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REVIEW

The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature

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The lymphatic system is composed of a vascular network of thin-walled capillaries that drain protein-rich lymph from the extracellular spaces within most organs. A continuous single-cell layer of overlapping endothelial cells lines the lymphatic capillaries, which lack a continuous basement membrane and are, therefore, highly permeable. Lymph returns to venous circulation via the larger lymphatic collecting vessels, which contain a muscular and adventitial layer, and the thoracic duct. The lymphatic system also includes lymphoid organs such as the lymph nodes, tonsils, Peyer's patches, spleen, and thymus, all of which play an important role in the immune response.

The lymphatic system develops in parallel with the blood vascular system through a process known as lymphangiogenesis, and lymphatic vessels are not normally present in avascular structures such as epidermis, hair, nails, cartilage, and cornea, nor in some vascularized organs such as brain and retina. Although studies of normal development and pathologic growth of the blood vascular system have thoroughly elucidated the molecular mechanisms that control these angiogenic processes (Gale and Yancopoulos 1999), studies of the lymphatic system have been hindered by the lack of specific lymphatic markers and growth factors. Consequently, our understanding of the development and function of the lymphatic system and its role in disease is still emerging.

Recently, the discovery of molecules that specifically control lymphatic development and lymphatic vessel growth (lymphangiogenesis) and the identification of new lymphatic endothelium-specific markers (Breiteneder-Geleff et al. 1999; Wigle and Oliver 1999; Jackson et al. 2001; Sleeman et al. 2001; Veikkola et al. 2001) have facilitated key scientific advances and provided new insights into the molecular mechanisms that control lymphatic development and function. These findings include the identification of specific genetic defects in certain hereditary diseases that are associated with lymphatic hypoplasia and dysfunction (i.e., lymphedemas; Milroy 1892; Meige 1898), and evidence that malignant tumors can directly activate lymphangiogenesis and lymphatic metastasis (Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001a; Stacker et al. 2001).

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Historical perspective

Lymphatic vessels were first described in the seventeenth century by Gasparo Aselli as "lacteae venae", or milky veins (Asellius 1627), and the embryonic development of lymphatics was extensively studied during the beginning of the last century. Since then, however, this field has advanced very slowly because of the lack of specific lymphatic markers, and the histogenetic origin of lymphatic vessels has remained a controversial issue.

Historically, the most widely accepted view of lymphatic development was proposed by Sabin in the early twentieth century (Sabin 1902, 1904). Following results obtained by ink injection experiments, Sabin proposed that isolated primitive lymph sacs originate from endothelial cells that bud from the veins during early development. The two jugular lymph sacs were proposed to develop in the junction of the subclavian and anterior cardinal veins by endothelial budding from the anterior cardinal veins (Sabin 1902, 1904). According to her view, the remaining lymph sacs originate from the mesonephric vein and those in the dorsomedial edge of the Wolffian bodies in the junction of the subclavian and anterior cardinal veins. The retroperitoneal lymph sac forms near the primitive inferior vena cava and mesonephric veins; the cisterna chyli forms near the Wolffian bodies; and the posterior lymph sacs appear near the junctions of the primitive iliac veins and the posterior cardinal veins (Fig. 1; Gray 1985).

The model proposed by Sabin indicated that the pe-

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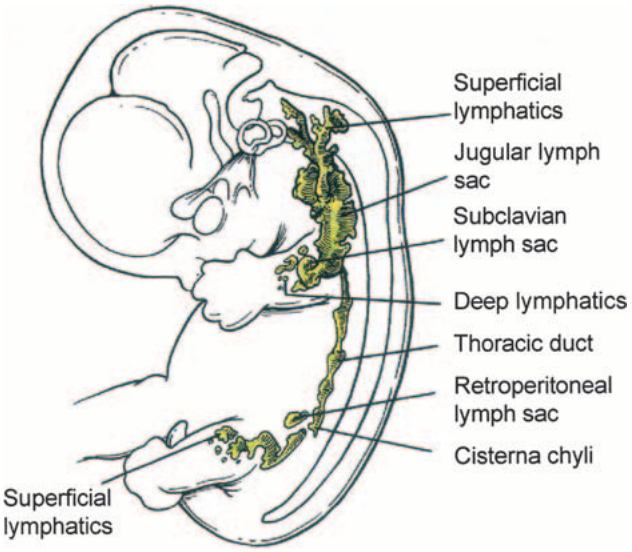

Figure 1. Schematic representation of the primitive lymphatic system showing the primary lymph sacs in a 42-day-old human embryo, after Sabin (reprinted from *Human Embryology*, by W.J. Larsen, 1993, Harcourt, NY; with permission from Harcourt International).

ripheral lymphatic system originates from the primary lymph sacs, then spreads by endothelial sprouting into the surrounding tissues and organs, where local capillaries are formed (Sabin 1902, 1904; Gray 1985). An alternative model suggested that the primary lymph sacs arise in the mesenchyme, independent of the veins, and secondarily establish venous connections (Huntington and McClure 1910). Support for this latter model has been recently obtained in birds, where it was proposed that the lymphatics of the early wing buds are derived not only by sprouting from the lymph sacs but also from the embryonic mesenchyme (Schneider et al. 1999).

In the developing mouse embryo, blood vessels are formed from mesodermally derived endothelial cell precursors (vasculogenesis). These vessels then grow by endothelial sprouting and splitting (angiogenesis). Because a variety of growth factors and receptors involved in these processes have been identified (Gale and Yancopoulos 1999; Yancopoulos et al. 2000), the molecular mechanisms that control the development of the vascular system are now being deciphered. For example, vascular endothelial growth factor (*VEGF*), the endothelial receptor tyrosine kinases *Tie1* and *Tie2*, and angiopoietin-1 (*Ang1*) and angiopoietin-2 (*Ang2*) participate in the process of vascular development. Results obtained from loss- and gain-of-function experiments performed with some of these factors indicated that *VEGF* and its receptors, *VEGFR-1* and *VEGFR-2*, are important for the proliferation, migration, and sprouting of endothelial cells (Risau 1997; Gale and Yancopoulos 1999). Angiopoietins and their receptor *Tie2* appear to play a later role by controlling the sprouting, remodeling, and maturation of the developing vasculature (Gale and Yancopoulos 1999). In contrast, the mechanisms involved in the development of the lymphatic system are still poorly characterized.

Lymphatic markers and the current view on lymphatic development: blood versus lymphatic vascular fate determination

Lymphatic and blood vasculature are difficult to differentiate when the histologic morphology of the two systems is the only basis on which the distinction is made. Recently, the identification of several markers that show different profiles of expression in blood and lymphatic vasculature has facilitated detailed analyses of the development and pathologic role of the lymphatic vasculature. The fact that so few lymphatic-specific markers have been identified thus far is a likely indication of the close structural and developmental relationship of the blood and lymphatic vasculature. In fact, the specificity of some of these markers is only acquired as embryonic development progresses, suggesting that the formation of the lymphatic vasculature is a stepwise process.

One of the first lymphatic markers to be identified was the vascular endothelial growth factor receptor-3 (*VEGFR-3*, also known as *Flt4*). The pattern of expression of *VEGFR-3* during murine development has provided additional support for Sabin's model; VEGFR-3 is initially expressed in angioblasts of murine head mesenchyme, dorsal aorta, cardinal vein, and allantois (Kaipainen et al. 1995; Dumont et al. 1998). At embryonic day 12.5 (E12.5), VEGFR-3 is expressed both in developing venous and in presumptive lymphatic endothelia, whereas in adult tissues, VEGFR-3 is largely restricted to the lymphatic endothelium (Kaipainen et al. 1995; Partanen et al. 2000). Overexpression of VEGF-C, a ligand of VEGFR-3, in the skin of transgenic mice results in hyperplasia of cutaneous lymphatic vessels (Jeltsch et al. 1997). The in vivo application of VEGF-C also stimulates blood vascular angiogenesis in the mouse cornea, likely via interaction with VEGFR-2 expressed on blood vessels (Cao et al. 1998).

The lack of a viable *VEGFR-3*-null mouse has hampered the analysis of the role of this growth factor receptor in the development of the lymphatic system. Inactivation of *VEGFR-3* causes cardiovascular failure and death of the embryo before the emergence of lymphatic vessels (Dumont et al. 1998). However, the identification of nonsense mutations in *VEGFR-3* in patients with hereditary lymphedema (Karkkainen et al. 2000) has provided support for an important role of this gene in lymphatic development. These findings suggest that *VEGFR-3* may play a role in the development of both the blood vascular and the lymphatic system (Kukk et al. 1996). VEGFR-3 is also expressed in some blood capillaries during tumor neovascularization and in wound granulation tissue (Valtola et al. 1999; Paavonen et al. 2000; Partanen et al. 2000); therefore, depending on the tissue and the developmental stage, this molecular marker alone may not precisely discriminate between blood and lymphatic vessels.

The lymphatic endothelial hyaluronan receptor

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(*LYVE-1*), a CD44 homolog, was recently identified as a specific cell surface protein of lymphatic endothelial cells and macrophages (Banerji et al. 1999; Jackson et al. 2001; Prevo et al. 2001). Immunostainings performed using antibodies against LYVE-1 and the blood vascular markers PAL-E (Skobe and Detmar 2000) and CD34 (Prevo et al. 2001) revealed that they show mutually exclusive vascular expression patterns (see also Fig. 4A, below). Recently, LYVE-1 expression has also been detected in liver sinusoidal endothelial cells (Carreira et al. 2001); however, LYVE-1 is not expressed by blood vessels in most organs including the skin (Skobe and Detmar 2000; Prevo et al. 2001).

Hyaluronan might regulate leukocyte migration through the lymphatic vasculature (Jackson et al. 2001). In addition, chemokines such as secondary lymphoid chemokine (SLC, also termed 6Ckine/exodus-2/CCL21), which is released by the lymphatic endothelium and interacts with the CC chemokine receptor 7 (Gunn et al. 1998, 1999; Zlotnik and Yoshie 2000), attract leukocytes toward the lymphatic vessels. As the development of the lymphatic vasculature advances, expression of SLC is first detectable at around E11.5 in mice (Wigle et al. 2002), and SLC becomes uniformly expressed in adult lymphatic endothelium (Gunn et al. 1998, 1999).

Podoplanin, a surface glycoprotein, has been recently described as a novel marker for the lymphatic vasculature. In humans, podoplanin is expressed in osteoblastic cells, kidney podocytes, lung alveolar type I cells, and lymphatic endothelial cells (Wetterwald et al. 1996), with a strong expression in the lymphatic vasculature of the skin (Breiteneder-Geleff et al. 1999). However, a detailed comparison of the expression of podoplanin with that of other lymphatic markers is still lacking, and expression of LYVE-1 was detected in only a subset of cultured podoplanin-positive endothelial cells (Makinen et al. 2001a).

Desmoplakin, a cytoplasmic protein that attaches intermediate filaments to the plasma membrane in epithelial cells, has also been reported to be a marker of the lymphatic endothelium. By immunoelectron microscopy, vessels reacting with an anti-desmoplakin antibody showed features characteristic of lymphatic vessels, including thin endothelial walls, incomplete basal lamina, open junctions, and overlapping endothelial cells. In contrast, blood vessels did not express desmoplakin (Ebata et al. 2001). A detailed comparison of desmoplakin expression with that of other lymphatic markers is not yet available.

To achieve a reliable differentiation between blood and lymphatic vasculature, combinations of positive and negative molecular markers for these tissues have been used. For example, the presence or absence of any of the aforementioned gene products, combined with different expression levels of vascular basement membrane markers such as laminin, collagen IV, and collagen XVIII (Skobe et al. 2001a), or that of the blood vascular surface antigens CD34 (see Fig. 4A, below) or PAL-E (Skobe and Detmar 2000), can be used to discern between the two systems.

The homeobox gene *Prox1* was originally cloned by homology to the *Drosophila* gene *prospero* (Oliver et al. 1993). Functional inactivation of *Prox1* in mice leads to embryonic lethality and causes phenotypic alterations of the lens, liver, and lymphatic vasculature (Wigle and Oliver 1999; Wigle et al. 1999, 2002; Sosa-Pineda et al. 2000). Targeted inactivation of *Prox1* resulted in the death of $Prox1^{+/-}$ mice within 2 to 3 d after birth in all but one of the tested genetic backgrounds (Wigle and Oliver 1999). The intestines of the heterozygous pups, in contrast to those of their wild-type littermates, were filled with chyle, the white fluid transported by the lymphatic vessels of the small intestine, a few hours before death (Wigle and Oliver 1999). This phenotype suggested a haploinsufficiency effect of *Prox1* during the normal development of the enteric lymphatic system; however, survival of a small percentage of heterozygous *Prox1* animals was obtained by crossing them into mouse strains of different genetic backgrounds (Wigle et al. 1999).

The detailed analysis of the *Prox1*-null mice revealed that the expression of *Prox1* in a restricted subpopulation of endothelial cells in the embryonic veins is required to promote lymphangiogenesis (Wigle and Oliver 1999). This analysis also determined that the initial localization of the Prox1-positive lymphatic endothelial cells in the cardinal vein and their subsequent migration from there occur in a polarized manner (Wigle and Oliver 1999). In *Prox1*-null mice, budding and sprouting of lymphatic endothelial cells from the veins appears unaffected at E10.5; however, this process is arrested prematurely at around E11.5–E12.0, and as a result, *Prox1*-null mice are devoid of lymphatic vasculature (Wigle and Oliver 1999).

Prox1-null embryos are the first mutants in which specific alterations of the development of the lymphatic vasculature were identified. The detailed analysis of *Prox1* expression in the lymphatic endothelium provided strong support for the original model proposed by Sabin (1902, 1904). Although Prox1 is expressed in a variety of cell types, among endothelial cells it is exclusively detected in embryonic lymphatic endothelial cells (Wigle and Oliver 1999) and in lymphatic vessels of adult tissues and tumors (Wigle et al. 2002).

A working model for embryonic lymphatic development in mammals

As mentioned above, several blood vascular markers are available, but only recently have markers of the lymphatic vasculature been identified. Remarkably, most of the blood vascular markers are also detected in the lymphatic vasculature (Sleeman et al. 2001). The level of expression of most of these markers in the lymphatic vasculature depends on the developmental stage of the embryo, the type of tissue being analyzed, or both. Similar considerations apply to other characteristics of the lymphatic vasculature such as the lack of a continuous basement membrane, which is reflected by the low expression of molecules such as laminin and collagen IV, or the low level of expression of surface antigens such as

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CD34. During embryogenesis, the different levels of expression of these markers probably reflect the state of differentiation or commitment of endothelial cells toward a lymphatic phenotype. For example, during early mouse development (E10.5–E11.5), the differences between the expression levels of laminin or CD34 in blood vasculature and in lymphatic vasculature are not as obvious as they are later in development (Sauter et al. 1998; Wigle et al. 2002).

Similarly, VEGFR-3 is expressed at comparable levels in blood and lymphatic vasculature during early embryonic development, but its expression later becomes down-regulated in the blood vasculature (Kaipainen et al. 1995; Wigle et al. 2002). Therefore, with the exception of the few lymphatic markers listed above, most available molecular markers can be detected in both blood and lymphatic vasculature during early embryonic development. In late embryonic and adult tissues, these markers become cell-type specific, a finding that suggests that blood and lymphatic vasculature have a common origin, or that one is derived from the other.

Because the lymphatic vasculature forms after the blood vasculature, and because only a few lymphatic-specific markers have been identified, one could argue that the expression of a limited number of additional genes in blood vascular endothelial cells is sufficient for the subsequent determination of the lymphatic vasculature. Support for this proposal has been provided by further characterization of the *Prox1*-null phenotype. Unlike the lymphatic endothelial cells that bud from the veins in E11.5 wild-type embryos, those of *Prox1*-null littermates do not coexpress any lymphatic markers. Instead, the mutant cells appear to have a blood vascular phenotype, as determined by the levels of expression of laminin and CD34 (Wigle et al. 2002). Therefore, *Prox1* activity may be required not only for maintenance of the budding of the venous endothelial cells but also for their differentiation to the lymphatic phenotype. These results suggest that during embryonic development, the default fate of the budding venous endothelial cells is to adopt the blood vascular phenotype; upon expression of Prox1 and other factors, these budding cells then switch to a lymphatic vasculature phenotype.

Based on the results presented above, a working model of the early embryonic steps leading to the development of the lymphatic vasculature has been proposed (Wigle et al. 2002). After the initial formation of the vascular system, venous endothelial cells become competent to respond to a lymphatic-inducing signal. The first indication that lymphangiogenesis has begun (lymphatic bias; Fig. 2) is the specific expression of Prox1 in a restricted subpopulation of endothelial cells located on one side of the anterior cardinal vein; in the mouse this expression occurs at ~E9.5 (Wigle and Oliver 1999). LYVE-1 is also expressed in endothelial cells in the cardinal veins at this stage, but not in a polarized manner. At ~E10.5, the restricted localization of Prox1 in the veins is still evident, and the first lymphatic endothelial cells have started to bud in a polarized manner.

All venous endothelial cells are probably initially bipotent, and the expression of at least Prox1 causes those cells to initiate the program of lymphatic differentiation.

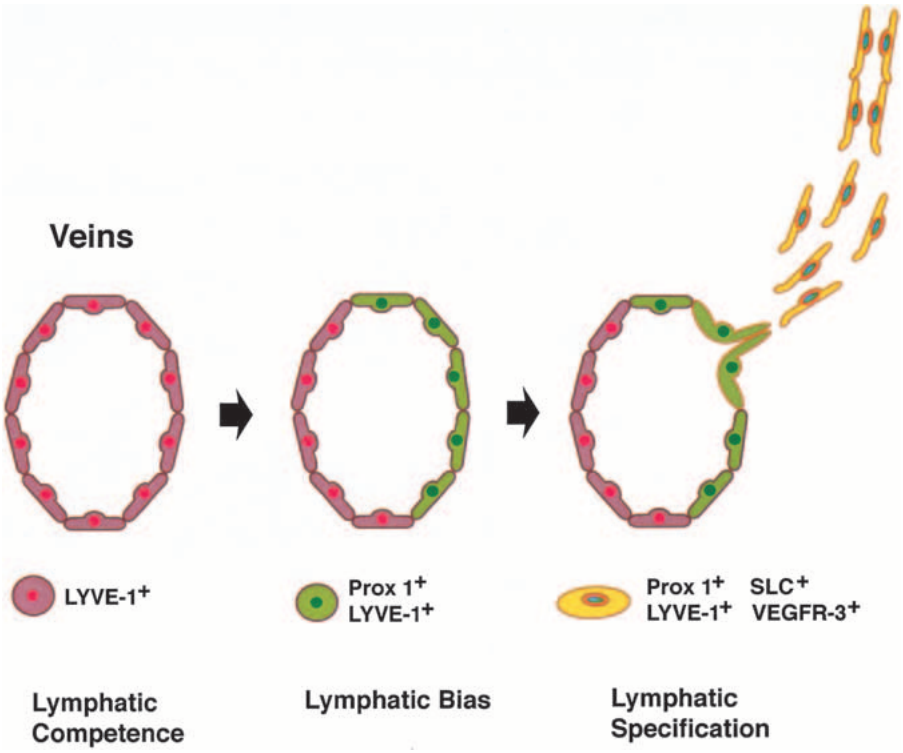

Figure 2. Summary of the proposed model for the embryonic development of the mammalian lymphatic vasculature.

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As development proceeds, the subpopulation of LYVE-1- and Prox1-positive endothelial cells starts to bud from the veins in an initially Prox1-independent manner. However, maintenance of the budding requires Prox1 activity. As the cells bud they start to express higher levels of additional lymphatic endothelial markers such as SLC and VEGFR-3, whereas the expression of VEGFR-3 decreases in blood vascular endothelial cells. The expression of Prox1, LYVE-1, SLC, and VEGFR-3 may indicate that the cells are irreversibly committed (specified) to the lymphatic pathway (Fig. 2).

Remaining questions about developmental lymphangiogenesis

The identification of lymphatic-specific markers will allow us to address many unanswered questions: Are lymphoangioblasts present in the mammalian embryo? Are venous endothelial cells initially pluripotent? If so, do pluripotent cells become committed to a lymphatic phenotype once Prox1 is expressed? Or does this commitment occur at an earlier stage in development? Is Prox1 activity sufficient to cause venous endothelial cells to bud from the veins and adopt a lymphatic phenotype? Is the polarized expression of Prox1 in the cardinal vein the consequence of a short-range signaling mechanism that operates only in that side of the vein, or does the polarized expression of Prox1 indicate that the cardinal vein already contains subpopulations of cells with previously determined lymphatic and vascular phenotypes?

Clinical implications of lymphatic system biology

In addition to its prominent role during embryonic development, lymphangiogenesis is also an essential feature of tissue repair and inflammatory reactions in most organs, and congenital or acquired dysfunctions of the lymphatic system, resulting in the formation of lymphedema, are frequent and often associated with impaired immune function (Witte et al. 2001). In addition, lymphangiogenesis is a common feature of vascular malformations (Witte et al. 2001). Finally, lymphatics are the primary conduit for malignant tumor dissemination to the regional lymph nodes, and recent evidence suggests an active role of malignant tumors in the induction of intratumoral and peritumoral lymphangiogenesis.

Lymphatic regeneration

Successful tissue repair requires the regrowth and reconnection of a functional lymphatic vascular system. Early studies showed the formation of lymphatic vessels in circumferential wounds in the rabbit, bridging the newly formed scar (Bellman and Oden 1958). In full-thickness skin wounds, ingrowth of new blood vessels (angiogenesis) into the newly formed granulation tissue largely dominates the delayed and comparatively less pronounced formation of new lymphatic vessels (Paavonen et al. 2000), which are predominantly located surrounding the blood vessel-rich granulation tissue. Lymphangiogenesis in the adult occurs by outgrowth from preexisting lymphatics (Clark and Clark 1932; Paavonen et al. 2000); it remains to be established whether lymphangiogenesis during tissue repair also involves the incorporation of progenitor cell populations, as in blood vessel angiogenesis (Rafii 2000), or the budding of lymphatic precursors from preexisting veins, similar to lymphatic development during embryogenesis. The recent discovery of specific lymphatic markers will greatly facilitate studies to address this issue in more detail.

During tissue repair, lymphatic vessels connect with lymphatic vessels, but not with blood vessels, and cultured lymphatic endothelial cells remain separated from blood vascular endothelial cells during tube formation of cocultured cells in vitro (Kriehuber et al. 2001). Whereas specific ephrins and their Eph receptors have been detected on arteries (Ephrin-B2) and veins (EphB4), specific molecules involved in lymphatic identity and homeotypic interactions remain to be identified. The establishment of well-characterized populations of cultured lymphatic- and blood-vessel-derived endothelial cells (Kriehuber et al. 2001; Makinen et al. 2001b) will now enable studies to identify such molecules. VEGF-C and VEGF-D, activating ligands of VEGFR-3, are prime candidates for molecules that control wound-associated lymphangiogenesis; however, despite the availability of a number of genetic mouse models for skin-specific overexpression or inhibition of bioactivity of VEGF-C and VEGF-D (Makinen et al. 2001a; Veikkola et al. 2001), the biological importance of these factors for tissue repair has not yet been established.

Very recent evidence suggests that VEGF, a major angiogenic molecule that is up-regulated during tissue repair (Brown et al. 1992), might also stimulate lymphangiogenesis under certain conditions, possibly via interaction with VEGFR-2 that is also expressed by lymphatic endothelial cells (Kriehuber et al. 2001; Makinen et al. 2001b). Moreover, subcutaneous injection of adenoviral VEGF constructs into mouse ear skin resulted both in enhanced formation of new blood vessels and in increased numbers of enlarged, proliferating lymphatic vessels (H.F. Dvorak, pers. comm.). However, because VEGF also potently induces vascular leakage and tissue edema, it remains to be established whether the lymphangiogenesis observed in conditions with enhanced VEGF tissue levels, including tissue repair and inflammation, is caused by direct activation of VEGFR-2 on lymphatic endothelium or by indirect stimulation of lymphangiogenesis by enhanced interstitial fluid accumulation.

Lymphedema

Our insights into the molecular and genetic mechanisms of lymphedema formation have been greatly enhanced over the last few years. This is mainly attributable to: (1) the discovery of gene mutations in two different types of lymphedema, (2) the identification of specific lymphangiogenesis factors and their receptors on lymphatic en-

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dothelium, and (3) the recent development of genetic mouse models for cutaneous lymphedema. Lymphedema is caused by an insufficient transport function of lymphatic vessels owing to lymphatic hypoplasia, impaired lymphatic function, or obstruction of lymph flow (Witte et al. 2001). Two recently identified lymphangiogenic factors, *VEGF-C* and *VEGF-D*, and their lymphatic receptor *VEGFR-3* most likely play an important role in the pathogenesis of at least some cases of lymphedema. Primary lymphedema has been classified as Milroy disease when present at birth (Milroy 1892), or as Meige disease, which develops predominantly after puberty (Meige 1898). Both diseases are characterized by a combination of dilated lymphatic capillaries and interstitial accumulation of lymph fluid leading to lymphedema. It has been shown that Milroy disease is linked, at least in some families, to the *VEGFR-3* locus on distal chromosome 5q (Ferrell et al. 1998; Witte et al. 1998; Evans et al. 1999). Subsequently, missense mutations in the *VEGFR-3* gene that interfere with the *VEGFR-3* tyrosine kinase signaling function were identified in several cases of hereditary, early-onset lymphedema (Karkkainen et al. 2000).

Whereas *VEGFR-3* inactivating mutations have been found in a relatively small number of cases of hereditary lymphedema thus far, additional supportive evidence for a role of *VEGFR-3* in the pathogenesis of lymphedema stems from experimental studies in transgenic mice with skin-specific overexpression of soluble VEGFR-3 using a *keratin 14* transgene promoter. In this genetic model, soluble VEGFR-3 is secreted at high levels by basal epidermal keratinocytes and binds both lymphangiogenesis factors, VEGF-C and VEGF-D (Fig. 3), thereby preventing them from activating VEGFR-3 on lymphatic endothelium (Makinen et al. 2001b). K14/soluble VEGFR-3 transgenic mice lack a functional cutaneous lymphatic system and are characterized by lymphedema formation in the skin.

Further experimental evidence for a role of the VEGF-C/VEGF-D/VEGFR-3 system in lymphedema formation has been provided by the identification of a heterozygous inactivating *VEGFR-3* mutation in the germ line of *Chy* mutant mice, which develop chylous ascites and lymphedematous limb swelling after birth (Karkkainen et al. 2001). Remarkably, therapeutic increase of tissue VEGF-C levels by virus-mediated gene therapy stimulated the growth of functional lymphatics in *Chy* mutant mice, suggesting that growth factor gene therapy might be applicable to at least some cases of human lymphedema (Karkkainen et al. 2001).

Despite the important role of *VEGFR-3* mutations in a subset of hereditary lymphedemas, primary lymphedemas are comprised of a heterogeneous group of diseases that can be associated with additional malformations of other organ systems. In one such disease entity, lymphedema-distichiasis, an autosomal-dominant disorder with congenital lymphedema, double rows of eyelashes (distichiasis), and other complications, inactivating mu-

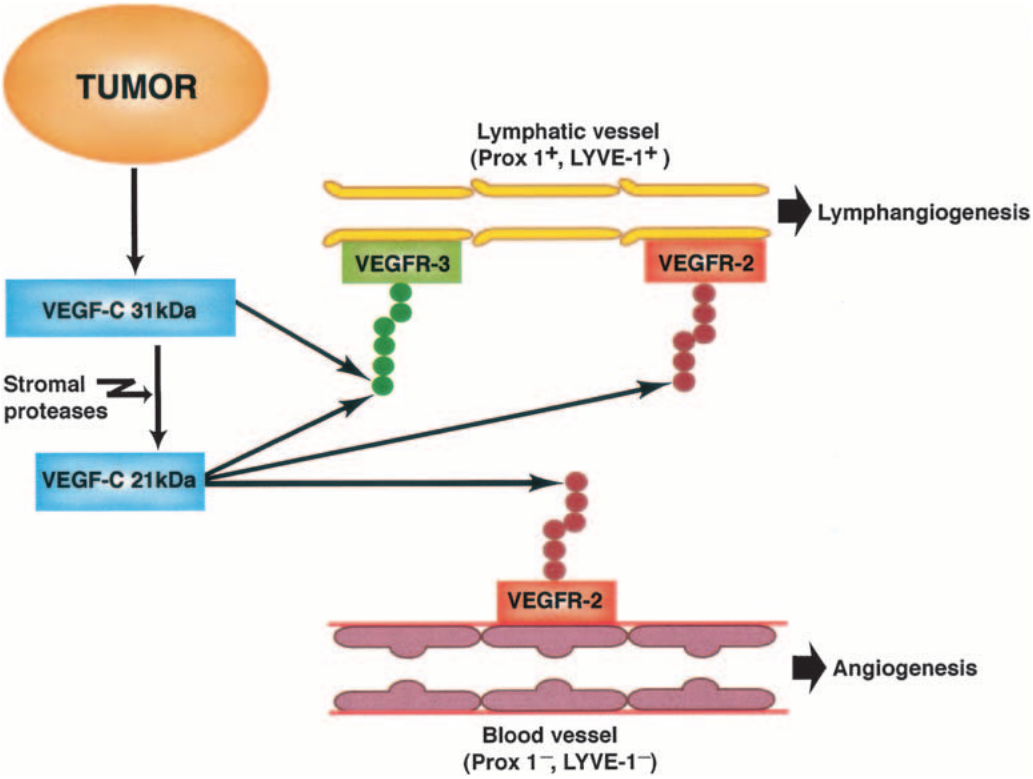

Figure 3. The differential effects of tumor-secreted VEGF-C on blood vascular angiogenesis versus lymphangiogenesis are dependent on proteolytic processing after secretion.

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tations in the *FOXC2* gene were identified in several families (Fang et al. 2000). *FOXC2* is a member of the forkhead/winged-helix family of transcription factors involved in diverse developmental pathways. Additional lymphatic-specific growth factor receptors, matrix molecule receptors, and transcription factors are likely involved in other cases of hereditary lymphedema and lymphatic malformations.

Tumor lymphangiogenesis

In most human cancers, the lymphatic system serves as the primary conduit for the metastatic spread of tumor cells to regional lymph nodes and, possibly, via the thoracic duct and the blood circulation to distant organs. Moreover, tumor cell metastasis to lymph nodes represents a major criterion for evaluating the prognosis of cancer patients and for the choice of additional chemotherapy and/or radiation therapy after excision of primary tumors. However, despite the importance of tumor-associated lymphatic vessels for cancer progression, little information has been available regarding the molecular mechanisms by which tumor cells gain access to the lymphatic system and consequently are able to spread. In fact, the presence of functional lymphatic vessels within tumors has been questioned because of the high interstitial pressure within most cancers (Jain 1989), and a widely held view has assigned the lymphatic system a passive role during the metastasis process (Folkman 1995; Carmeliet and Jain 2000).

The major reasons for this lack of insight into the early metastatic process have been: (1) the absence of specific markers for tumor-associated lymphatic vessels, (2) the lack of knowledge about specific lymphangiogenesis factors and the ligand–receptor systems that mediate tumor cell migration toward the lymphatics, and (3) the absence of experimental cancer metastasis models for the quantitative evaluation of lymph node metastasis even at the single-cell level. Based on the discovery of several lymphatic-specific markers, in particular of the hyaluronan receptor LYVE-1, and of several growth factors and chemokines involved in lymphatic growth and function, major scientific advances during the last year have provided new insights into the molecular mechanisms that control lymphatic metastasis. These experimental studies have also provided convincing evidence for an active role of malignant tumor cells in inducing peritumoral and intratumoral lymphangiogenesis, taking advantage of molecular mechanisms operative in the immune response, and for a potential role of tumor lymphangiogenesis as a novel prognostic marker for at least some types of human cancers.

Using an orthotopic human MDA-435 breast cancer model in immunosuppressed mice, it was shown that lymphatic vessels are, indeed, present both surrounding and within malignant tumors, and that overexpression of the lymphangiogenesis factor VEGF-C resulted in enhanced infiltration of breast cancers by proliferating lymphatic vessels that frequently contained cancer cells (Skobe et al. 2001a). Moreover, VEGF-C-induced tumor lymphangiogenesis resulted in enhanced tumor metastasis to regional lymph nodes, and the extent of lung metastasis was highly correlated with the extent of lymphangiogenesis of the primary tumor. Whereas VEGF-C selectively induced lymphangiogenesis, but not angiogenesis, in breast cancer models (Karpanen et al. 2001; Skobe et al. 2001a), subsequent studies of human malignant melanoma xenotransplants (Skobe et al. 2001b) revealed induction of both lymphangiogenesis and angiogenesis by tumor-derived VEGF-C. These apparently conflicting biological effects could be explained by the detection of the fully processed, mature 21-kD form of VEGF-C in melanomas (Skobe et al. 2001b). The 21-kD form is a cleavage product of the secreted 31-kD VEGF-C and activates both VEGFR-2, present on blood vascular endothelium, and VEGFR-3 (Joukov et al. 1997), predominantly expressed in lymphatic vessels, resulting in both lymphangiogenesis and vascular angiogenesis (Fig. 3). In contrast, only the secreted 31-kD form of VEGF-C, that selectively activates VEGFR-3, was found in the breast cancer model. These results indicate an important role of the in vivo processing of VEGF-C in lymphangiogenesis versus angiogenesis (Fig. 3).

The pro-metastatic role of growth factor-induced tumor lymphangiogenesis has also been reported in xenotransplant models of VEGF-D-transfected transformed human kidney cells (Stacker et al. 2001), of VEGF-C-transfected MCF-7 breast cancer cells (Karpanen et al. 2001), and in a genetic model of pancreas tumorigenesis in which VEGF-C expression, driven by the rat insulin promoter, was targeted to β-cells of the endocrine pancreas, resulting in enhanced rates of lymph node metastasis (Mandriota et al. 2001). Whereas these results indicate that tumor cells can actively induce tumor-associated lymphangiogenesis and lymphatic metastasis, a recent report indicates that lymphatic vessels might, in turn, actively promote tumor cell attraction and lymphatic metastasis (Wiley et al. 2001). Secondary lymphoid chemokine (SLC; 6Ckine/exodus-2/CCL21) is produced constitutively by lymphatic endothelial cells in the skin and other organs (Gunn et al. 1998) and attracts dendritic cells to the lymphatic vessels by interaction with the CCR7 receptor. CCR7 is also expressed by some human breast cancer and melanoma cell lines (Muller et al. 2001). When CCR7-transduced B16 malignant melanoma cells were injected into the footpads of mice, a >10-fold increase in the incidence of regional lymph node metastases was observed after 3 wk (Wiley et al. 2001), and metastasis was completely blocked by adding neutralizing anti-SLC antibodies. SLC, via the CCR7 receptor, selectively enhanced lymphatic metastasis, because CCR7-transduced and control B16 cells metastasized to the lung at the same frequency after intravenous injection (Wiley et al. 2001).

Taken together, these pioneering studies provide important new insights into the molecular control of lymphatic cancer metastasis. They also raise several new questions: (1) Is the lymphatic marker LYVE-1, that was used in most of these studies, specific for tumor-associated lymphatic vessels, or might LYVE-1 also be re-ex-

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pressed by tumor-associated blood vessels, similar to the reported re-expression of VEGFR-3 on some tumor blood vessels (Valtola et al. 1999)? (2) Is the presence of intratumoral lymphatic vessels restricted to tumor xenotransplant models because of trapping of lymphatic vessels in between growing tumor foci in xenografts that start from an injected cell population (Karpanen and Alitalo 2001), or can they also be detected during orthotopic, multistep carcinogenesis and in autochthonous human cancers? (3) Does tumor lymphangiogenesis also occur in human tumors, and will the quantification of tumor-associated lymphatic vessels serve as a new prognostic tool for determining the likelihood of primary human cancers for metastatic spread? (4) Are the molecular mechanisms that control lymphatic tumor metastasis also important for the survival of metastatic cancer cells within the lymphatic system or in distant organs, and might they, therefore, serve as new therapeutic targets for the treatment of advanced cancer?

The answers to some of these questions are beginning to emerge. LYVE-1 has indeed been shown to be specifically expressed by lymphatics in normal murine and human tissues (with the exception of liver sinusoidal endothelial cells that are strongly involved in hyaluronan uptake; Carreira et al. 2001) and in tumors of both murine and human origin (Wigle et al. 2002). In normal human skin, LYVE-1 is specifically expressed by lymphatic endothelium, but not by blood vascular endothelium that expresses the marker CD34 (Fig. 4A). In contrast to the proposed lymphatic marker podoplanin that is also expressed on some CD34-positive cutaneous endothelial cells (Kriehuber et al. 2001), we and others (Jackson et al. 2001) have consistently found that CD34-positive blood vessels are negative for LYVE-1 (Fig. 4A).

LYVE-1 is also specifically expressed by tumor-associated lymphatic vessels, and there is no evidence, thus far, that tumor-associated blood vessels reexpress *LYVE-1*. Double-staining for LYVE-1 and the lymphatic-specific transcription factor Prox1 revealed that all LYVE-1-positive lymphatic vessels within and surrounding human squamous cell carcinoma transplants also expressed Prox1 (Wigle et al. 2002). Similar results have been recently obtained during chemically induced multistep skin carcinogenesis in mice. This experimental model allows a detailed analysis of the successive stages of skin cancer development and has provided valuable insights into the importance of the blood vascular system for tumor progression and metastasis (Hawighorst et al. 2001). The squamous cell carcinomas that develop by malignant conversion from benign papillomas in this model are characterized by slow expansive growth. Similar to previously reported results in xenotransplant models (Skobe et al. 2001a), double stains for LYVE-1 and Prox1 revealed that both peritumoral as well as intratumoral lymphatic vessels were present in these tumors and that all LYVE-1-positive vessels expressed Prox1 (Fig. 4B). Proliferating lymphatic endothelial cells were found surrounding and within the tumors (Fig. 4C,D), showing that active lymphangiogenesis also occurs in orthotopic tumors.

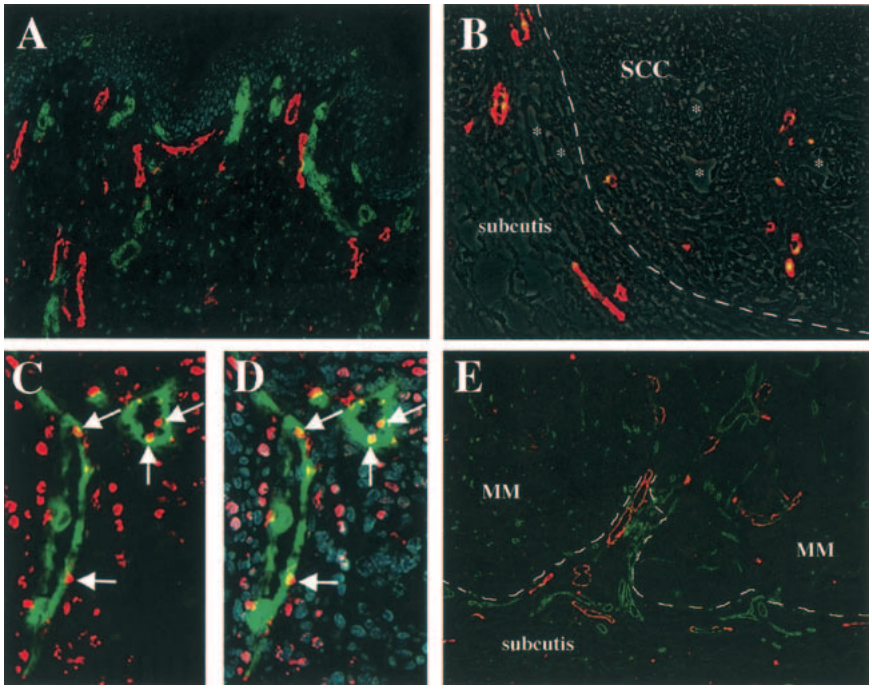

Figure 4. Specific detection of lymphatic and blood vessels in normal skin and in malignant murine and human tumors. (*A*) Mutually exclusive staining of lymphatic vessels with anti-LYVE-1 antibody (green) and of blood vessels with anti-CD34 antibody (red) in normal human neonatal foreskin. Nuclei are labeled blue (Hoechst stain). The epidermis is avascular. (*B*) Double-immunofluorescence stain of a chemically induced murine squamous cell carcinoma (SCC) for LYVE-1 (red) and Prox1 (green/yellow; nuclear stain) reveals peritumoral and intratumoral lymphatic vessels and confirms the specificity of LYVE-1 for tumor-associated lymphatics. Asterisks indicate blood vessels that do not express LYVE-1 or Prox1. The interrupted line indicates the border between the tumor and the subcutis. (*C*,*D*) Double immunofluorescence stain for LYVE-1 (green) and proliferating cell nuclear antigen (red) shows proliferating lymphatic endothelial cells (orange; arrows) within a murine squamous cell carcinoma. In *D*, a nuclear Hoechst stain (blue) shows proliferating tumor cells surrounding the lymphatic vessel. (*E*) Double immunofluorescence stain of a human malignant melanoma (MM) of the skin for the pan-vascular marker CD31 (green) and the lymphatic marker LYVE-1 (red/orange) reveals pronounced tumor angiogenesis (green vessels) and lymphangiogenesis (orange vessels), both surrounding and within (*upper right* corner) the tumor. The interrupted line indicates the border between the tumor and the surrounding tissue.

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Although mounting evidence shows that lymphangiogenesis occurs in experimental tumor models in mice, with important implications for tumor metastasis, it has been questioned whether lymphangiogenesis also occurs in human cancer and whether the presence of tumor-associated lymphatic vessels might have any functional consequences (Jain 1989; Carmeliet and Jain 2000; Carreira et al. 2001). Recent results suggest that, at least in certain types of human cancer, tumor lymphangiogenesis does occur and that the presence of tumor-associated lymphatic vessels is correlated with increased lymphatic tumor metastasis. Whereas no lymphatic capillaries were detected associated with invasive breast cancer (Jackson et al. 2001), proliferating intratumoral lymphatics have been detected in head and neck squamous cell carcinomas (Jackson et al. 2001). In addition, in oropharyngeal tumors, a high density of LYVE-1-positive lymphatic vessels correlated with the presence of regional lymph node metastases (Beasley et al. 2002). Recent studies using podoplanin as a lymphatic marker determined a significant correlation between the lymphatic microvascular density and the lymph node status in human breast cancer (Schoppmann et al. 2001); on the contrary, increased lymphatic microvessel density was associated with a favorable prognosis in early-stage cervical cancer (Birner et al. 2001). Although this is encouraging, further studies will be needed to compare the specificity of various lymphatic markers for tumor-associated lymphatic vessels and to evaluate the functionality of tumor-associated lymphatic vessels in more detail. Peritumoral and intratumoral lymphatic vessels are also found in human malignant melanomas (Fig. 4E), tumors that metastasize frequently and early to the regional lymph nodes via the lymphatic system (de Waal et al. 1997; Clarijs et al. 2001).

Conclusions

Finally, after more than 300 years since the initial description of the lymphatic vessels by Gasparo Aselli, some of the mechanisms controlling the normal and pathological development of the previously neglected lymphatic vasculature are being unraveled. The identification of specific markers for the lymphatic vessels has been instrumental in this advance. In addition, the recent concept of tumor lymphangiogenesis is starting to be considered as an important aspect of cancer metastasis. The future of this field of research is very promising and could eventually lead to better diagnosis and treatment of a variety of lymphatic disorders and certain types of cancer.

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