation, a "second hit"? It is possible that other genes can influence the expression of a vascular malformation in heterozygous carriers of a "predisposing" mutation. Such a genetic background effect has been shown in some knock-out mice. Crossing the $TF^{-/-}$ genotype from 129/Sv background into C57BL/6 line shifted the timepoint of death from midgestation to birth, although hemorrhage was still the cause of death (Toomey et al. 1997). In addition, a major modifier of embryonic lethality in TGF-$\beta1^{-/-}$ mice has been mapped to mouse chromosome 5 (Bonyadi et al. 1997). In a similar way, there could be a protein that could modulate the effects of the mutated TIE-2 receptor in developing blood vessels. The existence of such a protein is supported by the fact that penetrance of the identified TIE-2 mutation was 96%, not 100%, in the two families with the disorder (Vikkula et al. 1996). Moreover, there is genetic heterogeneity. We have analyzed families that are not linked to 9p; another gene(s) causing dominantly inherited VMs must therefore exist (L. M. Boon, J. B. Mulliken, B. R. Olsen, and M. Vikkula unpublished). Some VMs are characterized by a high number of glomus cells. These glomangiomas are sometimes inherited as an autosomal dominant trait (Rudolph 1993). The identification of this gene(s) may help us understand the cascade of signaling events involved in the etiopathogenesis of VMs and may clarify the reasons for the localized occurrence of lesions in carriers.

It would also be interesting to determine whether sporadic VMs are caused by the same TIE-2 mutation, as in the familial cases, or whether other TIE-2 mutations can have the same functional effects. In vivo studies are also needed. Given that homozygous Tie-2 knock-out mice die in utero, we are trying to construct a transgenic mouse expressing the human mutation. With this model, we could test pharmacological agents that block the effect of the Arg-to-Trp substitution in the TIE-2 receptor.

Signaling between ECs and SMCs may not be the same in all veins. As aforementioned, only veins in mucocutaneous tissues are affected in the individuals carrying the TIE-2 mutation Thus, it may be that this signaling pathway is different from, or less important, in other vessels. This belief is supported by the fact that there are families with VMs that occur predominantly in the central nervous system (OMIM #116860; familial "cavernous angioma"). Linkage studies of these families have determined a locus on the long arm of chromosome 7, not 9p (Dubovsky et al. 1995). The causative gene in the 7q-linked disorder has not yet been identified; thus nothing is known about how its function relates to EC-SMC signaling. There are also families with central nervous system VMs that do not link to 7q (Gunel et al. 1996). In summary, venous dysmorphogenesis is not caused by mutations in a single gene, but rather by mutations in a set of genes, the functions of which are probably related, yet could be quite different.

Capillary Malformation

Perhaps the most common cutaneous vascular anomaly in the general population involves capillary or venular-sized malformed vessels in the dermis. These lesions, often called port-wine stains (OMIM #163000) are reddish and macular and occur in 0.3% of newborns (Jacobs and Walton 1976). Unfortunately, capillary malformation (CM) is often confused with the very common fading macular stain of neonates, known in the literature as "stork bite," "salmon patch," or "angel's kiss." These harmless vascular birthmarks occur in 30% to 40% of newborns, usually in the nuchal region, eyelids, glabella, and lips, in order of frequency (Mulliken and Young 1988). These stains typically disappear within 1 year without leaving a trace. They are not considered dermatopathologic lesions; their etiology is unknown.

CM (port-wine stain) does not disappear. It gradually darkens from pink to purple, and some facial lesions exhibit soft tissue hypertrophy (see Figure 3 for a typical CM). Skeletal overgrowth in the region of the CM can also occur, especially in the maxillary region.

What little we know about CMs comes from histopathology. Hematoxylin-eosin staining shows that the anomaly consists of an increased number of abnormal ectatic capillary-like channels within the papillary dermis. The walls of these channels are thin, and the lining endothelial cells are flat (Barsky et al. 1980). It has been postulated that CM involves progressive dilatation of the cutaneous vascular plexus (Barsky et al. 1980). Using immunohistochemistry, Finley et al. (1982) did not detect differences in distribution of factor VIII, fibronectin, and type IV collagen. Smoller and Rosen (1986) showed that the density of pericapillary neurons is abnormal. They postulate that the diminished neuronal component may alter neural modulation of vascular tone and be responsible for the observed progressive ectasia.

Sturge-Weber syndrome (OMIM #185300) is composed of a CM located in the ophthalmic (V1) trigeminal neurotome in the face (sometimes V2 as well) and in the ocular choroid and ipsilateral leptomeninges. Enjolras et al. (1985) have hypothesized that the syndrome results from dysmorphogenesis (involving neural crest migration) of the cephalic neuroectoderm. Whether the etiopathogenesis of this syndrome is the same as for common CM is not known. Sturge-Weber syndrome is not believed to be hereditary. However, Unna nevus (a persistent nuchal and forehead CM; OMIM #163100) is inherited as an autosomal dominant trait with variable penetrance (Merlob and Reisner 1985, Zumkeller 1957).

The finding of an increased number of dilated small capillaries in this anomaly suggests that increasing knowledge of the triggers could provide clinicians with new peptides for induction and inhibition of angiogenesis. Basic studies of the beneficial effects of such factors, for example, VEGF, have moved from experimental treatment of induced ischemic animal models to clinical trials of peripheral arterial disease (Takeshita et al. 1994, Isner et al. 1996).

Arteriovenous Malformation

Arteriovenous malformation (AVM) is the most devastating and difficult to treat anomaly of vascular dysmorphogenesis. Sometimes these fast-flow vascular lesions present with congestive heart failure at birth. More commonly, an AVM remains quiescent until childhood or early adulthood when it begins to expand (see Figure 4 for typical

Figure 3. (A) A 20-year-old girl with a capillary malformation (CM) in the right V1-V2 distribution of the trigeminal nerve. The CM is red and flat. **(B)** CM from the lower lip of a 36-year-old female is composed of dilated capillaries (*arrows*) next to a large sebaceous gland (hematoxylin-eosin-safranine × 10).

AVM). Embolization, whenever possible followed by radical surgical resection, can "cure" the patient, but often this combined approach cannot be accomplished without mutilation or loss of critical tissue, for example, in AVMs of the brain, face, or extremities (Burrows and Fellows 1995, Kohout et al. 1998).

Morphologically, an AVM comprises microscopic connections from arteries to veins. The epicenter, called the nidus, is supplied by multiple feeding arteries. Most AVMs are sporadic; however, they are also seen in patients with hereditary hemorrhagic telangiectasia (HHT or Rendu-Osler-Weber syndrome, ROW; OMIM #187300). Two separate genes, identified as causing HHT, are as-

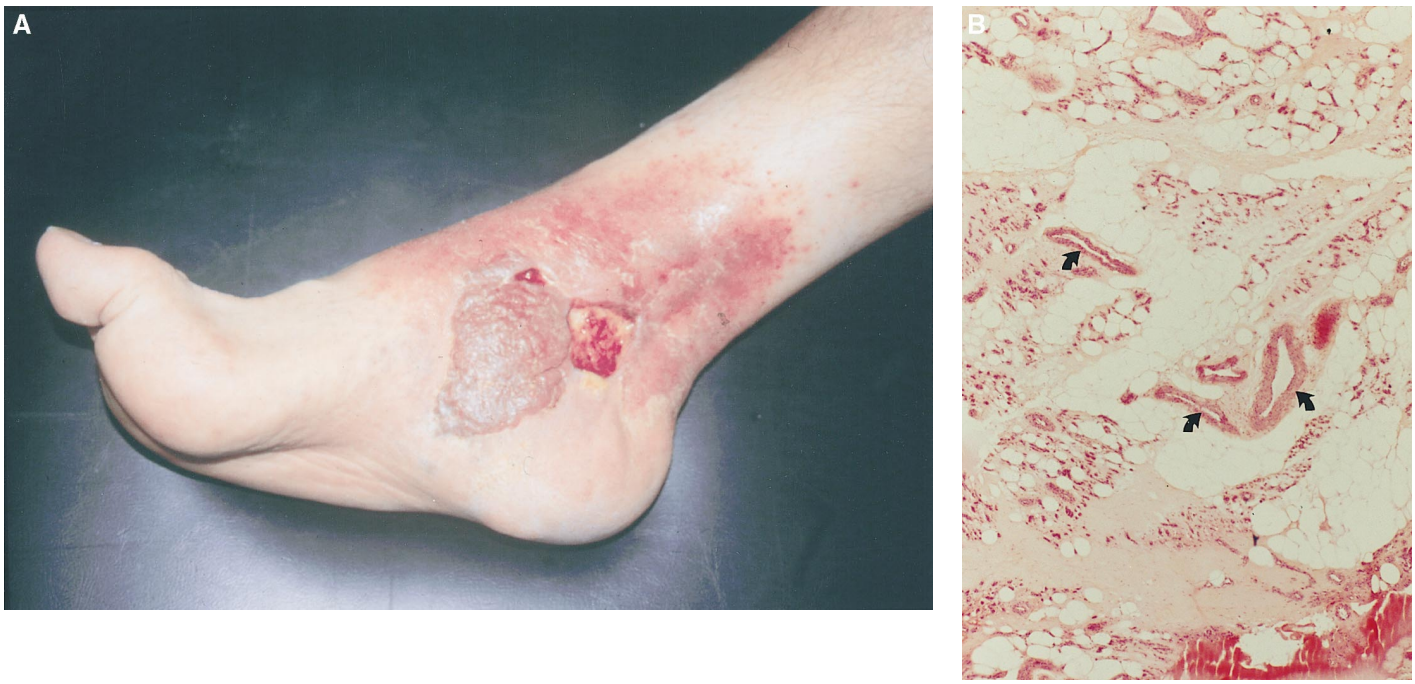

Figure 4. (A) A 50-year-old man with a diffuse arteriovenous malformation (AVM) and painful ulceration in the medial side of the right ankle. In addition to warm reddish blush, there is a prominent bruit and thrill. **(B)** AVM (from the forehead of a 24-year-old female) with multiple sclerotic veins (*arrows*) (hematoxylin-eosin × 2.5).

sociated with clinically distinct phenotypes (McAllister et al. 1994a, Shovlin et al. 1994, Vincent et al. 1995). HHT1, a locus in 9q34.1, is caused by mutations in endoglin, a TGF-β binding protein; these patients have a high frequency of pulmonary AVMs (McAllister et al. 1994b). In contrast, HHT2, located in 12q11–q14 (OMIM #600376), is caused by mutations in an activin receptor-like kinase, also a TGF-β binding protein. Patients with HHT2 rarely have pulmonary AVMs (Vincent et al. 1995, Johnson et al. 1996). It seems that the TGF-β signaling is important for the proper maintenance of capillaries. When this signaling pathway is altered, the capillary bed dilates, allowing direct arteriovenous flow and the formation of AVM. It is not known why mutations in activin receptor-like kinase produce fewer AVMs. The cutaneous telangiectasias seem to be similar to those caused by the endoglin mutations. Because the HHT1 mutations identified to date result in synthesis of shortened forms of endoglin or endoglin with amino acid substitutions, it is assumed that they cause either a dominant-negative effect or that they produce a non–membrane-bound, soluble form of endoglin that is still capable of binding TGF-β. In either instance, there would be failure of normal TGF-β effect on endothelial cells. This suggests that some AVMs might respond positively to (locally) administered TGF-β.

There is a report excluding both the HHT1 and HHT2 loci as the cause for HHT in one Italian family, evidence for locus heterogeneity (Piantanida et al. 1996). Other proteins are involved in the maintenance of normal capillary structure. The identification of this third gene and the construction of transgenic mouse models for HHT1 and HHT2 will advance our knowledge about the molecular events that cause destruction of the capillary network.

Lymphatic Malformations

Lymphatic-derived channels can also comprise abnormalities that are similar to those that occur in capillaries and veins. Lymphatic malformation (LM), often inaccurately called lymphangioma, is a defect of cutaneous and subcutaneous lymphatic vessels. An LM is composed of clusters of dilated lymphatic channels (vesicles) with both thick and thin muscular layers (smooth and skeletal), filled with clear proteinaceous fluid, and not connected to normal lymphatic vessels (Whimster 1976) (see Figure 5 for a typical LM). LMs may be sequestered segments of the primitive lymphatic system (Mulliken and Young 1988).

For reasons unknown, these lesions occur mostly in the cervicofacial area where they are commonly microcystic. In comparison, the more rare truncal lesions are usually macrocystic. LMs can also occur in solid organs, in skeletal tissue, and in multiple organ systems. Treatment is often limited to sequential surgical resection. There is also a role for sclerotherapy, using ethanol, ethibloc, and/or OK-43, particularly for macrocystic LM (Ogita et al. 1994).

There are few molecular clues for these lymphatic anomalies. To our knowledge, no inherited cases have been reported. Perhaps dominant mutations, even in heterozygotes, are lethal. In fact, lethal midline posterior cervical LM occurs with trisomy 13, and 18/21 (Greenberg et al. 1983, Garden et al. 1986). All sporadic cases of LM could be caused by de novo dominant mutations. Proteins involved in lymphangiogenesis, VEGF-C, VEGF-D, and their receptor VEGFR-3 (or FLT-4) are likely candidates (Kukk et al. 1996, Achen et al. 1998). VEGFR-3 expression is limited to lymphatic endothelium in adult mice (Kaipainen et al. 1995), and overexpression of VEGF-C

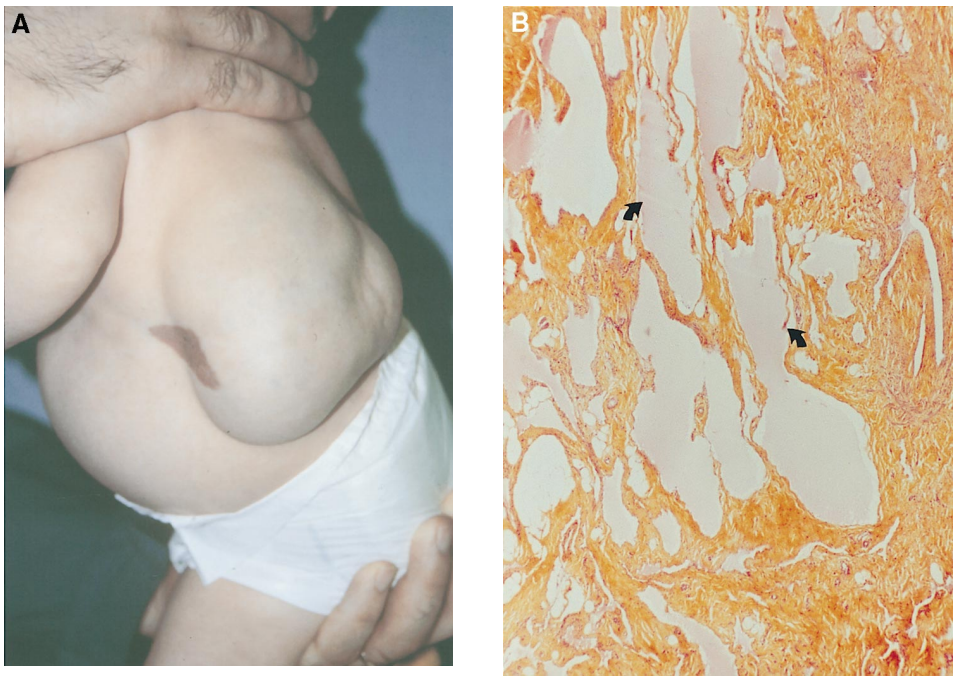

Figure 5. (A) A 2-year-old girl with a lymphatic malformation (LM) on left posterior thorax that easily transilluminates. A small reddish patch (CM), often associated with LM, is also seen. **(B)** LM, excised from the arm of a 15-year-old female, demonstrates large, irregular channels (*arrows*) with thin walls and intraluminal proteinaceous material (hematoxylin-eosin-safranine × 2.5).

in the skin of transgenic mice causes hyperplasia of adjacent lymphatic vessels (Jeltsch et al. 1997). Moreover, heterozygous loss of one allele of the related growth factor, VEGF, is lethal in mice (Carmeliet et al. 1996a). These findings support the concept of lethal germline mutations for these factors. Given that VEGF has been shown to be crucial for vasculogenesis, it is possible that VEGFR-3, with its ligands VEGF-C and VEGF-D, serves a similar function in lymphatic vessels.

Combined Malformations

A complex vascular malformation manifests clinical and histologic characteristics of several of the simple forms, comprising any combination of arterial, capillary, lymphatic, and venous components (see Figure 6 for a combined lymphatico-venous malformation, LVM). These combined lesions seem to be sporadic. They are more often located on the extremities than the trunk, and often only one side is affected, although bilateral involvement can occur. These observations suggest mosaicism for a de novo somatic mutation, perhaps affecting more than one gene.

The combined vascular malformations are often associated with osseous and/or cartilaginous overgrowth, although occasionally the affected extremity is smaller. Klippel-Trenaunay syndrome (KTS, OMIM #149000) is defined as an extensive combined malformation comprised of capillary, lymphatic, and/or venous malformations (CLVM) associated with overgrowth of the affected extremity (Klippel and Trenaunay 1900). There are several reports of sporadic cases of KTS, but only a few with familial clustering. Lindenauer (1965) described a brother and sister with an identical phenotype, and Aelvoet et al. (1992) reported on two families both having two individuals with KTS. In both families, the phenotype jumped over a generation, which argues for reduced penetrance. This is supported by the notion that in four families, the authors found increased occurrence of port-wine stains (CMs) in the relatives of KTS patients. CMs could, at least in these families, be caused by the same gene as the fully expressed KTS phenotype. Support for this concept comes from Ceballos Quintal and coworkers (1996), who reported a family in which the mother of a girl affected with KTS had a large CM on the back and "severe varicosities" without "clinical or radiological signs of limb hypertrophy." Because the maternal grandmother also developed severe varicosities in both legs at an early age, the authors suggested that KTS is inherited as an autosomal dominant trait with variable expressivity in their family (Ceballos Quintal et al. 1996).

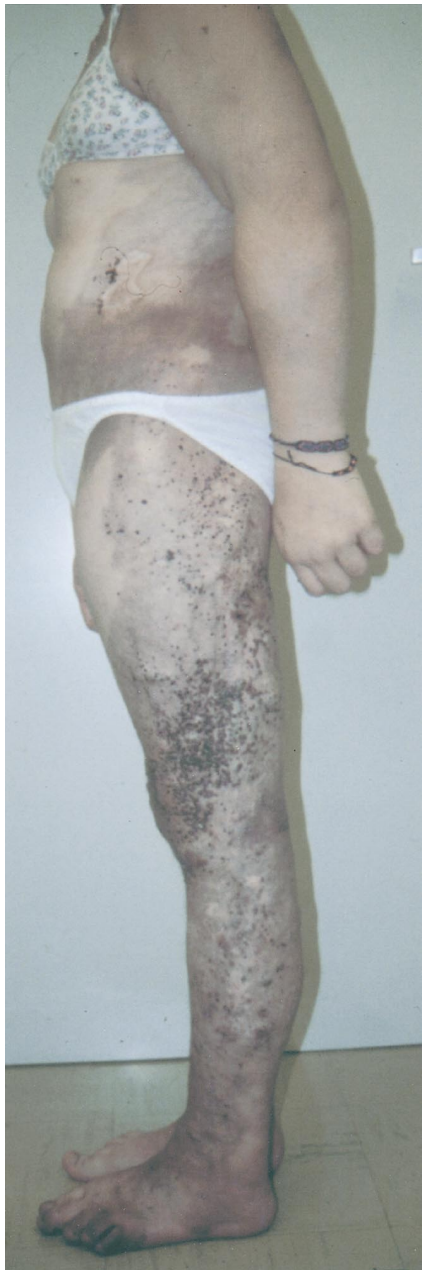

Figure 6. A 17-year-old girl with extensive combined capillary-lymphatico-venousmalformation (CLVM) of left lower extremity with overgrowth in girth and length (Klippel-Trenaunay syndrome).

In rare instances, a dominant genetic alteration has been associated with KTS. A chromosomal abnormality has been reported, that is, translocation between chromosomes 5 and 11 (Whelan et al. 1995). Much work must be done to decipher the molecular mechanisms leading to this complex phenotype, perhaps beginning with whether chromosomes 5 or 11 contain the causative gene(s).

Proteus syndrome is another multisystem disorder with a vascular component (OMIM #176920), characterized by macrocephaly, gigantism of hands and feet, hemihypertrophy of the body, lipomas, and capillary-venous malformations. Goodship et al. (1991) and Krüger et al. (1993) provide evidence of autosomal dominant inheritance with variable expression of the phenotype. The hypothesis of somatic mosaicism also has been proposed for this disorder (Cohen 1993). The majority of patients with Proteus are sporadic, and studies are just beginning to focus on the underlying mechanisms leading to this disorder. A similar complex vascular anomaly/overgrowth phenotype is Maffucci syndrome (OMIM #166000). This rare condition presents as raised cartilaginous overgrowths (enchondromas) in association with vascular malformations of the extremities resembling VMs (Maffucci 1881). It is tempting to suggest that Maffucci syndrome is a contiguous gene syndrome, involving genes important for venous morphogenesis and cartilaginous growth. Patients with Maffucci have a high frequency of malignant transformation, up to 20% to 30%. Could this be explained by loss of a tumor suppressor gene located in the vicinity of the gene(s) responsible for the VMs and enchondromas? Because the first identified gene causing VMs, TIE-2, is located on human chromosome 9 in a region containing the tumor suppressor genes P15 and P16, it is tempting to speculate whether some cases of Maffucci syndrome might be caused by mutations in this region.

• Future

Elucidation of the molecular basis of vascular anomalies provides insights into the cause of disorders and into the processes that regulate normal vascular development (Table 1). This research may lead to new approaches to control

Table 1. Histologic and molecular characteristics of vascular anomalies

Vascular anomaly	*Histologic characteristics*	*Molecular basis*
Hemangioma	Lobular architecture, plump, proliferating endothelium thickened basement membrane (see Figure 1B)	Upregulation of FGF and/or VEGF ? Downregulation of IFN ?
Cutaneo-mucosal venous malformation (VM)	Ectatic and serpiginous channels, flat endothelium, scattered smooth muscle cells (see Figure 2B)	TIE-2, Chr. 9p
Cerebral VM	See VM	Chr. 7q (gene unknown)
Capillary malformation (CM)	Dilated thin-walled capillaries (see Figure 3B), ↓ perivascular neural density	?
Arteriovenous malformation (AVM)	Dysmorphic arteries and sclerotic veins (see Figure 4B)	Endoglin ?, activin receptor-like kinase 1 ?
Ataxia-telangiectasia (AT)	See HHT1	ATM, Chr. 11q (phosphatidylinositol-3-like kinase)
Hereditary Hemorrhagic Telangiectasia (HHT1)	Dilated arterioles directly connecting with dilated venules, absent elastic fibers in arterioles & arteriolar-precapillary sphincters	Endoglin, Chr. 9q
HHT2	See HHT1	Activin receptor-like kinase 1, Chr. 12q
HHT3	See HHT1	Another TGF-β binding protein ?
Lymphatic malformation (LM)	Ectatic lymphatic channels lined by flat, nonproliferative endothelium (see Figure 5B)	VEGFR3 ?, VEGF-C ?, VEGF-D ?

vasculogenesis and angiogenesis in other diseases as well.

In addition to what has been learned from reverse genetic studies of human vascular malformations, important work is going on in other species, such as mouse and zebrafish. The zebrafish is a particularly exciting model for study. Large numbers of fish can easily be maintained in relatively small tanks, and their transparency makes it fairly easy to study the structure of their vasculature in vivo. Blood flow dynamics can be observed without invasive measurements, and screening for individuals with vascular phenotypes is easy (Stainier et al. 1996). A 2,5-cM genomic map of the zebrafish is already available (Knapik et al. 1996), thus it is also possible to do linkage mapping for causative genes. As large numbers of informative meioses can easily be obtained, it is possible to do refined mapping of a locus in a relatively short time. Thus it is feasible to identify the defective gene by positional cloning.

The differences in zebrafish physiology limit their usefulness to the study of human disorders. Nevertheless, this model system will undoubtedly provide detailed knowledge about the development, organization, and maturation of blood vessels. This knowledge, combined with understanding from murine models, will facilitate our understanding of human vascular disorders. Already, a number of genes that are expressed in blood vessel walls during development and/or in mature vessels have been mutated in mice. Many of these mutations, for example, knock-outs of PDGF-B, Tie-1 and Tie-2, result in homozygous lethal phenotypes with heterozygotes being unaffected, whereas others (VEGF) cause a lethal phenotype even in heterozygotes (Carmeliet et al. 1996a, Dumont et al. 1994, Leveen et al. 1994, Sato et al. 1995). Given that several human disorders are now recognized to be caused by mutations leading to dominant-negative or gain of function effects, it is also important to create transgenic mice that carry the murine equivalents. Such mice would serve as models of the human disorders and could be used for detailed studies of the pathophysiological processes that cause the phenotype. The murine models would also serve as valuable models for clinical testing of new therapies.

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PII S1050-1738(98)00024-3 **TCM**

Vascular Endothelial Growth Factor-C: A Growth Factor for Lymphatic and Blood Vascular Endothelial Cells

Berndt Enholm, Lotta Jussila, Marika Karkkainen, and Kari Alitalo*

The endothelial cells lining all vessels of the circulatory system have been recognized as key players in a variety of physiological and pathological settings. They act as regulators of vascular tone via the inducible nitric oxide system and in angiogenesis, the formation of blood vessels de novo. Aberrant regulation of endothelial cells contributes to tumor formation, atherosclerosis, and diseases such as psoriasis and rheumatoid arthritis. Among the most recently discovered growth factors for endothelial cells are newly isolated members of the platelet-derived growth factor/vascular endothelial growth factor (VEGF) family, VEGF-B, VEGF-C, and VEGF-D. VEGF-C is the ligand for the receptor tyrosine kinase VEGFR-3 (also known as Flt4), which is expressed predominantly in lymphatic endothelium of adult tissues, but a proteolytically processed form of VEGF-C can also activate VEGFR-2 of blood vessels. The lymphatic vessels have been known since the 17th century, but their specific roles in health and disease are still poorly understood. With the discovery of VEGF-C and its cognate receptor VEGFR-3, the regulation and functions of this important component of the circulatory system can be investigated. (Trends Cardiovasc Med 1998;8:292–297) © *1998, Elsevier Science Inc.*

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• Isolation and Cloning of Vascular Endothelial Growth Factor-C

Vascular endothelial growth factor-C (VEGF-C) was discovered as a factor in conditioned medium of PC-3 prostatic adenocarcinoma cells that stimulated tyrosine phosphorylation of VEGFR-3, a receptor tyrosine kinase closely related to the VEGF receptors VEGFR-1 and VEGFR-2 [previously named flt-1 and KDR/Flk-1, respectively (Ferrara and Davis-Smyth 1997, Joukov et al. 1996)] (Figure 1). Receptor-affinity chromatography using the VEGFR-3 extracellular domain led to the purification of VEGF-C. Partial amino acid sequence was obtained from the purified factor, and the cDNA encoding it was cloned from a library prepared from the PC-3 cells (Joukov et al. 1996). Independently, a sequence was identified in the expressed sequence tag (EST) database as being homologous with VEGF. Using the EST clone as probe, a full-length VEGF-C cDNA was isolated. The protein encoded by this cDNA was designated VEGF-related protein (Lee et al. 1996).

• Structure and Regulation of the Vascular Endothelial Growth Factor-C Gene

The human and mouse genes for VEGF-C both comprise more than 40 kb of genomic DNA and consist of seven exons, all of which contain coding sequences (Chilov et al. 1997). The VEGF-C gene was localized to human chromosome 4q34 and to the mouse chromosome 8 (Chilov et al. 1997, Paavonen et al. 1996). The VEGF homology domain of VEGF-C is encoded by exons 3 and 4. Exons 5 and 7 encode cysteine-rich motifs of the type C6XC10XCRC, and exon 6 encodes C10XCXCXC motifs typical of a silk protein (Chilov et al. 1997). The upstream sequences show promoter activity in reporter gene assays and contain conserved putative binding sites for Sp-1, AP-2, and NF-κB transcription factors, but no TATA box. The VEGF-C gene structure is thus assembled from exons encoding N- and C-terminal propeptides and distinct cysteine-rich domains, in addition to the VEGF homology domain.

VEGF-C mRNA was detected by Northern blotting from many embryonic and adult tissues. In adult humans,