

Lymphatic endothelium: morphological, molecular and functional properties

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The lymphatic microvasculature is uniquely adapted for the continuous removal of interstitial fluid and proteins, and is an important point of entry for leukocytes and tumor cells. The traditional view that lymphatic capillaries are passive participants in these tasks is currently being challenged. This overview highlights recent advances in our understanding of the molecular mechanisms underlying the formation and function of lymphatic vessels.

Introduction

The lymphatic system complements functions of the blood vascular system by regulating tissue fluid balance, facilitating interstitial protein transport, and serving immunological functions. Fluid and macromolecules that exit blood capillaries are collected from the interstitial space by lymphatic capillaries and returned back to the blood circulation through the network of larger lymphatics. Lymphatics are also responsible for absorption of fat from the gut. By directing leukocytes and antigens from tissues to lymph nodes, lymphatic vessels play an essential role in initiating the immune response. Although the lymphatic and blood vascular systems rely on each other for the maintenance of tissue homeostasis, they are structurally and functionally distinct entities.

Whereas the main function of large lymphatics is efficient transport of lymph back into the blood circulation, the lymphatic microvasculature is responsible for the uptake of components from the interstitium. Given their central role in regulating interstitial fluid pressure and cell trafficking, it is surprising that lymphatic endothelial cells (LECs) have until recently been poorly characterized, at least from a molecular point of view. This scenario is changing rapidly following the development of techniques for the isolation of pure LECs and the characterization of their molecular properties.

Structure–function relationships of the lymphatic capillary

Lymphatic capillaries are blind-ending vessels, comprised of a single, nonfenestrated endothelial cell layer, that is optimally

adapted for the uptake of fluid, macromolecules, and cells. Although LECs have many properties in common with the endothelium of blood vessels, they also have very distinct structural features that have been best characterized at the ultrastructural level. Lymphatic capillaries generally possess a more irregular and wider lumen than blood capillaries, and their endothelium is extremely attenuated. In contrast to blood vessels, lymphatic capillaries have an incomplete basement membrane and are not invested by pericytes. They are generally observed in a partially or fully collapsed state (Schmid-Schönbein, 1990a; Aukland and Reed, 1993). Unique to lymphatic capillaries are also overlapping intercellular junctions that are formed by the extensive superimposition of adjacent LECs. An increase in interstitial fluid pressure causes these junctions to open, thereby permitting the easy passage of fluid and particles into the vessel. As fluid enters the lumen, pressure differences across the vessel wall decrease and the junctions begin to close, preventing retrograde flow back into the interstitium (Fig. 1) (Schmid-Schönbein, 1990b; Ikomi and Schmid-Schönbein, 1996). Lymphatic capillary function is critically dependent on its connections with the ECM. LECs are attached to interstitial collagen by anchoring filaments, composed of elastic fibers (Leak and Burke, 1966; Gerli et al., 1990), which preserve functionality of lymphatics when interstitial pressure rises by preventing vessel collapse. The composition and organization of the ECM are thus also likely to play a critical role in lymphangiogenesis.

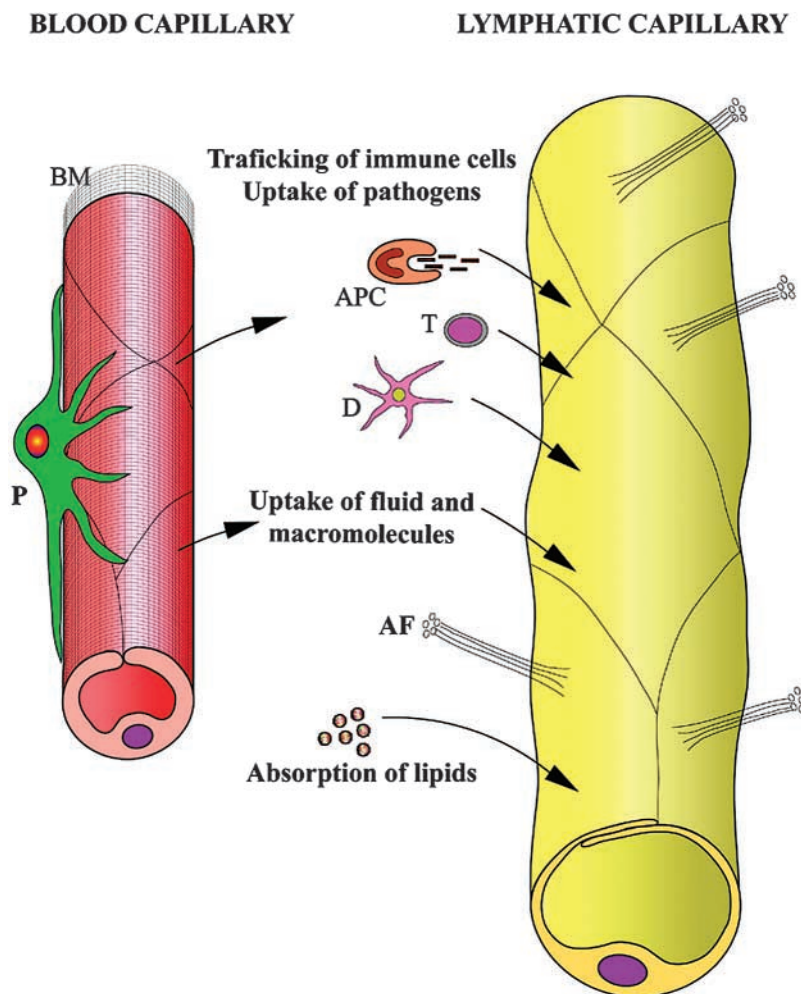
Molecular regulation of lymphatic vessel formation and differentiation

During development and wound healing, angiogenesis generally precedes lymphangiogenesis, implying the existence of distinct yet spatially and temporally coordinated regulatory mechanisms. Two members of the VEGF family, VEGF-C and VEGF-D, have been demonstrated to play a critical role in lymphangiogenesis via activation of VEGFR-3, which is expressed mainly by LECs in normal adult tissues (Joukov et al., 1996; Lee et al., 1996; Achen et al., 1998). VEGFR-3

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Abbreviations used in this paper: BEC, blood endothelial cell; CCL, chemokine ligand; CCR, chemokine receptor; CLEVER-1, common lymphatic endothelial and vascular endothelial receptor-1; LEC, lymphatic endothelial cell; MR, mannose receptor; VEGF, vascular endothelial growth factor.

Figure 1. Characteristic structure and function of the lymphatic microvasculature. The lymphatic capillary is uniquely adapted for the uptake of fluid, lipids, macromolecules, and cells from the interstitium. In contrast to the blood capillary, the lymphatic capillary has poorly developed basal lamina (BM) and is devoid of pericytes (P). Lymphatic endothelium is highly attenuated, and cells are connected directly to the interstitial collagen via anchoring filaments (AF). T, T cell; D, dendritic cell; APC, antigen presenting cell.



signaling is important for development of the embryonic lymphatic system, lymphatic regeneration in the adult, and tumor lymphangiogenesis (Alitalo and Carmeliet, 2002). VEGF-C and VEGF-D, when fully proteolytically processed, can also activate VEGFR-2 (Joukov et al., 1997), but whether VEGFR-2 plays a direct role in lymphangiogenesis is less clear.

VEGF-C also binds to a nonkinase receptor neuropilin-2 (NRP2) (Karkkainen et al., 2001), a classic receptor for class III semaphorins, which regulate chemorepulsive guidance of developing axons. Recent studies in NRP2-deficient mice demonstrated impeded development of lymphatic capillaries in most tissues, suggesting a role for NRP2 in LEC proliferation and, perhaps, guidance. NRP2 may cooperate with VEGFR-3 to mediate VEGF-C-dependent lymphangiogenesis (Yuan et al., 2002).

Finally, Ang2 is expressed by LECs (Petrova et al., 2002; Podgrabinska et al., 2002) and is required for the proper development of the lymphatic system (Gale et al., 2002). Mice deficient in Ang2 displayed disorganization and hypoplasia of lymphatic capillaries, and collecting lymphatic vessels were not properly invested by smooth muscle. As a result, Ang2 knockout mice developed severe lymphedema. Interestingly, the lymphatic phenotype caused by Ang2 deficiency was rescued by Ang1, suggesting redundant roles for these molecules in lymphatic development.

The homeobox transcription factor Prox-1 appears to be required for the commitment of endothelial cells to the lymphatic differentiation program (Wigle and Oliver, 1999; Wigle et al., 2002). Prox-1 expression in embryos localizes to a subpopulation of endothelial cells in embryonic veins, which are committed to the lymphatic pathway. Functional inactivation of Prox-1 in mice results in the arrest of lymphatic vessel development. In adult tissues, Prox-1 is expressed exclusively by LECs, and overexpression of Prox-1 in blood endothelial cells (BECs) down-regulated BEC-specific transcripts and up-regulated LEC-specific transcripts, thus conferring the lymphatic endothelial phenotype on these cells (Hong et al., 2002; Petrova et al., 2002). Most recent evidence suggested that the adaptor protein SLP76 and the tyrosine kinase syk, which are expressed primarily in hematopoietic cells, may also contribute to the anatomical separation of the blood and lymphatic vasculature (Abtahian et al., 2003).

Isolation and molecular characterization of LECs

Many attempts have been made in the past to isolate and culture LECs from a variety of species (Pepper, 2001). All of these studies have described isolation of the cells from large lymphatic vessels and have employed crude mechanical methods of cell separation. Since large lymphatics are supplied by a rich network of nutritive blood vessels, the purity of the isolated cell populations has remained in question.

Furthermore, given the heterogeneity of endothelial cells from different vascular beds, large vessel endothelial cells are likely to be inappropriate for the study of lymphatic capillary structure–function relationships. The identification of cell surface markers that reliably distinguish lymphatic endothelium from blood vascular endothelium (Sleeman et al., 2001) has led to the development of superior techniques for the isolation of pure lymphatic and blood vascular endothelial cells. LECs have been isolated by positive selection using antibodies to podoplanin (Kriehuber et al., 2001), VEGFR-3 (Makinen et al., 2001), or LYVE-1 (Podgrabinska et al., 2002), and by a negative selection with antibodies to CD34 (Hirakawa et al., 2003).

The above studies demonstrated that LECs and BECs retain their differentiated phenotypes in culture. LECs were distinguished by their homotypic association, selective responsiveness to VEGF-C in terms of growth, survival and morphogenesis, differential ECM requirements, and the distinct gene expression profile. LECs established by the different methods, however, exhibited certain differences in gene expression that may be attributed to the different source of tissues employed, i.e., adult versus neonatal skin. Alternatively, the different isolation strategies may select for specific subpopulations of LECs. LECs isolated using VEGFR-3 antibodies may be partly contaminated with BECs, since VEGFR-3 can also be expressed by the blood vascular endothelium (Partanen et al., 1999). Finally, isolated LECs were propagated under different conditions, which may further account for the variations in phenotype. It remains to be determined which purification strategy and culture conditions allow for optimal preservation of the lymphatic endothelial phenotype *in vitro*.

The availability of microvascular LECs now permits analyses of their molecular and functional characteristics. The molecular signature of LECs appears to reflect their unique functional characteristics and provides novel insight into the molecular basis of lymphatic function (Petrova et al., 2002; Podgrabinska et al., 2002; Hirakawa et al., 2003). For example, LECs express remarkably high levels of genes implicated in protein metabolism, sorting and trafficking (Podgrabinska et al., 2002). Genes with particularly high representation were those encoding proteins that control specificity of vesicle targeting and fusion, such as members of the SNARE family, rab GTPases, AAA ATPases, and sec-related proteins (Mellman and Warren, 2000), indicating the existence of a robust vesicular transport system. The lymphatic endothelium is characterized by an abundance of membrane invaginations and cytoplasmic vesicles (Leak, 1972, 1976), yet their functional significance has not been established. Intercellular clefts are considered to be a major pathway for the movement of fluid and proteins into lymphatics (Schmid-Schönbein, 1990b). However, some early studies also demonstrated the presence of interstitially injected molecular tracers within intracellular vesicles of LECs (Leak, 1972, 1976). In agreement with these findings, the results of the gene profiling studies suggest that, in addition to intercellular transport, transendothelial pathways may also be used as a mechanism for entry of molecules into lymphatics (Podgrabinska et al., 2002). This raises the intriguing possibility that lymphatics may have the capacity to selectively re-

move molecules from the interstitium and thereby actively control the composition of lymph and interstitial fluid.

Role of lymphatic vessels in tumor dissemination

The importance of the lymphatic system as a pathway for metastasis has been well recognized. Metastasis of most cancers occurs initially through the lymphatics and the extent of lymph node involvement is one of the most important prognostic indicators of patient outcome. Traditionally, the lymphatic system has not been considered to be actively involved in the process of metastasis. Tumor cells are believed to be passively carried into the lymphatic vessels with the interstitial fluid and proteins (Hartveit, 1990), and the prevailing view has been that lymphangiogenesis is not a part of tumorigenesis (Carmeliet and Jain, 2000; Leu et al., 2000; Padera et al., 2002).

Recent studies, however, have demonstrated enlarged lymphatic vessels and lymphangiogenesis in peritumoral areas of several human tumors using lymphatic endothelial markers (Stacker et al., 2002; Pepper et al., 2003). The number of tumor-associated lymphatics has been correlated with lymph node metastases, yet intratumoral lymphatics have so far been observed only in human head and neck cancers and in melanoma. The relative importance of preexisting versus newly-formed lymphatic vessels to lymphogenous metastasis is not understood. Although preexisting peritumoral lymphatics are likely to be sufficient for tumor spread, recruitment of lymphatic vessels into the close proximity of a tumor may increase the propensity of tumors to metastasize. Increased lymphatic vessel density and/or presence of intratumoral lymphatics should therefore be regarded as an additional pathway rather than a necessity for metastasis.

Notably, a large number of studies demonstrated a striking correlation between the VEGF-C expression in human tumors and lymph node metastases (Stacker et al., 2002; Pepper et al., 2003). Moreover, recent experimental studies using VEGF-C-overexpressing tumor cells have provided direct evidence for the causal role of VEGF-C in tumor lymphangiogenesis and lymphogenous metastasis (Mandriota et al., 2001; Skobe et al., 2001). Although an increase in lymphatic vessel density may promote tumor spread simply by creating more opportunities for metastatic tumor cells to leave the primary tumor site, lymphatic vessels may also play a more active role in metastasis. For example, soluble factors constitutively expressed by LECs may facilitate tumor cell invasion of lymphatic vessels. Activation of LECs by VEGF-C or other factors produced by a tumor could promote release of chemokines, which may attract tumor cells into the lymphatics. As the migration of cancer cells to regional lymph nodes resembles physiological migration of leukocytes, it is conceivable that the chemokine-mediated normal mechanisms of lymphocyte homing may also be used for metastatic dissemination.

Thus far, the importance of two chemokine receptors (CCRs) in lymph node metastasis has been established: CXCR4 and CCR7. CXCR4 was found to be up-regulated in malignant melanoma and in breast cancer, whereas its ligand CXCL12 is highly expressed in lymph nodes and other target organs for breast cancer metastasis. A neutralizing antibody to CXCR4 inhibited metastases to lymph

nodes and other organs, demonstrating a critical role for this chemokine/receptor system in mediating tumor cell homing (Muller et al., 2001). CCR7 and its ligands chemokines CCL19 and CCL21 are of crucial importance for the migration of lymphocytes and dendritic cells to lymph nodes. CCR7 was also found to be highly expressed by human malignant melanoma and breast cancer cells (Muller et al., 2001), and its expression has been associated with lymph node metastasis in gastric cancer (Mashino et al., 2002) and in nonsmall cell lung cancer (Takanami, 2003). Overexpression of CCR7 in a mouse model of melanoma enhanced metastases to lymph nodes, which could be blocked by neutralizing its ligand CCL21 (Wiley et al., 2001). CCL21 and several other chemokines are constitutively expressed by LECs (Kriehuber et al., 2001; Makinen et al., 2001; Podgrabinska et al., 2002), suggesting an active role for LECs in governing cell migration in normal physiology and in cancer. However, a direct role for lymphatic endothelium in the process still remains to be demonstrated.

Mechanisms mediating tumor cell transmigration across the lymphatic endothelium into this lymphatic vessels also remain obscure. The prevailing view has been that tumor cells passively enter lymphatics between intercellular junctions. Based on the differences in their structure, it has been assumed that the entry of cells into lymphatics is easier than into blood vessels. An alternative novel hypothesis is that tumor cell entry requires adhesive interactions with LECs. There is no direct experimental evidence in support of either concept.

Thus far, very few cell adhesion molecules expressed by LECs have been identified. Several genes encoding proteins that constitute adherens junctions, such as desmoplakin, plakoglobin, plakophilin 2, H-cadherin, and zona occludens 2, were identified in LECs by gene profiling (Petrova et al., 2002; Podgrabinska et al., 2002). Another junctional adhesion molecule belonging to the immunoglobulin superfamily, JAM-2, was found to be expressed in tight junctions of lymphatic vessels and was shown to facilitate lymphocyte transmigration (Aurrand-Lions et al., 2001; Johnson-Leger et al., 2002). The nature of the lymphatic endothelial junctions may indeed facilitate cell entry and the identification of adhesion molecules typical for lymphatic endothelium may be particularly important for the understanding of leukocyte trafficking and tumor metastasis via lymphatics.

In this regard, macrophage mannose receptor (MR) I expressed by LECs has been shown to mediate adhesion of lymphocytes to lymphatics in lymph nodes (Irjala et al., 2001). MR on LECs supports lymphocyte binding to lymphatic vessels in an L-selectin-dependent fashion, and this interaction has been suggested to control lymphocyte exit from the lymph nodes. MR was also found to be selectively expressed by cultured LECs (Petrova et al., 2002; Podgrabinska et al., 2002), and its presence on afferent lymphatics suggests its possible involvement also in lymphocyte exit from peripheral tissues. Common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) is another recently identified adhesion molecule implicated in binding of lymphocytes to LECs in lymph nodes (Irjala et al., 2003b). Because CLEVER-1 is an inducible vascular adhesion mole-

cule, it has been suggested to regulate migration of leukocytes to sites of inflammation. MR and CLEVER-1 expression have also been detected on peri- and intratumoral lymphatic vessels in human head and neck, and breast carcinomas (Irjala et al., 2003a). Notably, expression of MR on intratumoral lymphatic vessels was associated with increased lymph node metastases in breast cancer. These pioneering studies aid in shaping the new concept of a more active role of lymphatic vessels in cancer.

Summary and perspectives

Exquisitely detailed descriptive studies performed almost 100 years ago provided the basis for our understanding of the structure-function relationships in the lymphatic system. Today, we can truly speak of a renaissance in the field, owing to the identification of lymphatic specific markers and growth factors, as well as the sophistication of the techniques for isolation of pure LECs. The groundwork has thus been laid for study of the molecular mechanisms underlying the characteristic functions of lymphatic vasculature. Better understanding of the lymphatic endothelial properties and how they may be altered in inflammation and in cancer may open a new door to therapeutic interventions.

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