Vascular Growth Factors and Lymphangiogenesis

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Jussila, Lotta, and Kari Alitalo. Vascular Growth Factors and Lymphangiogenesis. Physiol Rev 82: 673–700, 2002; 10.1152/physrev.00005.2002.—Blood and lymphatic vessels develop in a parallel, but independent manner, and together form the circulatory system allowing the passage of fluid and delivering molecules within the body. Although the lymphatic vessels were discovered already 300 years ago, at the same time as the blood circulation was described, the lymphatic system has remained relatively neglected until recently. This is in part due to the difficulties in recognizing these vessels in tissues because of a lack of specific markers. Over the past few years, several molecules expressed specifically in the lymphatic endothelial cells have been characterized, and knowledge about the lymphatic system has started to accumulate again. The vascular endothelial growth factor (VEGF) family of growth factors and receptors is involved in the development and growth of the vascular endothelial system. Two of its family members, VEGF-C and VEGF-D, regulate the lymphatic endothelial cells via their receptor VEGFR-3. With the aid of these molecules, lymphatic endothelial cells can be isolated and cultured, allowing detailed studies of the molecular properties of these cells. Also the role of the lymphatic endothelium in immune responses and certain pathological conditions can be studied in more detail, as the blood and lymphatic vessels seem to be involved in many diseases in a coordinated manner. Discoveries made so far will be helpful in the diagnosis of certain vascular tumors, in the design of specific treatments for lymphedema, and in the prevention of metastatic tumor spread via the lymphatic system.
I. INTRODUCTION TO ANGIOGENESIS AND LYMPHANGIOGENESIS

Embryonic vascular development involves a complex series of events during which the endothelial cells differentiate, proliferate, migrate, and undergo maturation into an organized network of vessels (176, 177). The first step in the development of the blood vessels is called vasculogenesis, which is the process where endothelial cells are generated from their mesenchymal precursors and spontaneously assemble into tubules that fuse to form the primary vascular plexus of the embryo. Remodeling and expansion of these primary vessels into arteries, veins, and capillaries of different sizes is called angiogenesis. Although, by definition, vasculogenesis precedes angiogenesis, in practice the two processes continue in parallel during early development. Tissues that are vascularized by vasculogenesis are generally of endodermal origin (lung, pancreas, spleen, heart, and large blood vessels), while tissues of ectodermal and mesodermal derivation (such as the kidney and the brain) are vascularized primarily via angiogenesis.

The oxygen and nutrients supplied by the vascular system are crucial for cell function and survival. In fact, the cardiovascular system is the first organ system to develop in embryos, supplying oxygen and nutrients to the growing tissues. During organogenesis, the proximity of growing cells to the circulation is ensured by the coordinated growth of blood vessels and organ parenchyma. At the same time that the blood vessels form, the precardiac myoblasts develop by differentiating from the mesothelial cells and form the heart (179). On day 9 of mouse embryonic development (E9), the heart starts to beat and blood starts to circulate through the newly formed network of vessels (147). In the yolk sac blood islands, mesenchymal cells give rise to both endothelial and hematopoietic cells (37). These cells organize into clusters consisting of future endothelial cells in the outer layer surrounding hematopoietic cells. The endothelial cells then coalesce with those of the neighboring blood islands to form a primitive honeycomb-like blood vessel network, and the hematopoietic cells differentiate into erythrocytes. Later, hematopoiesis resumes in the embryo in the para-aortic region (AGM region) and in the fetal liver and finally in the bone marrow.

A complex orchestration of molecular regulators is needed for the blood vessels to grow. The vasculature begins as a plexus of primitive capillary tubes that are subsequently modified to generate the more complex vascular network of adults. Sprouting of new vessels from preexisting ones is the most frequent mechanism of angiogenesis in embryos, and it involves several sequential steps (223). First, the extracellular matrix components surrounding the endothelial cells are degraded locally by proteases produced by the endothelial cells. This allows the chemotactic migration of endothelial cells toward angiogenic stimuli. Subsequently, the endothelial cells in the midsection of the new vessels proliferate and form a lumen, and adjacent sprouts anastomose and form loops, which become perfused with circulating blood. The loops between the vessels can also form by another mechanism called intussusceptive growth, a form of angiogenesis involving the in situ remodeling of the vessels by protruding interstitial tissue columns. In this process, a large sinusoidal capillary can be divided into smaller capillaries, which then grow separately (176).

Although endothelial cells initiate angiogenesis, they cannot complete the process. Newly formed capillary sprouts are fragile and remain susceptible to remodeling as long as they lack appropriate perivascular structures. The maturation of new blood vessels into stable and functional vessels requires the accumulation of a basal lamina and recruitment of pericytes and smooth muscle cells to cover tightly the abluminal side of the vessel (Fig. 1) (93). The smooth muscle cells provide structural support to the larger vessels protecting the vessels against rupture and are important regulators of blood flow and pressure by their contractile abilities (22). Pericytes are absent or only loosely attached to vessels undergoing angiogenesis, suggesting that the mural cells stabilize nascent vessels by inhibiting endothelial cell proliferation and migration and by stimulating extracellular matrix production (19). Under certain conditions the pericytes support endothelial cell survival, for example, in the neonatal retina during hyperoxia-induced regression of the retinal vessels, where associated pericytes spare some of the vascular branches (19).

Smooth muscle cells are separated from the endothelial cells by a basement membrane and thus are not in direct contact with the endothelium. In contrast, the pericytes share their basement membrane with the endothelial cells and make direct contact with them through holes in the basement membrane. In sites where selective filtration is required, like in the kidney glomeruli and lung parenchyma, pericytes are selectively positioned to allow the fluid or gas exchange. Pericytes and smooth muscle cells may be derived from the mesenchyme variously by in situ differentiation, trans-differentiation from endothelial cells, from bone-marrow precursors or macrophages, soon after the endothelial cells have formed tubes. Like the endothelial cells, they proliferate and migrate in parallel to the growth of the vascular plexus (19, 37).

The vascular system is a highly heterogeneous and nonuniform organ system. It consists of an arterial and a venous system that differ structurally and functionally. It has been proposed that establishment of the identities of the arterial and venous vasculatures is under the control of related but distinct genetic programs (75, 218, 230).
Endothelial cells differ considerably in the arterial, capillary, and venous compartments, and there is further heterogeneity in the different organs (47). Recent molecular probing of the endothelial cell surface by phage display library panning in vivo has revealed striking molecular specificity for the availability of molecular determinants in different vascular endothelia (182). Endothelial cells in different vessels have distinct characteristics, such as fenestrations, cell junctions, enzymes, and carrier systems. For example, fenestrated endothelia are seen in places where extensive molecular exchange occurs across the blood vessel wall, such as in endocrine glands, choroid plexus, and kidney. Differentiation of endothelial cells is dependent on interactions with local parenchymal cells in the target tissues. Although it is not always known which cell type induces the organotypic differentiation of endothelial cells, the existence of such cell-cell interactions seems to be widely accepted.

A. Physiological and Pathological Angiogenesis

Angiogenesis is also required for the maintenance of the functional and structural integrity of tissues during postnatal life. Vasculogenesis is mainly restricted to early development, while new vessels in adults appear to be formed by angiogenesis (37). However, adults are apparently able to mobilize bone marrow-derived endothelial precursor cells for angiogenesis (174). In healthy adults, the endothelial cell turnover is usually very low, and the vascular endothelia are maintained in quiescence by a balance of positive and negative regulators of angiogenesis. Angiogenesis is limited to sites where the metabolic demands of the tissue are such that new blood vessels are needed. In wound healing, fracture repair, inflammation, folliculogenesis, and ovulation during the menstrual cycle, as well as in situations of ischemia, the positive regulators predominate, leading to the activation of an-
Angiogenic mechanisms (67). Cells suffering from hypoxia start to release angiogenic factors to establish better contact with the circulating blood. Metabolic stimuli, including hypoglycemia and low pH, also participate in the induction of vessel growth, but these mechanisms are less well known. In contrast to developmental angiogenesis, angiogenesis in adults originates mostly in mature blood vessels. In embryos, endothelial cells are loosely connected and actively growing, whereas in adults they are quiescent and encapsulated by a thick mural coat. Therefore, the blood vessels must first become destabilized to allow new growth. A carefully orchestrated activation of several signaling molecules is needed, which may differ in different tissues (37). In contrast to angiogenesis in embryos, there is often inflammation associated with adult angiogenesis, attracting monocytes/macrophages, platelets, mast cells, and other leukocytes.

Angiogenesis results in a higher capillary density, but also the larger vessels are modified by the lack of an adequate oxygen supply. In the case of acute or chronic occlusion of a major artery (coronary, femoral artery), preexisting arteriolar connections can be recruited by arteriogenic mechanisms to bypass the site of occlusion (37). Arteriogenesis produces rapid circumferential growth in the preexisting collateral vessels, which are less perfused with blood under normal flow conditions. These vessels have the ability to dramatically increase their lumen by proliferation of endothelial and smooth muscle cells (33). Recent findings suggest that endothelial precursors in the adult bone marrow also contribute to the expansion of preexisting collaterals (40). As a result of the increased collateral flow, endothelial cells recruit monocytes, which are capable of remodeling the media of the vessel wall. Activated endothelial cells then induce the regrowth of smooth muscle cells in the vessel wall. The smooth muscle cells synthesize a new elastic lamina to the enlarged collaterals. Once the mural cells have been recruited into the vessels, they further muscularize the nascent vasculature by sprouting or by migrating longitudinally along preexisting vessels. After a couple of months the new collateral artery is almost indistinguishable from a normal artery (37). Arteriogenesis differs from angiogenesis in several aspects, but the two processes also share certain mechanisms. For example, angiogenic factors, like fibroblast growth factor (FGF), can activate both mechanisms, but angiogenesis is induced by hypoxia whereas main driving force in arteriogenesis is inflammation (33). Arteriogenesis can counteract the damage in ischemic tissues, such as heart, brain, or limbs, and growth factors that are involved in the arteriogenesis hold great promise for treatment of patients with ischemic diseases, especially those that are poor candidates for mechanical revascularization or bypass surgery.

One of the most extensively studied forms of pathological angiogenesis is tumor angiogenesis (68). Like normal cells, tumor cells need to be located at a close distance from the blood vessels serving the metabolic demands of the growing tumor. The stage in tumor development when a solid tumor grows beyond a few millimeters in diameter and starts to generate its own microcirculation is called the angiogenic switch (70). It means the transition of an avascular tumor to a tumor with its own blood supply. At this stage the endothelial cells transit from a quiescent into an angiogenic state; the positive regulators are induced. Negative regulators can also decrease; for example, thrombospondin levels decrease in tumors upon loss of the p53 tumor suppressor gene (28, 91).

Tumor blood vessels are leaky and immature, at least partly because the pericytes and smooth muscle cells are usually poorly recruited to the tumors. The vessels are multilayered, protrude extensions bridging and splitting vessels, contain intercellular and transcellular holes, show relatively uncontrolled permeability, and undergo constant remodeling (68). These vessels resemble angiogenic vessels in other settings, such as in wound healing, with the exception that tumor vessels do not mature properly. Tumor angiogenesis is said to resemble a physiological response that is initiated but not terminated and is considerably more chaotic (53). Occasionally some of the endothelial cells in the tumor vessels are replaced by tumor cells, forming so-called mosaic blood vessels (44). Angiogenesis also takes place in other pathological conditions such as proliferative retinopathy, rheumatoid arthritis, psoriasis, and juvenile hemangioma (67).

B. Lymphangiogenesis

Lymphatic vessels are also part of the vascular circulatory system. The lymphatic system is made up of an extensive network of capillaries, collecting vessels, and ducts that permeate most of the organs (183). Unlike the blood vasculature which forms a continuous loop, the lymphatic system is an open ended, one-way transit system. These vessels collect the extravasated protein-rich fluid and lymphocytes from the tissues and transport them back into the circulation. From the lymphatic capillaries, the fluid is transferred to the collecting lymphatic vessels and ultimately into the venous circulation via the thoracic duct. Larger lymphatic vessels are surrounded by a muscular layer that contracts automatically when the vessel becomes stretched with fluid. In addition, external factors such as skeletal muscle contractions or arterial pulsation compresses the vessels and increases the efficiency of fluid transport.

In tissue sections, lymphatics resemble blood vessels but are generally thinner walled and more irregular. Lymphatics have a low intraluminal pressure and contain a bloodless fluid called lymph, which consists of interstitial
tissue fluid, macromolecules, and cells (227). The lymphatic vessels were discovered in 1627 by Gasparo Asellius (11), at about the same time that the blood circulation was described by William Harvey (89). However, compared with blood vascular studies, the lymphatic system has remained relatively neglected until the present day. This has been partly due to the difficulties in recognizing these vessels in tissues, due to a lack of specific markers. The only way of differentiating the lymphatic vessels has been based on morphology in histological samples and infusions of colored dyes, contrast agents, or macromolecules into the tissues. However, within the past few years molecules expressed specifically in the lymphatic endothelial cells have been found.

The lymphatic vessels also form part of the immune system by continuously transporting the white blood cells within the lymphoid organs (spleen, tonsils, thymus, Peyer patches, and lymph nodes) and bone marrow and transporting antigen-presenting cells. Mononuclear phagocytes and also lymphocytes patrolling the tissues enter the afferent lymph vessels and the lymph nodes to elicit primary immune responses before reentering the vasculature. Endothelial receptors and binding proteins are involved in this trafficking of specific lymphatic cell populations.

Lymphatic vessels start to develop in embryos around midgestation, in parallel with the development of blood vessels and most of the organs. When the embryo grows, these vessels are needed for the regulation of the interstitial tissue pressure. The origin of the lymphatic vessels has long been controversial. Historically, the best accepted view of lymphatic development is the one proposed by Sabin (184, 185). On the basis of the findings from her injection experiments, Sabin proposed that early in fetal development, isolated primitive lymph sacs originate by endothelial cell budding from embryonic veins. The two jugular lymph sacs develop from the junction of the subclavian and anterior cardinal veins. Later in development, the rest of the lymph sacs originate from the mesonephric veins, the veins of the Wolffian bodies, the primitive inferior vena cava and the junctions of the primitive iliac veins and the posterior cardinal veins. Sabin’s model proposes that the peripheral lymphatic system then spreads from these primary lymph sacs by endothelial sprouting into the surrounding tissues and organs where local lymphatic capillaries form.

An alternative model has suggested that the initial lymph sacs arise in the mesenchyme from precursor cells (“lymphangioblasts”), independent of the veins and secondarily establish venous connections (97). Although recent reports about the development of the lymphatic vessels support Sabin’s theory (52, 222), the existence of primitive lymphangioblasts, which can be recruited by the developing lymphatic vessels, has been shown at least in avian species (189). One should thus note that a combination of the two mechanisms is possible, whereby centrifugally sprouting lymphatic vessels anastomose with lymphatics developing from lymphangioblasts in tissues.

The lymphatic vessels differ in many ways from the blood vessels, but they also share many properties. Both vascular systems are lined by the endothelium, and the larger vessels are supported by a smooth muscle framework, particularly around luminal valves, which are present in the veins and in the large lymphatics (227). Both have vasa vasorum, the blood vessel network providing nutrition for the vessel wall. The smooth muscle layer in blood vessels controls the contractile tone of the vessels in response to vasoactive substances. Blood vessels have a continuous or fenestrated basement membrane and tight interendothelial junctions, which make the vessel wall selectively permeable to cells, fluids, and molecules, whereas lymphatic vessels have a relatively free import for interstitial fluid. Lymphatic endothelial cells have complex overlapping intercellular junctions and specialized anchoring filaments, which hold the vessel open as tissue pressure rises (227). It has been suggested that these properties provide the lymphatics a second valvular function, which permits fluid to enter from the interstitium into the initial lymph channels but prevents escape back out into the tissue (210). Liquid, macromolecules, and migrating cells pass through the blood capillary endothelia, enter the tissues, and are gradually absorbed into the lymphatic system. The fluid is transported via the lymphatic capillaries into the collecting vessels and through the lymph nodes, returning eventually to the circulation.

The blood vascular endothelium is a relatively leakproof, nonthrombogenic surface, with tightly regulated flow and intraluminal pressure, whereas the lymphatics in contrast are a low flow, a low-pressure system in close contact with the extracellular matrix. Lymph fluid does not contain red blood cells or platelets and is therefore much less coagulable than blood. The large lymphatic vessels with their smooth muscle have intrinsic contractility, which serves as a critical pumping force transporting lymph centrally toward the great veins. Compared with blood capillaries, the lymphatic capillaries send out fewer sprouts, anastomose less frequently, and show much less tendency to retract than undergo changes in size or form (227).

After the discovery of specific molecules regulating the lymphatic vessels, their role in certain pathological conditions has been extensively studied. Abnormal function of the lymphatic vessels is implicated in diseases such as lymphedema, inflammation, infectious and immune diseases, fibrosis, ascites, and tumors such as Kaposi’s sarcoma and lymphangioma/lymphangiomatosis. Perhaps most importantly, the lymphatic vessels are involved in tumor metastasis (reviewed in Refs. 110, 167). The identification of factors that promote tumor lym-
phangiogenesis and may promote the growth and spread of tumors via the lymphatic system is important for the development of new, effective treatments for cancer (108, 110, 145, 197, 201).

II. MOLECULAR REGULATION OF BLOOD AND LYMPHATIC VESSELS

Intercellular signaling mechanisms that govern the formation of blood and lymphatic vessels have emerged relatively recently. The complexity of endothelial cell development indicates that its regulation must involve many developmental and tissue-specific differentiation factors. Angiogenic signals are mediated by a number of growth factors and cytokines, and the balance between the positive and negative regulators maintains the adult vessels in a quiescent state (reviewed in Ref. 87). Whenever the balance is disturbed, the vessels react either by activating the angiogenic responses or regress by apoptosis when sufficient growth signals are not present. Interaction of angiogenic growth factors with their target cells triggers a cascade of steps, leading to the formation of blood vessels. Less is known about the regulation of the lymphatic vessels, although similar mechanisms seem to be involved.

Blood vessel development depends on members of the vascular endothelial growth factor (VEGF) family of proteins (Fig. 1). This family consists of VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF), which bind and activate cell surface receptor tyrosine kinases. Key signals regulating embryonic cell growth and differentiation, as well as remodeling and regulation of adult tissues, are mediated by the tyrosine kinase receptors (75, 109). The VEGF receptor (VEGFR) family includes VEGFR-1 (also known as Flt-1), VEGFR-2 (Flk-1), and VEGFR-3 (Flt4). Neuropilins 1 and 2 (NRP-1/-2) are another class of high-affinity nontyrosine kinase receptors for VEGFs on endothelial and neuronal cell surfaces (157, 169). Recently, additional molecules similar to VEGF and capable of increasing capillary permeability were found in snake venom, suggesting that the family may be even larger (79, 120). The receptors have partly overlapping but independent roles in the vascular development and maintenance, and the expression level of these genes modulates the abundance of different types of vessels in tissues. Other factors that are involved in the regulation of blood and lymphatic vessels are the angiopoietins and Tie-receptors, ephrins, and platelet-derived growth factors (PDGFs), which all act together in a coordinated manner during vessel formation (23, 37, 75). Interestingly, certain highly differentiated endothelia may have additional structurally unrelated regulators, such as EG-VEGF (127).

The regulation of blood vessels has been studied extensively over the past 10 years, but the molecular mechanisms behind lymphatic vessel growth have only been studied since 1995, when VEGFR-3, the first specific growth factor receptor of the lymphatic vessels, was found (105). Thereafter, a wealth of new information about the regulation of the growth of the lymphatics has been gained, and the factors known to regulate blood vessels have also been shown to be involved in the biology of the lymphatic vessels. Angiogenesis and lymphangiogenesis are thought to occur in parallel during embryonic development, and both may be important in adult physiological and pathological conditions. It is thus essential to understand the development and regulation of blood vessels to understand the biology of the lymphatic vessels. One must also consider blood and lymphatic vessels as collaborating parts of the circulatory system.

A. VEGF in Vasculogenesis and Angiogenesis

VEGF, discovered in 1989, is a major mediator of both vasculogenesis and angiogenesis (reviewed in Ref. 61). In endothelial cells, VEGF mediates mitogenic signals by activating VEGFR-1 and VEGFR-2 (62). VEGF is expressed as several isoforms consisting of polypeptides of different sizes (121, 145, 165, 183, 189 and 206 amino acid residues), which are all formed from the same gene by alternative splicing and differ in their ability to interact with extracellular matrix components and with NRP-1 (114, 130, 199, 209). These isoforms are thought to have distinct but overlapping functions in angiogenesis. VEGF is also known as vascular permeability factor, as it promotes the extravasation of fluid and plasma proteins, including fibrin, from the blood vessels (54, 190). The increase in microvascular permeability and tissue deposition of fibrin is considered to enhance the migration of endothelial cells in the extracellular matrix (55).

Two independent studies have shown that VEGF is essential for embryonic vasculogenesis and angiogenesis. Inactivation of only a single VEGF allele in mice resulted in embryonic lethality due to defective angiogenesis (38, 64). Also a reduced number of hematopoietic cells was observed. In mutant mice lacking the 164 and 188 amino acid isoforms of VEGF, one-half of the mice did not survive due to defects in, for example, postnatal angiogenesis in the myocardium, suggesting that the other forms of VEGF cannot completely replace the action of the others (42). Further studies suggest that in particular the heparin binding VEGF isoforms are required for efficient angiogenic sprouting and vascular morphogenesis (D. Shima, personal communication). Partial inhibition of VEGF by a soluble extracellular form of VEGFR-1 resulted in increased mortality and impaired organ development in the early postnatal period (81). It was shown
that in addition to proliferation VEGF is also required for
the survival of endothelial cells. Consistent with this,
other studies have also shown that VEGF supports the
survival in endothelial cells and induces the expression of
antiapoptotic proteins in endothelial cells (7, 18, 82).

VEGF is a major regulator of abnormal angiogenesis
(reviewed in Ref. 61). Consistent with this, the expression
of VEGF is potentiated in response to hypoxia and by
activated oncogenes as well as by a variety of cytokines
(85, 175, 193). VEGF is important in the etiology of several
diseases characterized by pathological angiogenesis such as
psoriasis, rheumatoid arthritis, and proliferative reti-
nopathy. Deregulated VEGF expression contributes to the
development of solid tumors by promoting tumor angio-
genesis (67). Tumor inhibition studies with neutralizing
anti-VEGF antibodies suggest that other angiogenic fac-
tors may also be involved (117). However, the VEGF
signaling pathway is currently considered to be one of the
most promising targets for the inhibition of tumor angi-
genesis.

B. Role of VEGF-B in the Myocardium

VEGF-B is structurally closely related to VEGF and
binds one of its receptors, VEGFR-1 (155). It has two
splice variants of which the 167 amino acid form binds to
heparan sulfates and NRP-1, while the other, of 186 amino
acid residues, is a freely secreted, soluble, and O-glyco-
sylated product (143). If the 186 amino acid form is pro-
teolytically cleaved, NRP-1 binding epitopes are exposed
in this isoform as well. Both VEGF-B isoforms are able to
form heterodimers with VEGF, and perhaps with other
growth factors. This adds diversity to their biological
roles by allowing a variety of combinations for cellular
signal transduction. During development, VEGF-B may
modulate the biological activities of VEGF, either by
forming heterodimers or by controlling the bioavailability
of VEGF (156).

VEGF-B is produced in large quantities by the develop-
ing myocardium and by muscle, bone, pancreas, adre-
nal gland, and the smooth muscle cell layer of several
large vessels, but not by endothelial cells (1). VEGF-B is
likely to act in a paracrine fashion as its receptor is almost
exclusively located on endothelial cells. VEGF-B is a very
weak endothelial cell mitogen when produced in mamma-
lian cells (155), but otherwise its biological role is still
unclear. It is possible that some of the biological activity
of VEGF-B produced in mammalian cells can be attrib-
uted to VEGF/VEGF-B heterodimers.

Mice lacking a functional VEGF-B gene are healthy
and fertile, but depending on the genetic background may
have a first degree heart block (conduction defect) or
reduced heart size (2, 17). The knockout mice display a
striking vascular dysfunction after coronary occlusion,
and they show impaired recovery from experimentally
induced myocardial ischemia (17). Considering such re-
sults, it is interesting to note that while VEGFR-1 and
VEGFR-2 were expressed rather uniformly in the devel-
oping vasculature, only VEGFR-1 was prominently ex-
pressed in the human fetal coronary endothelium (166).
These results suggest a role for VEGF-B in the coronary
vasculature and potential clinical use in therapeutic an-
giogenesis.

C. PlGF Is Essential in Pathological Angiogenesis

PlGF was discovered in the human placenta, and it is
\sim 50\% homologous to VEGF (139). Three splice isoforms
of PlGF have been published, and PlGF-2 at least com-
petes with VEGF\textsubscript{165} for binding to VEGFR-1 (35, 140, 163).
This is considered to increase the proportion of VEGF
available to activate VEGFR-2, thereby potentiating the
angiogenic properties of VEGF (163). A lack of PlGF has
no effect on embryonic development, even in combina-
tion with a loss of VEGF-B (41). However, loss of PlGF
impairs angiogenesis associated with tumors, ischemia,
myocardial infarcts, and experimental retinopathy and
leads to prolonged healing of incisional skin wounds (41).
During collateral growth after ligation of the femoral ar-
tery, PlGF was found to be essential for plasma extravasa-
sion, monocyte recruitment, and the growth of endothelial
and smooth muscle cells. These results indicate that
PlGF activates membrane-bound VEGFR-1 and specific-
ally potentiates the angiogenic response to VEGF. In
contrast to the essential role of VEGF in physiological and
pathological angiogenesis, the role of PlGF is restricted to
pathological vessel formation and is therefore a possible
target for therapy.

PlGF is not needed to enhance VEGF signaling in
embryonic vascular development. This is probably be-
cause VEGF is upregulated in response to a lack of PlGF.
This suggests that PlGF serves as an inert regulator of the
VEGF activity during development. The need for ampli-
fication of VEGF responses in adult pathological angiogen-
esis might be explained by the requirement of stronger
responses to VEGF than in embryonic angiogenesis (41).
When new blood vessels form in adults, endothelial cells
may become more responsive to VEGF by upregulating
PlGF and VEGFR-1. PlGF and VEGFR-1 are minimally
expressed in normal quiescent adult vasculature, but both
are markedly upregulated in pathological conditions.
Also, the membrane localization of VEGFR-1 is increased
in pathological angiogenesis compared with embryonic
angiogenesis. PlGF is also a chemoattractant for inflam-
matory cells, which are hallmarks of pathological angio-
genesis and collateral growth. PlGF may contribute to
vessel growth in adults by mobilizing bone marrow-de-
rived mononuclear cells. There may also be a synergism
between PlGF and VEGF-B in pathological angiogenesis.
D. VEGF-C and VEGF-D Coordinate the Development of Blood Vascular and Lymphatic Endothelia

VEGF-C was cloned from human prostate carcinoma cells, and its mature form consisting of the VEGF homology domain is 30% identical to VEGF165 (101). VEGF-C is synthesized as a preproprotein and from which a stepwise proteolytic processing generates several forms, with sequentially increasing binding and activity for its receptors, VEGFR-2 and VEGFR-3 (102). Like VEGF, VEGF-C stimulates the migration of endothelial cells and increases vascular permeability and endothelial cell proliferation but at higher concentrations than VEGF. These signals for endothelial cells are probably mediated through VEGFR-2 in blood vascular endothelial cells, and generally via VEGFR-3 in the lymphatic endothelial cells (102, 105).

Unlike VEGF, the expression of VEGF-C does not appear to be regulated by hypoxia (58) but is increased in response to proinflammatory cytokines, suggesting a role in inflammatory responses (178). VEGF-C, along with VEGFR-3, is also prominently expressed by activated macrophages (196; S. Mustjoki, personal communication). The pattern of VEGF-C expression in embryos suggests that it plays a role in the development of the lymphatic vessels, since a paracrine expression pattern is seen between VEGF-C and VEGFR-3 at sites where the first lymphatic sprouts occur (123). Conversely, VEGF-C is already expressed before the emergence of the lymphatics, which also suggests its involvement in vasculoangiogenesis during early development.

VEGF-C can regulate physiological and pathological blood vessel growth in vivo. It is able to stimulate angiogenesis in the mouse cornea and in the hindlimb ischemia model (36, 228). On the other hand, VEGF-C has been shown to regulate the growth of lymphatic vessels in various experimental models. Overexpression of VEGF-C in skin keratinocytes leads to dermal lymphatic vessel hyperplasia (Fig. 2, A and B) (100). Signaling via VEGFR-3 alone was shown to be sufficient for the hyperplasia, since transgenic mice overexpressing a mutant form of VEGF-C, which has lost its capacity to bind VEGFR-2 and only binds and activates VEGF-3 (VEGF-C156S), was able to induce a similar phenotype (216). VEGF-C was also studied in the mature, differentiated chorioallantoic membrane (CAM), which contains lymphatic vessels mainly around arterioles and veins (154). In this assay, VEGF-C acts as a highly specific lymphangiogenic factor. However, when VEGF-C was applied to the early CAM, where the lymphatics have not yet developed, it promoted angiogenesis. The angiogenic versus lymphangiogenic responses to VEGF-C may depend on the degree of proteolytic processing of its precursor and on the expression of its receptors in the blood versus lymphatic endothelial cells of the target tissue. VEGF-C also has synergistic effects with VEGF, during the induction of angiogenesis, and this effect is more prominent in cells expressing both of its receptors (168). In addition, VEGF-C can compete with VEGF in binding to VEGFR-2.

VEGF-D (also known as c-fos-induced growth factor or FIGF) is the most recently discovered member of the mammalian VEGF family (3). It shares 61% sequence identity with VEGF-C, and these two growth factors bind to the same receptors on human endothelial cells. VEGF-D is proteolytically processed similarly to VEGF-C, and the proteolytic processing also appears to regulate VEGF-D biological activity and receptor specificity (202). Interestingly, in mice, VEGF-D binds only to VEGFR-3, suggesting that VEGF-D may have a somewhat different function in
mouse and human (12). This is uncommon within the VEGF family as these homologous and evolutionary conserved growth factors are assumed to exhibit similar receptor binding characteristics in different species.

VEGF-D has been shown to be able to stimulate the proliferation of endothelial cells, and it shows angiogenic properties in vitro and in vivo (146). Like VEGF-C, it was also shown to be lymphangiogenic when overexpressed in skin keratinocytes (216). Little is known about the expression of VEGF-D in physiological conditions, but its mRNA has been observed in the developing melanocytes and fibroblasts, lung mesenchyme, and the adult vascular wall (4).

The exact roles of VEGF-C and VEGF-D during embryonic vascular development are still unknown due to the lack of gene deletion studies. In adults, VEGF-C and VEGF-D may regulate the responses of the lymphatic vessels in inflammatory processes and in the regeneration of tissues after trauma, but they may also have important roles in physiological and pathological angiogenesis in various conditions. VEGF-C and VEGF-D may also affect the fluid dynamics in lymphatic vessels and be involved in the formation of valves and recruitment of smooth muscle cells to the developing lymphatic collecting vessels. Unpublished data indicate that VEGF-C and VEGF-D can heterodimerize (M. Jeltsch, personal communication), as has been reported for PlGF and VEGF as well as VEGF-B and VEGF, making their biological properties even more diverse (34, 156).

E. Orf Virus VEGF-E Promotes Angiogenesis

A VEGF homolog, VEGF-E, was recently discovered in the genome of the parapoxvirus, Orf virus, that infects sheep, goats, and occasionally humans (138). Infection by this virus causes proliferative skin lesions in which extensive papillary proliferation and dilation are prominent histological features. Several strains of the virus encode different VEGF-E variants, which bind specifically to VEGFR-2 and NRP-1 and are able to stimulate endothelial cell mitogenesis and vascular permeability (153, 225). VEGF-E is not essential for viral replication but rather plays an important role in modulating the host environment during infection.

F. VEGF Receptors in Endothelial Cell Proliferation, Migration, and Survival

All VEGFRs are characterized by seven extracellular immunoglobulin homology domains (Ig) (Fig. 1), of which the second and third are critical for ligand binding and the first three domains are necessary for establishment of full binding affinity (15, 49, 73, 142). In VEGFR-3, the fifth Ig homology domain is proteolytically cleaved, but the fragments remain together via disulfide bonds. These receptors control many aspects of vascular growth and have partially overlapping expression patterns in the developing vasculature in embryos but show a more restricted expression in adults.

VEGFR-1 and VEGFR-2 are important in blood vascular endothelial cell proliferation, migration, and survival. Mice carrying a homozygous disruption in either of the two VEGF receptors die during early development due to defects in both vasculogenesis and angiogenesis. Embryos lacking functional VEGFR-2 die without mature endothelial or hematopoietic cells (191). Primitive hematopoietic and endothelial progenitors arise normally within the yolk sac blood islands from precursors cells that express VEGFR-2. In contrast, VEGFR-1-deficient mice have normal hematopoietic progenitor cells and endothelial cells that migrate and proliferate but do not assemble into tubes and functional vessels (71). More recent studies have shown that an excessive proliferation of endothelial progenitors is the main factor leading to this disorganization (72). This supports the view that VEGFR-1 is a negative regulator of VEGF-induced vasculogenesis in embryos.

Although VEGFR-1 alone has been shown to induce weak mitogenic signals in vitro (125), it is thought that VEGFR-2 is the major receptor transducing the effects of VEGF in endothelial cells. For example, VEGF-E and site-directed mutants of VEGF, which bind only to VEGFR-2, stimulate endothelial cells similarly to VEGF (82, 116, 148, 225). VEGF also provides survival signals for endothelial cells via VEGFR-2 (82). Outside of the vascular system, VEGFR-1 is expressed in monocytes and macrophages, placental trophoblasts and renal mesangial cells, and VEGFR-2 in hematopoietic stem cells, megakaryocytes, and platelets and retinal progenitor cells (14, 45, 46, 112, 232). Despite the importance of these receptors during embryonic blood vessel development, VEGFR-1 and VEGFR-2 appear to be downregulated in the quiescent adult endothelium.

VEGFR-3 was cloned from a human leukemia cell line and human placenta (76, 161). Two isoforms of VEGFR-3 have been described, designated VEGFR-3s (short) and VEGFR-3l (long), which differ as a result of alternative splicing. The long form is the predominant form in most tissues. An endogenous retroviral genome appears responsible for the short isoform in humans, but this form is missing from mice (96). In adults, the expression of VEGFR-3 is mainly restricted to lymphatic endothelial cells and in hematopoietic cells of monocytic lineage, where it serves as a molecular marker for these vessels (Fig. 2, C and D) (103, 105). In embryos VEGFR-3 is initially expressed in all vasculature, but during development its expression in blood vessels decreases and becomes restricted to the developing lymphatic vessels (Fig. 3) (105). VEGFR-3-deficient embryos die as a result...
of a defect in the remodeling of the primary vascular network and cardiovascular failure at midgestation, before the lymphatic vessels start to develop (52). Interestingly, the differentiation of endothelial cells, the formation of the primary vascular plexus, and vascular sprouting were not disturbed by the absence of VEGFR-3 signaling, but the embryos had severe anemia due to impaired yolk sac hematopoiesis (86). These results suggest that VEGFR-3 plays a dual role, in embryos in cardiovascular development before the emergence of the lymphatic vessels and in adults in the regulation of the lymphatic vessels.

The majority of blood vessel endothelial cell populations around midgestation are positive for both VEGFR-2 and VEGFR-3, suggesting that these receptors have essential roles in angiogenesis (86). Also, VEGFR-1 is seen in the developing vasculature at this stage (71). Endothelial cells are apparently activated by both VEGF and VEGF-C at this stage, but it is not known whether these receptors transduce similar signals. The VEGF/VEGFR-2 system appears to be responsible for most of the growth signals for vascular endothelial cells, but it has also been proposed that VEGF-C induces proliferation of embryonic vascular endothelial cells through VEGFR-2. VEGF-C signaling through VEGFR-2 and VEGFR-3 may thus have distinct roles in embryogenic vasculogenesis (86). In addition, in VEGF-deficient mice, some endothelial cells survive, and this may be promoted by VEGF-C.

In adults, VEGFR-3 is expressed in a subset of capillary endothelia, although it is absent in endothelia of all large blood vessels (165). Although lymphatic vessels develop from large veins in the embryonic jugular, retroperitoneal, and perimesonephric regions, only a few adult tissues retain VEGF-3 expression in the venous endothelia. Such endothelia are seen in the veins of the cartilage channels, vertebral bodies, venous canals of the adrenal medulla, and the splenic venous sinuses (165). Differences in VEGFR-3 expression are also seen between continuous and discontinuous endothelia, the former being negative and the latter positive for VEGFR-3, suggesting that this receptor plays a role in the endothelial transport functions in fenestrated capillaries. VEGFR-3 is also present in the fenestrated endothelia, and it is possible that VEGF-C and VEGF-D secreted by the neuroendocrine cells signal via both receptors in this specific subtype of capillaries in endocrine organs. VEGFR-3 is seen also in endothelia at sites of hematopoiesis or blood cell trafficking, such as in the sinusoids of liver, spleen, and bone marrow, suggesting that it has a regulatory role in the transendothelial translocation of hematopoietic cells (165). Also some nonendothelial expression of VEGF-3 has been observed in embryonic notochordal cells and in the trophoblasts of the placenta (165, 224).

VEGFR-3 is activated in the blood vessel endothelium in certain pathological conditions, and upregulation of VEGF-C/VEGF-D ligands may accompany this (4, 186, 214). Similarly, VEGFR-2 can be expressed by both blood vascular and lymphatic endothelia (166). During wound healing, acute inflammation is followed by the deposition of fibrin and connective tissue and the growth of blood vessels into the granulation tissue. Most blood vessels then regress as the wound is remodeled into scar tissue.

FIG. 3. VEGFR-3 expression during the development of the lymphatic vessels. Blood vascular and lymphatic vessels can be visualized in stained sections from VEGFR-3 knock-out heterozygous embryos (VEGFR-3-LacZ), in which the marker gene reveals sites of VEGFR-3 expression (blue). Early in mouse development (embryonic day 9.5, E9.5) VEGFR-3 is expressed uniformly in the whole developing vasculature (A). After the blood vessels have been established, the VEGFR-3-positive lymphatic vessels (arrow) start to sprout from preexisting veins (B). Later, VEGFR-3 expression is further downregulated in the blood vessels (arrowhead in C) and becomes restricted to the lymphatic vessels (arrow in C). The lymphatic vessel network in the skin of E15.5 mouse embryo is shown in whole mount staining (D). Scale bar in D is for A–D; A, 30 μm; B, 100 μm; C, 125 μm; D, 80 μm.
VEGFR-3-positive lymphatic vessels have been observed to sprout from preexisting lymphatics and grow into the granulation tissue in healing skin wounds (160). These lymphatic vessels persisted in the wound for some time but regressed as the healing proceeded. This suggests that transient lymphangiogenesis is needed during wound healing, in parallel with angiogenesis, and it is possible that inflammatory cells such as macrophages secrete relevant lymphangiogenic factors. On the other hand, no lymphatic vessels were seen in chronic human wounds (160). The absence of lymphatic vessels may contribute to the impaired healing in these conditions. The angiogenic vessels in wound healing remained negative for VEGFR-3, suggesting that there are differences in the regulation of angiogenesis in various pathological conditions.

Little is known about the characteristic features of lymphatic endothelial cells, mainly because isolated lymphatic endothelial cells have not been available for molecular studies. Recently, primary cultures of human dermal endothelial cells were shown to consist of distinct lines of blood vascular and lymphatic endothelial cells (Fig. 4) (121, 144). Cells of the lymphatic lineage could be isolated by antibodies against VEGFR-3 or podoplanin, another marker for lymphatic endothelium. Signaling via VEGFR-3 was shown to be critical for growth, migration, and survival of the isolated lymphatic endothelial cells (144). Also, VEGFR-2 was detected in the lymphatic endothelial cells, suggesting that activation of both VEGF-C receptors may be required for their maximal survival (144, 166). The isolation and culturing of lymphatic endothelial cells now allow more detailed studies of the molecular properties of these interesting cells.

**G. Neuropilins Modulate VEGF Activities**

NRP-1 and NRP-2 are transmembrane receptor proteins that are required for axon guidance and, according to recent discoveries, also for the regulation of angiogenesis (83, 198, 199). Both neuropilins bind certain isoforms of VEGF, VEGF-E, PlGF-2, and VEGF-B (143, 149, 199, 225). NRP-1 is expressed in the tips of actively growing axons of particular classes of neurons (74), but also in the blood vascular endothelial cells and in certain tumor cells (198, 199). NRP-1 enhances VEGF165 binding to VEGFR-2 and VEGF-mediated chemotaxis. Embryos lacking functional NRP-1 die due to defects in VEGF-mediated angiogenesis and subsequent cardiovascular failure (113), and ectopic overexpression of NRP-1 leads to an excess of dilated blood vessels and hemorrhage, apparently due to inappropriate VEGF activity (119). Recently, NRP-2 has been shown to bind VEGF-C and to be expressed together with VEGFR-3 in the endothelial cells of a subpopulation of the lymphatic vessels (106).

**H. Angiopoietins and Tie-2 Are Involved in Vessel Stabilization and Maintenance**

Tie-1 and Tie-2 (Tek) are expressed in endothelial cells throughout embryonic development as well as in hematopoietic progenitor cells (50). Gene targeting experiments indicate that Tie-1 and Tie-2 are essential to the angiogenic expansion of the vasculature during development. In mouse embryos lacking the Tie-2 receptor, endothelial cells are present in slightly reduced numbers.
and are assembled into tubes, but the vessels remain immature, lacking branching networks and proper organization into a hierarchy of large and small vessels (51, 187). The vessels lack intimate encapsulation by periendothelial support cells, and the endocardium is only loosely attached to the myocardium. Thus Tie-2 appears to control the capability of endothelial cells to recruit stromal cells, which stabilize the vessel structure and modulate the function of blood vessels (87). Tie-1 is required cell autonomously for endothelial cell survival and extension of the vascular network during the later part of embryogenesis (173, 187). Vasculogenesis proceeds normally in embryos lacking both Tie-1 and Tie-2, since the angioblasts differentiate normally (172). It appears that one of the earliest critical functions of these receptors concerns endocardial development but that rescue of the embryos is possible if one bypasses the critical period using transgenic expression of Tie-2 (D. Dumont, personal communication).

Angiopoietin (Ang)-1 and Ang-2 bind to Tie-2 with similar affinities, but only Ang-1 can activate the receptor directly. Ang-2 is capable of inhibiting the effects of Ang-1 in endothelial cells in short-term experiments. However, if endothelial cells of human umbilical vein are stimulated with Ang-2 for longer periods, activation of the Tie-2 receptor is obtained (206). Ang-2 is also capable of stimulating Tie-2 in transfected nonendothelial cells. Thus Ang-2 has both agonistic and antagonistic properties, which may relate to its ability to dimerize or oligomerize less efficiently than Ang-1, or to binding to an inhibitor that needs to be downregulated.

Ang-1 is widely expressed in both embryonic and adult tissues (205). Ang-2 is also expressed in embryos around large vessels, but in adults the expression pattern is restricted to sites of physiological angiogenesis, where vascular remodeling occurs (141). Transgenic overexpression of Ang-2 under the Tie-2 promoter in the embryonic endothelium indicates that Ang-2 inhibits the recruitment of supporting perivascular cells, resulting in a phenotype similar to that of the Ang-1 knockout embryos (205). In adults, Ang-2 allows vascular remodeling, which otherwise is restricted by encapsulation by the basement membrane and periendothelial support cells. When the expression of Ang-1 overcomes that of Ang-2, such remodeling ceases and vessels stabilize (reviewed in Ref. 75).

Several lines of evidence indicate that there is significant collaboration between VEGF, Ang-2, and Ang-1 in angiogenic processes. Vascular regression is associated with very high levels of Ang-2 in the absence of activating survival signals from VEGF. In the skin of transgenic mice, VEGF increases the number of capillaries, whereas Ang-1 causes a massive enlargement of postcapillary venules (208). Interestingly, Ang-1 was able to prevent the permeability effects of VEGF (207). Coexpression of both factors was required to obtain an increased number of large vessels in transgenic mice. Surprisingly, a combinatorial function of Ang-1 and Tie-1 was critical for the development of the right-side venous system, but not for the left-side venous system (135). Identification of the ligand(s) for the Tie-1 receptor should provide further insights into the mechanistic basis for this asymmetric regulation of vascular development. Ang-2 may also be involved in the regulation of lymphatic vessels, since knock-out mice lacking functional Ang-2 have chylos ascites with a disorganized and leaky lymphatic vasculature (G. Thurston and G. Yancopoulos, personal communication).

I. PDGFs Are Involved in Recruitment of Perivascular Structures

The PDGF family consists of homodimers or heterodimers made from the pairwise assembly of the related PDGF polypeptide chains (reviewed in Ref. 92). These effects of the PDGFs depend on the target cell type, in particular on the cell’s repertoire of PDGF receptors. The dimeric, active receptors consist of α- and β-subtypes of the PDGF tyrosine kinase receptor. The α-receptor can bind PDGF-A, PDGF-B, and PDGF-C chains, whereas the β-receptor is selective for the PDGF-B and PDGF-D chains (21, 23, 132). On the basis of the gene-deficient studies, both receptors are essential for embryonic development. PDGF-A and PDGF-α are prominently expressed at sites of epithelium-mesenchyme interaction, whereas PDGF-B takes part in blood vessel development (94, 133, 159). PDGF-D is the first known PDGFR-β-specific ligand, and its unique receptor specificity indicates that it may be important in the development and pathophysiology of several organs. The expression of PDGF-C and PDGF-D in the arterial wall and cultured vascular cells suggests that they can transduce proliferation/migration signals to pericytes and smooth muscle cells (213). The expression patterns of PDGF-A and PDGF-C are compatible with overlapping functions, but the situation with PDGF-B and PDGF-D is not that well characterized yet.

The association of supporting smooth muscle cells or pericytes with angiogenic vessels has been suggested to regulate endothelial proliferation, survival, sprouting, and differentiation. During blood vessel development, PDGF-B is expressed in endothelial cells, while pericytes and smooth muscle cells covering the blood vessels express PDGFR-β, indicating paracrine signaling between these two cell types (94, 133). Targeted gene deletion studies of PDGF-B or PDGFR-β gave similar phenotypes. In both mouse strains, blood vessel development was deficient because of the inability of blood vessels to attract pericytes (93, 94, 133). Also the development of the renal and hematopoietic systems was affected (131, 200). Lack of pericytes in embryonic angiogenesis led to hyperplasia of
endothelial cells, supporting the notion that pericytes inhibit endothelial cell proliferation (93).

Smooth muscle cell proliferation in response to the release of growth factors from neighboring cells is one mechanism postulated to account for the development of atherosclerotic lesions. PDGFs may be involved in initiation and progression of atherosclerotic changes in arterial intima by promoting proliferation of smooth muscle cells of the vascular wall (24, 150). These molecules may also have an important role in tumor biology, since expression of the mRNA of the receptors and ligands A and B has been observed in a wide variety of human tumors (92).

J. Ephrins and Ephs Are Involved in Arteriovenous Differentiation

Before the heart starts to beat and circulate blood, the vascular hierarchy must be organized and arteries and veins must be ready to properly transport blood. In studies of the ephrin family of molecules it has become obvious that the fate of the endothelial cell is already marked in early embryonic development when the whole endothelium is still rather uniform in nature (reviewed in Ref. 230). Unlike ligands for other receptor tyrosine kinases, the ephrins cannot act as soluble mediators but are membrane bound to activate their receptors. The Eph receptor family consists of at least 14 members. The receptors, and their ligands, can be divided into two subclasses, A and B (230). Ephrin-B ligands are transmembrane proteins and bind to receptors of the Eph-B subclass. Ephrin-B2 was shown to mark future arteries while its receptor Eph-B4 reciprocally marks the venous endothelium (218). Furthermore, embryos lacking Ephrin-B2 displayed severe defects in vascular remodeling in both arterial and venous domains. These findings provide some of the earliest known markers distinguishing the arterial and venous endothelia. These data for the first time show the existence of bidirectional signaling between these vessel types. This suggests that the molecular differences are at least in part programmed genetically in arterial versus venous endothelia and that these differences may be critical to normal development of the vasculature.

As the ephrins and Eph receptors distinguish arterial versus venous endothelial cells in the primary vascular plexus and are membrane bound, they interact with each other at sites of cell-to-cell contact, and the interactions are considered to lead to cell retraction. This signaling may happen at the junction of arterial and venous cells, or when the developing tubules pass each other and come in contact with adjacent sprouts during early remodeling of embryonic vasculature (230). These local interactions may ensure that new angiogenic sprouts fuse only with their counterpart branches. Inhibitory interactions may also help fusion to occur between the right type of vessels during remodeling, e.g., preventing fusion of arterial and venous structures and ensuring remodeling into a proper capillary network. On the other hand, coexpression of ligands and receptors in the same vessels may provide stimulatory signals to endothelial cells and promote sprouting and morphogenesis resulting in formation of functional vessels (5).

Other members of the Eph family are also critical for the remodeling of the vascular network (5). It has been also shown that the interactions of ephrins and Ephs are not restricted to the arterial/venous boundary but occur throughout vasculature and in mesenchymal cells adjacent to the blood vessels. These endothelial-mesenchymal contact zones may be critical for the patterning of the vasculature.

III. MARKERS OF THE LYMPHATIC ENDOTHELIUM

A major advance in the field of lymphangiogenesis has come from the discovery of lymphatic endothelium-specific markers (Table 1). VEGFR-3 was the first mole-

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Protein Class</th>
<th>Biological Effect</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-3</td>
<td>Receptor tyrosine kinase on endothelial cell</td>
<td>Lymphangiogenesis, survival of LEC</td>
<td>105</td>
</tr>
<tr>
<td>LYVE-1</td>
<td>Receptor for extracellular matrix glycosaminoglycan</td>
<td>Transport of HA from tissues to lymph nodes</td>
<td>13</td>
</tr>
<tr>
<td>PROX-1</td>
<td>Transcription factor</td>
<td>Developmental lymphangiogenesis</td>
<td>222</td>
</tr>
<tr>
<td>Podoplanin</td>
<td>Integral membrane protein</td>
<td>Unknown</td>
<td>30</td>
</tr>
<tr>
<td>β-Chemokine receptor D6</td>
<td>Chemokine receptor in afferent lymphatics</td>
<td>Leukocyte recirculation</td>
<td>151</td>
</tr>
<tr>
<td>Desmoplakin</td>
<td>Component of intercellular adhering junction in LECs</td>
<td>Adhesion of LECs</td>
<td>188</td>
</tr>
<tr>
<td>Macrophage mannose receptor</td>
<td>Receptor in macrophages</td>
<td>Phagocytosis of microbes, viral endocytosis</td>
<td>134</td>
</tr>
</tbody>
</table>

LEC, lymphatic endothelial cell; HA, hyaluronan.
cule found to be expressed in the lymphatic endothelium, but further studies have revealed that it is also expressed in a subset of blood vessels and in addition can be reactivated in the angiogenic vessels in certain pathological conditions (165, 214). Podoplanin is a glomerular podocyte membrane mucoprotein, which occurs together with VEGFR-3 in the lymphatic endothelium and in benign vascular tumors and angiosarcomas, but is also expressed in certain nonendothelial cells, such as osteoblastic cells, kidney podocytes, and lung alveolar type I cells (29, 30). Podoplanin is present in small lymphatics but not in the larger ones having smooth muscle cells, and all the blood vessels as well as high endothelial venules in the lymph nodes are negative for podoplanin expression. Prox-1 is a homeobox transcription factor gene product involved in the growth and elongation of the lymphatic vessel sprouts during development (222). Consistent with this, Prox +/− heterozygous newborns develop chylous ascites and die shortly after birth (222). Prox-1 is also expressed in non-endothelial cells in the lens, heart, liver, pancreas, and nervous system. The third marker, lymphatic vessel endothelial HA receptor-1 (LYVE-1), is a receptor for extracellular matrix/lymphatic fluid glycosaminoglycan in lymphatic endothelial cells (13). This molecule, which is related to the CD44 receptor for hyaluronan, is distributed equally among the luminal and abluminal surfaces of lymphatic vessels and is involved in the uptake of hyaluronan by lymphatic endothelial cells and its transport from the tissues to the lymph (171). However, LYVE-1 is not completely specific for the lymphatic endothelial cells, as it is also present, e.g., in normal hepatic blood sinusoidal endothelial cells (43).

Recently, a β-chemokine receptor D6 was shown to be present in a subset of lymphatic vessels in the skin, intestine, and lymphoid tissues (151). Interestingly, lymphatic vessels in most of the organs remained negative for D6 immunoreactivity. The existence of this receptor on only a subset of lymphatics suggests a functional heterogeneity within the lymphatic vasculature. Consistent with this, recent findings revealed the coexpression of NRP-2 and VEGFR-3 in the lymphatic vessels of the intestine, whereas dermal lymphatic vessels did not show NRP-2 expression (106). The mannose receptor of macrophages is also expressed by lymphatic endothelium in addition to macrophages and other nonendothelial cells (134). The biological role of this receptor in lymphatic vessels is not known, but it may play a role in inflammation and immunity. 5′-Nucleotidase and desmoplakin have also been used to distinguish the lymphatic from the blood vascular endothelium (188, 212), and because lymphatic capillaries lack a continuous basement membrane, immunohistochemistry for extracellular matrix components type IV collagen and laminin have been used to distinguish them from blood capillaries (16). VEGFR-2 is occasionally expressed in lymphatic endothelia (166), and Tie-1 and Tie-2 may also have a role in lymphatic vessel regulation, as they also appear in lymphatic endothelia (103, 166) and mice deficient for the Ang-2 have a lymphatic phenotype (G. Thurston and G. Yancopoulos, unpublished data). In cultured lymphatic endothelial cells, the VEGFR-3, LYVE-1, and podoplanin were essentially located in the same cells (144). In addition, VEGFR-1, VEGFR-2, and VEGFR-3 were expressed in the same cultured lymphatic endothelial cells.

The markers of lymphatic endothelium show overlapping expression patterns in the lymphatic vessels of most benign tissues. However, recent findings have revealed that at least VEGFR-3 and LYVE-1 can be expressed in blood vascular endothelia in physiological conditions (43, 165). More information needs to be obtained to clarify the specificity of these markers in the lymphatic vessels in disease processes. Until then, several markers should be used to confirm the staining of lymphatic vessels in pathological conditions.

IV. DISEASES OF THE LYMPHATIC VESSELS

Pathological processes similar to those that affect blood vessels, such as thrombosis, inflammation, vessel wall hypertrophy, and sclerosis, may also occur to some extent in lymphatic vessels. However, the slow flow of the lymphatic fluid makes lymphatic disorders less acute in character (227). Lymphatic vessel defects are associated with intense fibrosis and overgrowth rather than the dramatic occlusive events such as occur in blood vessels when the blood flow is interrupted.

A. Lymphedema, a Failure of Lymph Transport

An important function of the lymphatic vessels is to regulate the pressure of interstitial fluid in tissues by transporting excess fluid back into the circulation. Edema represents an imbalance between lymph formation and its absorption into the lymphatic vessels. Clinical situations in which the lymphatic system is involved include lymphedema due to impaired lymphatic drainage. This can be caused by inflammatory or neoplastic obstruction of the lymphatic vessels including accumulation of ascites fluid due to lymphatic obstruction in peritoneal carcinomatosis or edema of the arm after surgery or radiotherapy for breast cancer. Lymphatic filariasis, globally the second leading cause of permanent and long-term disability, is a parasitic infection in the lymphatic vessels, which leads to abnormal transport function, massive edema, and deformation of the limbs (227).

Primary lymphedema is a rare developmental disorder in which the transport failure of the cutaneous lymphatic vessels results in interstitial lymph fluid accumulation. Chronic lymphatic dysfunction gradually results in
thickening of the skin, accumulation of adipose tissue, and dermal fibrosis of the affected area (129). Recently, several groups have reported linkage of congenital lymphedema (Milroy’s disease) to the VEGFR-3 gene (59, 66, 226) with autosomal dominant inheritance. This mutation was shown to lead to reduced VEGFR-3 tyrosine kinase activity and subsequent failure in transducing sufficient physiological VEGF-C/VEGF-D signals to the lymphatic endothelial cells (107). In all lymphedema families studied, the affected individuals had only one mutant allele (98, 107). This is compatible with the results that inactivation of both VEGFR-3 alleles in mice is embryonic lethal (52).

The mutation affecting the biological activity of VEGFR-3 is probably one cause of primary lymphedema, but some other lymphedema genes also exist, for example, FOXC2 (60). Identification of genetic markers and high-risk members of lymphedema families would facilitate the identification and management of environmental factors influencing the expression and severity of lymphedema. In particular, these findings permit better-informed genetic counseling in affected families and effective therapeutic regimens for lymphedema.

B. Kaposi’s Sarcoma Originates From Lymphatic Endothelia

Kaposi’s sarcoma is a multicentric neoplasm consisting of multiple vascular nodules appearing in the skin, mucous membranes, and viscera. Typically, the tumor respects tissue planes and is rarely invasive. Molecular and epidemiological studies indicate that development of Kaposi’s sarcoma is associated with infection by the human herpesvirus-8 (HHV-8) (204). However, predominantly individuals with specific conditions of immunodysregulation, especially acquired immunodeficiency syndrome patients, develop Kaposi’s sarcoma. The nodules are characterized by clusters of spindle-shaped tumor cells and by prominent vasculature consisting of small, irregular, endothelial-lined spaces. It is thought not to be a neoplastic transformation of cells in the classic sense, but rather a manifestation of excessive proliferation of the spindle cells. A central question in the pathogenesis of Kaposi’s sarcoma has long been, which cell type in early lesions gives rise to the uniform tumor cells of late nodules (77). The spindle cells are most likely endothelial in origin, but there has been controversy as to whether they are of lymphatic or blood vascular derivation. The pathobiology of Kaposi’s sarcoma is complex, and several vascular growth factors have been reported to be expressed in the nodules, suggesting that the balance of angiogenic/lymphangiogenic molecules is behind this tumor type (77).

Several reports support the idea that the cells in Kaposi’s sarcoma are of lymphatic endothelial origin. The spindle cells and cells lining the irregular vascular spaces are positive for both VEGFR-3 and podoplanin (30, 103). In addition, the spindle cells have been shown to express VEGFR-2, Ang-2, Tie-1, and Tie-2 (31, 164, 195).

C. Vascular Tumors Express VEGFR-3 in the Endothelium

Vascular malformations are structural anomalies of the vascular system and may be composed of arteries, capillaries, veins, lymphatics, or combinations of them (78). They are often congenital benign lesions present at birth, but their enlargement ceases with the growth of the patient. An activating mutation in the Tie-2 receptor has been shown to cause inherited venous malformations (217). Altered Tie-2 regulation leads to abnormal venous growth or remodeling due to local uncoupling between proliferation and differentiation of endothelial and smooth muscle cells. This resembles the defects in the transgenic mice overexpressing Ang-1 (207, 208). An activating mutation of Tie-2 or overexpression of its activating ligand are both apparently able to cause similar changes in vascular remodeling (reviewed in Ref. 69).

Vascular tumors consist of a broad morphological spectrum from hamartomas to malignant neoplasia. They can be divided into benign tumors (hemangiomas) and malignant vascular tumors (angiosarcoma) and to the tumors of lymphatic vessels (lymphangioma) and perivascular cells (glomus tumors). The molecular characteristics of these tumors are so far mostly unknown, but VEGF and its receptors have been shown to be expressed in the endothelial cells of these tumors (180). Although normal mesenchymal tissues show VEGFR-3 in the lymphatic endothelial cells, cells of benign and malignant vascular tumors show widespread VEGFR-3 expression, suggesting that VEGFR-3 is upregulated in the proliferating blood vascular endothelial cells. This is consistent with the VEGFR-3 expression in the embryonic developing vessels, as well as reactivation in adult angiogenic blood vessels (52, 214). Although strong expression of VEGFR-3 would be consistent with lymphatic differentiation, the extensive erythrocyte content in the vascular lumina of these lesions supports the idea that VEGFR-3 expression in these tumors reflects a proliferative vascular phenotype rather than a lymphatic phenotype. The expression of VEGFR-3 among vascular proliferations demonstrates that blood vessel endothelia can acquire VEGFR-3 expression independently of lymphatic vascular differentiation.

Classification of angiosarcomas is mostly based on morphological criteria. It has been suggested that some of the angiosarcomas contain components of a lymphatic lineage, but since the antibodies used for the detection of blood endothelia show overlapping staining of lymphatic...
endothelia, there is no proof of this. Some cell populations in angiosarcomas are positive for podoplanin, which retains its lymphatic endothelial cell specificity in vascular tumors, supporting the idea of mixed expression of both blood and lymphatic phenotypes in angiosarcomas (30). As expression of VEGFR-3 is seen in the majority of benign and malignant vascular tumors (164), it could thus be used as a lineage marker to identify endothelial cell differentiation in the tumors. However, further studies are needed to evaluate the sensitivity and specificity of both VEGFR-3 and podoplanin in the malignant transformation of blood or lymphatic endothelial cells.

Lymphangiomas result from abnormal development of lymphatic vessels, which prevents lymph fluid draining from the affected area. Lymphangiomas can originate in most organs, although they are most often found in the soft tissues of the head and neck (cystic hygroma) and axilla. They consist of a benign multicystic mass of dilated networks of lymphatic channels. Both VEGFR-3 and podoplanin specifically stain the endothelia of lymphangiomas and could be used for diagnostic purposes (30, 103, 164).

V. TUMORIGENESIS AND METASTASIS

Cancer is a disease involving dynamic changes to the genome. Mutations produce oncogenes that gain a dominant function, and tumor suppressor genes become inactivated and lose function (reviewed in Ref. 88). Lots of evidence indicates that tumorigenesis in humans is a multistep process, and these steps reflect the genetic alterations that drive the progressive transformation. Cancer cells have defects in regulatory circuits that govern normal proliferation and homeostasis. Whereas normal cells require mitogenic signals before they can move from a quiescent state into an active proliferative stage, malignant cells are self-sufficient for the growth signals and insensitive to the growth-inhibitory signals, which normally operate to maintain cellular quiescence and tissue homeostasis. Tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from the normal tissue microenvironment. They develop various strategies to avoid terminal differentiation and are therefore more capable of proliferating than the well-differentiated benign cells. Most soluble mitogenic growth factors are produced by one cell type to stimulate the proliferation of another, whereas many cancer cells acquire the ability to synthesize their own growth factors leading to autocrine stimulation. It has long been thought that tumors are independent from the surrounding cells and their action, but now it seems more likely that cancer development depends on interactions between tumor cells and their benign neighbors. Tumors and metastases tend to harbor complex mixtures of several cell types that collaborate to create a malignant growth, including fibroblasts, immune cells, and endothelial cells (68, 88).

The cells within aberrant proliferative lesions initially lack angiogenic ability, preventing their expansion. The ability to induce and sustain angiogenesis seems to be acquired in discrete steps during tumor development via an angiogenic switch from vascular quiescence to proliferation (88). In studies of transgenic mouse tumorogenesis (Rip-Tag), angiogenesis was found to be activated in midstage lesions before the appearance of full-blown tumors (70). These observations indicate that neovascularization is necessary for the rapid clonal expansion associated with the formation of macroscopic tumors. Tumors appear to activate the angiogenic switch by changing the balance of angiogenic inducers and inhibitors (20, 68). Another regulatory mechanism is the function of proteases, which can control the availability of angiogenic activators and inhibitors stored in the extracellular matrix (48). There is also evidence of heterogeneity of angiogenic activity in malignant neoplasms, where this seems to be regulated by the organ microenvironment (194).

Tissue hypoxia is a fundamental angiogenic stimulus characteristic also of malignant tumors. The VEGF gene has been shown to contain specific hypoxia-responsive elements and to be upregulated in response to low oxygen tension (65). Ang-2 levels are also increased by hypoxia, suggesting a collaboration of VEGF and Ang-2 in the regulation of neovascularization of ischemic tissue (reviewed in Ref. 126). Tumor-derived signals such as VEGF may specifically induce Ang-2 expression in tumor endothelia, and this may be one important component in angiogenic switch and in the formation of an endogenous tumor microcirculation.

A. Lymphatic Vasculature and Growth Factors in Tumors

As tumors need neovascularization to grow, microvascular density has been used as a measure of tumor angiogenesis, and its correlations to tumor growth, metastasis, and prognosis have been studied (221). Levels of VEGF are upregulated in a large number of human tumors (for review, see Ref. 65), and inhibition of VEGF activity results in the suppression of growth of a wide variety of tumor cell lines in murine models (63). It was long thought that lymphatic vessels may be lost, collapsed, or could not penetrate in the expanding primary tumors because they cannot survive in the high solid stress inside the tumors (reviewed in Ref. 167). However, recently some intratumoral lymphatic vessels have been observed (201), and an interesting question now is whether or not lymphatic vessel density, compared with blood vascular density, is related to prognosis and metastatic spread.
Few data are available on the influence of lymphatic microvessel density on survival in cancer, because until recently there was no reliable immunohistological marker for the lymphatic endothelium. In ovarian cancer, the lymphatic vessel density had no influence on the progression of the disease, and in cervical cancer an increased amount of lymphatic vessels may even be associated with a favorable prognosis (25, 26). It is likely that human tumors demonstrate heterogeneity with regard to the presence or absence of intratumoral lymphatics. The nature of the marker may also influence the determination of the lymphatic vessel density. VEGFR-3 has been seen in the endothelial cells of the proliferating vasculature in solid tumors and vascular tumor cells of endothelial origin (164, 214) and therefore cannot be used alone to confirm the intratumoral lymphatic vessel density. Also LYVE-1 has been shown to be expressed by the sinusoidal blood vessels of the liver (43). Therefore, until the specificity of these markers in lymphatic vessels during tumor progression and metastases has been clarified, multiple markers should be used in elucidating correlation between lymphatic vessel density and tumor growth, metastases, and prognosis.

All VEGFRs are present in tumor neovasculation, and tumor cells have been reported to be able to secrete VEGF, VEGF-B, VEGF-C, and VEGF-D (4, 65, 186). However, the angiogenic switch is thought to be carefully regulated, and at least some specific genetic events in tumor progression correlate with lymphatic metastasis, suggesting that a "lymphangiogenic switch" mechanism is also a formal possibility.

**B. Mechanisms of Blood Vascular and Lymphatic Metastasis**

The capacity to spread enables cancer cells to escape the primary tumor mass and colonize new areas in the body, where nutrients and space are not limiting. Tumor cell dissemination is mediated by mechanisms including local tissue invasion, lymphatic spread, hematogenous spread or direct seeding of body cavities or surfaces (47). Although the biochemical mechanisms are not completely understood, it is thought that the metastatic spread of a tumor is not a random process. Distinct patterns of metastasis can be discerned that vary from tumor type to tumor type. A common pattern for carcinomas is that regional lymph nodes are often the first sites to develop metastases either draining via preexisting afferent lymphatic vessels and/or via newly formed lymphatic capillaries. This pattern of metastasis is central to the utility of the sentinel lymphadenectomy as a surgical technique. However, not all tumors and tumor types metastasize to the regional lymph nodes first. The mechanisms determining whether regional lymph nodes or other sites first develop metastases remain poorly understood. In fact, most disseminated tumor cells have a limited life span, and only a few develop into clinically detectable micrometastases. Nevertheless, identification of those occult tumors cells, and prevention of their growth and spread would be of great clinical significance.

The presence of a metastasis in a lymph node does not necessarily mean that the tumor cells have been arrived via the lymphatic vessels (215). Intra-lymphatic tumor cells may pass directly into the blood vascular system through venolymphatic communications; that has been observed in certain organs. Also, an increase in the number of tumor cells in the blood circulation should also raise the frequency of the lymph node metastases. This pathway may be related to the increased number of microvessels in the tumors and therefore cannot be considered as lymphatic vessel-mediated metastasis.

**C. VEGF-C, VEGF-D, and Tumor Metastases**

VEGFR-3 may play an important role in the formation of tumor-induced neovascularization, since it is expressed in capillary vessels during tumor angiogenesis (164, 214). Inactivation of VEGFR-3 by neutralizing antibodies suppressed tumor growth by destabilizing large vessels in tumor xenografts in mice. Microhemorrhages were seen in these vessels, suggesting that VEGFR-3 could be involved in maintaining the integrity of the endothelial cell lining in the neovasculature (122). Frequent VEGFR-3 antibody administration was required for the suppression of tumor growth, but the architecture of the nonangiogenic blood and lymphatic vessels remained unaffected. It has also been shown that even a prolonged suppression of VEGF activity in adult mice has no effect on the maintenance of the vascular system, although it suppressed angiogenesis severely in embryos (81). The fully established blood and lymphatic vessels seem to be resistant to treatment with these kinds of antiangiogenic agents.

Recent work using experimental models has highlighted the role of VEGF-C and VEGF-D in tumor biology. Transgenic mice overexpressing VEGF-C in β-cells of the endocrine pancreas (Rip-VEGF-C, rat insulin promoter) developed extensive lymphangiogenesis around the endocrine islets of Langerhans (145). Furthermore, when tumors were induced in these VEGF-C overexpressing islets, by mating the mice with transgenic mice expressing the simian virus 40 T-antigen oncogene in the β-cells (Rip1-Tag2), metastatic tumor cell aggregates of β-cell origin were observed in the surrounding lymphatic vessels. These mice also frequently developed metastases in the lymph nodes, which drain the pancreas, whereas tumors in mice lacking the VEGF-C transgene never metastasized, nor were tumor cells observed inside the lymphatic vessels (145). VEGF-C overexpressed by the
tumors did not significantly alter tumor volume, transition from adenoma to carcinoma or tumor angiogenesis, but interestingly the tumor incidence was increased, for as yet unknown reasons. Similarly, human breast cancer cells expressing ectopic VEGF-C were shown to induce lymphangiogenesis in and around the orthotopically implanted tumors (Fig. 5A) (111, 197). However, VEGF-C did not have a significant effect on angiogenesis, although it increased tumor growth. Increased spreading of the cells to the regional lymph nodes was observed, and the degree of tumor lymphangiogenesis correlated with lymph node metastases (M. M. Mattila, personal communication). VEGF-C-induced tumor growth, lymphangiogenesis, and intralymphatic tumor growth was inhibited by adenoviral expression of the soluble VEGFR-3 receptor (Fig. 5B) (111). However, although VEGF-C is present, it is not always sufficient to induce the formation of functional lymphatic vessels (128). There may be variation between different tumor types, and the solid stress exerted by the growing tumor may prevent the growth of lymphangiogenic sprouts into the tumor.

VEGF-D was also shown to promote the metastatic spread of tumor cells via the lymphatics (201). In addition to lymphangiogenesis and increased metastases, the tumors secreting VEGF-D also had an increased growth rate and tumor angiogenesis. The growth of the tumor, angiogenesis, and formation of metastases were inhibited by neutralizing antibodies against the bioactive region of VEGF-D. The differences between the tumor angiogenic properties of VEGF-C and VEGF-D may be due to differences in their proteolytic processing in different tumors. Some of the heterogeneity in the effects of these growth factors may also result from variable expression of their receptors, VEGFR-2 and VEGFR-3, on the blood vascular and lymphatic endothelia. In particular, in the above case, enhanced tumor angiogenesis was probably obtained for VEGF-D because of its increased proteolytic processing, which resulted in an increased affinity to VEGFR-2 (201),

**FIG. 5. Development of metastasis via the lymphatic vessels.** Overexpression of VEGF-C in xenotransplanted human breast carcinoma cells in mice leads to tumor lymphangiogenesis and accumulation of metastatic tumor cells in newly formed peritumoral lymphatic vessels (stained for the lymphatic marker LYVE1; arrows in A). Such lymphangiogenesis and intralymphatic tumor growth was prevented by adenoviral delivery of a soluble VEGFR-3 protein (B). Scale bar in B is for A and B, 200 μm.
compared with the VEGF-C models. Consistent with this, the vascular effects of VEGF-C in experimental tumors were reduced, when the mice were treated with blocking antibodies against VEGFR-2 (104). Also, intratumoral lymphatic vessels were observed in tumor xenografts (111, 197, 201), but not in the transgenic tumors (145), which may be at least partially explained by the trapping of vessels in between the rapidly growing tumor foci in the xenografts. On the basis of these observations, tumor vessel formation can be dissected into pathways that preferentially activate angiogenesis (driven by VEGFR-2) and pathways that preferentially activate lymphangiogenesis (driven by VEGFR-3), although there is evidence that the receptors share overlapping expression patterns (166).

Activation of lymphatic endothelial cells by tumor cell secreted factors may promote the interaction of tumor cells with the lymphatic endothelial cells, and thereby facilitate tumor cell entry into the lymphatics. In spite of the increased metastatic tendency of VEGF-C overexpressing tumor cells, metastases were only seen in about one-third of the tumor-bearing mice. Overall, on the basis of studies in which VEGF-C or VEGF-D has been overexpressed in tumors, one could suggest that there are additional, rate-limiting steps in the metastatic process. The simplest explanation for the metastasis-enhancing effects of VEGF-C and VEGF-D is that they eliminate one rate-limiting step by increasing the surface area between invading tumor cells, which is in contact with the hyperplastic lymphatic endothelium. However, they could also facilitate metastasis by increasing vascular permeability, by changing the adhesive properties or cytokine or chemokine expression patterns of the lymphatic endothelium. VEGF-C and VEGF-D secreted by the tumor cells could also have important effects on the tumor interstitial fluid pressure. Both can increase vascular leak, but not as efficiently as VEGF; a parallel increase in lymphangiogenesis could alleviate this effect. The increased interstitial fluid pressure could be a major determinant of tumor cell seeding into the blood vascular and lymphatic circulation, especially since recent studies have shown that a proportion of the lumen of tumor blood vessels themselves consists of tumor cells (44, 90).

It is still unknown whether VEGF-C or VEGF-D expression also promotes lymphangiogenesis in human tumors, and if so, does this increase the rate of metastasis to the lymph nodes. VEGF-C expression has been detected in about one-half of human cancers analyzed (186). In breast cancer, VEGF-C expression seems to correlate with lymph node-positive tumors, whereas VEGF-D may be expressed predominantly in inflammatory breast carcinomas, suggesting that these growth factors have distinct roles in various tumors despite their biochemical similarities (124). A number of reports have described a correlation between VEGF-C expression in human tumors and the formation of metastases in regional lymph nodes. So far, VEGF-C levels in primary tumors have been shown to correlate significantly with lymph node metastases in thyroid, prostate, gastric, colorectal, lung, and esophageal carcinomas (6, 32, 118, 152, 211, 231). Less is known about the presence of VEGF-D in human tumors, but VEGF-D was shown to be upregulated in human melanomas compared with melanocytes (4). In melanomas, VEGF-D was detected in the tumor cells and in vessels adjacent to immunopositive tumor cells, but not in vessels distant from the tumors. This suggests that VEGF-D binds to the endothelial cells of nearby vessels and contributes in a paracrine manner to the regulation of tumor angiogenesis.

It is not known to what extent tumor cell secreted factors are directly responsible for the large lymphatic vessels occasionally detected around human tumors. Inflammatory cells for example could contribute to the lymphangiogenesis, as VEGF-C is chemotactic for macrophages and readily induced by proinflammatory cytokines (58, 178). Dilated and engorged peritumoral lymphatics may function poorly because of the obstruction of the lymphatic vessel by tumor cells. There is evidence that the lymph flow in peritumoral lymphatics can change to lymphaticovenous communication or reverse lateral flow (229). It may be that lymph retention and reflux of lymph induced by vessel obstruction favors the metastasis to occur by direct seeding to body cavities or by hematogenous spread. It is not clear whether the newly formed lymphatic vessels mature in a similar way to the blood vessels or whether they are more prone to tumor cell invasion, for example, because of differences in the expression of adhesion receptors. VEGF is known to be able to upregulate the expression of adhesion molecules in the vasculature, but such a role for VEGF-C and VEGF-D is not known.

Although it seems evident that both VEGF-C and VEGF-D can induce the growth of new lymphatic vessels, several questions remain unanswered regarding tumor lymphangiogenesis and metastasis. For example, it is not known whether it is sufficient for preexisting lymphatic vessels to expand by circumferential growth or whether new vessels are required for the enhancement of the metastatic process. On the other hand, lymphatic vessels may either actively penetrate into existing tumors or become trapped between expanding tumor foci. The intratumoral lymphatic vessels observed are usually collapsed due to the high tissue solid stress on solid tumors; this may impair their transport capacity. Also, as in angiogenesis, lymphangiogenesis may occur by several mechanisms, and different regulatory factors may be involved.
VI. IMPLICATIONS FOR THE HUMAN DISEASE THERAPY

A. Antiangiogenic and Antimetastatic Therapy

Despite advances in surgery, radiotherapy, and chemotherapy, the prognosis of many cancers remains poor. One of the goals of gene therapy in cancer treatment is to target the therapeutic gene to all tumor cells, as each untreated tumor cell has the potential to progress and to metastasize. The purpose of combining conventional cancer therapy with antiangiogenic agents is that the antivascular effects of the chemotherapy and radiotherapy are selectively enhanced in the cells of newly formed vessels, for example, when survival signals mediated by VEGF are blocked (61, 115). However, one needs also to consider the unwanted toxic effects of the cancer therapy on the vasculature, some of which could be alleviated by provision of vascular survival factors (162). Therapy resistance in tumor cells depends on tumor cell heterogeneity, genetic instability, and a high mutation rate. Compared with conventional cytostatics, there may well be less of a risk of resistance to antiangiogenic agents, since the endothelial cell compartment is assumed to be genetically more stable and have a lower mutation rate than the tumor cells (27, 61). However, the immature nature of tumor blood vessels should provide a therapeutic window where the tumor vascular endothelium can be targeted leaving the rest of the vasculature intact.

Several antiangiogenic agents, alone or in combination with conventional therapies, have advanced to clinical trials. Many of them target angiogenic growth factors, their receptors, or downstream signaling. For example, neutralizing antibodies against VEGF or VEGFR-2 have been used in the treatment of various solid tumors with and without combination with traditional cancer therapy (39). Although preclinical results are promising, it is not yet clear how antiangiogenic therapies will perform clinically. As mechanisms of angiogenesis differ in various tissues, therapeutic inhibition of angiogenesis needs to be modified for each target tissue (63). There is evidence indicating that different types of tumors have distinct molecular mechanisms to activate the angiogenic switch. Whether a single antiangiogenic molecule will suffice to treat all tumor types, or whether an ensemble of such molecules needs to be developed, remains to be seen. Toxic or vaso-occlusive therapy has also been used to target directly tumor vasculature (8, 56, 84).

The differences between the surface molecules of blood vascular and lymphatic endothelia can be taken into account when targeting therapeutic agents selectively to tumor lymphatic vessels. This would increase the potency of the drug in the target tissue and limit the possibility of side effects (9, 181). Methods such as cDNA microarray analysis and phage display screening have been used to identify such markers. The targeting of lymphatic vessels in human tumors would help in imaging these vessels and facilitate studies into the role of lymphatic vessels in the metastatic processes. Anticancer drugs specifically targeted to peritumoral lymphatic vessels could be used to inhibit lymphatic metastasis. However, the destruction of these vessels would further elevate the high interstitial fluid pressure inside the tumors, impairing the delivery of other drugs. Because VEGF-D expression has been shown to become upregulated by direct cell-cell contacts, the increased intratumoral pressure could increase close contacts between the tumor cells and lead to a compensatory increase of the lymphangiogenic growth factor levels (158). Increased intratumoral fluid pressure could also enhance the likelihood of hematogenous metastasis (39, 203).

B. Gene and Recombinant Protein Therapy of Myocardial and Peripheral Ischemia

Ischemic heart disease stems from poor oxygenation of the heart muscle as a consequence of coronary vessel obstruction (47). Promoting angiogenesis in this situation may have a positive impact by increasing collateral vessel formation. Similarly, ischemia in the lower limb would also be alleviated from such an improvement. Animal studies on proangiogenic therapies on these conditions have shown promising results (63). However, studies of Buschmann and Schaper (33) have shown that collateral formation is critically dependent on arteriogenesis, which is a distinct process from angiogenesis.

Various angiogenic approaches to treating ischemic diseases are already in clinical trials (63, 99). Many of them involve the delivery of VEGF to ischemic tissue to stimulate the growth of new vessels. One outstanding question is whether a single angiogenic factor can promote functional and sustainable angiogenesis, or if a combination of angiogenic molecules is required. For example, vessels induced by VEGF are leaky and tortuous, so it may be possible to control leakiness by combining VEGF with Ang-1, as was done in a mouse model (207). Recombinant VEGF-C may also be used as a therapeutic angiogenic growth factor in the treatment of tissue ischemia, possibly even in combination with VEGF (95). The angiogenic activity of VEGF-C in ischemic conditions may relate to the increased expression of VEGFR-2 and the presence of relatively high endogenous VEGF levels in such conditions. On the other hand, lymphangiogenesis has never been studied in ischemia, but no evidence exists at present concerning the possible role of hypoxia in the regulation of the lymphatic vessels. The findings that VEGF has an important role in bone angiogenesis and endochondral bone formation suggest that these factors...
could also be used to enhance revascularization in orthopedic conditions such as nonhealing fractures (80).

An important question concerning the proangiogenic therapies is how the therapeutic molecules should be administered. Is it possible to deliver a potent molecule like VEGF in therapeutic quantities without causing toxic side effects, like hypotension or edema? Suitable methods and routes of therapy would also avoid the infiltration of inflammatory cells, such as macrophages, which express VEGFR-1. It is not clear for how long these factors should be administered, whether the therapy leads to a functional vasculature and whether the vessels will regress upon the completion of therapy. On the other hand, the studies of Dvorak and co-workers (170) clearly show that at least some of the vessels generated in response to VEGF gene therapy eventually stabilize and acquire periendothelial structures. Such stabilization of vessels may depend on the level of intraluminal blood flow. However, concern about potential side effects, such as inappropriate blood vessel growth in patients with diabetic retinopathy or solid tumors, has decreased the enthusiasm for the use of these powerful agents (220).

New endothelial cells must be recruited in the beginning of angiogenesis. This recruitment is from two possible sources, preexisting vessel endothelia and endothelial precursor cells from the blood circulation (174). The existence of angioblast-like circulating endothelial precursor cells in adults has only recently been suggested, and their role in supporting postnatal angiogenesis is under intensive investigation (174). VEGFR-2 has been suggested as a marker for these cells. The ex vivo expansion of these precursors would be useful for the promotion of vascular healing, for provision of suitable cellular coatings for vascular grafts, or for the delivery of toxins to the tumor vascular bed (174). Mobilization of such precursors from bone marrow could also accelerate healing at sites of vascular trauma.

C. Therapeutic Lymphangiogenesis

At present, lymphedema is treated by manual lymphatic drainage and by compressive garments. The discovery of specific genes involved in the regulation of lymphatic vessels and in the pathology of lymphedema should make the design of more targeted treatments for this disease possible. Because transgenic VEGF-C/D overexpression is able to induce the postnatal growth of new lymphatic vessels in the skin (100, 216), these molecules may also be useful in lymphedema patients. Subcutaneous adenoviral gene transfer of VEGF-C in mice has already been shown to induce lymphangiogenesis within 2 wk of treatment (57). A mouse model (Chy), which mimics human lymphedema, allows the study of possible gene therapies (106). These mice, like the human patients, have a heterozygous mutation in the VEGFR-3 gene, resulting in partial loss of VEGFR-3 activity (106, 136, 137). This impairs the development of the cutaneous lymphatic vasculature and leads to hypoplastic, nonfunctional vessels. Secondary to this, there is thickening of the skin as well as accumulation of adipose tissue and dermal fibrosis similar to the lymphedema patients.

The effect of VEGF-C was explored by both gene therapy and transgene approaches in the Chy model (106). When VEGF-C was overexpressed in the skin of Chy mice, growth of functional cutaneous lymphatic vessels was induced, suggesting that VEGF-C/D gene therapy may be applicable to human lymphedema. Such therapy could also be used in nonhereditary, regional forms of lymphedema resulting from trauma, surgery, or lymphatic vessel destruction after filariasis. Because VEGFR-3 signaling plays a role in lymphatic endothelial cell survival (142, 144), long-term growth factor expression may be needed to obtain lymphangiogenesis and maintain these vessels in chronic lymphedema. The functional characteristics of the newly formed lymphatic vessels, for example, their connections to draining lymphatic vessels, still require additional studies.

The larger connecting lymphatic vessels, the lymphatic vessels of the visceral organs, as well as the VEGF-3-positive fenestrated blood vessels appeared normal in the lymphedema mice (106). Consistent with this, skin is the most affected organ also in lymphedema patients. Interestingly, the lymphatic vessels of transgenic mice expressing a soluble, circulating form of VEGFR-3 (which results in reduced VEGF-C/D signaling via their lymphatic endothelial receptor) regress before birth, but start to regrow later during the postnatal period in the internal organs (142). However, in the skin, the lymphatic vasculature remains hypoplastic. These results support the view that the cutaneous lymphatic vessels are regulated differently from those in other organs and that besides VEGFR-3, there are additional signals for growth and maturation of the lymphatic endothelium postnatally. For example, it could be that the extracellular matrix provides signals via the integrins, which activate the VEGFR-3 pathway (219). NRP-2, which binds VEGF-C and is expressed in the lymphatic vessels of internal organs but not in the skin, may also be one such factor (106). It is possible that NRP-2 is involved in VEGFR-3-mediated signal transduction at sites where the two receptors are coexpressed, which is similar to what has been reported for NRP-1 regulation of VEGFR-2-mediated signals (199).

The mechanisms of lymphangiogenesis in adults have not been elucidated. The generation of lymphatic vessels could in principle require endothelial cell sprouting from, or splitting of, preexisting lymphatic or blood vessels; in situ differentiation of endothelial cells; or recruitment and lymphatic differentiation of endothelial precursor cells, as has been described in other models.
Results obtained so far are comparable to the processes of sprouting from preexisting vessels and splitting of large dilated lymphatic vessels, which are seen after VEGF-C treatment (57). However, upregulation of VEGFR-2 and VEGFR-3 on endothelial cells was seen in response to VEGF-C, raising the interesting possibility that endothelial cells in blood vessels may also participate in lymphangiogenesis by the process of migration and transdifferentiation. Furthermore, at least in the avian system, precursor cells or lymphangioblasts have been demonstrated, from which new lymphatic growth may initiate (189).

VII. CONCLUSIONS

The development and regulation of endothelial cells require the orchestration of many growth factors in a carefully coordinated manner. Blood and lymphatic vessels are formed in an interactive manner during embryonic development, but both vessel types are maintained in a rather quiescent state in adults and are active only in sites of new tissue growth. Angiogenesis has been extensively studied for over a decade, but lymphangiogenesis is a relatively new topic for vascular biology. The discovery of specific molecules involved in the biology of the lymphatic vessels now enables a more extensive study of the many roles of the lymphatic vessels. VEGF-C and VEGF-D both stimulate the growth of lymphatic vessels, being the first growth factors found for lymphatic endothelial cells. Similarities between the regulation of blood and lymphatic vessels have been observed, and these two vessel systems seem to work in a tightly regulated manner. Lymphangiogenesis may occur in sites of angiogenesis, either following the growth of blood vessels, as in wound healing, or independently as has been observed in experimental tumor models and in inflamed tissue (D. McDonald, personal communication). The findings made so far on lymphatic regulation will be helpful in the diagnosis of certain vascular tumors and in designing specific treatments for lymphedema. Regulation of the blood and lymphatic vessels and some possible therapeutic approaches have been summarized in Figure 6. Many promising experiments have been done concerning the inhibition of metastatic spread of tumor cells via the lymphatic vasculature. The isolation and culture of lymphatic endothelial cells now offer better tools to study the molecular characteristics of these cells. Encouraging results on the therapeutic use of angiogenic and lymphangiogenic molecules have been obtained in experimental models, and ongoing and future clinical trials will show the therapeutic potential of the molecules of VEGF family of growth factors and receptors.

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