Immunohistochemical Differentiation between Lymphatic Vessels and Blood Vessels — Use of Anti-Basement Membrane Antibodies and Anti-Factor VIII-Related Antigen*

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Summary. Several immunohistochemical methods using Factor VIII-Related antigen (FVIII:Ag), laminin, Type IV collagen and fibronectin antisera were applied for the purpose of differentiating rat lymphatics from blood vessels by light and electron microscopy. Weibel-Palade bodies (WPB) were demonstrated in both types of vessels by conventional electron microscopy. The immunoreactivity to laminin and Type IV collagen in blood vessels showed a strong, continuous, linear subendothelial staining pattern in contrast to lymphatic vessels in which immunoreactivity was absent or weak in paraffin-embedded sections stained with the indirect immunoperoxidase technique. A positive reaction for fibronectin was observed in all extra-vascular tissue spaces as well as in lymphatics and blood vessels. FVIII:Ag and WPB were present in both lymphatic and blood endothelial cells. FVIII:Ag antiserum labeled with gold particles was observed only in the vacuoles which were assumed to be identical with WPB as demonstrated by our conventional electron microscopy.

We conclude that the immunohistochemical method using laminin and Type IV collagen antisera is a reliable and practical way to differentiate lymphatic vessels from blood vessels by light microscopy.

In surgical pathology it is well recognized that vascular invasion is an important prognostic factor in malignant disease, and the morphological analysis of vessels invaded by tumors provides many suggestions for the study of the metastatic modes of malignant cells or the origin of tumors. It is extremely difficult to determine whether invaded capillaries are blood capillaries, lymphatic capillaries or artefactual tissue spaces which sometimes mimic vascular spaces in conventional hematoxylin and eosin sections, whereas large vessels usually do not provide much of a problem of recognition in these sections. Many electron microscopic studies have already shown the ultrastructural differences between lymphatic capillaries and blood capillaries; blood capillaries possess continuous basal lamina, pericytes and tight junctions, while the characteristic features of lymphatic capillaries are the presence of anchoring filaments and no basal lamina at the endothelial-interstitial interface (CASLEY-SMITH and FLOREY, 1961; LEAK and BURKE, 1966; LEAK, 1987; OTSUKI and MAGARI, 1987; MAGARI, 1987). However, electron microscopy can hardly be said to be a routine method, because it takes much time to prepare ultrathin sections embedded in epoxy resin. Recently, some studies have shown that immunohistochemical staining of basement membrane components (BARSKY et al., 1983; HULTBERG and SVANHOLM, 1989) and FVIII:Ag (MUKAI, ROSAI and BURGDORF, 1980; LITTLE et al., 1986) makes it possible to distinguish blood capillaries from lymphatic capillaries. In this paper, we focused our attention on WPB and FVIII:Ag as endothelial markers and basement membrane components present in the endothelial-interstitial interface of lymphatics and blood vessels, and confirmed the location of WPB and the differences in immunoreactivity of FVIII:Ag, laminin, Type IV collagen and fibronectin in the two types of vessels by light and electron microscopy.

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MATERIALS AND METHODS

Eleven adult female Wistar rats, 5-7 weeks old (body weight 80-100 g), obtained from the Clea Laboratories, Inc., Japan, were anesthetized with intraperitoneal Nembutal (40 mg/kg). Tissues for immunohistochemical examination were fixed by perfusion via the left ventricle with 100 ml of heparinized saline followed by 200 ml of 4% formaldehyde in phosphate buffer (PB) (pH 7.4). Tissues for conventional electron microscopy were perfused with 200 ml of Karnovsky fixative (KARNOVSKY, 1965). At the end of the perfusion the tissue specimens were taken from the subcutaneous layer in the neck including abundant arteries, arterioles, veins, venules, blood capillaries, lymphatic collecting vessels and lymphatic capillaries, and from the aorta with the thoracic duct and surrounding fatty tissue. The specimens were cut into small pieces and kept for 3-12 h in the same fixative.

The immunoreactivity to laminin, Type IV collagen and fibronectin was examined in paraffin-embedded sections. Fixed materials were dehydrated in the usual way and embedded in paraffin. Dewaxed and rehydrated paraffin-embedded sections 6 μm thick were treated with 0.4% pepsin (Sigma Chemical Co., U.S.A.) to enhance the availability of the antigens (EKBLOM et al., 1982; BARKSY et al., 1983). They were then exposed to 0.1% hydrogen peroxide in methanol to inactive endogenous peroxidase, stained by an indirect immunoperoxidase technique (GREGSON, DAVEY and PRENTICE, 1979) and examined under light microscopy. The following commercially available antisera were used: rabbit anti-mouse laminin, anti-bovine Type IV collagen antiserum (both from Advance Co. Ltd., Japan) and rabbit anti-mouse fibronectin antiserum (Paesel & Lorei, West Germany) in dilutions from 1:100 to 1:500 with phosphate buffered saline (PBS) containing 1% normal goat serum (NGS). After incubation for 1 h with primary monoclonal antibodies, tissue sections were incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Cappel Laboratories, U.S.A.) diluted 1:100 with 1% NGS in 0.01 M PBS for 1 h. Peroxidase activity was revealed by exposing the sections to a solution of 3', 3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., U.S.A.) containing 0.05% DAB, 0.01% H2O2 in 0.05 M Tris-HCl buffer (DAB solution) at pH 7.6 for 5 to 10 min. The sections were counterstained with 1% methyl green and examined by light microscopy. All procedures were carried out at room temperature. Some formalin-fixed materials were pre pared for electron microscopy. They were cut into serial sections 40-60 μm thick on a Dosaka microslicer at 4°C. These thick sections were stained by the above-mentioned indirect immunoperoxidase technique. Following incubation with DAB solution, they were post-fixed with 1% osmium tetroxide in 0.1 M PB for 1 h, stained further with 1% uranyl acetate in 70% ethanol for 40 min, and then dehydrated in the usual way and embedded in Epon 812. Ultrathin sections 60-80 nm thick were cut and examined by electron microscopy. Control sections were incubated with 1% NGS in 0.01 M PBS and examined for non-specific staining.

The antigen sites of basement membrane proteins and FVIIIIR:Ag were examined in ultrathin 60-80 nm sections by a post-embedding immunogold staining method. Fixed materials were embedded in LR White without passage in osmium tetroxide, and dehydrated in a graded ethanol series at 4°C. LR White infiltration of the tissues was carried out in small bottles placed in a rotating device at 4°C, with a 1 : 1 LR White: 90% ethanol mixture for 1 h before being transferred to 100% LR White for 1 h with one change. Tissues were then embedded in gelatin capsules. The resin was polymerized at 50°C for 24 h in an accurate oven. LR White sections mounted on Formvar-coated nickel grids were preincubated for 5 min face down on drops of washing buffer containing 0.1% bovine serum albumin (BSA), 0.05% Tween 20 and 0.5 M NaCl in 0.05 M Tris-HCl buffer and transferred to a drop of 5% NGS in washing buffer for 15 min. They were then incubated on a drop of rabbit anti-human FVIIIIR:Ag (DAKO, Denmark), antisemur against laminin, Type IV collagen or fibronectin, or nonimmune serum as a control, each one of which was diluted 1:100. Excess unbound primary antibody was then washed off the grids by passing them sequentially across three drops of washing buffer, and incubation was continued with goat anti-rabbit IgG bound to 5 nm gold particles (Auroprobe EM GAR G5; Janssen, Belgium) in a 1:20 dilution. After being washed to remove excess unbound gold particles, the grids were transferred to a drop of distilled water. The sections were then stained for 4 min with uranyl acetate and for 15 sec with lead citrate. Double immunogold staining was carried out to compare simultaneously the antigen sites of the three basement membrane proteins in the same ultrathin sections, and the above mentioned method was repeated twice for double immunogold staining. The combination of each antisemur and size of immunogold particles was as follows: laminin antiserum with 5 nm gold particles, Type IV collagen antiserum with 15 nm or 15 nm
gold particles, and fibronectin antiserum with 15 nm gold particles.

Conventional electron microscopy was utilized to examine the location of WPB in lymphatic and blood vessel endothelial cells, and of lamina densa in the two types of vessels. The specimens fixed with Karnovsky fixative were post-fixed with 1% osmium tetroxide in 0.1 M PB. They were routinely dehydrated and embedded in Epon 812. Ultrathin 60-80 nm sections were double-stained with uranyl acetate and lead citrate, and examined with a Hitachi H300 electron microscope.

RESULTS

Conventional electron microscopy confirmed the presence of continuous lamina densa with electron density around blood vessels (Fig. 1), nerve fibers, smooth muscle cells and fat cells, in contrast lymphatic capillaries generally possessed no lamina densa around endothelial cells. However, in lymphatic collecting vessels disrupted lamina densa was occasionally noted, and especially the valvular portions showed a clear lamina densa which was similar to that of blood vessels (Fig. 2).

Laminin and Type IV collagen immunoreactivity was demonstrated around blood vessels, in addition to nerve fibers, smooth muscle cells and fat cells by the indirect immunoperoxidase technique. The staining pattern of blood vessels for laminin or Type IV collagen (Fig. 3) was consistently linear in cross or longitudinal section through vessels. In tangential sections through blood vessels they were stained in a focally discontinuous pattern for the two types of anti-serum. Immuno-electron microscopic observations of blood vessels revealed that laminin or Type IV collagen antiserum conjugated to 5 nm gold particles was abundantly present in the endothelial-
interstitial interface and arranged in a belt-like pattern (Fig. 4). High magnification of electron micrographs showed that these antisera labeled with 5 nm gold particles were located mainly on the lamina densa (Fig. 5) and were confirmed to coexist by double immunogold staining. Lymphatic capillaries, in contrast to blood capillaries, generally exhibited a very weak reaction, if any staining for laminin or Type IV collagen dose exist (Fig. 3). Lymphatic collecting vessels possessed weakly focal staining at places around the wall, and an especially intense reaction was observed in the areas with valve (Figs. 3, 6). Immuno-electron microscopy showed that laminin and Type IV collagen antisera labeled with 5 nm gold particles were scattered sparsely in the other areas of lymphatic endothelial cells (Fig. 7).

Immunoreactivity for fibronectin was demonstrated by the immunoperoxidase method in all extravascular tissue spaces as well as in lymphatics and blood vessels (Fig. 8), so it was very difficult to differentiate blood vessels from lymphatic vessels by this method. The immunolocation of fibronectin in

![Image](image-url)
the basal lamina by immunogold staining showed no typical pattern such as belt-like staining for laminin or Type IV collagen in blood vessels. Double immunogold staining of the three basement membrane proteins revealed clear differences among them, for example, laminin antiserum labeled with 5 nm gold particles was arranged in a belt-like pattern around the endothelial cells of blood vessels, while fibronectin antiserum labeled with 15 nm gold particles was distributed throughout the endothelial-Interstitial interface (Fig. 9). In artefactual tissue spaces, which may mimic blood or lymphatic capillaries, these basement membrane proteins were not stained by either immunoperoxidase or immunogold staining techniques.

**Fig. 3.** Immunoperoxidase staining of Type IV collagen in the blood vessels (B) is consistently linear, whereas lymphatic vessels (Ly) exhibit a weak reaction or none whatsoever. Note strong immunostaining in the valvular portion of the lymphatic vessel (arrow). × 650

**Fig. 4.** Part of an arteriole shows the immunolocatation of laminin at high magnification. Laminin antiserum conjugated to 5 nm gold particles are located in a belt-like pattern beneath the endothelial cell (E) and the smooth muscle cell (M). × 52,400

**Fig. 5.** Part of a blood capillary exhibits the immunolocatation of laminin at high magnification. Laminin antiserum labeled with 5 nm gold particles are located only on the lamina densa (arrows). × 46,400
Conventional electron microscopy revealed that WPB generally were present in the endothelial cells of all types of blood vessels (Fig. 10), thoracic duct (Fig. 11), and even lymphatic capillaries (Fig. 12). The majority of WPB were observed just beneath the luminal plasma membrane and occasionally lay close to vesicles, caveolae and Golgi complexes. Some aortic endothelial cells contained numerous WPB (Fig. 10). The density of WPB tended to be correlated with the caliber of the lymphatics and blood vessels. WPB appeared as rod-shaped bodies in longitudinal sections and the matrix contained many tubules which were paralleled the long axis of the rods. In cross sections, the WPB appeared as circular bodies possessing numerous densely-packed vesicles in the matrix (Fig. 11).

The immunolocation of Anti-FVIIIR:Ag was confirmed not only in blood vessels and lymphatic...
collecting vessels but also in lymphatic capillaries (Fig. 13) by electron microscopy. Anti-FVIIIIR:Ag conjugated to 5 nm gold particles was observed most frequently in aortic endothelial cells and was located only in the vacuoles in the cytoplasm (Fig. 14). All controls for immunoperoxidase and immunogold staining methods failed to exhibit specific staining.

DISCUSSION

Various methods have been employed in the past to differentiate lymphatic vessels from blood vessels. Most of the methods have focused mainly on the endothelial cells or the luminal contents of the vessels. These authors directly stained nonspecific alkaline phosphatase, aminopeptidase, peroxidase, acid-p-nitrophenyl phosphatase for the detection of blood vessels (NISHIDA and OHKUMA, 1984; OHKUMA and NISHIDA, 1987) and 5'-nucleotidase (VETTER, 1970; KATO and MIYAUChI, 1989) for the detection of lymphatic vessels, or observed dye injected into the vessels. These enzyme histochemical methods used to identify the vasculature are inferior to the immunohistochemical method with respect to specific staining; the former simultaneously stains numerous other structures (ENGEL and CUNNINGHAM, 1970; KHAN, 1979). The direct injection of dye into the vascular bed or lymphatic bed has several disadvantages: the contaminated extravascular tissue spaces and the limited expression of lymphatics or blood vessels by the injected dye. Moreover, the limitations in sensitivity with the enzyme histochemical method and the direct injection of dye lie in their reliance on the presence of endothelial cells and a lumen. If the lumen is surrounded by thin endothelial cells or is collapsed, it is very difficult to distinguish between lymphatic vessels and blood vessels by these methods.

Recently, several papers have appeared reporting the expression of FVIIIIR:Ag by blood endothelial

Fig. 8. Immunoperoxidase staining of fibronectin exhibits no specific location in contrast to that of Type IV collagen as shown in Fig. 3, and a positive reaction is observed in extra-vascular tissue spaces as well as lymphatic vessels (Ly) and blood vessels (B). ×670

Fig. 9. Electron micrograph simultaneously showing the immunolocalization of laminin and fibronectin using double immunogold staining technique in a venule. Laminin antisemur labeled with 5 nm gold particles is located in a linear pattern beneath the endothelial cell (E) and the smooth muscle cell (M) in contrast to immunostaining of fibronectin shown by 15 nm gold particles throughout the endothelial-interstitial interface. ×71,100
cells (Mukai, Rosai and Burgdorf, 1980; Bettelheim, Mitchell and Gusterson, 1984; Little et al., 1986). Immunohistochemical studies using anti-FVIII:Ag antiserum have confirmed the presence of FVIII:Ag in the endothelial cells of blood vessels, and we have found this method to be unsurpassed for specific staining. However, there is much discussion as to whether FVIII:Ag is located only in blood vessel endothelial cells or in both lymphatic and blood vessel endothelial cells. Our immunoelectron microscopic study of FVIII:Ag reveals the immunolocation of this antigen in the two types of

![Fig. 10. electron micrograph showing part of an artery. The endothelial cell possesses abundant Weibel-Palade bodies and vacuoles. Note continuously thick lamina densa (arrows) beneath the endothelial cell and the smooth muscle cells (M) as shown in Fig. 1. ×35,700](image)

![Fig. 11. Part of a thoracic duct showing Weibel-Palade bodies in the cytoplasm near the luminal plasma membrane. The matrix of Weibel-Palade bodies contains many tubules which are paralleled the long axis of the rods in longitudinal section (arrowhead), and circular or oval vesicles in cross sections (arrows). ×34,800](image)
vessels and even in lymphatic capillaries. Our data are in accord with the results published by Burgdorf, Mukai and Rosai (1981), Svanholm, Nielsen and Hauge (1984), Lee, De Lellis and Wolfe (1986), Magari and Ito (1988) and Hultberg and Svanholm (1989), but are not in agreement with those of Mukai, Rosai and Burgdorf (1980) and Little et al. (1986), who reported negative staining of lymphatic endothelial cells.

It is well recognized that WPB contain FVIII:Ag and histamine (Sakariasen, Bolhuis and Sixma, 1979; Kagawa and Fujimoto, 1987). In this study,

Fig. 12. Electron micrograph showing a Weibel-Palade body in the thin endothelial cell of a lymphatic capillary. Note the absence of lamina densa beneath the lymphatic endothelial cell. ×31,700

Fig. 13. Electron micrograph exhibiting the immunolocalization of Factor VIII-Related antigen in a lymphatic capillary. The antiserum against Factor VIII-Related antigen conjugated to 5 nm gold particles is present only in a vacuole (arrow) of the endothelial cell. ×45,300

Fig. 14. Electron micrograph showing the immunolocalization of Factor VIII-Related antigen in an aorta. Five nm gold particles conjugated to Factor VIII-Related antigen are especially aggregated in a large vacuole (arrow). L lumen of the aorta. ×45,600
anti-FVIIIR:Ag labeled with gold particles was seen only in vacuoles which were assumed to be identical with WPB, as confirmed by our conventional electron microscopy, but not on the endoplasmic reticulum or filaments of the extra-cellular matrix, as reported by Wagner, Olmsted and Marder (1982) and Rand et al. (1982). These contradictions about the different antigen sites for FVIIIR: Ag may be explained on the basis of different fixatives and the specificity of the antisera and the immunogold particles used. The above-mentioned findings indicate that the presence of WPB or positive immunoreactivity in the endothelial cells does not necessarily define a vessel as a blood vessel, because these endothelial markers are generally observed in both lymphatics and blood vessels.

Our immunoperoxidase method using laminin and Type IV collagen antiserum reveals clear differences in immunoreactivity to both types of antiserum between lymphatics and blood vessels; the blood vessels show strong, linear subendothelial staining in all directional sections through the vessels, while lymphatic vessels show negative or weak immunoreactivity. These results are supported by Barsky et al. (1983) and Hultberg and Svanholm (1989) and have been corroborated by our ultrastructural demonstration of continuous lamina densa around blood vessels and lack of lamina densa around lymphatic vessels. Moreover, the most characteristic finding is the presence of a continuous lamina densa and a strong, linear immunostaining pattern in the valvular portions of lymphatic collecting vessels. The materials used for the immunoperoxidase method were fixed with paraformaldehyde and embedded in paraffin—the common procedure for anatomists, pathologists and clinicians. Laminin and Type IV collagen antigenicities were reasonably well preserved in the sections, although considerable loss of antigenicity can occur in paraffin-embedded tissue. We propose that the immunohistochemical method using laminin and Type IV collagen antisera in paraffin-embedded sections is a reliable and practical way to distinguish lymphatic vessels from blood vessels by light microscopy.

There are some objections to the immunolocalization of laminin and Type IV collagen in basal lamina. Our double immunogold staining method reveals that laminin and Type IV collagen conjugated to gold particles are present mainly on the lamina densa and are assumed to coexist on it. Our data are in good accord with the results of Grant and Leblond (1988), but are not in agreement with Courtoy, Timple and Farquhar (1982), who stated that laminin is probably located in the lamina rara in contrast to Type IV collagen which is present in the lamina densa.

The immunoperoxidase method using fibronectin antiserum stained extra-vascular tissue spaces in addition to lymphatics and blood vessels, so failed to distinguish lymphatic vessels from blood vessels in this study. This result at the light microscopic level correlates well with our electron microscopic findings of fibronectin antiserum labeled with gold particles which were distributed throughout the endothelial-interstitial interface, and the double immunogold staining method reveals clear differences in the immunolocalization of fibronectin and laminin or Type IV collagen. The fibronectin antiserum used in this study may recognize all fibronectin of plasma and tissue origin produced by endothelial cells, epithelial cells or mesangial matrix, as reported by Courtoy, Timple and Farquhar (1982).

REFERENCES


