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Laboratory Investigation
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Brief Method

Application of a New 5'-Nase Monoclonal Antibody Specific for Lymphatic Endothelial Cells

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5'-nucleotidase (5'-Nase) histochemical staining has proved to be an effective method in differentiating initial lymphatics from blood capillaries, based on its much higher activity on lymphatic than on blood vascular endothelium (Kato et al, 1996). A considerable variability in the enzyme activity of lymphatic endothelial cells, however, was revealed in different organs and tissues, eg, a reduced expression for lymphatic endothelium in the developing gastric wall and an overexpression for uterine lymphatics in pregnant monkeys (Ji, 1998; Ji and Kato, 2003). When 5'-Nase antigenity rather than its activity is considered, 5'-Nase mAb specific for lymphatic endothelium, instead of adenosine 5'-monophosphate, can serve immunohistochemically as a useful marker for cell selection and in vitro cultivation. Under these circumstances, the enzyme histochemistry cannot detect under- or overexpressed 5'-Nase activity. This deficiency can be made up by immunohistochemistry with an effective 5'-Nase-mAb to determine the existence and site of the antigen specific for lymphatic endothelial cells rather than other parenchymal cells. The aim of this contribution is to produce a sensitive and specific marker for the identification of lymphatic endothelial cells.

5'-Nase from Crotalus atrox venom (EC 3.1.3.5, Sigma, St. Louis, Missouri) was directly injected into the spleen of male BALB/c mice (5 to 8 weeks old) for the first immunization. The spleen cells were prepared after the booster injection and fused with X63-Ag8.653 myeloma cell line (Kohler and Milstein, 1975). Following hybridoma selection, screening of the supernatants from growing cells was initially performed by ELISA and on cryosections by the indirect streptavidin-biotin-peroxidase complex method. One of the clones, JC815, was selected from positive hybridoma wells by limiting dilution (0.25 cells per well) in the presence of BALB/c thymocytes used as feeder cells. Ascites tumor fluid was produced by using female BALB/c mice and purified by the protein A–Sepharose chromatography. For immunoblotting protein samples of 5'-Nase and cultured rat thoracic duct endothelial cells (ThDECs) homogenized in lysis buffer were processed with SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane at 200 mA for 3 hours. The trimmed sheets were incubated with JC815 (IgG and ascites diluted in 1:800) and then reacted with goat antimouse immunoglobulins and streptavidin-biotin-peroxidase solution. A water rinse was used to stop 0.03% 3,3'-diaminobenzidine tetrahydrochloride (DAB) development of the sheets. For immunohistochemistry, cultured ThDECs and 5- to 7-μm thick cryosections from mouse and rat tissues were incubated with JC815 diluted in 1:200 to 1:400 and with biotinylated conjugated rabbit antimouse IgG. DAB visualization was partly processed by 2% OsO_4 fixation for transmission electron microscopy (TEM) to identify JC815 reactivity. Postembedding staining ultrasections were incubated with JC815 diluted in 1:400 and then with goat antimouse IgG bound to 5-nm gold particles (BBInternational, Cardiff, United Kingdom). Negative controls were incubated with 1% normal goat serum in 0.1 M PBS rather than JC815. Positive controls were treated with 5'-Nase histochemical staining to demonstrate the enzyme activity for lymphatic endothelial cells.

JC815 immunoreactivity was distinctly expressed on the lymphatic vessels of the stomach, diaphragm, skin, thoracic duct, tongue, pancreas, and liver from mice and rats, in comparison with 5'-Nase staining controls. In the tongue, JC815 strongly stained the subepithelial lymphatic endothelium and was similar to the 5'-Nase staining pattern (Fig. 1, A and B). In the liver, JC815-expressing lymphatic vessels with thin endothelial walls and irregular contours were located in the interlobular connective tissue (Fig. 1C). Lymphatic vessels in hybridoma-induced pancreatic tumors showed uneven JC815 immunoreactivity, in whose lumen there were numerous metastatic hybridoma cells (Fig. 1D). In the confluent monolayer of ThDECs, the dividing and multinuclear cultured cells were characterized by a uniform cobblestone appearance, showing various reactive

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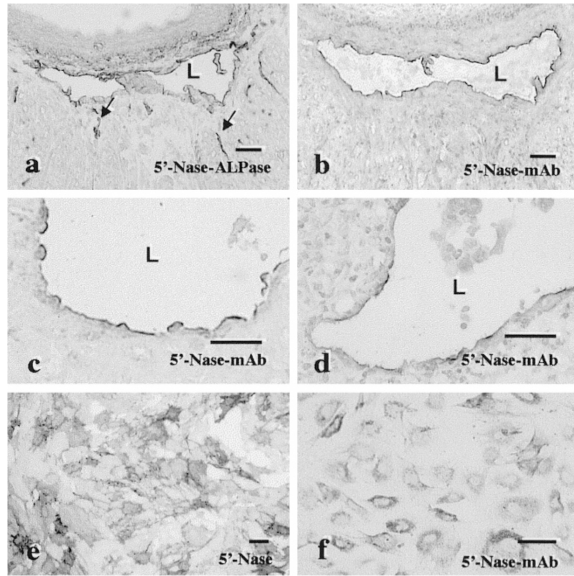

Figure 1.

Photomicrographs of cryosections (A to D) and thoracic duct endothelial cells (ThDECs) (E and F). (A and B) A 5'-nucleotidase (5'-Nase)–positive lymphatic vessel, around which are several ALPase-positive blood vessels (*arrows*) (A), is strongly stained with 5'-Nase-mAb beneath the squamous epithelium of the mouse tongue (B). (C and D) Interlobular lymphatic vessels show 5'-Nase-mAb expression in the rat liver (C) and in the hybridoma-induced pancreatic tumor of the mouse (D). Numerous metastatic tumor cells are seen within the lymphatic vessel (D). (E and F) ThDECs show different staining intensities with 5'-Nase-lead staining procedure (E); immunocytochemical localization of 5'-Nase-mAb demonstrates a granular reaction product, but the nucleus appears negative (F). *L* = lymphatic vessel. A to D, bars = 50 μm; E and F, bars = 100 μm.

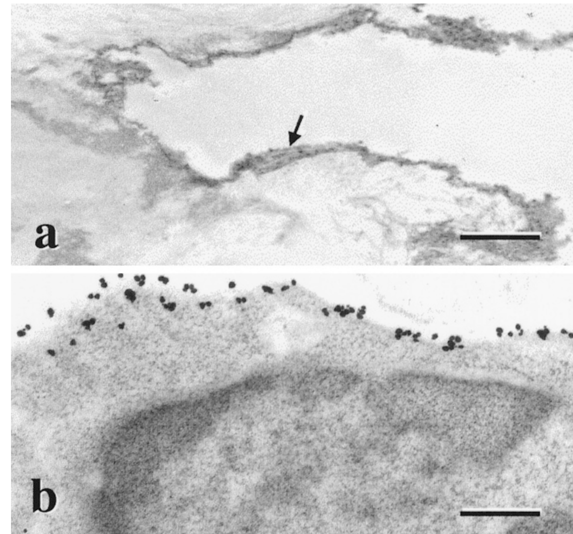

Figure 2.

Transmission electron microscopy views of immunocytochemical staining in lymphatic endothelial cells of the rat tongue (A) and the hybridoma-induced intestinal tumor of the mouse (B). (A) With a pre-embedding technique, immunoreactive deposition of 5'-nucleotidase (5'-Nase)-mAb is detected on the endothelial cells and is scattered into an interdigitating junction (*arrow*). (B) With postembedding staining, 5'-Nase-mAb immunogold particles with silver enhancement are attached to the luminal surface of endothelial cells. A, bar = 2 μm; B, bar = 0.5 μm.

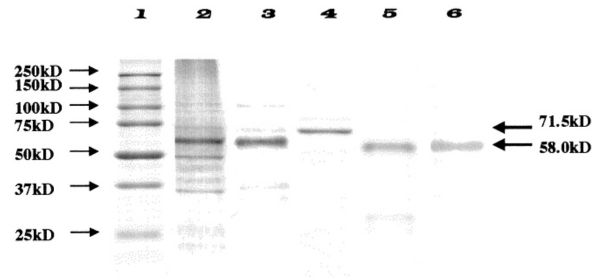

Figure 3.

Analysis of 5'-nucleotidase (5'-Nase) antigen and 5'-Nase-mAb. The SDS-PAGE gels are prestained (Lane 1) or stained with Coomassie blue (Lanes 2, 4, and 5), and the immunoblotting sheets (Lanes 3 and 6) test for 5'-Nase antigenic specificity and 5'-Nase-mAb effectivity by indirect peroxidase method. Lane 1, prestained precision standard molecular marker. Extract from primary cultured thoracic duct endothelial cells is submitted to SDS-PAGE (Lane 2) and transferred to nitrocellulose strip by electroblotting (Lane 3), in which a strong positive binding of extract with 5'-Nase-mAb is revealed. Lane 4, electrophoresis of 5'-Nase-mAb shows a single band. Lanes 5 and 6, 5'-Nase-mAb–positive band in the immunoblotting sheet (Lane 6) is transferred from 5'-Nase antigen on SDS-PAGE gel (Lane 5).

intensities when processed by 5'-Nase-lead enzyme cytochemistry. Immunocytochemical localization of JC815 revealed a granular reaction product on the ThDECs, in contrast to extensively diffuse staining for 5'-Nase (Fig. 1, E and F). In TEM, immunoreactive deposition of JC815 was detected on the endothelial cells and dispersively extended into an interdigitating junction (Fig. 2A). JC815 immunogold particles with silver enhancement were attached to the luminal surface of endothelial cells (Fig. 2B). JC815 revealed a constant single protein band of 71,500 Da in molecular weight, a strongly recognized 5'-Nase antigen band noted at 58,000 Da, and the antigen from ThDECs extract (Fig. 3). JC815 clearly reacted with the submucosal and serosal lymphatic vessels of the small intestine and colon, and the initial lymphatics immediately beneath peritoneal mesothelium. JC815 showed no obvious staining in the central lymphatic vessels of intestines but occasionally reacted with small blood vessels.

The occurrence of a series of specific antibodies that recognize previously undefined different antigens of lymphatic endothelial cells has greatly expedited the investigation on the relationship between lymphangiogenesis and tumor cell metastasis. Widespread application of lymphatic endothelial markers, such as the receptor of vascular endothelial growth factor C, the lymphatic vessel endothelial hyaluronan receptor-1, podoplanin, and Prox-1 would shed light on different biological functions of the endothelial cell (Ji and Kato, 2003; Paavonen et al, 2000; Prevo et al, 2001; Wigle et al, 2002). Unfortunately, none of the monoclonal antibodies recognizing lymphatic endothelium has been found to be absolutely specific for this cell type so far, especially in the early embryonic stage and poorly differentiated malignant tumor. In the present study, JC815 recognizes the endothelium of almost all lymphatic vessels with a predominance over small blood vasculatures in easily detectable amounts on cultured endothelial cells and on a panel of cryosections of different tissues in mice and rats. The tissue reactivity of JC815 immunohistochemistry showed high similarity in specificity to that of 5'-Nase

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enzyme histochemistry, suggesting that the epitope detected by JC815 is located on molecules from 5'-Nase antigen of lymphatic endothelial cells. The new mAb JC815 described herein promises to be important in elucidating function-structural characteristics and in studying proliferation and differentiation of lymphatic endothelial cells. Therefore, the combination of JC815 with other endothelial markers, and enzyme histochemistry with immunohistochemistry, might open a new possibility for lymphatic investigation.

References

Ji RC (1998). Enzyme-histochemical investigations on the lymphatics of the intrinsic uterine wall in the monkey. Biomedical Res 19:347–355.

Ji RC and Kato S (2003). Lymphatic network and lymphangiogenesis in the gastric wall. J Histochem Cytochem 51:331–338.

Kato S, Itonaga I, Ji RC, and Miura M (1996). Enzyme triple staining for differentiation of lymphatics from venous and arterial capillaries. Lymphology 29:15–19.

Kohler G and Milstein C (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497.

Paavonen K, Puolakkainen P, Jussila L, Jahkola T, and Alitalo K (2000). Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. Am J Pathol 156: 1499–1504.

Prevo R, Banerji S, Ferguson DJ, Clasper S, and Jackson DG (2001). Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. J Biol Chem 276:19420–19430.

Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, Jackson DG, and Oliver G (2002). An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J 21:1505–1513.

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