Histol Histopathol (2005) 20: 155-175

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Characteristics of lymphatic endothelial cells in physiological and pathological conditions

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Summary. Impairment of lymphatic structure and function, e.g., inadequate endothelial permeability and intercellular openings, abnormal lymphangiogenesis and overexpression for immunoreactive agents, will result in tumor metastasis, autoimmune response alteration and accumulation of interstitial fluid and proteins. Recently, several novel molecules have been identified that allow a more precise distinction between lymphatic and blood vascular endothelium. The differences in expression of endothelial markers on the lymphatic vessel strongly suggest the possibility that there will be important divergence in the differentiating and regenerating responses in lymphatic behavior to various pathological processes. Undoubtfully, molecular techniques would also lead to the definition of unique markers found on lymphatic endothelial cells (LECs) in lymphatic-associated diseases which are mostly involved in lymphangiogenesis. This review is mainly concentrated on the characteristics of LECs in diabetes, wound healing, lymphedema and tumor, especially in the experimental models that have offered insight into the LEC role in these diseases affecting the lymphatic system. Increased knowledge of the molecular signaling pathways driving lymphatic development and lymphangiogenesis should boost the impact of therapeutics on the diseases. Although the field about the mechanisms that control the formation and lineage-specific differentiation and function of lymphatic vessels has experienced rapid progress in the past few years, an understanding of the basis of the differences and their implications in the pathological conditions will require much more investigation.

Key words: Lymphaticc endothelial cell, Diabetes, Wound healing, Lymphedema, Tumor

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Introduction

The lymphatic system, a one-way drainage system, transports filtered, synthesized, or absorbed macromolecules and excess interstitial fluid from tissue space to the blood circulation and directs lymphocytes and antigen-presenting cells (APCs) from the lymphatic vessels to the lymph nodes to initiate cellular immunity. Progress in our molecular understanding has been possible because of the development of important tools that allow us to assess the roles of specific markers for LECs. Recently, the application of a group of new lymphatic markers, e.g., VEGFR-3, LYVE-1, podoplanin and *Prox-1* has shed light on different biological functions of these endothelial cells (Prevo et al., 2001; Rodriguez-Niedenfuhr et al., 2001; Wigle et al., 2002). The LECs of various sizes express different signals for these markers on their surfaces. This probably reflects subtle differences in the function of the LECs of the various vessels and their abilities to respond to different types of stimuli (Saaristo et al., 2002). As both initial and collecting vessels are needed for lymph drainage, most attention has been paid to the development of lymphatic vessels in the embryonic and differentiated tissues (Wilting et al., 1999). In addition, lymphangiogenesis is closely related to the malformation and dysfunction of the lymphatic system, which has been demonstrated by growth factors and other molecules in tumor, wound healing and lymphedema in humans and animal models (Skobe and Detmar, 2000; Jackson et al., 2001; Sleeman et al., 2001; Karkkainen and Alitalo, 2002; Jeltsch et al., 2003). In diabetes, lymphatic vessels participate in the migration of APCs, which is regulated by numerous adhesion molecules and chemokines expressed on LECs (Martín-Fontecha et al., 2003). Therefore, impairment of lymphatic function, which results in abnormal permeability and inadequate transport of fluid, macromolecules, or cells from the interstitium, is associated with a variety of diseases and leads to tissue edema, fibrosis, impaired immunity (Witte et al., 2001) and defective remodeling and maturation of lymphatic structures due to improper

reactions for molecular signals. The phenotypes in ultrastructural default are displayed in lymphatic walls and the surrounding matrix, especially in the intercellular junctions and vesicles of LECs. Although the alterations of LECs are regarded to be causative for the detrimental effect on these disorders, the mechanisms whereby LECs are involved in the whole pathological process are still uncertain.

The purpose of this study is to provide a brief overview of the structure and function of the lymphatic vessels, to summarize some of new evidence in lymphatic-associated diseases, and to draw attention to aspects of LECs in which different immunoactivities and molecular agents are thought to be expressed.

Morphological and biological features of LECs

Lymphatic structures

Initial lymphatics show extensive networks, obvious valve-like structures and numerous blind-ends and functionally benefit lymph formation, transport and redistribution. The lymphatic endothelial wall, consisting of a single flattened cell layer with irregular or absent basement membrane, is ultrastructurally characterized by end-to-end, overlapping and interdigitating junctions (Ji and Kato, 1997a,b). Tissue fluid and particles freely enter into initial lymphatics through large gaps between adjacent endothelial cells, so-called open junctions, and by vesicular transport through the endothelial cells themselves. Micropinocytotic vesicles are abundant in the endothelial cytoplasm, which might also predominate in normal lymph formation (O'Morchoe, 1997), but this changes in pathological conditions. The fold of endothelial cytoplasm, which was found to abluminally entrap immune cells, may be involved in providing a pathway across the lymphatic vessels in diabetic pancreas (Qu et al., 2003). Initial lymphatics lined with endothelial cells lack supporting structures, such as smooth muscle cells and pericytes occurring in the collecting lymphatics and blood vessels. The outer surface of the slender lymphatics is fixed by anchoring filaments into the surrounding connective matrix to maintain lumen patency (Leak and Burke, 1968). These initial lymphatics are easily obstructed by the cancer cell emboli to become extremely enlarged in the hybridoma-induced tumor tissues (Ji et al., 2003), and to form a potential pathway for tumor metastasis due to the above-mentioned structural features.

Collecting lymphatic vessels have clearly visible valves to prevent lymph retrograde flow and are interspaced by lymph nodes. They are relatively impermeable and play important roles not only in the communication between intra- and extra-organ lymphatics (Ji and Kato, 1997b), but also in the regulation of draining tissue fluids in the interstitium, especially in the prevention of edema during chronic inflammation processes. All the lymph passes through one or more lymph nodes where the lymphatic vessels form sinuses in which water and other micromolecules freely exchange between the lymph and blood compartments. Regional lymph nodes are a common site of tumor metastases, and the presence or absence of tumor cells in regional lymph nodes is an extremely important prognostic factor for predicting survival. A sentinel lymph node, which receives lymph drainage directly from a tumor site (Uren et al., 2003) biopsy has rapidly entered the clinical mainstream for melanoma and breast carcinoma.

Unlike the widely accepted criteria for distinguishing between lymphatic and blood capillaries, there are no referential criteria for evaluating peripheral lymphatic development depending on the structural changes. The whole mount images of ongoing embryonic and adult lymphangiogenesis suggest that the mechanisms of lymphangiogenesis may be very similar to those of angiogenesis (Risau, 1997), in that lymphangiogenesis appears to proceed via vessel enlargement, sprouting and splitting (Saaristo et al., 2002). However, the distribution and general structures of lymphatics, including the lymphatic networks, blind-ends, islands, intercellular junctions and intracellular organelles may help us to analyze lymphatic localization (Ji and Kato, 1997b). Numerous small islands extend and interconnect to form simple networks, where they send out many branches and connect with nearby networks to form complex networks. This indicates that the occurrence of lymphatic islands may be one of the basic steps in lymphatic development. In the adult mammals, lymphatic islands have been regarded as an active proliferating vascular unit corresponding to the importance of lymph drainage in some organs due to the physiological and pathological requirement (Kato et al., 1993; Ji et al., 1996).

In vitro, dividing and multinucleated cultured LECs represent a uniform, cobblestone appearance and show different biological activities (Fig. 1a,b). An *in vitro* study on LECs would be beneficial for the identification of molecules involved in adhesive interactions with other cells (e.g., lymphocytes and tumor cells), and for the application of differential gene expression to identify molecular differences between lymphatic and blood vessels.

Lymphatic endothelial markers

The differences, rather than the similarities, between the endothelial cells of lymphatic and blood vessels have been stressed since numerous lymphatic endothelial markers came out, which include two overlapping categories, i.e., differentiating and functional.

The receptor for the vascular endothelial growth factor-C (VEGF-C), VEGFR-3 (also known as Flt-4), plays a remarkable role in lymphatic development, in hereditary and acquired lymphedema, and putatively in lymphatic metastasis (Sleeman, 2000; Karkkainen et al., 2001a,b). VEGFR-3 is expressed predominantly in the

LECs that line the inner surface of lymphatic vessels (Kaipainen et al., 1995; Ji and Kato, 2003) to selectively label lymphatic endothelia in several normal tissues (Jussila et al., 1998). VEGF-C and VEGF-D are the two currently known ligands for VEGFR-3. Overexpression of VEGF-C and VEGF-D in the skin of transgenic mice induces the formation of a hyperplastic lymphatic network (Veikkola et al., 2001). VEGF-C156S is a viral vector for a *VEGFR-3*-specific mutant form of VEGF-C, and essentially lacks the blood vascular effects of native VEGF-C. Stimulation by VEGF-C156S potently induces lymphangiogenesis in transgenic embryos and after virus-mediated gene transfer (Joukov et al., 1998; Saaristo et al., 2002), although its capability for inducing the newly-formed lymphatic sprouts in adults is less pronounced than with native VEGF-C, indicating that lymphatic growth is regulated via VEGFR-3. Inhibition of VEGFR-3 signaling using soluble VEGFR-3, which

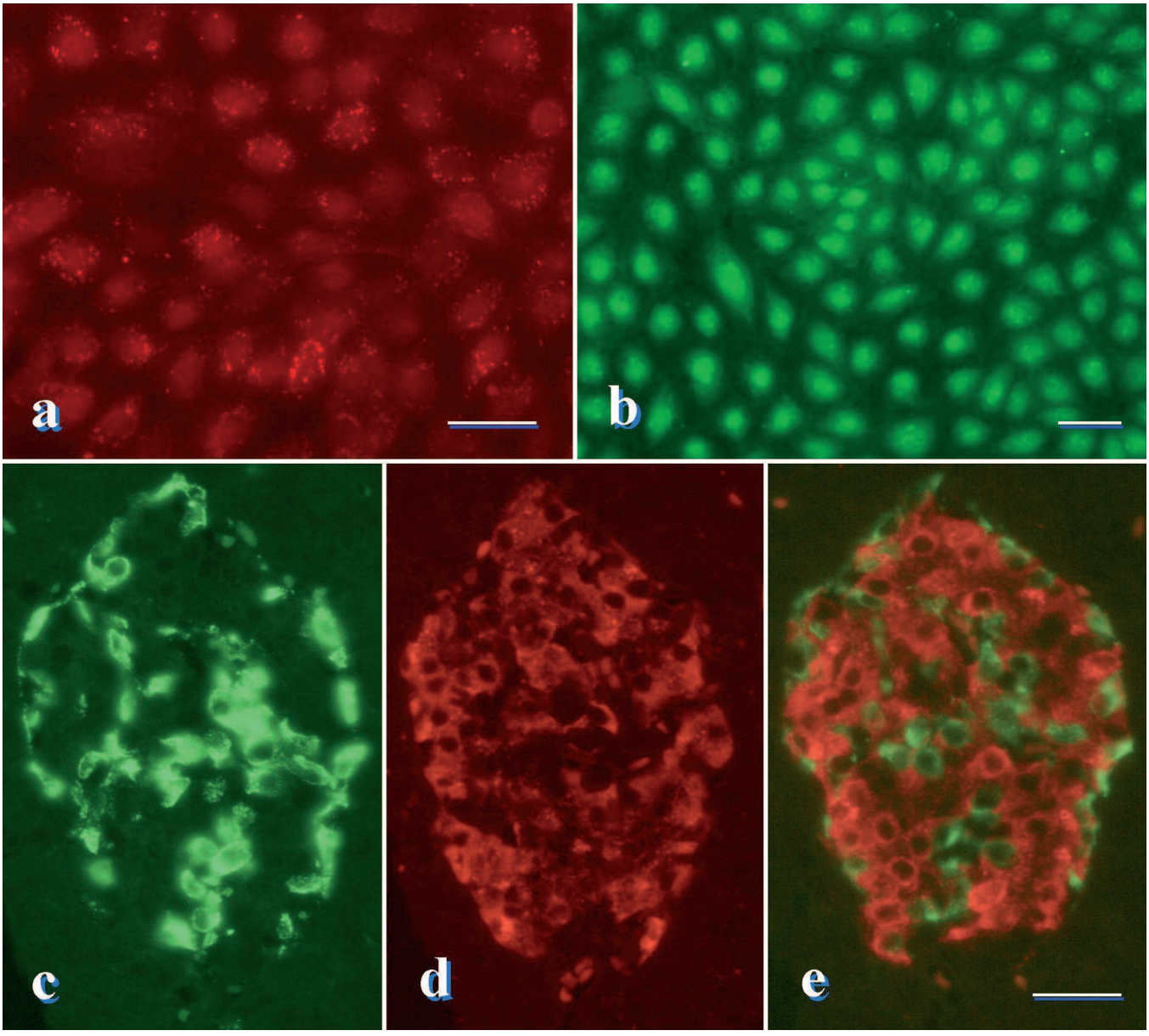


Fig. 1. Fluorescence micrographs of cultured thoracic duct endothelial cells in the Wistar rat **(a,b)** and the islet of Langerhans in the non-obese diabetic (NOD) mice **(c,d,e)**. **a,b.** *In vitro*, dividing and multinucleated cultured LECs represent a uniform, cobblestone appearance, being intensely immunoreactive for JC815 (5'-Nase mAb) **(a)** and the endothelial nitric oxide synthase **(b)**. **c,d,e.** VEGFR-3 (**c**, green) and insulin (**d**, red) double-fluorescence imaging **(e)** shows that VEGFR-3 signals are located in certain endocrine cells, probably except ß-cells of the islet. Bars: 50 µm.

competes for ligand binding with the endogenous receptors, led to lymphatic regression in several organs of the transgenic mice (Makinen et al., 2001a). In addition, prenatal VEGFR-3 expression was found on both venous and lymphatic endothelium, but became restricted to LECs during development (Kaipainen et al., 1995), thus supporting the theory of a venous origin of lymphatic vessels. VEGFR-3 is also expressed on the proliferating adult vascular endothelium (e.g., tumor vasculature), the epithelium of pancreatic ducts and some endocrine cells (Figs. 1c-e, 2a,b). From this viewpoint, it seems clear that VEGFR-3 should not be regarded as a specific LEC marker, because it is almost impossible to reliably differentiate lymphatics from angiogenic capillaries (Partanen et al., 1999). In coordination with VEGFR-3, Ang-2 appears to be required for proper lymphangiogenesis in the regulation of the lymphatic endothelial-periendothelial cell interactions (Veikkola et al., 2003), whereas signals mediated by Tie-2 are needed for the subsequent remodeling and maturation of these vessels (Gale et al., 2002).

Podoplanin, an integral plasma membrane glycoprotein of podocytes, has been found to be colocalized with VEGFR-3, selectively expressed in the normal lymphatic endothelium in the skin, kidney and pancreas (Fig. 2c), and in vascular tumors of lymphatic origin (Breiteneder-Geleff et al., 1999; Weninger et al., 1999). In immunohistochemical analysis, podoplanin shows specific staining mainly on the luminal surface of LECs and in a minor proportion on the abluminal surface and intercellular regions (Kriehuber et al., 2001), but does not appear in adult vascular endothelium or proliferating adult vascular endothelium as present in malignant vascular tumors (Breiteneder-Geleff et al., 1999).

The homeobox gene *Prox-1* acts as a cell proliferation inducer and a fate determination factor for

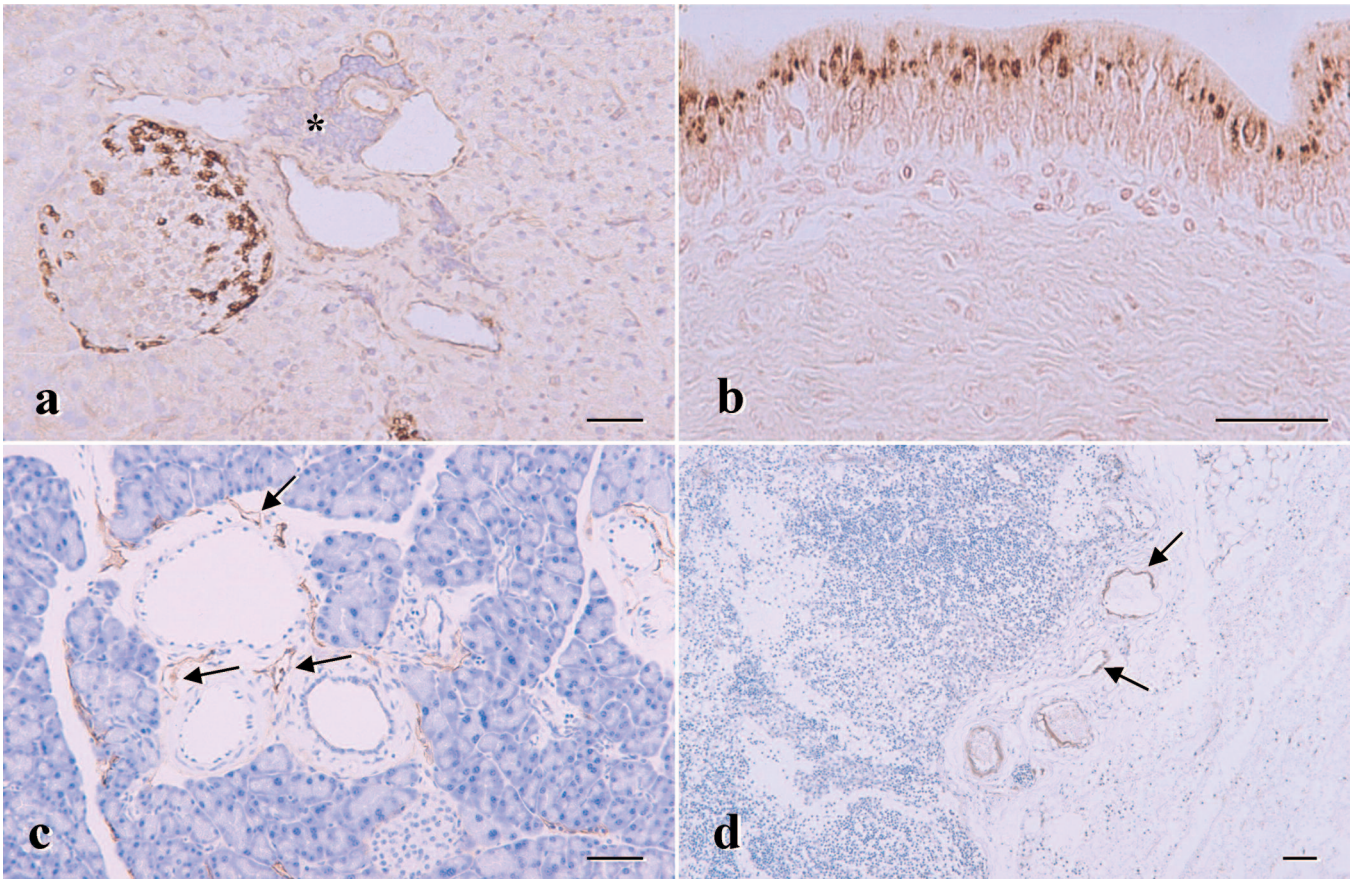


Fig. 2. Histochemistry of VEGFR-3 **(a,b)**, podoplanin **(c)** and Prox-1 **(d)** in the tissue sections. **a,b.** VEGFR-3-expressing cells **(a)** in the islet of Langerhans of NOD mice administrated with complete Freund's adjuvant, and VEGFR-3-expressing epithelial cells **(d)** in the monkey pancreatic duct. The pancreatic islet cells seem to have a very strong expression on VEGFR-3 near the duct, blood vascular and lymphatic vessels and near the side of the infiltrating area (asterisk) in the NOD mice. **c.** Podoplanin expression is detected in the interlobular lymphatic vessels (arrows) of the non-insulitic NOD pancreas. **d.** Immunoreactivity of Prox-1 is represented in the esophageal lymphatics (arrows) of the monkey, where numerous cell types are infiltrated. Bars: 50 μm.

the LECs (Petrova et al., 2002), involved in regulating lymphatic development by budding and sprouting during embryogenesis (Wigle and Oliver, 1999). VEGFR-3 is located on the surface of the LECs, whereas *Prox-1* is found in the nucleus. The lymphatic-specific transcription factor *Prox-1* expression is mutually exclusive with that of the blood vascular marker PAL-E, and is found in tissues of healthy adults and lymphedema patients (Wilting et al., 2002) as well as in the esophageal lymphatics of the monkey (Fig. 2d).

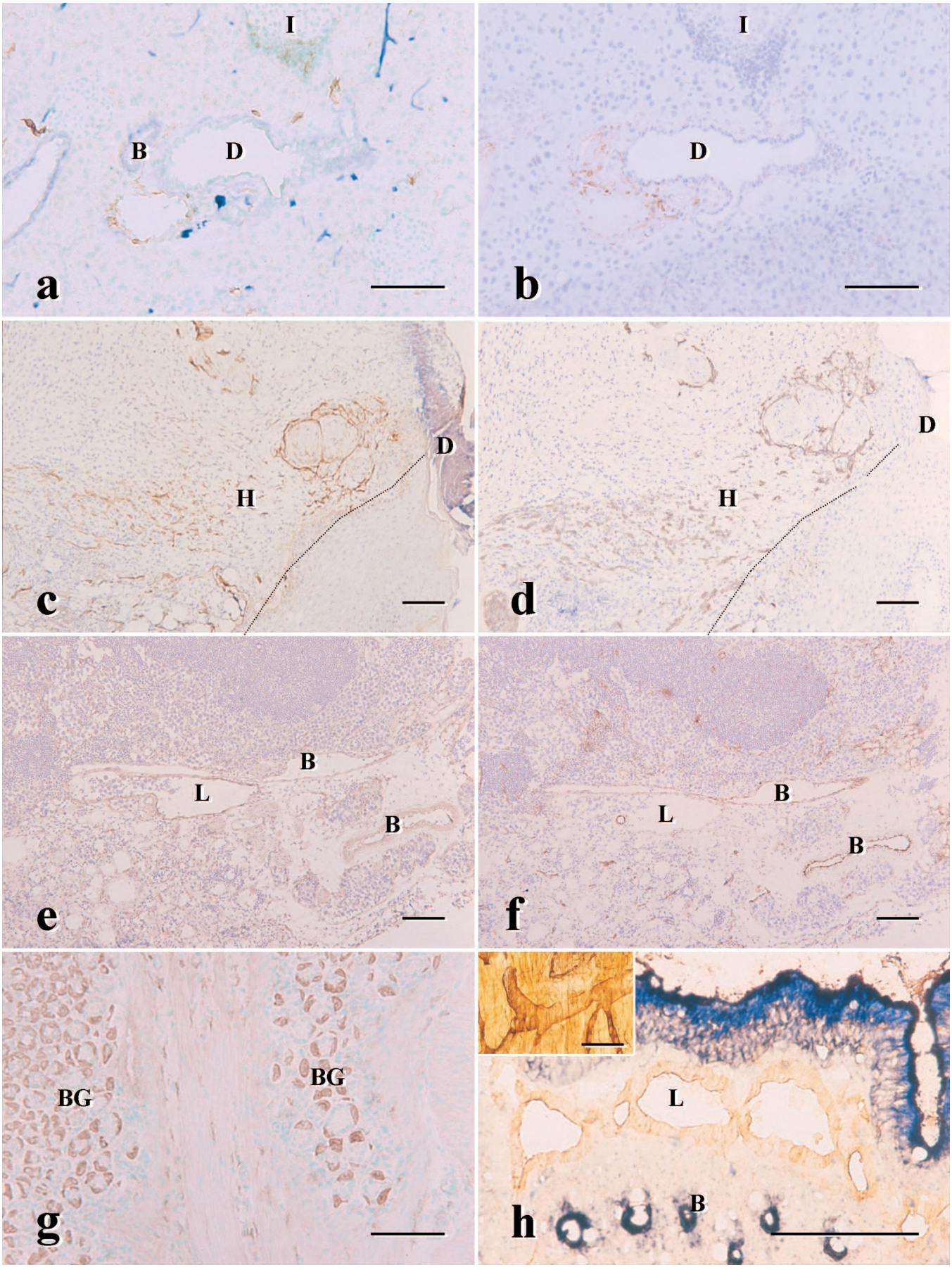
Likewise, the discovery of the lymphatic endothelial hyaluronan receptor-1 (LYVE-1) as a specific marker for both normal and tumor-associated lymphatics has now paved the road to study tumor lymphangiogenesis not only in experimental tumor models, but also in spontaneously-arising human tumors (Dadras et al., 2003). Differential immunohistochemistry for LYVE-1 and *Prox-1* has confirmed that LYVE-1 is selectively expressed by lymphatic vessels, but not by blood vessels, in murine and in human tumors (Hawighorst et al., 2002; Oliver and Detmar, 2002). LYVE-1 appears to be consistently exposed to the luminal and abluminal surfaces of LECs in a number of normal tissues (Banerji et al., 1999; Prevo et al., 2001), suggesting that it may shuttle across the lymphatic endothelium to transport hyaluronan from tissue to lymph by transcytosis. Hyaluronan is a key mediator of cell migration both during embryonic morphogenesis and in adult processes such as wound healing and tumor metastasis (Knudson and Knudson, 1993). Further studies are needed to develop anti-LYVE-1 function-blocking antibodies and LYVE-1-knockout mice to explore the role of this fascinating receptor in hyaluronan transport, leukocyte migration and tumor metastasis (Jackson et al., 2001).

5'-nucleotidase (5'-Nase), an adenylate and guanylate cyclase, has been widely used in various tissues of several mammalian species to study the distribution and fine structure of lymphatic vessels with special reference to their relationship with alkaline phosphatase (ALPase)-positive blood vessels (Kato and Miyaguchi, 1989; Werner and Schunke, 1989; Kato, 2000; Ji and Kato, 2001). With cerium-based 5'-Nase histochemical staining, the reaction granules are evenly decorated on the luminal and abluminal surfaces of LECs and extend into the intercellular interface. The catalytic site of the membrane-related ecto-enzyme makes significant contributions to endothelial functions, particularly in proliferation and migration (Zimmermann, 1992), and to lymphocyte signal transduction (Thompson et al., 1989). However, our previous studies revealed that obvious changes in 5'-Nase activity occur in embryonic and differentiated tissues, as well as in disordered tissues, e.g., a reduced expression for lymphatic endothelia in developing gastric wall and lymphostatic intestine and an overexpression of uterine lymphatics during the pregnancy (Ji and Kato, 1997b, 2000, 2001; Ji, 1998). There still remains a considerable variability with regard to both lymphatic structure and 5'-Nase staining intensity in different organs and animals. Recently, a novel JC815, 5'-Nase monoclonal antibody (mAb) has been produced to recognize lymphatic vessels in easily detectable amounts on cultured endothelial cells (Fig. 1a) and on a panel of cryosections of different tissues (Ji et al., 2003). The tissue reactivity for JC815 shows high similarity in specificity to that of 5'-Nase enzyme. The immunoelectron microscopy indicates a relation of antigenic determinant with cell membrane. When 5'-Nase antigenity rather than its activity is considered, 5'-Nase mAb specific for LECs, instead of adenosine 5'-monophosphate, can serve immunohistochemically as a useful marker for cell selection and *in vitro* cultivation. It should thus be noted that a combination of enzyme- and immunohistochemistry might provide a new approach for lymphatic investigation.

With the exception of regional variations in the lymphatic endothelial morphology, the biological basis of the difference between lymphatic and blood vascular endothelial cells is not fully clear. Components of the vascular wall, basal lamina, pericyte, vascular smooth muscle cell and fibroblast also contain more or less specific antigens which may participate in the formation of developing vessel-like structures. Therefore, many routine markers including type IV collagen, laminin, endothelial nitric oxide synthase and a pan-endothelial marker, platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), still have potential utility for analyzing endothelial cells of lymphatic and blood vessels, depending on the degree of reaction intensity or expression level rather than the consistent qualitative difference (Ji et al., 2004). In this way, leukocyte migration from the extracellular matrix to the lymphatic lumen is mediated by CD31, and alteration in neovascularization patterning is indicated by factor VIII-related antigen (Ribatti et al., 1999; Sawa et al., 1999).

Secondary lymphoid-tissue chemokine (SLC/CCL21, also known as TCA4, 6Ckine) is expressed in secondary lymphoid organs, and mediates the chemotaxis of lymphocytes and DCs via its receptor, the CC chemokine receptor 7 (CCR7) (Saeki et al., 1999). CCL21 is expressed in intestinal lymphatics of normal mice (Gunn et al., 1998) and in pancreatic lymphatics of the RIP-BLC1 transgenic mice (Luther et al., 2000) and diabetic mice (Fig. 3a,b). A postembedding immunogold technique displays CCL21 reaction product on the luminal and abluminal surfaces and peri-nuclear areas of LECs in diabetic mice (Qu et al., 2004). The expression of CCL21 on LECs suggests that the migration of lymphocytes from tissues into efferent lymphatics might be an active process mediated by this molecule (Gunn et al., 1998). In addition, CCL21 has been assumed to be involved in recruiting mature dendritic cells (DCs) from afferent lymphatics to the T-cell area of lymph nodes (Cyster, 1999).

A panel of proposed markers for lymphatic vessels may greatly contribute to the molecular mechanisms that control survival, activation and proliferation of LECs in the regenerating and differentiating tissues, and will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probably help us to understand the pathogenesis of different diseases in which lymphatic vessels are involved (Table 1). Therefore, a greater emphasis should be placed upon the different biological nature of these endothelial markers and local environments for the lymphatic function. These LEC modulators will be further mentioned in sections on lymphatic-related disorders.

Derangement of lymphatic vessels and extracellular matrix

The interstitial-endothelial interface plays an integral role in lymphatic function, as fluid equilibrium is controlled by the cooperation of both lymphatic function and extracellular matrix. While several factors and mechanisms involved in blood endothelial cell-periendothelial cell interactions have been characterized, such mechanisms are poorly understood in the lymphatic vasculature. The elasticity and hydration of a tissue is determined by the composition and organization of the extracellular matrix; e.g., collagen provides structural framework, and proteoglycan largely determines water content and resistance to fluid transport. Extensive and chronic degradation of the extracellular matrix, e.g., by hyaluronidase, induces a collapse of lymphatic vessels and eventually renders them nonresponsive to the changes in the interstitium and therefore causes dysfunction (Negrini et al., 1996). Hence, the functioning of lymphatic vessels is critically dependent on the extracellular matrix composition, geometry, and integrity (Skobe and Detmar, 2000). Sprouting and differential growth of the endothelial cells with the intervention of the connective matrix have been implicated as a novel understanding in remodeling and maturation of developing lymphatic vessels in several physiological and pathological processes (Enholm et al., 2001; Ji and Kato, 2003). The heterogeneity of surrounding components indicates that fibroblasts and primitive mesenchymal cells may be essential factors for lymphatic regeneration in the woven mesenchymal tissue. It is becoming clear that multiple molecules are necessary to ensure proper differentiation and patterning of LECs into a functional lymphatic system (Jain and Padera, 2003). The extracellular matrix, glycosaminoglycan hyaluronan, is an abundant component of skin and mesenchymal tissues where it facilitates cell migration during wound healing, inflammation, and embryonic development by forming a pericellular matrix surrounding fibroblast and epithelial cells, reducing the level of intercellular adhesion (Brown et al., 1999). A series of recent data propose that chemokines may act in concert with the extracellular matrix, resulting in the migration of a given cell subset, either within the thymus or at the entrance into and/or exit from the organ (Savino et al., 2002). Endothelial-periendothelial cell interactions can regulate gene expression in the LECs, and therefore smooth muscle cell interactions are likely to modulate the LEC responses to lymphangiogenic stimuli (Veikkola et al.,

Table 1. Histochemical evaluation and function of lymphatic endothelial markers.

MARKERS	HISTOCHEMISTRY				BIOLOGICAL SIGNIFICANCE	REFERENCES
	LV	(Lu	Ablu)	BV		
VEFGR-3	++	+	+/-	+	Receptor of vascular endothelial growth factor	Kaipainen et al., 1995; Ji and Kato, 2003
Prox-1	***	-	-	*	Vertebrate homologue of *Drosophila prospero* gene	Wigle and Oliver, 1999; Wilting et al., 2002
Podoplanin	+++	+	+	-	Integral plasma membrane glycoprotein of podocytes	Breiteneder-Geleff et al., 1999; Kriehuber et al., 2001
LYVE-1	+++	+	+	+	Lymphatic endothelial hyaluronan receptor	Banerij et al., 1999; Prevo et al., 2001
5'-Nase	+++	+	+	+/-	Adenylate and guanylate cyclase	Kato and Miyauchi, 1989; Werner and Schunke, 1989
JC815	++	+	+	+/-	Anti-5'-Nase mAb	Ji et al., 2003
CCL21	++	+	+	+	Secondary lymphoid-tissue chemokine	Gunn et al., 1998; Qu et al., 2004

LV: lymphatics; Lu: luminal; Ablu: abluminal; BV: blood vessels.

Fig. 3. Histochemical staining micrographs in the tissues of different animal models. **a,b.** In 17-week-old NOD mice, an interlobular 5'-Nase-positive lymphatic vessel **(a)** shows relatively weak and uneven CCL21 **(b)** expression, which is also seen in the blood vessel (B), and infiltrating cells surrounding the islet (I) and duct (D). **c,d.** In a 5-day wound-healing mouse, numerous accumulated vasculatures with expression of 5'-Nase **(c)** and CD31 **(d)** are located along the wound edge (a dotted line), running irregularly from the hypodermis (H) to the dermis (D). **e,f.** In the hybridoma-induced intestinal tumor of the mouse, numerous metastatic cells are accumulated in the VEGFR-3-positive lymphatic vessel **(e)**, which shows extremely faint CD31 immunoreactivity in contrast with intense staining in the blood vessels **(f). g**. In the hybridoma-induced gastric tumor of the mouse, VEGF-C-expressing cells are detected in the basal portion of glands (BG). **h.** In the lymphostasis model with blockage of rat thoracic ducts, the subcutaneous lymphatic vessels with weak 5'-Nase activity in the duodenum become extremely dilated, in contrast to ALPase-positive blood vessels. Inset is a microphotograph of the whole mount preparation in the subcutaneous layer, indicating an enlarged lymphatic vessel connecting with numerous fine initial lymphatics. L: lymphatic vessel; B: blood vessel. Bars: 100 μm.

2003). In an *in vitro* experiment, up-regulation of VEGFR-3 expression in the cultured blood vascular endothelial cells may have been due to lack of smooth muscle cells or some extracellular matrix components (Makinen et al., 2001b). This indicates that an appropriate extracellular matrix provides additional signals for the adhesion, survival and proliferation of these cells. However, the mechanisms responsible for hyaluronan transport across the lymphatic endothelium, the receptors involved in its uptake and transport within the lymphatic vessels and the maintenance of tissue integrity need to be studied.

The lymphatic vessels are the victims of a variety of changes, including partial failure to develop with resultant lymphedematous and fibrotic changes in various organ systems and a number of degenerative conditions, e.g. lymphangitis and lymphangiosclerosis. Many secondary changes of lymphatic vessels are obviously due to obstructions and raised intraluminal pressures. One of the most common findings in areas of tissue damage is a dilatation of lymphatic vessels (lymphangiectasia), which may lead to an inability of the valves to prevent retrograde flow. Moreover, high intraluminal pressures are associated with an increase in the number of open junctions between endothelial cells with the loss of luminal contents into the surrounding tissues (Casley-Smith, 1980). However, dilated lymphatics have been observed without any apparent obstructions within the vessels. In contrast to the situation with obstructions, the intralymphatic pressure is not elevated in post-surgical lymphedemas, which provides some interesting problems in this regard since edema often develops months or years after the lymph nodes and vessels have been removed. Lymph drainage is restored by the regenerating vessels and yet for some reason the vessels become fibrotic and dilated with lymphostasis and, ultimately, edema formation (Olszewski, 1973). Since lymph flow, protein and fat absorption in the initial lymphatics are closely related to each other, myogenic and neurogenic activities of lymphatics play an important role in the regulation of the interstitial volume in many tissues and organs (Ji and Kato, 2000). An increased permeability to large molecules in the lymphatic vessel represents a large number of pathophysiological states, e.g., the leakage occurs through gaps between endothelial cells in acute lymphedema. The cause of the increased permeability may in some cases be due to direct damage of the vessels and in others to increased intralymphatic pressures. In addition, some inflammatory mediators (mast cell products) and cellular components may induce these changes as well (Carr et al., 1980).

In normal tissues, extracellular matrix fibers are ideally arranged for directing fluid into lymphatic vessels (Ryan, 1989). In tumors, the extracellular matrix composition and organization is frequently altered, and it is possible that fluid channels in tumor stroma do not direct fluid into tumor lymphatics in an organized manner (Pepper et al., 2003). The preference of melanoma cells for adhering to the extracellular fibronectin of endothelial cells may be a reason for the privileged invasion of the melanoma cells in the lymphatic system, because the extracellular matrix is not protected by a continuous basal lamina like blood vessels (Duenne and Werner, 2000). In addition, interstitial flow can drive the formation of fluid channels along which LECs migrate, proliferate, and finally reorganize into a functional capillary network (Boardman and Swartz, 2003).

In *Prox-1*-null mice, budding and sprouting of LECs from the vein is arrested at around E11.5-E12.0 (Wigle and Oliver, 1999). As the development proceeds, the subpopulation of LYVE-1- and *Prox-1*-positive endothelial cells starts to bud from the veins in an initially *Prox-1*-independent manner. As the cells bud they start to express higher levels of additional LEC markers such as CCL21 and VEGFR-3, whereas the expression of VEGFR-3 decreases in blood vascular endothelia (Oliver and Detmar, 2002). Therefore, there is ample agreement that the blood and lymphatic vasculature systems have highly similar features in endothelial cells and share the expression of many common markers. In contrast to a great body of current knowledge on vascular diseases and angiogenesis, the application of new approaches on the studies of lymphatic-related disorder and lymphangiogenesis would bring valuable information on the relationship between LECs and the surrounding matrix components.

Lymphatic vessels in pathological processes

There is now a wealth of evidence that lymphangiogenesis is modulated by multi-factors and takes place in various pathological processes as diverse as lymphedema, growing tumor and wound healing (Kampfer et al., 2001; Padera et al., 2002; Ji et al, 2003; Yoon et al., 2003). LECs have several morphological and functional features, differing from blood vascular endothelia in normal and diseased tissues (Ji et al., 1996; Kriehuber et al., 2001; Kim and Dumont, 2003). The identification of vascular endothelial growth factors, especially VEGF-C, encourages not only basic investigation in regenerating and differentiating tissues but also its perspective use in molecular therapeutics clinically. Lymphangiogenesis-dependent physiological and pathological conditions would not be restricted to embryogenesis, but would extend to the growth of new lymphatics into tumors and granulation tissues and into an area of inflammation. Moreover, a few progressive points concerning the active role of LECs in autoimmune response mediated by several adhesion molecules and chemokines are also worthy of emphasis.

Diabetes

Insulin-dependent diabetes mellitus (IDDM, Type I diabetes) results from progressive destruction of the insulin-producing cells, the islets of Langerhans, as a

consequence of a ß-cell-directed autoimmune process (Tisch and Miyauchi, 1996). The presence and involvement of various subpopulations of lymphocytes, DC cells and macrophages have been predominantly described in IDDM processes (Jansen et al., 1994). As the exogenous and endogenous factors contributing to the pathogenesis of human diabetes are complex and multiple, diabetologists have been studying spontaneous diabetes in the animal kingdom where the genetic and environmental factors contributing to the etiology of the disease can be controlled and simplified (Tochino, 1986). Much of our understanding of the pathogenesis of ß-cell destruction in Type I diabetes has been obtained through the study on the nonobese diabetic (NOD) mice (Delovitch and Singh, 1997). NOD mice develop diabetic signs characterized by hyperglycemia, polyuria, weight loss, insulin deficiency and destructive insulitis. The infiltration of massive mononuclear cells mainly consisting of a large number of lymphocytes and some macrophages around and into the pancreatic islet, results in insulitis, which is the most prominent pathological lesion and precedes the onset of diabetes. A vast amount of new data suggests that the animal model of human Type I diabetes can contribute to the investigation of the genetic and morphological features and the immune mechanism in the etiopathogenesis of diabetes.

Studies on the relationship of insulitic development with blood vascular changes have made great progress in diabetes (Zatz and Brenner, 1986; Tooke, 1995), but little is known about the morphological properties of pancreatic lymphatics and about the dynamic migration of immune cells via the lymphatic wall due to the difficulty in distinguishing intra-organic initial lymphatics from blood capillaries. Recently, our investigation by using combined 5'-Nase and CCL21 histochemical approaches has demonstrated that transendothelial migration of DCs and lymphocytes through lymphatic vessels may be facilitated by structural and functional alterations during pathological processes of the insulitis in NOD mice (Qu et al., 2003). Open junctions between adjacent endothelial cells and increment of cytoplasmic protrusions and vesicles might be actively involved in the removal of destroyed cell components and transport of immune cells. In the non-insulitic stage, the 5'-Nase product is evenly distributed on the surface of LECs with weak expression of CCL21. 5'-Nase acts on transmembrane signaling to regulate lymphocyte function-associated antigen-1 (LFA-1)-medicated lymphocyte binding and emigration (Zimmermann, 1992). 5'-Nase activity on lymphatic vessels is slightly reduced with the development of insulitis, whereas the increment of blood glucose value appears to be consistent with an increasing CCL21 expression on the endothelial lining, especially on the surface of LECs adjacent to the infiltrating islet and tissue. This suggests that endothelial cells might secrete or contain CCL21, being induced by inflammatory stimuli to a greater or less extent (Hjelmstrom et al., 2000). In severe insulitis, lymphatic vessels show obvious reduced 5'-Nase activity, indicating that alteration of endothelial cell components may directly induce functional expression on cell membranes to influence exchange of interstitial tissue fluids, modification of permeability and absorptive capacity of lymphatic vessels.

In the prediabetic NOD mice, APCs, such as DCs and macrophages, infiltrate peri-ductal and vascular spaces adjacent to the islet of Langerhans. DCs are first located around swollen parainsular vessels as early as 4 weeks old. With insulitis appearance, the number of DCs increases in para- and intra-islets until 17 weeks, where lymphocytes have also accumulated (Shinomiya et al., 2000). These cells capture self-antigen and migrate via afferent lymphatics into the T-cell zones of lymph nodes and provide stimulation to naive lymphocytes originating from the thymus, thus eliciting continuous generation of memory T-lymphocytes against autoantigen (Yoon et al., 1999). Activated or self-reactive T-lymphocytes are implicated as effector cells for the destruction of insulin-producing ß-cells. These migratory properties of lymphocytes and DCs are regulated by various adhesion molecules and chemokines expressed on blood vascular and lymphatic vessels (Newman, 1997; Saeki et al., 1999; Martín-Fontecha et al., 2003).

Lymphoid tissue frequently forms at sites of inflammation, especially in chronic autoimmune diseases, such as in the pancreas of diabetics and in the joint synovium of rheumatoid arthritis (Hjelmstrom, 2001; Schrama et al., 2001). Different tissues produce different repertoires of “inflammatory chemokines” in order to attract appropriate subsets of effector cells into the tissue which respond to the particular pathogen or type of tissue damage (Cyster, 1999). CCL21 is chemotactic for both mature DC and naive T cells, suggesting that it may be an important factor for the colocalization of these two cell types to facilitate immune response initiation (Chan et al., 1999; Martín-Fontecha et al., 2003). Generally, mobilization of immune cells into lymphatics, whether being in a steady or antigen-triggered state, is a passive phenomenon due to the absorbing effect of the negative pressure in lymphatic vessels of the normal tissues. In chronic inflammation, adhesion molecules and chemokines expressed on LECs, however, become the initial guidance to migration of lymphocytes and DCs in lymphatic vessels. Immature DCs express receptors for inflammatory chemokines and, upon induction of maturation, up-regulate CCR7, which drives their migration to the lymphatics (Dieu et al., 1998). The increased expression of CCL21 and CCR7 promotes molecular interactions of DCs with LECs, directing the migration of DCs to lymph nodes through lymphatic vessels. The CCL21 can regulate homeostasis of CD4, but not CD8 T cells. In the prediabetic NOD mice, CCL21 mRNA appears in the islets of Langerhans, in the infiltrates and in the vascular walls, whereas it is seen at extremely low levels in the pancreas of healthy

wild-type mice (Hjelmstrom et al., 2000). During diabetic processes, CCL21 is widely expressed in the lymphatic vessel, infiltrating islets and adjacent to inflammatory infiltrates (Fig. 3a,b). DCs and lymphocytes gradually accumulate in the perivascular area and in the peri- or para-insular areas. In the absence of CCR7 ligands, transferred CD4 T cells fail to expand in lymphopenic hosts, whereas in the presence of CCL21 overexpression, homeostatic CD4 T cell proliferation occurs even in nonlymphopenic recipients (Ploix et al., 2001). In the BDC2.5 TCR-Tg NOD mice, the peri-insular infiltrates histologically resemble secondary lymphoid tissue, especially mucosa-associated lymphoid tissue (e.g., Peyer's patches) (Rosmalen et al., 2000). The expression pattern is similar to that observed in the spleen and pancreatic lymph nodes of wild-type animals, where CCL21 activity is detected on stromal cells in T-cell zone and high endothelial venules (Luther et al., 2002). The mice homozygous for the paucity of lymph node T-cell (plt) mutation lack expression of CCL21 and have defects in T lymphocyte homing and DC localization in spleen and lymph nodes (Gunn et al., 1999). In the transgenic mice with islet ß-cell-specific expression of TCA4/SLC, the recruitment of lymphocytes and DCs to pancreatic islets appears to be sufficient to trigger development of organized lymphoid tissue. TCA4/SLC transgenic expression can drive lymphoid neogenesis under the conditions where the majority of recruited cells show a naive phenotype (Fan et al., 2000). These observations propose that the lymphatic vessel not only contributes to removing excess fluid because of microcirculation disturbances, but also plays a pathogenic role in the maintenance and possible worsening of the diabetes. It is thus interesting in future studies to test whether blocking CCL21 function is sufficient to prevent development of diabetes in NOD mice.

Complete Freund's adjuvant (CFA), one of the most commonly used immunomodulators, has well-documented protective effects from several autoimmune diseases in humans and animal models (McInerney et al., 1991; Jansen et al., 1994; Kahn et al., 2001). Many autoimmune diseases are initiated and maintained by sensitized and activated autoreactive T cells, which destroy target cells harboring corresponding tissue-specific antigens. Antigen presentation by professional APCs, particularly DCs, is of key importance for the initiation of primary immune responses. DCs presenting self-antigens are potent inducers of autoreactive T cells, and help to maintain a locally peripheral immune response. CFA appears to influence the initiation and maintenance of the autoimmune response by a distinct regulatory mechanism, probably depending on two factors; the sufficient autoantigen and the islet-associated lymphoid-like tissue (Ludewig et al., 1998; Yoon et al., 1999). In the NOD mice, administration of CFA can definitely inhibit insulitis and prevent occurrence of diabetes by reducing the infiltration by different subsets of mononuclear cells. The transendothelial migration of DCs and T lymphocytes through pancreatic lymphatics and the immunoreaction to islet antigens might be suppressed, due to down-expression of CCL21 and CD31 on the LECs and improved lymphatic structures, such as disappearance of open junctions (Qu et al., 2004). The decreased chemotactical affinity between lymphatics and infiltrating cells suggests that the entry of activated DCs into lymphatic vessels with a chemotactic gradient is inhibited to reduce the efficiency of islet antigen-specific T cell responses. The expression of glutamic acid decarboxylase (GAD), a pancreatic ß-cell autoantigen, is required for the development of autoimmune diabetes in humans and NOD mice. ß-cell-specific suppression of GAD expression in two lines of antisense GAD transgenic NOD mice has prevented autoimmune diabetes (Yoon et al., 1999). The immune responses induced with antigen in CFA are markedly augmented in the presence of inducible NO synthase (iNOS) inhibitors or when using iNOS knockout mice (Kahn et al., 2001). The evidence that the time frame of CFA efficacy is coincident with a period of emerging insulitis and ß cell damage in NOD mice suggests that useful immune regulation of the damaging infiltrating cells occurs before 10 weeks (Delovitch and Singh, 1997). A recent experiment has indicated that natural killer (NK) cells may mediate the protective effects of CFA, possibly through down-regulation of autoreactive cytotoxic T cells, and that the stimulation of NK cells seems to provide an approach to the prevention of autoimmune diabetes (Lee et al., 2004). The relationship between pancreatic lymphatics, infiltrating cells ($CD11c^+$, $CD4^+$ and $CD4/80^+$) and insulitic development is helpful in elucidating the possible role of CFA in the autoimmune pathological process (Fig. 4).

The overexpression of chemokines CCL21 and adhesion molecules CD31 on pancreas may participate in the establishment of the insulitic lesion in the NOD mice, although the role of other chemokines and adhesion molecules cannot be ruled out. CD31 expression on the intercellular junctions and surfaces of LECs, subsets of DCs and T lymphocytes shows homophilic CD31 interaction between DCs and LECs. The striking anatomic localization of CD31 suggests that it is involved in the process of DC and lymphocyte recruitment and transmigration (Newman, 1997). The blockage of CD31 effectively inhibits leukocyte transmigration, indicating that CD31 molecules on both the endothelial cell as well as the leukocyte side contribute to bidirectional transmembrane signal transduction (Muller et al., 1993). CD31/PECAM-1-mediated homophilic interactions may support physiological, chemokine-independent transendothelial migration of naive T lymphocytes into sites of antigenic priming (Zocchi et al., 1996). The application of a number of novel molecular markers is expected to further explore the altered LEC components and functional expressions on cell membranes which influence permeable modification and the absorptive

capacity during the diabetic progress.

Wound healing

Wound healing represents a highly ordered interaction of cellular and biochemical events which are characterized by chemotactic migration of inflammatory cells, re-epithelialization, fibroblast ingrowth, extracellular matrix deposition, angiogenesis, lymphangiogenesis and remodeling repair (Martin, 1997; Enholm et al., 2001; Kampfer et al., 2001; Inkinen et al., 2003; Yonekawa et al., 2003). These processes are driven by a complex mixture of growth factors, especially by a strong induction of VEGF expression during the reparative phases until the granulation tissue normally occurs.

In a porcine model of cutaneous wound healing, VEGFR-3-positive lymphatic vessels appear concurrently with blood vessels (Paavonen et al., 2000), suggesting that angiogenesis and lymphangiogenesis are coordinately regulated. In wounds, acute inflammation with increased permeability is followed by the deposition of a provisional fibrin and connective tissue matrix, proliferation and migration of endothelial cells, vessel formation and remodeling. The regenerating signals of VEGFR-3 on the LECs, appearing as early as 3 to 5 days after injury in the granulation and hypodermal tissues, can be detected up to day 14, and regress earlier than the blood vessels (Paavonen et al., 2000; Ji et al., 2004). VEGFR-3-positive lymphatics are distinct from the PAL-E/laminin/vWF-positive vessels and are fewer in number than the blood vessels at the wound edge. The expression pattern of VEGFR-3 is extremely uneven on the lymphatic wall, indicating that endothelial sprouting might begin from its up-expressing side. VEGFR-3-immunogold is mainly labeled on the

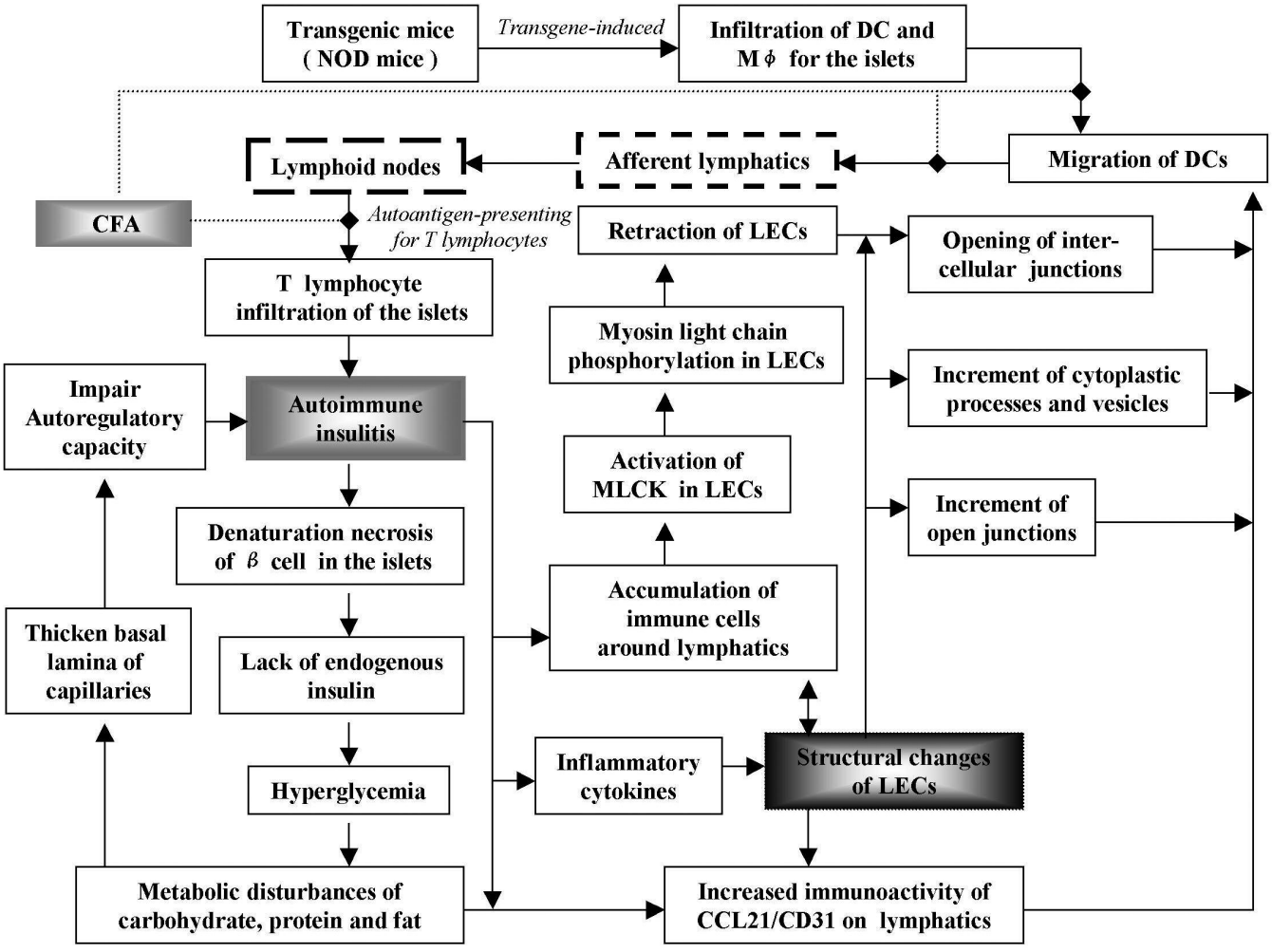

Fig. 4. Relationship between insulitis, hyperglycemia and lymphatic vessels in the pancreas of NOD mice. **NOD mice: nonobese diabetic mice; DC: dendritic cell; ø: macrophage; CCL21: secondary lymphoid tissue chemokine; LECs: lymphatic endothelial cells; MLCK: myosin light chain kinase.

lymphatic luminal surface, suggesting that the receptor may actively participate in migration and sprouting of endothelial cells in the loose mesenchymal tissue (Ji et al., 2004). The wound bridging by lymphangiogenesis sequentially appears in an irregular course at sites where there is vigorous angiogenesis and least tissue reaction. In chronic inflammatory wounds, VEGFR-3 becomes up-regulated in the blood vascular endothelium. The proliferation of LECs, characterized by intercalated and circumferential growth, could contribute to the vascular enlargement by VEGF-C mediator (Saaristo et al., 2002). The occurrence of newly formed lymphatics is just as prominent a feature as angiogenesis in the skin healing wounds, although the intra-organic lymphatics usually show obvious morphological and functional variations. While the lymphatic vessel becomes mature, the wall is slender and irregular, and the endothelium protrudes into the lumen and adjacent connective tissue. Intercellular junctions undergo a morphological change from simple end-to-end to overlapping and interdigitating, which appear to be in good agreement with our previous observations in early embryonic tissues (Ji and Kato, 2003). The simple junctions may facilitate separation, spreading and migration of endothelial cells during lymphatic remodeling in compliance with tissue repair patterns. Changes in the synthesis and breakdown of extracellular matrix components are known to play a crucial role in tissue remodeling during inflammation and wound healing (Yonekawa et al., 2003). However, no obvious change of 5'-Nase activity in newly-formed lymphatic structures occurs in the wound repair as in the embryonic development. It thus seems quite evident that a causative factor of lymphatic-related disorders should be concentrated on the endothelial cells.

The endothelial growth of lymphatics is delayed where wound healing is interrupted by any appreciable infectious reaction. The healing repair in the myocardium in cardiac lymph-obstructed dogs is markedly altered and prolonged. The interference appears to result from persistent local edema and delayed removal of local debris and inflammatory cells. In the wound-healing tissues, there are few elastic or collagen fibers surrounding newly formed lymphatic vasculatures, although 5'-Nase activity is definitely confined to the endothelium (Ji et al., 2004). The 5'-Nase enzyme interacts with both laminin and fibronectin to involve cell migration on these substances (Stochaj et al., 1990). Apparently, simple mechanical means, such as collagen bundles can influence or block the sprouting and growth of lymphatic vessels. The observation in the ear of guinea pigs has revealed that some opening up of pre-existing collateral channels are followed by sprouting growth of new vessels after injury (Yong, 1980), which is compatible with our findings that numerous accumulated lymphatic-like vasculatures with expression of 5'-Nase and CD31 are located along the wound edge, running irregularly from the hypodermis to dermis (Fig. 3c,d). These vasculatures may include pre-existing enlarged lymphatics and newly formed structures for active VEGF-C-mediated endothelial sprouting response, which are definitely essential in reducing tissue edema in healing wounds. Interestingly, the blood vessel has to reestablish before it directly induces proliferation of LECs and sprouting of new lymphatic vasculatures via the secretion of VEGF-C (Kriehuber et al., 2001). Whatever the cause of disparity in growth rate between blood capillaries and initial lymphatics, it can create a temporary disturbance of tissue fluid equilibrium. This equilibrium will be restored by way of both ingrowth of lymphatic vessels and gradual loss of vascularity in the regenerating tissues.

VEGFR-3 remains predominant expression in the lymphatics in normal adult tissue and wound-healing skin, although it is largely absent from the blood vessels. As VEGF-C and VEGF-D are localized on vascular smooth muscle in adult tissues (Achen et al., 2001) and VEGF levels are elevated in cutaneous wounds (Yao et al., 2001), these growth factors are available for coordinating angiogenesis and lymphangiogenesis as a result of activation by plasmin during wound healing. Indeed, the overexpression of VEGF-C in the skin of transgenic mice results in hyperplasia of the lymphatic networks without obvious effects on blood vessels (Jeltsch et al., 1997). In wounds of diabetic (db/db) mice, the overexpression of Ang-2 in the presence of significantly reduced VEGF (Frank et al., 1995) is associated with a dramatic decrease in vascular endothelial cell numbers within the granulation tissue compared with normal healing as assessed by analysis of the endothelial-specific markers CD31 and vWF, whereas the lymphatic endothelium remains stable as determined by VEGFR-3 expression (Kampfer et al., 2001). The expression of cell-surface proteins and the molecular identification by CD31 and vWF immunolocalization are helpful in analyzing functional properties of endothelial cells and interstitial environment. It is thus clear that efficient lymphangiogenesis may be dependent on a mixture of growth factors, including an intense induction of VEGF-C, cytokines, adhesion molecules and their receptors in wound healing.

It should be emphasized that wound healing in the embryo may be simple, efficient, and more perfect than in adult tissue, resulting in rapid and scarless repair. For this reason, the next few years will be exciting as we test whether we can improve on nature and induce adult wounds to heal like embryonic wounds without delay, without scarring, and with full regeneration of hairs and glands (Martin, 1997).

Tumor

Currently, two essentially conflicting views in the dissemination of tumor cells from the primary site have been advanced. The first maintains that tumors metastasize solely by invasion of pre-existing lymphatics

at the tumor margin (peritumoral lymphatics) (Carmeliet and Jain, 2000; William et al., 2003) due to the high pressures found within the tumor (Jain, 1994). The second maintains that tumors metastasize by promoting newly formed lymphatics within the tumor parenchyma (intratumoral lymphatics) (Pepper, 2001). Some authors argue against tumor-induced lymphangiogenesis, while others have observed dilated lymphatics in the vincinity of tumors. Without doubt, vessel growth, increased permeability, and changes of interstitial tissue fluid pressure and flow could form a prerequisite for metastatic spread occurring after the tumor cells have progressed to an invasive phenotype (Jussila et al., 1998).

The presence and potential function of lymphatic vessels in tumors has remained controversial, mostly due to the lack of molecular markers to reliably distinguish the lymphatics and blood vessels. The discovery of LEC markers has, for the first time, allowed the unambiguous characterization of tumor lymphatics and the assessment of lymphangiogenesis during tumor progression. VEGF-C produced by tumor cells stimulates the lymphangiogenesis and dilation of lymphatic vessels by activating VEGFR-3, and serves as a survival factor for LECs to induce metastases by increasing the functional lymphatic surface area in the tumor margin. Mature VEGF-C also increases the permeability of lymphatic vessels as well as the migration and proliferation of endothelial cells (Joukov et al., 1997). In an experimental model, tumors containing elevated levels of VEGF-C and displaying high metastatic capability, most likely did not metastasize via intratumoral lymphatics, as these internal structures were non-functional, in contrast to the functional lymphatic periphery surrounding the tumor (Straume et al., 2003). Enlarged and dilated lymphatic vessels, in which endothelial proliferation has often been observed, are very frequently present in peritumoral areas of many tumor types. In another experimental observation, LYVE-1-positive structures in the tumor seem to be collapsed and become filled with tumor cells that occlude the lumen, with very few structures having an open lumen (Padera et al., 2002). An explanation for the absence of functional lymphatics within tumors may be that neoplastic cells grown in a confined space generate mechanical stress or interstitial hypertension, which may compress the newly formed lymphatic channels inside the tumor parenchyma (Helmlinger et al., 1997) and interfere with the delivery of therapeutic agents (Jain, 1994). Because of increased interstitial pressure, lymphatic vessels cannot in general penetrate the tumor stroma, although tumors may be able to induce their growth displayed by the rapid growth and regression of lymphatic vessels. Adversely, peritumoral lymphatics potentially provide low stress and pressure, and large volume flow which is well suited for tumor cell extravasation and dissemination (Swartz and Skobe, 2001). It seems reasonable to assume that functional lymphatics in the tumor margin alone are sufficient for lymphatic metastasis and should be targeted therapeutically (Padera et al., 2002). Generally, initial lymphatics are partially or fully collapsed under normal circumstances, because of the lack of smooth muscle coverage and the low pressure within the lymphatic system (Ryan, 1989; Aukland and Reed, 1993). Increased demand for fluid transport in tumor tissues results in the widening of lymphatic lumina; if overdistended, lymphatics become dysfunctional. The functional state of lymphatic vessels cannot be deduced from their morphology, because an open lumen can indicate both vessels with dysfunction as well as normally functioning vessels with increased load. Consequently, functional impairment of lymphatic vessels in terms of transport or uptake of fluid or functional overload might become worse because of an increase in VEGF-C-induced vascular permeability and intratumoral interstitial pressure, destruction of lymphatic networks by invading tumor cells and valve dysfunction.

Although intratumoral lymphatics have been proposed to be nonfunctional in tumor models, univariate proportional hazard analysis reveals that the presence of intratumoral lymphatics is a significant risk factor for the development of lymph node metastasis in patients with cutaneous melanoma (Dadras et al., 2003). In contrast, detailed analysis of lymphatic structures in human head and neck cancers and in melanoma shows that intratumoral lymphatics play a large role in determining the occurrence of relapse, severity of the disease, and sensitivity to therapy (Beasley et al., 2002; Maula et al., 2003). The formation of an intratumoral lymphatic network, whether fully functional in fluid transport or not, provides the most direct route for metastatic tumor spread by increased opportunities for tumor cells to leave the primary tumor site. Furthermore, tumors may secrete factors that promote lymphatic reactions in a similar way that they produce angiogenic factors. Strong angiogenesis reflected by the high vascular density, may increase lymphatic spread of the tumor cells indirectly. Lymphangiogenesis with an increased density was also found in the stromal tissue at the periphery of tumors (Jussila et al., 1998). Tumor spread via the lymphatic vascular bed may be facilitated by the high intrinsic lymphatic density in the tissue in which the tumor arises (Pepper et al., 2003). Although pre-exiting peritumoral lymphatics provide a readily accessible and sufficient avenue for tumor dissemination, recruitment of lymphatic vessels into the close proximity of a tumor may increase the propensity of the tumor to metastasize. Therefore, increased lymphatic vessel density as well as the presence of intratumoral lymphatics should be regarded as an additional pathway rather than a necessity for metastases.

Recently, studies in a series of tumor models have provided fundamental evidence for the importance of tumor-secreted cytokines, such as VEGF-C and/or VEGF-D in tumor lymphangiogenesis and metastases

via lymphatic vessels to regional lymph nodes (Karpanen et al., 2001; Mandriota et al., 2001; Papoutsi et al., 2001; Skobe et al., 2001a,b; Stacker et al., 2001; Krishnan et al., 2003). The development of a series of specific antibodies that recognize previously undefined different antigens on endothelial cells has greatly expedited the investigation on lymphatic growth and regeneration. VEGF-C and -D binding to VEGF receptors on LECs induce proliferation and growth of newly formed lymphatics, whose process is similar to the well-known mechanism of angiogenesis. In the skin melanoma model, lymphatic vessels are frequently enlarged surrounding VEGF-C-overexpressing tumor, and numerous small lymphatic vessels are scattered within the tumor, but are not found in the control tumor (Skobe et al., 2001a). The tumor-derived VEGF-C has potential ability to induce both lymphangiogenesis and angiogenesis. In the breast cancer model, however, VEGF-C overexpression selectively induces intratumoral lymphangiogenesis, leading to increased tumor metastasis, without obvious effects on angiogenesis (Skobe et al., 2001b). Tumor and stromal cells can contribute to the expression of VEGF-C and VEGF-D, suggesting a close tumor cell-host interaction (Krishnan et al., 2003). In the hybridoma-induced intestinal tumor of the mouse, numerous metastatic cells are accumulated in the VEGFR-3-positive lymphatic vessel, which shows extremely faint CD31 immunoreactivity in contrast with intense staining in the blood vessels (Fig. 3e,f). VEGF-C-expressing cells are significantly distributed in the basal portion of the glands rather than LCEs in the hybridoma-induced gastric tumor (Fig. 3g). In certain metastatic human tumors, VEGF-C and VEGF-D are involved in tumor lymphangiogenesis, suggesting that they are up-regulated (Achen et al., 2001), which is considered to be one mechanism whereby tumor metastasis via the lymphatic system can be potentiated. Other mechanisms that can contribute to lymphatic metastasis include chemotatic or chemokinetic stimulation of tumor cells to enter the lymphatics and the passive transport of tumor cells into the lymphatics through interstitial fluid flow. Future work will be directed at functionally determining the contribution of each of these mechanisms to lymphatic metastasis.

Identification of an enzyme that activates the lymphangiogenic growth factors will facilitate development of inhibitors of metastasis. As lymphangiogenic growth factors promote the metastatic spread of cancer via the lymphatics, the proteolytic activation of these molecules represents a potential target for antimetastatic agents (McColl et al., 2003). The proteolytic cleavage removes the propeptides from pre-pro-peptide forms to generate the mature forms, which consist of dimers of the VEGF homology domain and bind receptors with much greater affinity than the full-length forms. However, VEGFR-3 seems to lack specific expression on lymphatic capillaries but is critical for formation of large vessels in the early embryo (Dumont et al., 1998). In the adult, VEGFR-3 is predominantly restricted to LECs, where activation of this receptor induces lymphangiogenesis. VEGFR-3 can be up-regulated on the endothelium of blood vessels in tumor (Veikkola et al., 2001). Furthermore, the blockade of VEGF-C by using a soluble VEGFR-3 extracellular domain inhibits peritumor lymphangiogenesis (Karpanen et al., 2001). A neutralizing VEGF-D mAb, which blocks binding to both VEGFR-2 and VEGFR-3 (Achen et al., 2000) has inhibited endothelial cell growth of both blood and lymphatic vessels, as well as lymphatic metastatic spread in a mouse tumor model. The forms of VEGF-C and VEGF-D used in the experimental studies have the potential to activate VEGFR-2 in addition to VEGFR-3, and thus it cannot be determined whether or not VEGFR-3 activation is specifically responsible for the observed effects on metastasis (Krishnan et al., 2003). Moreover, the incidence of regional lymph node metastases has greatly increased by using a footpad injection of CCR7-tranduced B16 malignant melanoma cells in mice. The expression of a single chemokine receptor gene, CCR7, increased metastases of murine melanoma (B16 cell) to draining lymph nodes, suggesting that cancer cells may co-opt normal mechanisms of lymph modes homing for metastatic dissemination (Wiley et al., 2001). Therefore, as tumor-induced lymphangiogenesis has been associated with enhanced metastasis to lymph nodes (Nathanson, 2003), the risk of enhanced growth and spread of dormant metastases needs to be carefully evaluated. The mechanisms by which malignant tumors leave the primary tumor site, invade lymphatics, and metastasize to regional lymph nodes are complex and interrelated. Several problems touched upon are still far from being solved: 1) Whether expressions of VEGF-C and VEGF-D *in vitro* accurately represent the *in vivo* situation owing to the fact that some of these studies based their conclusions on cultured tumor cells; 2) Has the role of the peritumoral lymphatics been over-emphasized in contrast with that of the intratumoral lymphatics in metastasis of the tumor cells? If not, how do we evaluate the role of the intratumoral lymphatic vessels in the tumor growth and therapeutic target?; 3) Is the high intratumoral parenchymal pressure the sole reason which makes the intratumoral lymphatics dysfunctional?; 4) How tumor cells enter, interact with, and are transported within lymphatic vessels. Are the mechanisms of fluid and particle uptake into lymphatic vessels different from the mechanisms of tumor cell entry into the lymphatics (Skobe et al., 2001a)?; 5) What are the differences in the lymphangiogenesis of the tumor and embryonic tissues ? If there are differences, what are the morphological and molecular characteristics of the LEC phenotypes in newly-formed vessels?; and 6) Whether lymphangiogenesis and pre-existing lymphatics, including lymphatic enlargement, are equally involved in tumor metastasis? Otherwise, the VEGFR-3 signaling system is wholly unnecessary to modulate the integrity/function of tumor-associated mature lymphatic vessels.

The identification of LECs in poorly-differentiated malignant tumors as in early embryonic tissues should be carefully undertaken, although the endothelial markers are useful to identify peritumoral and intratumoral lymphatics and to visualize the ingrowth of tumor cells into the lumen of lymphatics. Introduction of the useful marker for visualizing initial lymphatics has opened new possibility for lymphangiogenetic study in developing tissues and its relation with tumor cell metastasis. Exciting studies involving the pathophysiology of interstitial fluid pressure in tumors and the peritumoral extracellular matrix have focused on lymphatic flow and tumor microenvironment and microcirculation.

Lymphedema

Many concepts in the fields of tissue fluid dynamics, permeability of initial lymphatics, and lymph formation are based on the premise that the protein concentration and colloid osmotic pressure of lymph and tissue fluid are identical. It is obvious then, that edema could occur if either too much lymph is formed relative to the transport capabilities of lymphatics or if the ability of lymphatics to transport lymph is compromised. When the lymphatic pathway is congenitally absent or becomes blocked or obliterated, plasma normally escaping from the bloodstream gradually accumulates as protein-rich edema or effusion. This phenomenon of static insufficiency or low output failure of lymph flow is generally referred to as 'lymphedema', which is defined as primary (congenital) or secondary (acquired) chronic tissue swelling. In primary lymphedema, the superficial or subcutaneous lymphatic vessels are usually hypoplastic or aplastic, and fail to transport the lymph fluid into the venous circulation. A protein-rich fluid accumulates in the interstitial space, leading to tissue fibrosis and adipose degeneration, interference with wound healing, and susceptibility to infections, such as lymphangitis (Szuba and Rockson, 1998). Noninherited secondary lymphedema develops when the lymphatic vessels are damaged by surgery, radiation, infection or trauma, in which malignancy-associated lymphedema may result from tumor infiltration of the lymphatics or from treatment intervention (Browse and Stewart, 1985), although the mechanisms underlying the development of lymphedema are not yet fully understood. Furthermore, accumulation of stagnant, protein-rich fluid into the interstitial matrix between cells reduces the delivery of oxygen and other molecules to cells and attenuates immune responses in tissues.

In recent years, a number of genetic mouse models have been developed with the enhanced molecular understanding on lymphedema, in which FOXC2-null mice, Chy mice, K14-VEGFR-3-Ig mice and T1α/podoplanin mice greatly contribute to genetic mechanisms of lymphedema formation and lymphatic development controlled by multiple factors, especially VEGFR-3 and FOXC2. In human primary disorders, lymphedema distichiasis is associated with gene FOXC2; this forkhead/winged helix family transcription factor suffering from multiple insertions and deletions predicts defects in DNA binding and transcriptional activation (Finegold et al., 2001). The development of human hereditary lymphedema phenotype is associated with heterozygous missense mutation of the Flt4 gene, which leads to insufficient VEGFR-3 signal transduction or to inactivate VEGFR-3 tyrosine kinase catalytic function (Ferrell et al., 1998), suggesting a direct evidence of the link between VEGFR-3 and lymphedema. Genetic disruption of FOXC2 in mice results in a number of phenotypes that mirror the human pathology (Kume et al., 2001). However, the overt lymphedema in humans is not as robustly recapitulated in the mouse model. Subcutaneous injection of adenovirus or adeno-associated virus encoding VEGF-C can generate lymphatic vessels in the skin of normal mice (Enholm et al., 2001) and in a mouse model (Chy mouse) of primary lymphedema (Karkkainen et al., 2001b). Chy mice develop chylous ascites after birth; like the lymphedema patients these mice have a heterozygous inactivating VEGFR-3 mutation within the kinase domain and edema of the lower extremities because of a lack of subcutaneous, but not visceral lymphatic vessels. Inactivating missense point mutations in one VEGFR-3 allele lead to chronic lymphedema. Furthermore, by using viral gene delivery and transgenic approaches, VEGFR-3 ligand overexpression is elucidated to induce the growth of functional cutaneous lymphatic vessels in the Chy mice, indicating the possible therapeutic effect of VEGF-C/D in hereditary lymphedema (Karkkainen et al., 2001b).

In addition, a chimeric protein consisting of the ligand-binding portion of the extracellular part of VEGFR-3, joined to the Fc domain of immunoglobulin (Ig) γ-chain (VEGFR-3-Ig) neutralizes the activity of VEGF-C and VEGF-D and inhibits the formation of the dermal lymphatic vasculature when expressed in mouse epidermis under the keratin-14 (K14) promoter (Makinen et al., 2001a). In K14-VEGFR-3-Ig mice, the inhibition of VEGF-C and/or VEGF-D binding to VEGFR-3 during development leads to apoptosis of the LECs, disruption of the lymphatic network and signs of dermal fibrosis. Overexpression of a soluble VEGFR-3 in mice induces regression of lymphatic vessels during embryonic development and has features of human lymphedema, without any apparent effects on the blood vascular networks (Makinen et al., 2001a). The absence of lymphatic vessels in the skin of K14-VEGFR-3-Ig mice is associated with a thickening of the dermis and subcutaneous layer, a disorder caused by insufficiency of the lymphatic system and characterized by a swelling of the extremities of increasing severity (Karkkainen et al., 2001b). Functionally, a lack of macromolecular transport occurs in the dermis, especially in older mice (Makinen et al., 2001a). T1α/podoplanin$^{-/-}$ mice are characterized by congenital lymphedema, as manifested by the pronounced swelling of the limbs at birth. Intradermal

dye injection into the foot pads of T1α/podoplanin$^{-/-}$ mice reveals several enlarged, plump lymphatic vessels, but fails to visualize the characteristic dermal capillary networks which occur in wild-type and in T1α/podoplanin$^{+/-}$ mice (Schacht et al., 2003). However, histological examination reveals the presence of dermal capillaries in T1α/podoplanin$^{-/-}$ mice and these findings indicate an insufficient formation of anastomosing lymphatic vessels between the superficial and subcutaneous lymphatic networks. The transmembrane glycoprotein T1α/podoplanin is required to control different aspects of normal lymphatic vasculature formation. Lack of T1α/podoplanin leads to alterations in the final patterning of the lymphatic vasculature as well as in lymph transport (Schacht et al., 2003).

In hereditary lymphedema with reduced *VEGFR-3* signaling in heterozygous-affected individuals (Irrthum et al., 2000), genes that would induce VEGFR-3 signaling specifically in the lymphatic endothelium might improve the growth and function of lymphatic vessels without side effects in other tissues. Gene transfer of naked plasmid DNA-encoding human VEGF-C (phVEGF-C) in animal models of secondary lymphedema promotes selective proliferation of functional lymphatics associated with increased lymph drainage and decreased skin thickening (Yoon et al., 2003). Similar effects have been obtained by administration of a single dose of recombinant VEGF-C in the same model of acquired lymphedema in the rabbit ear (Szuba et al., 2002).

It is still unclear as to whether VEGFs are involved in the regeneration of post-surgical lymphedema. Prevention or reduction of fibrotic alteration is very important in lymphedema therapy, since the secondary change can drive edema into a vicious cycle by increasing interstitial solid pressure (by fibrofatty deposition) and thus collapse already reduced or impaired lymphatic vessels (Yoon et al., 2003). Radiotherapy of the breast and the number of lymph nodes removed are the most important prognostic factors in the occurrence of lymphedema after treatment for breast carcinoma (Herd-Smith et al., 2001). The development of fibrosis as a consequence of radiotherapy induces lymphatic vessel constriction, which most likely decreases the filter function of the lymph nodes and alters the immune response. It has been proposed that mastectomy patients may benefit from stimulation of lymphangiogenesis in the region of lymph node removal to aid fluid drainage and prevent side effects associated with breast cancer. Therapeutic approaches to achieve this could be based on gene therapy or direct protein application to administer VEGF-C or VEGF-D to affected sites. Recently, experiments in animal models and analysis of genetic lesions in human hereditary lymphedema have indicated that the VEGF-C/VEGF-D/VEGFR-3 signaling system drives lymphatic hyperplasia and/or lymphangiogenesis during embryonic development (Irrthum et al., 2000; Veikkola et al., 2001) and in adult tissues (Enholm, 2001). It seems reasonable to deduce that the regrowth of lymphatics after surgery induced by VEGF-C may help to prevent subsequent lymphatic obstruction in view of the fact that VEGF-C is a necessary and sufficient signal for the growth of LECs. Therefore, more information from ongoing trials on lymphedema after sentinel lymph-node biopsy is needed, especially when associated with radiotherapy. One might assume that treatment of lymphedema in the arm after axillary lymphadenectomy in association with breast cancer surgery may pose a problem because it could enhance the growth and spread of dormant metastasis. However, the half-life of VEGF-C in the blood circulation is short (Veikkola et al., 2001), and local VEGF-C therapy is thus likely to function without systemic effects.

The feature common to all types of obstructive lymphostasis is an impairment of the organ's lymphatic transport capacity. The small intestine is a main target organ for elucidating the effects of thoracic duct blockage on the endothelial cells of initial lymphatics. Taking this into account, the alterations of endothelial cell function and morphology might be the key points for analyzing the mechanisms involved in lymphatic disorders. Conspicuous effects on the intestinal lymphatics by lymphostasis result in dilation and dysfunction of collecting lymphatics and intralymphatic valves and lymph retention in intestinal lymphatics for several days (Fig. 3h). Functionally, the endothelial cells of initial lymphatics represent reduced 5'-Nase and endothelial nitric oxide synthase (Ji and Kato, 2001). Prolonged obstruction of intestinal lymph flow will progressively aggravate peripheral lymphostasis and lymphatic incompetence. However, according to our observations, TD blockage effects on intestinal LECs are temporary and last for about 6 weeks after ligation. The gradual recovery of LEC structure and function might be due to the observation that effective circulation is established by a remarkable regenerative capacity of smaller lymphatics, rapid development of collateral pathways around the blockage and a relatively high rate of formation of lymphovenous anastomoses (Piller and Clodius, 1980). The possibility that lymph vessels may proliferate or grow to adapt a long-term increase in the rate of lymph flow has received limited attention. Although it has become clear in recent years that lymph is pumped actively by intrinsic contractions of lymphatic vessels, the relative roles of the intrinsic and extrinsic lymphatic pumps have still not been determined quantitatively. During blockage of the lymph flow, numerous open junctions between endothelial cells appear in the intestinal lymphatics. Open junctions of the initial lymphatics fail to close or become permanently closed, failing in their ability to concentrate proteins in the lymphatic vessels. Acute hydrostatic edema has been suggested to correlate with apoptosis in microvascular endothelial cells (Gotoh et al., 2000). Further detailed studies on newly-formed initial lymphatics are necessary for a better understanding of the role of various growth

factors and cytokines in regulating lymphatic permeability during lymphatic obstruction and lymphangiogenesis. Moreover, it remains to be explored whether lymphostasis is a pivotal factor in inducing apoptosis of LECs or not.

Lymphedema as a result of low output of the lymph circulation can be congenital or acquired and gives rises to swelling, scarring, immune dysregulation and malnutrition. Both the induction and inhibition of lymphangiogenesis are of clinical interest. Patients suffering from lymphedema may benefit from VEGF-C-induced therapeutic lymphangiogenesis. Anti-VEGF-C strategies may result in inhibition of lymphangiogenesis, which is beneficial to patients with lymphangiosarcoma.

Concluding remarks

There is an increasing need to study the heterogeneity of endothelial cells in order to understand the specificity of chemokines, the vasoactive agent and growth factors that control the behavior and growth of lymphatic vessels. Currently, great advances have been made at the molecular level, thereby pushing forward our understanding of lymphatic function. Coupled with this knowledge is the possibility of elucidating a number of pathological states that are associated with defective lymphatic function (Kim and Dumont, 2003), although conclusive evidence in humans rather than animal models are eagerly hoped for. Therefore, manipulation of VEGF-C/VEGF-D/VEGFR-3-, *Prox-1*-, Syk/SLP-76-, Podoplanin/Ang-2/Nrp-2-signaling pathways (Abtahian et al., 2003; Folkman and Kaipainen, 2004) should offer the opportunity for therapeutic strategies designed to inhibit or stimulate growth of lymphatic vessels in conditions such as lymphedema, cancer and infectious diseases. Perhaps more importantly, it will also open the door to new discoveries concerning the important and often overlooked role of the lymphatic system in physiology and pathology.

Acknowledgements. The author would like to thank Profs. Seiji Kato and Yoshihisa Fujikura, Associate Prof. Keisuke Ina, Drs. Hirokazu Kitamura, Mei Cao, Peng Qu, and Mr. Tooru Kajiwara, Shuji Tatsukawa, Hiroaki Kawazato, and Ms. Miyuki Maki, Xiao-Ou Ji and Aiko Yasuda for their kind assistance.

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Accepted July 13, 2004