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The lymphatic vascular system, a one-way drainage system, plays an important role in transportation of excess interstitial fluid and absorbed macromolecules from tissues back to blood circulation, as well as in many pathological processes including tumor metastasis and lymphedema. The structural organization and fine distribution of lymphatic vessels in the tissues are very important in the pathological physiology of a variety of microcirculartory disorders, infectious diseases and cancer. A process known as lymphatic development (lymphangiogenesis) is closely related to malformation and dysfunction of the lymphatic system. In the past few years the discovery of new lymphatic endothelial cell (LEC)-specific markers has now provided new insights into the molecular mechanisms that control lymphatic development and function [1]. We herein review the histochemical progresses in the field of lymphangiogenesis, with special emphasis on the novel and reliable LEC markers, such as 5´-nucleotidase and VEG-C/VEGFR-3, as well as on experimental lymphatic regeneration[2,3].

Lymphangiogenesis; Lymphatic regeneration; Histochemistry; Lymphatic markers; 5´-nucleotidase

L 5´-nucleotidase (5´-Nase), an adenylate and guanylate in the metabolism of nucleotides, has widely been employed as a marker of cell membranes in differentiating initial lymphatics from blood capillaries, based on its much higher activity on lymphatic than on blood vascular endothelium[4,5]. In addition, blood endothelia, especially artery and arterial capillaries, have comparatively strong alkaline phosphatase (ALPase) activity. Thus, 5´-Nase-ALPase double staining has provided an effective method to distinguish two kinds of vessels[6,7]. Furthermore, dipeptidyl amminopeptidase IV (DAPase) activity is markedly higher in the endothelium of the venous part of the capillaries and venules. Therefore, DAPase-ALPase double or DAPase-ALPase-5´-Nase triple staining for differentiating venous and arterial capillaries and/or lymphatics is of benefit to an understanding of pathological implications not only in laboratory animals but also in humans[8].

SEM examination of 5´-Nase-stained tissues on whole-mount preparations or tissue blocks allowed a precise analysis of the abluminal aspects and three-dimensional structure of the lymphatic network [9,10]. Adequate treatment with NAOH could visualize the three-dimensional structures of the immunohistochemial stained lymphatics in both secondary emission and backscattered images[11].

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When 5´-Nase antigenecity rather than its activity is considered, 5´-Nase mAb specific for the lymphatic endothelium, instead of adenosine monophosphate, can serve immunohistochemically as a useful marker for cell selection and in vitro cultivation. JC815 immunoreactivity was distinctly expressed on the lymphatic vessels of several mammalian tissues, in comparison with 5´-Nase staining control [12]. In the pancreas, JC815 strongly stained the interlobular lymphatic endothelium and was similar to the 5´-Nase staining pattern. Furthermore, in the rat tongue, immunohistochemical analysis of ecto-5´-Nase (CD73) is assumed to provide not only reassessment of the validity of 5´-Nase as a lymphatic endothelial marker, but also new information including technical benefit. The 5´-Nase activity, CD73 immunoreactivity and hybridization signals for its mRNA were colocalized in the lymphatic vessels, including central lacteals of the small intestine, suggesting that 5´-Nase is actually produced in

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LECs and allocated to their cell membrane as an enzyme to regulate lymph production and flow [13]. These findings support our view that 5´-Nase is potential marker of lymphatics and its histochemical methods is useful not only for demonstrating of lymphatics, but also for examining the functional roles and dynamics of 5´-Nase in LECs in physiological and pathological conditions.

A highly glycosylated class III cell surface tyrosine-kinase receptor, VEGFR-3[14,15] was preferentially immunolocalized in the structures corresponding to the 5´-Nase-positive lymphatics in the lesion, and the immunoreaction products were ultrastructurally distributed on the cell membrane of lymphatic vessels. In contrast, immunostaining for VEGF-C demonstrated significant reaction products in many stromal cells, which predominantly demonstrated signals for VEGF-C mRNA in in situ hybridization [13]. Therefore, the combination of enzyme histochemistry and immunohistochemistry, especially 5´–Nase mAb with other LEC markers, and may give a new possibilitys for lymphatic investigation.

Initial lymphatics show extensive networks, obious valve-like structures and numerous blind–ends. These structural features undoubtedly help us to analyze lymphatic localization, and to understand the vessel sprouting and splitting. Functionally, endothelial cells of newly-formed lymphatic-like structures usually show extremely low 5´-Nase activity in early embryonic tissue [16]. Interrupted weak or absent staining of endothelial cells was seen on newly-formed lymphatic-like structures in the early stage, although no 5´-Nase reaction product was observed in blood vascular endothelial cells. Lymphatic vessels in the rat stomach reveal increased 5´-Nase activity as the animals grew [17]. Thus, 5´-Nase staining appears to be impractical for distinguishing developing lymphatics and blood vessels. On the other hand, in the early developing gastric wall, antiVEGFR-3 was expressed in a cluster of circular lymphatic-like structures, which were gathered together in several groups [17]. VEGFR--3 makes it possible to identify developing and regenerating lymphatic vasculature by localizing the antigen. VEGFR-3 binding to endothelial cells showed variations in staining intensity in the lymphatic wall and among samples. Developing endothelial cells of lymphatic and blood vasculatures react with VEGFR-3 during the early embryonic stage, although immature lymphatic vessels were usually stained with VEGFR-3 staining more intensely than typical lymphatic vessels. This suggests that LECs are similar in molecular physiological features to blood vascular endothelia, and probably originate from the sprouting of small venous structures. However, the opinion is not contrary to the observation that lymphangiogenesis originates from lymphatic vessels [18]. The present findings are in close agreement with the view that VEGFR-3 is widely expressed to the lymphatic endothelium at later developmental stages and in postnatal life [19]. Many circular and incomplete lymphatic-like structures expressing VEGFR-3 show an obvious accumulation, indicating that lymphangiogenesis occurs sequentially in definite regions in the early embryonic stage, although the staining results for cell proliferation should be carefully analized.

Many concepts in the fields of tissue fluid dynamics, permeability of initial lymphatics, and lymph formation are base on the functional characteristics of LECs, which keep an identical in proteins concentration and colloid osmotic pressure of lymph and tissue fluid. The effects of experimentally induced lymphedema have been studied in the extremities and internal organs of various animals in the light of the clinical understandings of lymphedema[20,21]. After thoracic duct (TD) blockage in rats, the mucosal and submucousal compartments of the small intestine in lymphostasis showed tortuous lymphatic networks and saccular dilations of the lymphatic vessels surrounded by fibrinoid materials, swollen collagen fibers, and focal accumulation of mononuclear cells. A tendency for reduced 5´–Nase activity in the TD endothelial cells became visible when the lymph flow was obstructed by TD blockage [22]. During TD blockage-induced lymphostasis, the 5´-Nase reaction product was almost undiscernible as a continuous demarcation of the endothelial layer within 2 weeks. Interestingly, the reduced 5´-Nase activity appeared earlier in the intramural intestinal lymphatics than in the TD itself. Prolonged obstruction of intestinal lymph flow will progressively aggravate peripheral lymphostasis and lymphatic incompetence. The effect of TD blockage on endothelial cells of the intestinal lymphatics and TD was temporary and lasted for about 6 weeks af-

ter ligation. The gradual recovery of the structure and function of the endothelial cells might be due to the observation that effective circulation is established by the marked regenerative capacity of smaller lymphatics, rapid development of collateral pathways around the blockage, and relatively high rate of lympho-venosus anastomose formation[23].

Wound healing skin

Wound healing skins in mice were processed for 5′-Nase and VEGFR-3 histochemical staining to distinguish lymphatics from blood capillaries and analyze lymphangiogenesis[3]. In the wound skin of the mice, anti-VEGFR-3 immunopositive signals unevenly appeared in 5′-Nase-positive lymphatic vessels in the subcutaneous tissue 3–5 days after injury. Numerous accumulated vasculatures were stained for 5′-Nase and PECAM-1/CD31, extending irregularly along the wound edge on days 7 to 15. Ultrastructural changes in lymphatic vessels developed at different stages, from lymphatic–like structures to newly-formed lymphatic vessels with an extremely thin and indented wall. The generating signals of VEGFR-3 on LECs appeared as early as three days after injury in the subcutaneous tissue, much earlier than in the dermis. The expression pattern of VEGFR-3 in regenerating tissues was extremely uneven on the lymphatic wall, which indicating that endothelial sprouting might begin from its up-expressing side. The most noteworthy finding was that numerous circular and irregular lymphatic-like structures with VEGFR-3 expression were distributed in the dermal and subcutaneous tissues along the wound edge. With the maturation of lymphatic vessels, the wall became slender and irregular, and the endothelium protruded into the lumen and adjacent connective tissue. Intercellular junctions underwent a morphological change from simple end-to end to overlapping and interdigitating. The simple junction might facilitate separation, spreading and migration of endothelial cells during lymphatic remodeling in compliance with tissue repair patterns. This finding appeared to be in good agreement with our previous observations in the early embryonic tissues of the monkey [17]. However, 5′-Nase activity in the endothelial cells of newly-formed lymphatic vasculature is low during the wound healing process.

Combined histochemical staining for 5′-Nase and VEGFR-3 with multiple endothelial cell markers is useful to study regenerating lymphatics and their relationship with blood vessels [3]. VEGFR–3–expressing vasculatures occurred in the dermal–subcutaneous transitional area at the early stage of wound injury, whereas a 5′-Nase-positive lymphatic structure along the wound edge underwent morphological changes. These findings indicated that sprouting and growth of regenerating lymphatic vessels are active processes in the healing tissue response.

Regrowing an intestinal muscle coat after myectomy

The lymphatic regrowth from the surviving vessels in the severed stumps of the intestine occurred behind the regeneration of other tissue elements. including blood vessels [24]. The vascular arcades and terminal expansions are presumed to serve as growth points in lymphangiogenesis, as in angiogenesis[25]. The unusual ultrastructural characteristics of the regrowing LECs filopodium-like cytoplasmic projections and numerous intracellular thin filaments, probably indicate the high regenerative and migratory potential of the cells to establish new vascular channel.

The enzyme histochemistry for 5′-Nase demonstrated the manner of lymphatic regrowth, which was established by vascular sprouting from preexisting lymphatics and structural changes in the endothelial cells indicating their high migratory potential. The expression of 5′-Nase in the regenerating lymphatics was increased in proportion to their growth. These findings suggest that 5′-Nase may be correlated to the functional maturation of the regenerating lymphatics, because it is thought to facilitate membrane tr ▮

transaction of the intestinal muscle coat affords a useful experimental model for the investigation of lymphatic regeneration in tissue repair, and that the interstitium may play a crucial role in lymphangiogenesis through VEGF-C molecular regulation.

With regard to adhesion, differentiation, and migration of endothelial cells, some elements, including 5´-Nase and eNOS[22]. the extracellular matrix of the basement membrane, and components of the surrounding connective tissues, are very important factors in the formation of developing vasculature. More recently, several other proposed markers for LECs, e.g., LYVE-1, a homologue of the CD44 hyaluron receptor[27,28], podoplanin[29,30], and Prox1[31,32], have emerged, but they remain questions about their reliability and specificity, and need further study. However, co-expression of these new markers with so-called routine differentiating reagents, such as laminin, collagen type IV, and α-smooth muscle actin, is definitely helpful in analyzing the functional-structural properties of endothelial cells of both lymphatic and blood vessels.

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