

Scanning Electron Microscopic Study of Canine Lymphatic Vessels and Their Valves

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Summary

The morphology of canine thoracic duct, peripheral collecting lymphatics and their valves was determined using scanning and transmission electron microscopy (SEM and TEM). Information from surface and subsurface structures was derived simultaneously in the SEM by using a heavy metal stain combined with backscattered electron imaging. The peripheral lymphatics and thoracic duct were covered by a continuous sheet of endothelial cells, the nuclei of which were slightly raised, ovoid in shape with coarsely granular chromatin and long axes oriented parallel to the direction of flow. The immediate subendothelial connective tissue in the thoracic duct was primarily smooth muscle, whereas in the peripheral lymphatics reticular and elastic fibers predominated. Valves were seen in both peripheral and central lymphatics. These were covered with endothelium similar to the collecting lymphatic vessels, were usually bicuspid and had cusps that inserted either at a single point or overlapped prior to a wide based insertion. A complex network of connective tissue and elastic fibers was seen in valves and in the vessel walls adjacent to the valves. The similarities and dissimilarities of the SEM appearances of lymphatics, arteries and veins are discussed.

Scanning electron microscopy (SEM) has been used extensively in the study of normal and pathologic processes in arteries, and to a lesser degree, to study the surface of veins (1-8). However, relatively few SEM studies have been reported on lymphatic vessels. *Leak* (9) in his discussion of the pulmonary lymphatic system shows scanning electron micrographs of several pulmonary lymphatic vessels and portions of their valves. Fine wrinkling of the endothelial surface is noted in his photographs. *Ohkuma* (10) studied a lymphangioma circumscripta removed from the perianal region of a

two year old female by scanning electron microscopy. The luminal surface of the ramifying vessels showed parallel, slightly raised, endothelial cells with occasional papillary luminal projections and fine surface wrinkling. Given the paucity of information about lymphatics, the purpose of this study was to describe the surface endothelium and immediate subendothelial structures of the thoracic duct, peripheral collecting lymphatics and their valves in the dog.

Materials and Methods

Three mongrel male dogs weighing between 14 and 20 kilograms were anesthetized using intravenous sodium pentobarbital (30 mg/kg initially) with supplementation as necessary. Two bundles of prenodal lymphatics, just distal to the popliteal lymph node, parallel to and on either side of the saphenous vein were exposed with a dermal flap. Both bundles were occluded with a suture just distal to the popliteal lymph node. The paw was flexed passively in a gentle fashion allowing the lymphatics to fill. The lymphatics were carefully isolated and a 2-3 cm vascular segment chosen with the minimum number of collaterals. This vessel was cannulated distally in a cephalad direction using polyethylene tubing with an outside diameter of .024 in. After placing a small hole in the proximal portion of the lymphatic wall, the vessel was perfused with normal saline for approximately one minute. It was then perfused with *Karnovsky's* solution at a pressure of 1 cm H₂O for 10 min. The portion of the vessel containing the small hole was occluded

with a suture. The vessel and its cannula were excised together and placed in *Karnovsky's* fixative still inflated at 1 cm H₂O for another 30 to 60 min. The dogs were then intubated with endotracheal tubes and a left posterior lateral thoracotomy was made through the fifth and sixth intercostal space. The left lung was retracted anteriorly and a part of the visceral pleura removed. Four to 5 cm of the thoracic duct was isolated in a position anterior and slightly lateral to the spine and posterolateral to the aorta. The distal portion of the thoracic duct was cannulated in a cephalad direction and handled in a fashion similar to the peripheral lymphatics. After fixing for at least 24 hrs, several segments of the vessels were processed for SEM. They were rinsed with distilled water overnight and half were stained with *Willard's* modification of *Gormori's* methenamine silver stain (11); half were left unstained. Both groups were dehydrated in graded ethanol solutions and in graded ethanol amyl acetate mixtures, critical point dried, and sputter coated with carbon to a thickness of 200 Å. They were examined with an Etec Autoscan Scanning Electron Microscope equipped with a backscatter electron

detector. Accelerating voltages of 2.5 to 20 KV were used for secondary electron imaging and 20 KV for backscattered electron imaging. Portions of the specimen also were processed for transmission electron microscopy (TEM). They were embedded in an EPON mixture, sectioned at 700 Å on a LKB microtome, stained with uranyl acetate and lead citrate and examined with a Phillips 201 transmission electron microscope.

Results

Vessels

The scanning electron microscopic appearances of the lymphatics were similar in all three dogs (Fig. 1a). The surfaces of both the thoracic duct and peripheral collecting lymphatics were covered with a continuous sheet of endothelial cells, the nuclei of which were slightly raised, ovoid in shape and had their long axes oriented parallel to the direction of lymph flow. The borders between adjacent endothelial cells were indistinct. Numerous fine ridges and furrows were seen. The larger ridges were interpreted as being underlying connective tissue fibers (12) whereas the smaller furrows appeared to be produced by folding of the endothe-

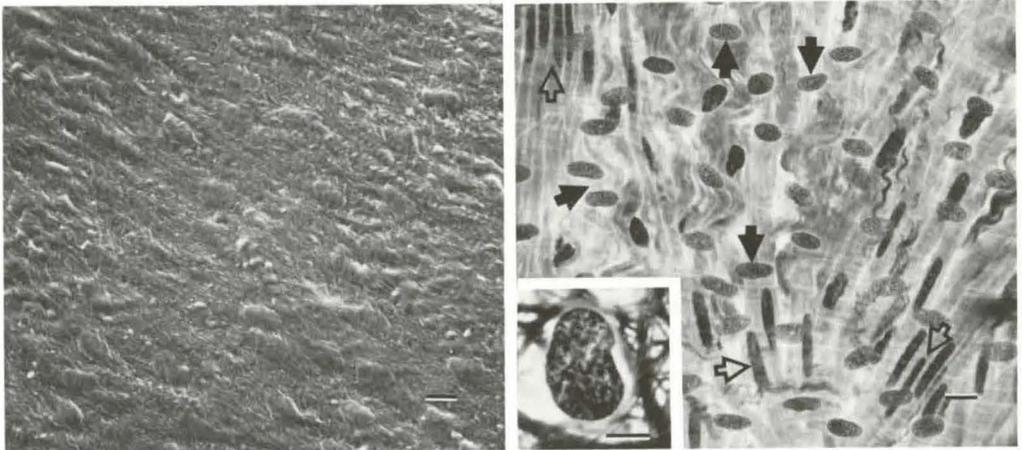


Fig. 1a Left SEM of the thoracic duct surface in an unstained specimen examined in the secondary electron mode demonstrating slightly raised endothelial nuclei with longitudinal axes parallel to the direction of flow (lower right to upper left). The fine furrowing of the surface is apparent. Marker = 10 μ m.

Fig. 1b Right SEM of a stained portion of the thoracic duct from an adjacent area to 1a in a stained specimen examined in the backscattered electron mode demonstrating the uniform endothelial cell nuclei (solid arrows) and elongated hyperchromatic smooth muscle nuclei (open arrows). Marker = 10 μ m.

Insert is higher power demonstrating uniform granular nuclear chromatin, Marker = 5 μ m.

lial cell cytoplasm (Fig. 2). These furrows also were seen in specimens that were not cannulated and perfused but fixed by direct immersion into *Karnovsky's* solution. Small round cytoplasmic projections also were seen in many areas (Fig. 2 arrows). Occasionally a second type of endothelial cell was observed (Fig. 8). These were characterized by markedly raised, oval elongated nuclei with longitudinal coarse furrowing of the cytoplasm. They lacked the characteristic irregular fine furrows of the endothelium seen throughout the main body of the vessel (Figs. 1 and 2). This second type of endothelium was observed predominately on the endothelial surfaces of valves and rarely on the surface of the lymphatic vessel wall. Using the silver stain and the backscatter electron mode of the SEM the longitudinal orientation of the endothelial cell nuclei was seen clearly (Fig. 1b). In this mode the image contrast is dependent upon differences in average atomic number (11). Thus the nuclear chromatin and connective tissue fibers which are selectively stained with silver stand out from the surrounding tissues. This allows better depth perception of the

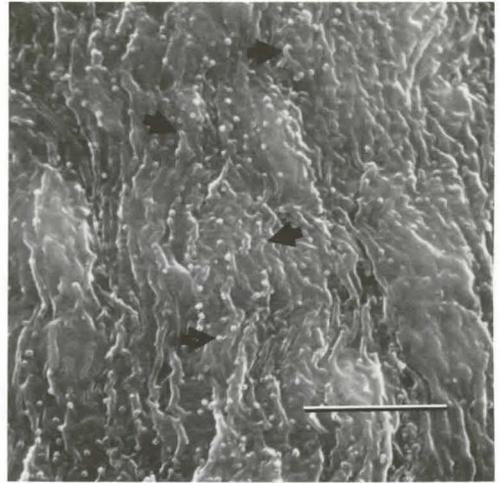


Fig. 2 SEM of the thoracic duct surface in an unstained specimen examined in the secondary electron mode showing fine cytoplasmic furrowing and numerous round cytoplasmic microprojections (arrows). Marker = 10 μm .

sample and visualization of structures immediately beneath the surface. At high magnification in the backscatter mode the nuclear

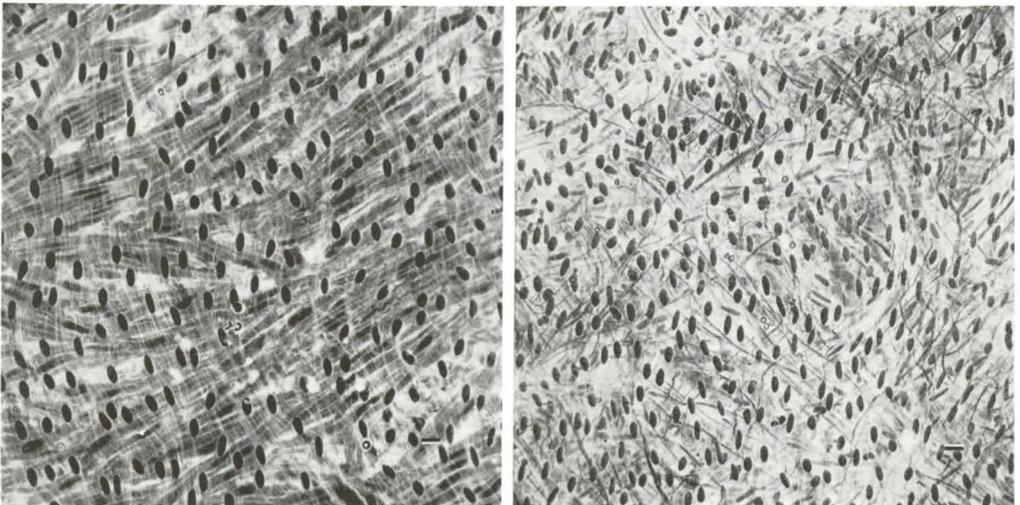


Fig. 3 SEM of a stained portion of thoracic duct left and peripheral lymphatic right examined in the backscattered electron imaging mode demonstrating the uniform parallel endothelial nuclei with long axes parallel to the direction of flow (oval cells). Numerous smooth muscle bundles are seen in the thoracic duct, whereas elastic and reticulin fibers predominate in the peripheral lymphatic. Direction of flow is vertical. Marker = 10 μm .

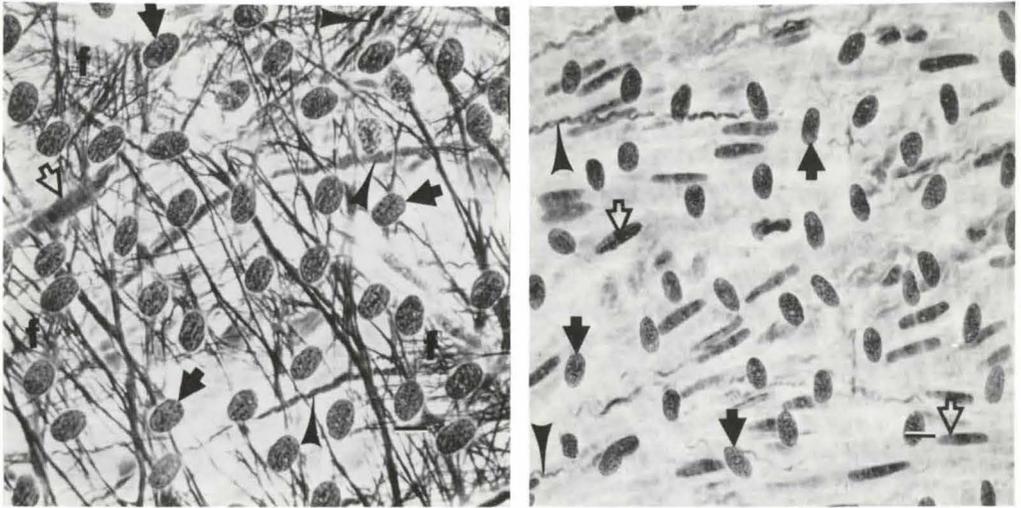


Fig. 4 SEM of two different areas of thoracic duct in two stained specimens examined in the backscattered electron mode demonstrating the uniform endothelial cell nuclei (solid arrows), elongated smooth muscle cells (open arrows), wavy elastic fibers (solid arrowheads) and reticular fibers (f). Direction of flow is vertical. Markers = 10 μ m. Photographs used with permission of SEM Inc. AMF O'Hare, IL. 60666 USA.

chromatin was seen to be coarsely granular and uniformly distributed throughout the nucleus (Fig. 1b insert). A second population of connective tissue cells with elongated nuclei were visible beneath the endothelium (Fig. 1b open arrows). These ran singly and in bundles with an orientation oblique to the direction of flow. Fine reticular and elastic fibers were seen interspersed with these cells. The immediate subendothelial connective tissue in the thoracic duct was composed predominately of smooth muscle bundles with an occasional elastic fiber (Figs. 1b and 3) while in the peripheral lymphatics the subendothelial connective tissue was composed predominately of elastic and reticular fibers with occasional smooth muscle (Fig. 3). The amount of superficial smooth muscle, elastic tissue and connective tissue fibers in the thoracic duct and peripheral lymphatics was extremely variable. This is illustrated in Figures 1b, 3 and 4; however, the predominant patterns were those previously stated.

The SEM appearances of the lymphatic sub-surface were correlated with transmission electron microscopy (TEM) of the same vessels. TEM of the thoracic duct showed slightly

raised endothelial cells and nuclei with numerous elastic and collagen fibers immediately beneath the surface (Fig. 5). Cytoplasmic intraluminal projections corresponding to the cytoplasmic blebs seen in the SEM also were demonstrated by TEM. The dark staining elongated subendothelial connective tissue bundles visible by scanning electron microscopy were identified as smooth muscle cells (Fig. 5b).

Valves

The majority of valves seen in both the thoracic duct and peripheral lymphatic vessels were bicuspid, though a single tricuspid valve was observed in one thoracic duct specimen. Fig. 6a illustrates a typical bicuspid valve from a peripheral lymphatic vessel. The valves were covered with a continuous layer of endothelial cells, similar to that found in the collecting lymphatic vessels. At the base of the valve the longitudinal axes of the endothelial nuclei were oriented in the direction of lymph flow whereas at or near the free valvular margins they lay parallel to the edge (Figs. 7 and 8). Backscattered electron imaging of the same valve seen in Fig. 6a (Fig. 6b) showed connective tissue fibers running through

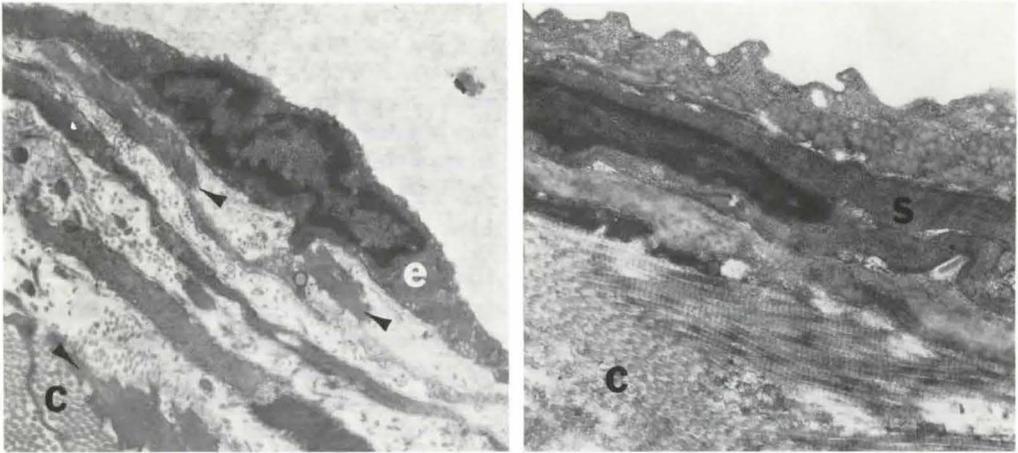


Fig. 5 (a and b) TEM of the thoracic duct wall showing a slightly raised endothelial cell with its nucleus (e), fragments of elastic tissue (solid arrowheads), bundles of collagen (c) and a portion of a smooth muscle cell (s). 10,000 magnification.

the cusp. The density of the fibers was increased at the base where they appeared to form a fibrous annulus. This is shown in greater detail in Figure 7. Connective tissue fibers also were seen running almost perpendicular to the free margin of the cusps in the midportion of the valve (Fig. 8b). At the site of attachment of the valve cusp into the ad-

jacent wall a marked increase in elastic fibers was observed (Fig. 9). The larger fibers extended out from the insertion of the valve in a radial fashion. However, at the insertion of one cusp there was an increase in connective tissue fibers and smooth muscle, but no elastic tissue in the adjacent vessel wall. The wall adjacent to the valves appeared to be fortified

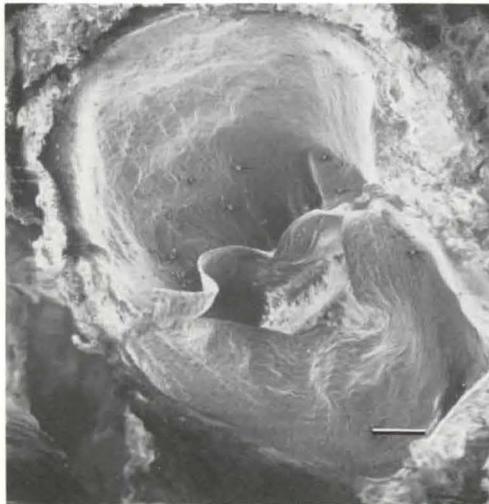


Fig. 6a SEM of a peripheral lymphatic valve in a stained specimen examined in the secondary electron mode demonstrating the typical bivalvular cusps. Marker = 100 μ m.



Fig. 6b SEM of the same valve, lower cusp examined in the backscattered electron mode showing increased density of connective tissue fibers which appear to form a fibrous annulus in the cusp base (arrows). Marker = 30 μ m.

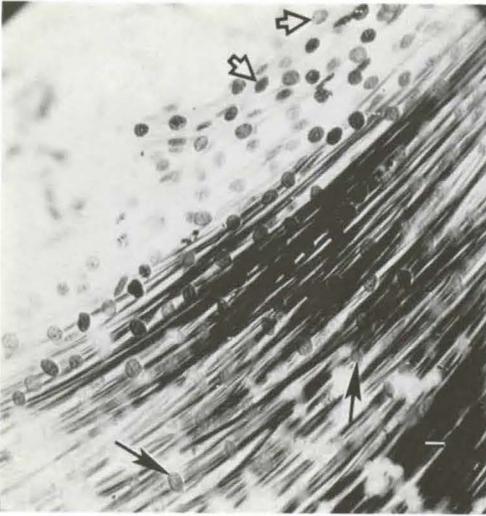


Fig. 7 SEM of a valve cusp from a peripheral lymphatic near its insertion in a stained specimen examined in the backscattered electron mode. The longitudinal axes of the endothelial cell nuclei lie parallel to the direction of lymph flow at the cusp base (solid arrows) and parallel to the cusp edge near the cusp margin (open arrows). Connective tissue fibers are also visible. Marker = 10 μm .

by increased numbers of wavy elastic and fine reticular fibers (Fig. 10). Two types of insertions of valve cusps were seen. The first and most common type had converging margins that inserted at a single point (Fig. 11a), while in the second type one cusp margin overlapped the other prior to a wide based insertion (Fig. 11b).

Connecting Vessels

Several interconnecting vessels were studied. Connecting vessels opened obliquely into the main lymphatic channel (Fig. 12). The longitudinal axes of the endothelial cell nuclei were orientated parallel to the direction of flow in the interconnecting or tributary vessels except at the upstream margin at the junction of the two vessels where the longitudinal nuclei were orientated tangential to the circumference of the inserting vessel (Fig. 12 arrows).

Discussion

This is the first detailed SEM study of lymphatic vessels and their valves. Our findings concur with the information on lymphatic vessels viewed by dissecting, light and electron

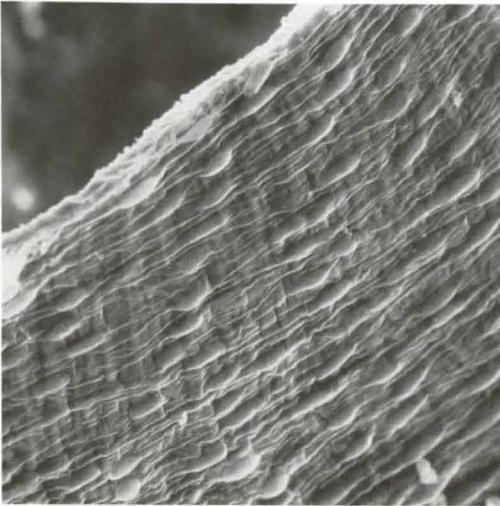


Fig. 8 SEM of the central portion of a peripheral valve cusp in a stained specimen examined in the secondary (left) and backscattered electron modes (right). The longitudinal endothelial cells and their nuclei run parallel to the cusp edge. Connective tissue fibers almost perpendicular to the free margin of the cusps are present (arrows). Marker = 10 μm .

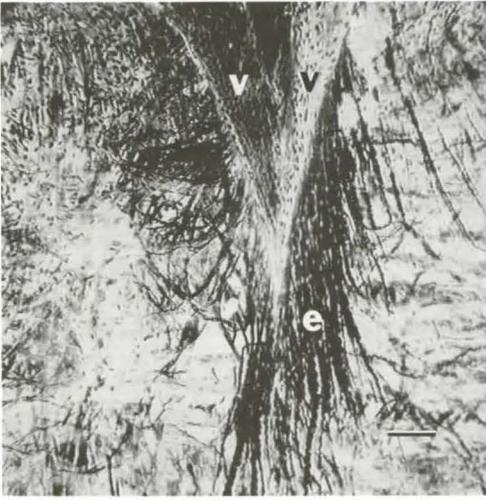


Fig. 9 SEM of a stained specimen of a thoracic duct examined in the backscattered electron mode demonstrating an increased density of elastic fibers (e) in the region of the insertion of the valve cusps (v). Marker = 100 μ m.



Fig. 10 Backscatter SEM of thoracic duct in a stained specimen from an area adjacent to a valve cusp insertion demonstrating an increased density of wavy elastic fibers. Marker = 10 μ m.

microscopic methods (13–16). The thoracic duct and collecting lymphatics are delicate vessels with an intima, media and adventitia similar to arteries and veins. The intima consists of a single continuous layer of endothelial cells resting on a basement lamina (9, 14).

Prominent endothelial cells with nuclei and fine cytoplasmic processes project into the lumen. These fine cytoplasmic processes also have been illustrated by *Leak* (15) and by *Boggon and Palfrey* (13). Collagen and elastic fibers provide a filamentous network up to

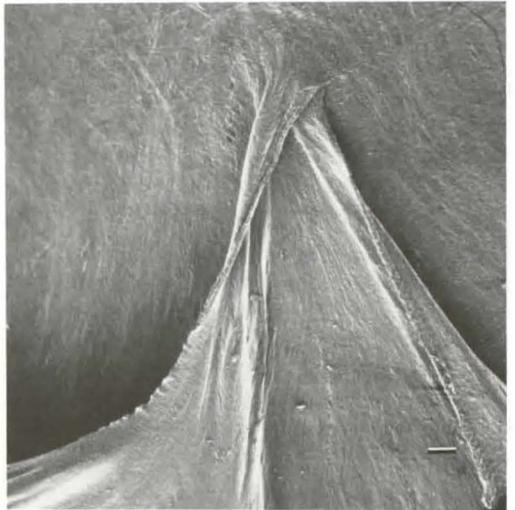
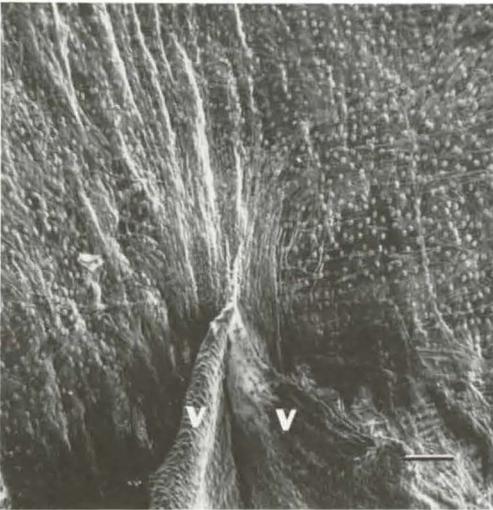


Fig. 11 (a and b) SEM of a stained specimen of thoracic duct demonstrating two types of valve cusp insertion. The one on the left has converging cusp margins (v) inserting at a single point (photographed at 20 kv.) while the one on the right has a wide based overlapping insertion (photographed at 2.5 kv.). Markers = 100 μ m.

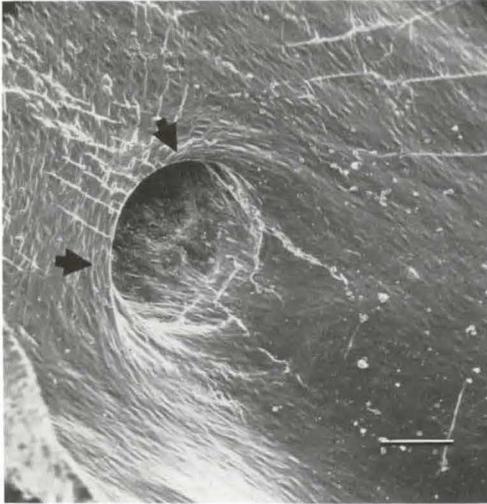


Fig. 12 SEM of peripheral collecting lymphatic demonstrating two interconnecting or tributary vessels examined in the secondary electron mode. The longitudinal axes of the endothelial cells lie parallel to the direction of flow (upper left to lower right) except at the upstream junction of the two vessels where the longitudinal axes are oriented tangential to the circumference of the inserting vessel (arrows). Marker = 100 μ m.

5 μ m wide between the smooth muscle of the tunica media and basement lamina (9, 13, 14). The media is composed of several layers of smooth muscle, 25 to 70 μ m thick, and collagen. The smooth muscle cells vary in size and in direction of their long axes. There is no clear division into circular and longitudinal bundles; the fibers run obliquely or may be arranged in a spiral fashion along the length around the vessel. The tunica adventitia consists of connective tissue components.

Our results also agree with the findings of Schipp which were summarized by Barrowman (16): "The smooth muscle of lymphatic vessels is characteristically found in the regions between valves, while at the origin of the valves the lymphatic wall has little or no smooth muscle". We found smooth muscle only in the area of one cusp leaflet and never in the valve itself.

In an earlier study (12) we compared the SEM characteristics of canine collecting lymphatics to anatomically equivalent arteries and veins.

We were able to show that the lymphatic surface was unique and differed in several important respects from the other vessels. Firstly the endothelial cell density of lymphatic vessels is much less than that for either arteries and veins prepared at physiologic pressures. Secondly, the endothelial cell nuclei of lymphatics differ from those of arteries and veins with respect to both shape and chromatin distribution. Venous endothelial nuclei are more angular in outline and less elongated than those of either arteries or lymphatics. Venous and arterial endothelial cell nuclei show a fine chromatin pattern and deep grooves. The grooves were orientated predominantly parallel to the direction of blood flow in the veins and randomly orientated in the arteries. The lymphatic endothelial nuclei did not show grooving, except in very rare instances, and exhibited a more coarsely granular and uniform distribution of their chromatin than either arteries or veins. Lastly, the density and distribution of superficial subendothelial connective tissue elements as viewed in the backscatter mode of the SEM are dissimilar. Reticular and elastic fibers were readily visible immediately beneath the endothelial surface of lymphatics together with bundles of obliquely orientated smooth muscle fibers. Such structures are infrequently seen in veins and do not appear in arteries.

This study demonstrates the value of combining surface information with information derived from the immediate subsurface. The depth to which structures can be visualized with backscatter electron imaging depends on the accelerating voltage and on the average atomic number of the material to be examined. Correlations of the backscattered electron images in the SEM with TEM sections from adjacent areas of the same case showed that structures could be visualized to a depth of 10 μ m. Greater penetration could be achieved by using higher accelerating voltages. We feel that this technique could be applied to any biological specimen to study changes in the surface and immediate subsurface areas, such as in atherosclerosis where lesions are found close to the surface.

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