Demonstration of the Rat Lymphatic Vessels Using Immunochemistry and In Situ Hybridization for 5'-Nucleotidase

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ABSTRACT

The cellular distribution of 5'-nucleotidase (5'-Nase) was studied in the rat tissues at the levels of protein and mRNA by use of immunohistochemistry and in situ hybridization, and compared with that of reaction products by enzyme-histochemistry. 5'-Nase activity, immunoreactivity of ecto-5'-Nase (CD73) and hybridization signals for its mRNA were colocalized in the lymphatic vessels including central lactic of the small intestine, suggesting that 5'-Nase is actually produced in the lymphatic endothelial cells and allocated to their cell membrane as an enzyme to regulate lymph production and flow. Double immunostaining for CD73 and Factor VIII-related antigen clearly showed the distinction and relationship between lymphatics and the blood vessels. The present findings support our view that 5'-Nase is an available marker of lymphatics, and indicate the usefulness of the histochemical methods for 5'-Nase not only for demonstration of lymphatics, but also for examination of the functional roles and dynamics of 5'-Nase in the lymphatic endothelial cells in physiological and pathological conditions.

The lymphatic system in several organs has been investigated by a variety of methods involving injection of colored substances and electron microscopy. In addition, recent studies described the occurrence of novel markers for lymphatic vessels, such as vascular endothelial growth factor receptor (VEGFR)-3 (3, 5, 7), LYVE-1 (1, 10) and Proxl (16, 17).

We have developed an enzyme-histochemical method for 5'-nucleotidase (5'-Nase) to visualize the structure and distribution of lymphatic vessels in various tissues (6, 12), but the expression of the protein in the lymphatics remains to be elucidated. Immunohistochemical analysis of 5'-Nase is assumed to provide not only reassessment of the validity of 5'-Nase as a lymphatic endothelial marker, but also a new information including technical benefit. The present study, therefore, demonstrates the expression of 5'-Nase in the lymphatic vessels by employing immunohistochemistry and in situ hybridization techniques.

MATERIALS AND METHODS

Five Wistar rats of both sexes (150-250 g) were used in this study. They were allowed standard laboratory animal chow and water ad libitum under routine laboratory conditions. All experiments were adhered to the Guidelines for Animal Experimentation, Oita Medical University.

Tissue Preparation. After the rats were sacrificed by exsanguinations under deep anesthesia with sodium pentobarbital (50 mg/kg), the jejunal segments with mesentery and tongues were removed and placed in a 0.1 M cold phosphate buffer (pH 7.4).

For light and fluorescence microscopy, some tissue pieces of jejunal segments and tongues were rapidly frozen in liquid nitrogen to prepare serial tissue sections of 20 μm thickness, whereas mesentery was fixed with acetone for 3 h at 4°C in a stretched...
state. The sections were immersed in ice-cold acetone for 10 min before processing for histochemical staining.

For electron microscopy, some small tissue pieces of the intestine were fix ed in periodate-lysine-parafomaldehyde (PLP) solution for 3 h at 4°C and immersed in 0.1 M phosphate buffered saline (PBS) containing 30% sucrose. The tissues were rapidly frozen in liquid nitrogen and sectioned at a 20 μm thickness.

**Enzyme-histochemistry.** Some tissue sections were processed for enzyme-histochemical staining for 5'-nucleotidase (5'-Nase) activity (6, 12). After rinsing in 0.1 M cacodylate buffer (pH 7.2), the specimens were incubated in the reaction medium for 5'-Nase for 20 min at 37°C with 5'-adenosine monophosphate (AMP) (Sigma Chemical, St. Louis, MO, USA) as a substrate, lead nitrate (TAAB Laboratories Equipment, Berkshire, England) as a capture agent, and 2 mM t-tetramisole (Sigma) as an inhibitor of nonspecific alkaline phosphatase. After washing with distilled water, the tissues were treated with 1% ammonium sulfide solution for 1 min at room temperature, and examined under a light microscope (Olympus, BX-60, Tokyo).

Control staining for 5'-Nase reaction was carried out by incubation with medium containing 50 mM NiCl₂, a potent inhibitor of this enzyme, and gave completely negative results.

**Immunohistochemistry.** Some tissue sections were immersed for 20 min in 0.3% H₂O in PBS containing 0.01% sodium azide at room temperature to block the endogenous peroxidase activity. After being rinsed in PBS, the sections were incubated in 10% normal rabbit serum for 15 min and then in mouse monoclonal antibody against rat CD73 (ecto-5'-nucleotidase) (5F/b9; diluted 1:100; BD Biosciences PharMingen, San Diego, USA) at 4°C overnight. Following a rinse in PBS, they were treated with a biotinylated rabbit anti-mouse immunoglobulins, followed by peroxidase-conjugated streptavidin (Histofine, Nichirei, Tokyo, Japan). The site of the immunoreaction was visualized by the diaminobenzidine (DAB) reaction, and examined with a light microscope (Olympus, BX-60).

For electron microscopic observation, the tissues fixed with PLP solution were immunostained as mentioned above, and postfixed in 1% OsO₄ for 30 min. They were dehydrated in a graded ethanol series, and embedded in Epon 812. Ultrathin sections were prepared and stained with uranyl acetate to be examined in a JEM 1200 EX transmission electron microscope (TEM) (JEOL, Tokyo).

The other tissue sections and whole-mount preparations of the mesentery were processed for double immunostaining for CD73 and Factor VIII-related antigen (von-Willebrand Factor). The sections were incubated in 10% normal goat serum, and then in a mixture of mouse monoclonal anti-CD73 (diluted 1:100; BD Biosciences PharMingen) and polyclonal rabbit anti-Factor VIII-related antigen (diluted 1:200; DAKO Cytomation, Glostrup, Denmark) antibodies at 4°C overnight. After rinsing with PBS, they were treated with a mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:100; Jackson Immuno Research, West Grove, USA) and indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG (diluted 1:400; Jackson Immuno Research) for 1 h at room temperature. The stained tissues were mounted in fluorescent mounting medium (DAKO Cytomation) and viewed with a fluorescence microscope (Olympus, BX-60).

Control immunostaining was carried out by the same procedure, except for utilization of a non-immunized serum, or an antiserum absorbed with an excess amount of antigens (50–100 μg/ml diluted antiserum), instead of the corresponding antibodies, and no specific reaction products were seen.

**In situ hybridization.** Some tissue sections of the jejunum were processed for in situ hybridization histochemistry to examine the cellular distribution of 5'-Nase mRNA signals.

Two kinds of antisense oligonucleotide probes were prepared in this study, which were complementary to nucleotide residues 693 to 737 (TGGCACAC TTCATGAAACCTCTGGCTAGATCCAGGCCA CACTGG) and 1078 to 1122 (TGCCCAGAAGGTGAGAGGCGTGGACGTCGTGGGAGGACA CAC) of the rat 5'-Nase cDNA (accession No. J05214) (8). We used two types of each nucleotide: one was labeled with biotin, the other was unlabeled for control hybridization.

The non-fixed tissue sections were fixed with 4% paraformaldehyde solution for 10 min and acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). The tissues were incubated in endogenous biotin blocking agent (DAKO Cytomation), and then in 0.3% H₂O₂ in methanol to block the endogenous peroxidase activity. They were prehybridized for 2 h at room temperature in a hybridization solution, containing 50% formamide, 30 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.6 M NaCl, 1 x Denhardt's solution, 0.25% so-
Lymphatics by Histochemistry for 5′-Nase
dium dodecyl sulfate (SDS), 10% dextran sulfate and 200 μg/ml yeast tRNA, followed by hybridization with the biotinylated oligonucleotide probes (1.5 μg/ml) in a hybridization solution containing 100 mM dithiotreitol for 12 h at 42°C. The specimens were rinsed in 2x SSC (1xSSC: 150 mM NaCl and 15 mM sodium citrate) containing 0.1% sarkosyl for 30 min at room temperature and then twice in 0.1x SSC containing 0.1% sarkosyl for 40 min at 55°C, and then in 0.05 M Tris buffered-saline (pH 7.6) containing 0.1% Tween 20 for 10 min. To detect the biotin-bound hybridized probe, the tissues were subsequently processed for peroxidase catalyzed deposition of biotinylated tyramid with a streptavidin peroxidase method (GenPoint System, DAKO Cytomation). The site of the hybridization was visualized by DAB reaction.

Control hybridization was carried out using the same procedure, except for utilization of the labeled probes with a 20-fold excess amount of the respective unlabeled probes, and gave no specific signals.

RESULTS

Enzyme-histochemistry
Enzyme-histochemical staining for 5′-Nase demonstrated reaction products in the lymphatic vessels in the tissue sections of the rat jejunum (Figs. 1a, 3a), as described in our previous studies (11, 14). In contrast, significantly lower enzyme activity was detected in the blood vessels, some free cells and certain smooth muscle cells in the jejunum (Figs. 1a, 3a). The 5′-Nase-positive lymphatics being rod in shape extended in the lamina propria of the villi in the jejunum, whereas those in the other regions of the intestine appeared as irregularly-shaped spaces (Figs. 1a, 3a).

Immunohistochemistry
Immunostaining using anti-CD73 (ecto-5′-Nase) antibody demonstrated the lymphatics with intense immunoreactivity in the rat jejunum, while poor immunoreaction products were seen in the blood vessels around the lymphatics and in some other kinds of cells (Figs. 1b, c). Histochemical examination on serial tissue sections further showed that the distribution of CD73-immunoreactivity exclusively corresponded to that of the enzyme activity (Figs. 1a, b, 3a, c). The immunoreaction products for CD73 were preferentially localized at the cell membrane, especially at the luminal surface of the lymphatic endothelial cells (Figs. 1c, d).

Double immunostaining for CD73 and Factor VIII-related antigen allowed a precise analysis of the discrimination and relationship between the lymphatics and blood vessels (Fig. 2). The CD73-immunoreactivity was intensely shown in the lymphatics, although some blood vessels near the lymphatics were moderately immunoreactive (Figs. 2 a, d, g). In contrast, the immunoreaction products for Factor VIII-related antigen were selectively localized in the blood vessels, but not in the lymphatics (Figs. 2b, e, h).

The jejunal villi revealed the central lacteals displaying strong immunoreaction to CD73 and the blood vessels with Factor VIII-related antigen-immunoreactivity around the lacteals (Figs. 2a-c), as reported in previous SEM study (9).

In the tongue, the blood vessels immunoreactive for Factor VIII-related antigen were often distributed around irregularly-shaped lymphatics with intense CD73-immunoreactivity (Figs. 2d-f).

In the mesentery, slender CD73-immunopositive lymphatics ran adjacent to the blood vessels with Factor VIII-related antigen-immunoreactivity (Figs. 2g-i).

In situ hybridization
In situ hybridization demonstrated consistent labeling in the tissue sections of the rat jejunum (Fig. 3b). 5′-Nase mRNA was preferentially expressed in the lymphatic vessels including central lacteals (Fig.3b). Little or no signals were seen in any other elements including blood vessels. The distribution of signals for 5′-Nase mRNA well coincided with that of reaction products of 5′-Nase activity and/or CD73-immunoreactivity (Figs. 3a-c).

DISCUSSION

The present study firstly demonstrated the cellular distribution of 5′-Nase at the levels of protein and mRNA, and compared it with that of the enzyme activity in the rat tissues.

Colocalization of enzyme activity, protein and mRNA of 5′-Nase in the lymphatic endothelial cells indicates that 5′-Nase is actually produced in lymphatic endothelial cells and disposed as their cell membrane-bound protein for displaying the enzymatic activity. This finding supports our idea that 5′-Nase is an available marker of lymphatic vessels (6, 12, 14), although 5′-Nase is also contained in a variety of tissues and cells, including renal tubules (4) and some leukocytes (2). Furthermore, the present histochemical methods can be used to examine the dynamics and functional roles of 5′-Nase in the lym-
Fig. 1  Light (a-c) and transmission electron (d) micrographs on tissue sections of the rat jejunum. a: S'-Nase staining. Lm, lamina propria mucosae; Sm, submucosa; ML, muscle layer. bar, 100 μm. b: Immunostaining for CD73 on a section adjacent to Fig. 1a. S'-Nase-positive lymphatics are also stained for CD73. bar, 100 μm. c: Endothelial cells of a central lacteal (CL) show intense immunoreaction to CD73. bar, 50 μm. d: The CD73-immunoreaction products are densely seen on luminal surfaces of endothelial cells (E) of a central lacteal (CL). bar, 1 μm.
Fig. 2  Fluorescence micrographs of double immunostaining for CD73 (a, d, g, green) and Factor VIII-related antigen (b, e, h, red) on tissue sections of rat jejunal villi (a-c) and of tongue (d-f) and a whole-mount preparation of mesentery (g-i). Figs. c, f and i show merged images. a-c: A central lacteal (CL) shows an intense immunoreaction to CD73, whereas blood capillaries (arrowheads) are intensely immunoreactive for Factor VIII-related antigen, but less intensely for CD73. bar, 100 μm. d-f: Blood capillaries (arrowheads), being intensely immunoreactive for Factor VIII-related antigen and less intensely for CD73, are seen around CD73-immunopositive lymphatic (arrow). Ep, epithelium. bar, 100 μm. g-i: CD73-immunopositive lymphatics (arrows) run adjacent to Factor VIII-related antigen-immunopositive blood vessels (BV). A thin blood vessel (arrowhead) is intensely immunoreactive for Factor VIII-related antigen, but faintly for CD73. bar, 200 μm

Lymphatic endothelial cells in various physiological and pathological conditions.

Dense distribution of the deposits of 5'-Nase-immunoreactivity, as of the enzyme activity (13, 14), on the luminal surface of the lymphatic endothelium suggests that 5'-Nase might serve membrane transport of chemical component of lymph involving adenosine to regulate the composition of lymph, and...
Fig. 3 Light micrographs of enzyme-histochemistry for 5'-Nase activity (a), in situ hybridization for 5'-Nase mRNA (b), and immunohistochemistry for CD73 (c) on serial tissue sections of the rat jejunal villus. 5'-Nase activity, signals for 5'-Nase mRNA and CD73-immunoreactivity are colocalized in endothelial cells of a central lacteal (CL). bars, 50 μm

Therefore the lymph flow in normal condition. However, further examination is required for confirmation of this view.

The enzyme-histochemical staining for 5'-Nase activity which we established is considered to be useful for visualizing the lymphatics, as has been previously reported (6,12,14). However, it is often difficult due to some reasons to combine this method with other staining procedure except for some enzyme-histochemical techniques (12, 13). Double immunostaining for CD73 and Factor VIII-related antigen is able to ensure not only the distinction, but also demonstration of the relationship between the lymphatics and blood vessels, though the blood vessels occasionally exhibited some CD73-immunoreactivity. The present results further indicate the usefulness of the immunohistochemical method using antibodies against a tissue element and CD73 for evaluation of its relation to the lymphatic vessel.

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REFERENCES

8. Misumi Y., Ogata S., Hirose S. and Ikeda Y. (1990) Primary structure of rat liver 5'-nucleotidase deduced from the
cDNA. J. Biol. Chem. 265, 2178–2183.