Lymphology 26 (1993) 67-78

TERMINAL ENDOTHELIAL CELLS OF LYMPH CAPILLARIES AS ACTIVE TRANSPORT STRUCTURES INVOLVED IN THE FORMATION OF LYMPH IN RAT SKIN

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ABSTRACT

Electron microscopic examination of lymph capillaries of the dermal papillae of rat scalp skin revealed continuous extension of the lymph collection system into 4 to 10 micron diameter lumen capillaries with thin walls, scant basement lamellae (membranes), and blind-endings of 1 to 4 micron lumen diameter within endothelial-type cells. These terminal endothelial cells also displayed intracytoplasmic channels and pinocytotic vesicles, extensive cytoplasmic processes and a high cytoplasmic volume-percent of mitochondria suggesting active transport capabilities of lymphatic endothelia. The mitochondrial cytoplasmic volume-percent (mean 14.5%) exceeded that present in blood capillary endothelial cells of the rat brain (the anatomic substrate of the blood-brain barrier), that have a volume-percent of mitochondria of 10 to 12% (1). Active transport processes centered in such endothelial cells could account for a portion of lymph formation, and explain the continued accumulation of lymphedema distal to blocked lymphatic collection ducts when lymphatic intraluminal pressure is greatly increased. The small lumen diameter capillaries, which correspond spatially to the prelymphatics of other authors (2), typically converge in groups of three to form larger diameter lymph capillaries corresponding to the lymph "initials" previously described (2,3).

Ultrastructural investigations of lymphatic collecting vessels have defined many properties of lymph conduits with diameters of 15 microns and larger that differ from blood capillaries in diverse tissues of humans and other mammals (e.g., 2-5). The structural organization of the light microscopically visible "blind-end" lymph collecting system of the human skin has been recently reviewed and a new nomenclature proposed for a hierarchy of sizes of interconnected lymphatic collection conduits of 50 micron and larger diameter, principally in the subpapillary dermis (6).

Ultrastructural contributions to elucidation of the still controversial mechanism of lymph formation by lymphatic capillaries include the findings of patent (gap) intercellular endothelial cell junctions (7-11), intraendothelial channels (12-14), pinocytotic vesicles (11,15-18) and speculation on the role of collagenous attachments of the lymph capillary walls to the intracellular matrix in passively pulling the endothelial gaps open to admit excess tissue water and protein (7,19-21). A recent study of guinea pig mesentery with lymphographic techniques confirmed the presence of "prelymphatic channels" feeding into lymphatic capillaries of 15 micron or

WH Oldendorf died December 1992

slightly greater diameter, but continuity of the endothelial lining of the "channels" was apparently incomplete (2). Other studies have also deduced the presence of feeder channels visualized by light microscopic techniques emptying into the lymphatic collection system in several systems (e.g., 22-26), but these "prelymphatics" have not been regarded as an integral component of the lymph formation apparatus.

Lymphatic endothelial capillary-lining cells were of interest to us as a possible example of a capillary endothelium exhibiting minimal active transport characteristics, based on the classical conceptions of the lymph capillary as a passive conduit filled by osmotic or hydrodynamic forces (27,28), for comparison with blood-brain barrier function capillary endothelia. The present ultrastructural investigation of the lymphatic capillaries of rat skin revealed evidence of smaller lumen-diameter, endothelial-lined capillaries, connected with the lymph collecting duct system and sharing many ultrastructural characteristic of lymph capillaries, than had previously been documented. These short capillary segments featured luminal endings within the cytoplasm of terminal endothelial cells having several features suggestive of active or facilitated transport function, including a high mitochondrial volume-percent of the cytoplasm (1). Stereometric analyses of the mitochondrial volume-percent of cytoplasm of the small lymphatic capillaries were compared with blood capillaries of the same tissue sections to delineate the possible active transport qualities of these structures.

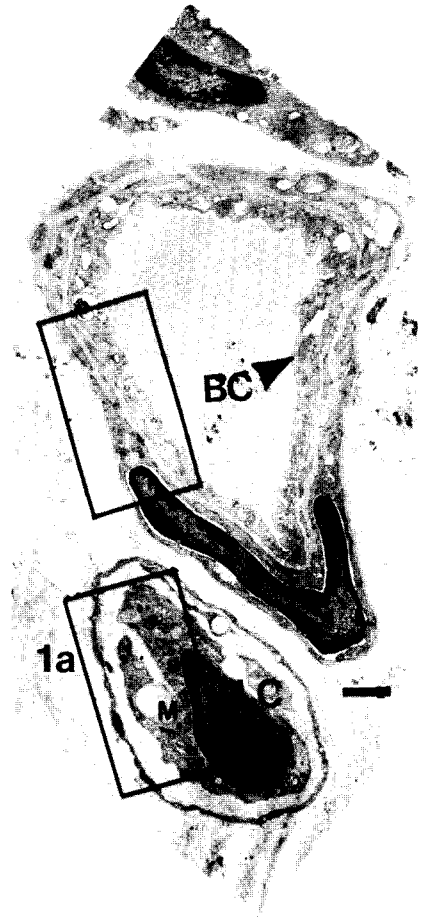

Fig. 1. (a) Cross section of adjacent lymph capillary (LC) of 5-6 microns diameter and blood capillary (BC) of 6-7 microns diameter, near the apex of a rat skin dermal papilla. The lymph capillary contains a macrophage (M). A mitochondrion is indicated in the blood capillary endothelial cell (arrowhead). The capillaries are surrounded by a field of collagenous fiber bundles of intermeshed orientation. Magnification bar=1.5 micron.

MATERIALS AND METHODS

Scalp skin specimens were obtained from six chloral hydrate-anesthetized 250 to 400 gram adult Wistar rats of both sexes after intracardiac perfusion fixation for brain preservation for other experiments. Fixation was performed by perfusion with 1.25% glutaraldehyde/1% paraformaldehyde in pH 7.2, 0.12M phosphate buffer for 10 minutes after a brief McEwen's buffer preperfusion. This was followed by immersion fixation of 5 to 10, 1mm^3 blocks of scalp skin from each rat for 4 hours in the same fixative and post-fixation in 2% osmium tetroxide/phosphate buffer with 8% dextrose added, for several hours. Each specimen was embedded in Epon 812, oriented to cut perpendicular to the central axis of the dermal papillae, parallel to the plane of the skin, and trimmed of all hair follicles. Random sections, 10 micron or more

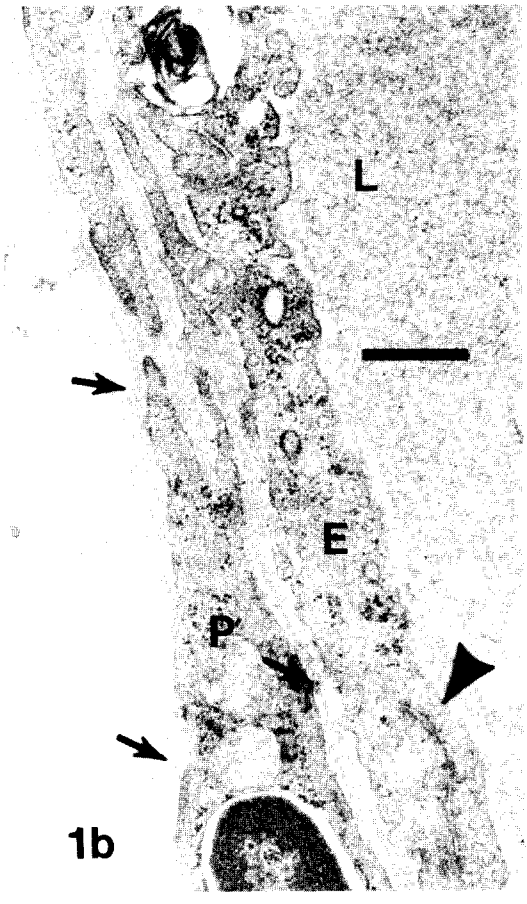

Fig. 1. (b) Detail of a segment of the blood capillary wall, as outlined in Fig. 1a. Basement lamellar material adjoins the endothelial cell (E) and pericyte (P) displaying a uniform width and characteristic fibrillary texture (arrows). Pinocytotic vesicles, but no fenestrations, are typical. An overlapping endothelial cell seam is indicated by an arrowhead. Magnification bar=0.75 micron.

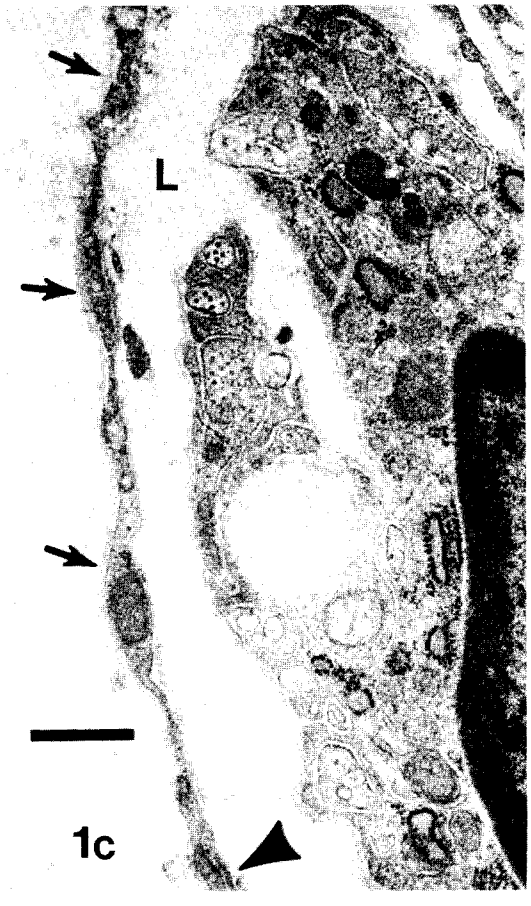

Fig. 1. (c) Detail of a segment of the lymph capillary wall, indicated in Fig. 1a. Basement lamellar material is scant and irregular (arrows), luminal (L) content is granular and moderately dense, and the endothelial wall is attenuated (about 0.1 micron). An overlapping endothelial cell seam is indicated by an arrowhead. Magnification bar=0.75 micron.

apart, were collected after sectioning on an LKB II microtome with a diamond knife, stained with lead citrate (29) and saturated uranyl acetate, and examined in a Siemens 1A Elmiscope. Micrographs were prepared of every complete round to oval lymph and blood capillary profile found in a section to avoid selection bias, at original magnifications of 4000 to 8000x. Mitochondrial volume-percentage was determined by cutting the endothelial cell cytoplasmic profiles from the micrograph, and mitochondria from the cell, and weighing the resulting photographic paper profiles, exclusive of nuclei present (1). Stereometric considerations (30-32) provide that proportional measurements of a sufficient number of randomly chosen cross sections (area) of a structure reflect the proportion of the whole volume that it occupies, in three dimensions. Student's t test was applied to the data from measurements from 30 to 35 capillaries of each type considered to deter-

mine significance of the differences of the means of mitochondrial volume-percent in the endothelial cells.

RESULTS

Fig. 1 illustrates the ultrastructural criteria applied to distinguish blood capillaries from the several sizes of lymph capillaries found within the confines of the rat dermal papilla (rete peg). Each dermal papilla contained interwoven bundles of collagen fibers, a branched blood capillary loop, and a central, "blindly-ending," 50 micron diameter lumen extension of the lymph collecting duct system of the dermis, as schematically diagrammed in *Fig. 2.* Smaller lymph capillaries connect with the central collecting duct at several sites, and spread rather uniformly through the interwoven bundles of collagenous connective tissue filling the dermal papilla and surrounding the blood capillary loop. The 50 micron lumen-diameter vessel corresponds to the "initial lymph sinus" of skin, the smallest lymph vessel recognized in the light microscopic study of Wenzel-Hora and coworkers (6). For the present report, lymph capillaries were considered to be lymph conduits with lymph formation capabilities, of lumen diameter less than 50 microns. Lymph "initials," with lumen diameters of 15 to 40 micron, reported by many authors (e.g., 2) from a variety of tissues and species, correspond to lymph capillaries using this definition. Lymph collecting vessels (ducts) were larger lumen diameter lymph conduits [initial lymph sinuses, precollectors, and collectors (6)], which function primarily in

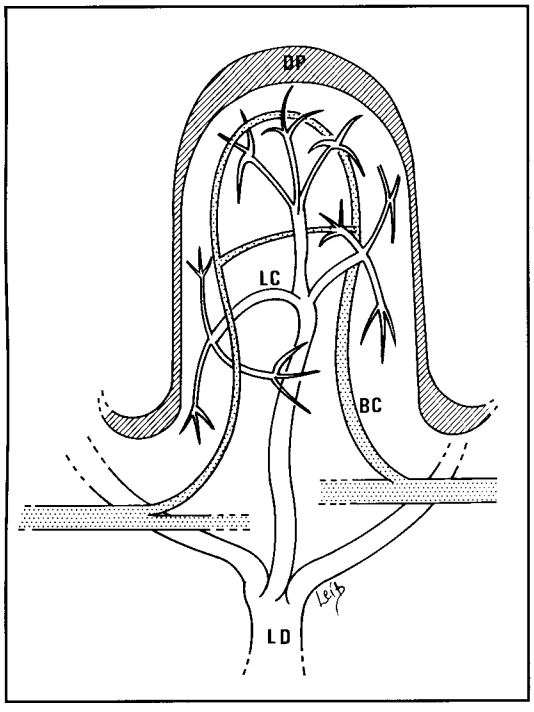

Fig. 2. Schematic diagram of the relationship of the several orders of increasing size lymphatic capillaries (LC) of the dermal papilla (DP), the blood capillary loop (BC) extending into the papilla, and the lymphatic collecting duct (LD) leading into the dermis.

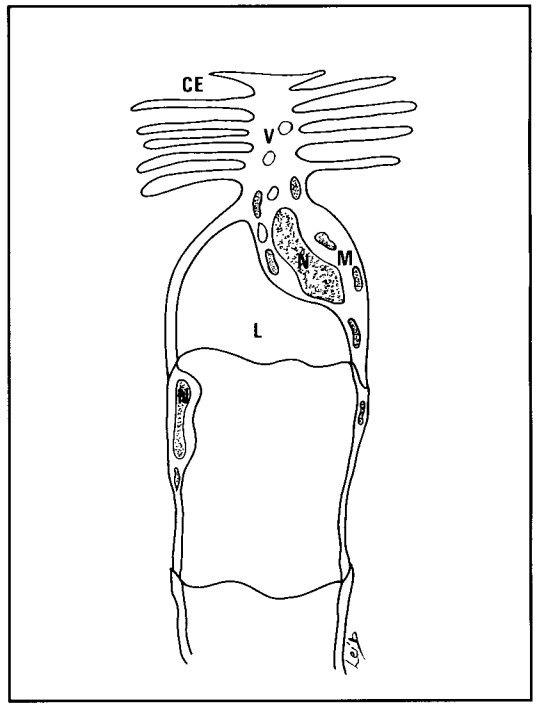

Fig. 3. Schematic diagram of the structure of the terminal small lymphatic capillary, showing the blindly-ending lumen (L) within an endothelial cell, the cell's surface-increasing cytoplasmic extensions (CE), cytoplasmic vacuoles (V); and location of the mitochondrial "pool" (M) and nuclei (N) in endothelial cells.

conduction of lymph away from the site of lymph formation. *Fig. 2* was formulated from the observations of several sections from the central portions of a number of dermal papillae, although serial sections were not taken. Apparently two or three orders of lymph capillaries of increasing size were formed by the confluence of, usually, three lymph capillaries of the next smaller order, ultimately leading into the central lymph collecting duct. The approximate diameters of the increasing sizes of lymph capillaries found within the rat scalp dermal papillae were 4 to 10 micron, 15 to 20 micron, 25 to 30 micron, and 40 to 50 micron for the central duct extending into the subpapillary area. Perfusion fixation of the skin *in situ* on the intact animals minimized mechanical stresses of the tissue before fixation, which may produce distortions in cell junction morphology and lymphatic lumen sizes in specimens subjected to manipulations or injections of tissue before fixation.

Fig. 3 diagrams the distal terminus of the smallest lumen lymph capillary, showing the lumen origin within an endothelial cell, as illustrated in the micrographs of *Figs. 4, 5, and 6*. The terminal endothelial cell was connected by overlapping, non-"tight" junctions with the penultimate endothelial cell, which formed a continuous capillary lumen of 4 to 10 microns diameter. This smallest lumen diameter segment of capillary was typically two to several endothelial cells in length.

Rarely, as in *Fig. 1*, the blood capillary loop and small lymph capillary profiles were closely adjacent, enabling clear demonstration of the differences between the capillary types in the prominence of the basement lamella (membrane), luminal content, and width of the endothelial cell wall. Small lumen-diameter lymph capillaries were distinguished from blood capillaries of similar size by several ultrastructural features, as previously defined for larger diameter lymphatic capillaries (4,16): much less prominent basement lamellar layer adherent to the abluminal surface of the endothelial cells, thinner endothelial wall and luminal content with a less flocculent, more granular texture than the perfused blood capillaries (*Fig. 1a,1b,1c*). The basement lamella of lymph capillaries appeared to be composed of the same type of fibrillar material as blood capillaries, but was disposed in a more irregular, thinner layer, with less dense packing of fibrils, and frequent divergence of some of the fibrils into the adjacent connective tissue. The thickness of the endothelial wall of all lymphatic capillaries was less than 0.1 micron, compared to 0.3 to 0.6 micron or more in blood capillaries of the dermal papilla. The lumen walls in the terminal endothelial cells (*Figs. 4,5,6*) were typically wider than 0.1 micron, but were bounded by the characteristic lymphatic type of basement lamella. Luminal content of blood vessels, after perfusion fixation as described, presented a uniform, moderately dense, flocculent appearance, as illustrated in *Fig. 1*; occasional erythrocytes and platelet fragments were still found in some of the blood capillaries. The luminal content of lymphatic capillaries appeared more granular than that in blood capillaries, and varied more in density (compare *Fig. 1* and *Fig. 4*). Luminal content of the terminal endothelial cell lumen was consistently less electron-dense than more proximal segments of the lymphatic capillary. Occasional leukocytes were found in lymphatic capillaries.

The lymph capillary lumen was typically 1 to 4 microns in diameter within the terminal lymphatic endothelial cell. The minimum blood capillary diameter in circular profiles was 5 microns, with striking uniformity of width and density of the basement lamella of the appearance illustrated in *Fig. 1b*. Cytoplasmic vesicles of the pinocytotic type (60 to 90nm) were found more frequently in blood capillaries, but also occurred in lymph capillaries. Pinocytotic vesicles were rather rare in lymph capillaries of diameter greater than 7 microns, but were prominent in the cytoplasm of the terminal lymph endothelial cells (*Figs. 4,5*). Blood capillary endothelial cell junctions typically showed slight overlap,

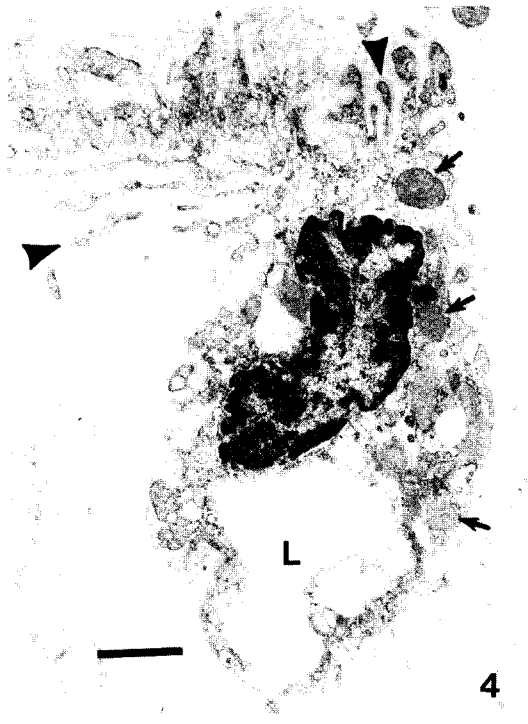

Fig. 4. Micrograph of a lymphatic terminal endothelial cell oriented to display the parallel cytoplasmic elaborations of the cell body (arrowheads). The cell body contains several large mitochondria (arrows), the nucleus, and a number of clear vacuoles of varying sizes. The lumen of the lymphatic capillary within the endothelial cell is 2.5x4 micron diameter. The basement lamella is similar to Fig. 1c appearance. Magnification bar = 2 microns.

without the juxtamarginal linear segments of increased membrane density of adjoining endothelial plasma membranes identifying a junctional apparatus of the "tight junction." Skin blood capillaries lacked the fenestrations through the endothelial cell wall that are seen in muscle capillaries (1). Lymphatic capillaries also displayed principally overlapping junctions, without tight junction specialization. Some non-overlapping, end-to-end endothelial junctions, and very rare open (gap) junctions, were found in lymph capillaries of less than 10 micron diameter. In larger lymph capillaries of the dermal papillae, typically formed by the confluence of three smaller capillaries (*Fig. 7*), open junctions were found slightly more often, but may represent the rupture of end-to-end or overlapping junctions by intraluminal pressure increase from the cutting of tissue during preparation prior to embedding (4,16). Some of the smallest diameter lymph capillaries had no visible seams joining edges of the endothelial cytoplasm to enclose the capillary lumen, and were assumed to individually form the lumen by an invagination through the cytoplasm, as seen in the terminal endothelial cells (*Figs. 4-6*). Valve-like structures were found at the confluence of small lumen-diameter lymph capillaries forming a larger lymph capillary, formed by the unattached proximal borders of the endothelial cells (*Fig. 7*), but were not seen at intercellular junctions of two endothelial cells forming a continuous diameter lumen, in capillaries of 4 to 25 micron lumen size.

Mitochondrial "pools" of several large mitochondria were typically found surrounding the nucleus in the cell body of the terminal endothelial cell. Fields of intracytoplasmic channels or caveolae (*Fig. 5*) and pinocytotic-type vesicles were continuous with the capillary lumen in some profiles. The distal portion of the terminal endothelial cell, opposite the lumen-forming end, appeared flattened, and often displayed an array of uniformly close-spaced, parallel cytoplasmic extensions into the collagenous background substance (*Fig. 4,6*). The central cytoplasm of the cell body frequently contained several clear vacuoles of various sizes, including some that appeared to adjoin or were continuous with the capillary lumen (*Fig. 4*). External to the endothelial cell capillary walls, pericytes with a similar basement lamella layer on the plasma membrane were frequently seen, but may represent cellular extensions from the endothelial cell out of the plane of section (*Figs. 4-6*). The basement lamellae of the terminal endothelial cells appeared to attach directly to 300nm wide fibrils, which may be collagen fibrils (*Fig. 6*); elastin fibrils of 10nm diameter (15) were not recognized.

The terminal endothelial cells of the lymph capillaries were distinguished by a high

mitochondrial volume-percent of the cytoplasm, exclusive of the nuclei, compared to larger lumen-diameter lymph capillaries of 5 to 10 micron diameter, and lymph capillaries with complete lumen profile diameters of up to 25 micron. Diameters were estimated from the average of perpendicular measurements of each lumen. Lymph capillaries with lumen diameters greater than 25 micron were usually greatly flattened, and could not be reliably measured for size, or the lumen could not be completely included in micrographs for adequate stereometric analysis, so were not considered in the mitochondrial volume analysis. "Small" and "large" lumen diameter groups of lymph capillaries referred to in the mitochondrial volume-percent determinations, therefore, represented the two smallest lumen sizes of capillaries in the dermal papillae. Small capillaries of 10 micron and less diameter, including the terminal endothelial cells, were found to have a volume percent of mean 14.5% and larger capillaries of 11 to 25 micron diameter lumen had a mean mitochondrial volume percent of 9.6%. Blood capillaries of the rat dermal papillae, determined in the same sections of tissue, had a mitochondrial volume-percent of 4.5%. These differences were statistically significant (see *Table 1*).

DISCUSSION

The presence of a previously unrecognized system of 4 to 10 micron lumen diameter lymphatic capillaries which originate from the lymph "initial sinuses" of the lymphatic collecting duct system in rat skin dermal papillae, with endothelial features suggesting active transport capabilities, is reported here. These small lumen-diameter capillaries display the same ultrastructural features that distinguish larger lymphatic capillaries from the small blood capillaries within the same dermal papilla structure: thin, fibrillar texture basement lamella (membrane), thin endothelial walls, and distinctive luminal content. Ichikawa and his coworkers report small,

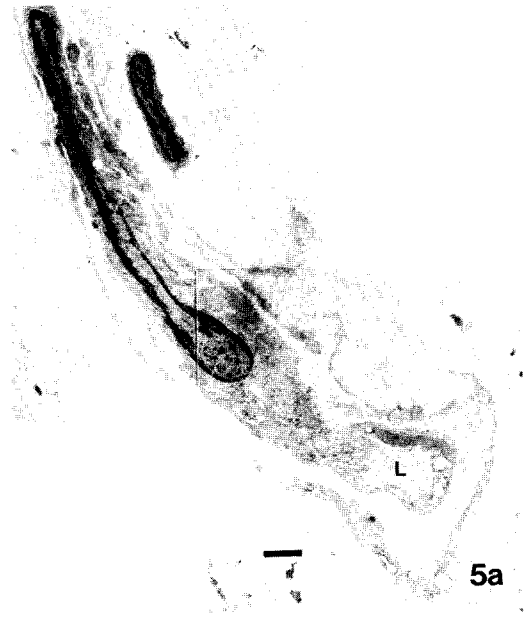

Fig. 5. (a) Micrograph of a terminal endothelial cell oriented perpendicular to the plane of section illustration in Figs. 3 and 4, so that the cytoplasmic elaborations are not visible. The lymphatic basement lamella is variable in thickness. The capillary lumen is 2 micron in largest diameter. The cell body shows mitochondria, nucleus, clear vacuoles and abundant cytoplasmic pinocytotic vesicles. Magnification bar = 1 micron. (b) Detail of the cell body adjacent to the capillary lumen shows pinocytotic vesicles and associated channel-like structures (caveolae). A Weibel-Palade body in the adjacent "pericyte" (arrow) and the basement lamella similar to the endothelial cell may indicate that this cytoplasm is an extension of the endothelial cell out of the plane of section. Magnification bar = 1 micron.

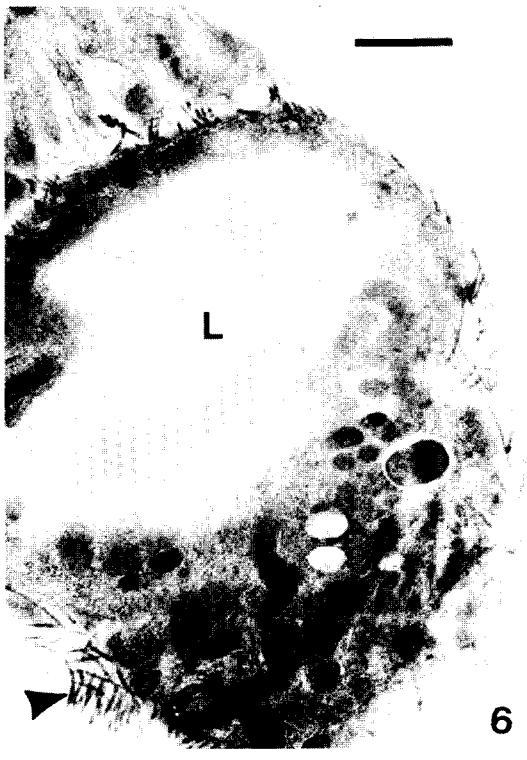

Fig. 6. Micrograph of another terminal endothelial cell, with parallel cytoplasmic elaborations connection to the cell body out of the plane of section; the characteristic thin, wispy basement lamellar material outlines the cytoplasmic folds. Non-striated, randomly oriented fibrils of about 300nm width attach all around the luminal portion of the cell, suggesting collagen fibrils (arrowheads). The capillary lumen of 5 micron diameter is surrounded by several large mitochondria (arrows). Magnification bar = 1 micron.

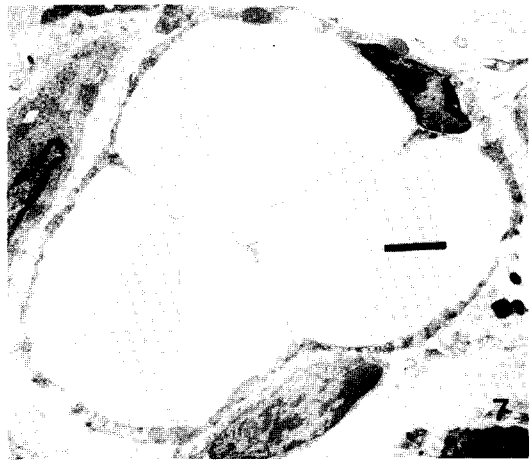

Fig. 7. Junction of three 7 to 10 micron lymphatic capillaries to form the next larger order of collecting capillary in the dermal papilla, with the diameter of the whole confluence about 20 microns. Mitochondria are frequent, and the basement membrane has the typical appearance of lymphatic vessel basement lamellae. The intraluminal trailing ends of the converging endothelial cells may perform a valve like function to prevent backflow with intraluminal pressure increase. Magnification bar = 1.5 micron.

single endothelial cell lymphatic "spines" as extensions of (15-20 x 3-5μ lumen diameter) lymph capillaries in dog cardiac papillary muscle (33); this finding suggests that the pattern of the terminal small lymphatic capillaries where lymph is actually formed varies considerably in different tissues and species.

The small-lumen diameter lymph capillaries of rat scalp skin terminate within endothelial cells featuring an increased cytoplasmic mitochondrial volume-percent (14.5%), compared to the endothelial cells of the slightly larger size lymphatic capillaries formed by the convergence of the smallest diameter capillary segments (9.6%), and compared to endothelial cells of adjacent blood capillaries (4.5%). Active and facilitated transport processes, requiring energy expenditure and associated with relatively high mitochondrial volume in the cell, occur in blood capillary endothelial cells of the blood-brain barrier regions of the mammalian brain (1,32). The mitochondrial volume-percent of blood-brain barrier endothelial cells is of the same magnitude as small lymphatic capillaries of rat skin (10 to 12%), whereas more permeable blood capillaries of several other organs of the laboratory rat, such as skeletal muscle, and kidney, have mitochondrial volume-percentages (2 to 5%) comparable to blood capillaries of the rat scalp skin (4.5%) when examined by similar methods (1).

The terminal endothelial cells of the smallest lymph capillaries in rat dermal papillae also display increased plasma membrane surface area at the interstitial

interface, obtained by an array of parallel cytoplasmic evaginations into the collagenous substratum. Numerous clear cytoplasmic vacuoles, pinocytotic-size vesicles, and empty-appearing cytoplasmic channels impinging on the capillary lumen, suggestive of fluid passage through the cytoplasm, are prominent features in these cells. These characteristics of the terminal endothelia cells of the small lymphatic capillaries suggest that such cells participate in active transport of water and protein molecules from the substance of the gel-sol intercellular matrix material into the lymphatic lumen. Bulk flow of water and proteins from the intercellular matrix through the vacuolar-vesicular channels and selective active/facilitated transport through receptors on the surface of the cytoplasmic evaginations may both occur through this cell, and to a lesser degree, through the endothelial cells of more proximal lymphatic capillaries.

The contribution of such transport of lymph-forming materials into the termini of the lymph collecting duct system would account for some, or most, of the volume of lymph fluid, independent of osmotic and/or hydrostatic pressure gradients classically assumed to account for lymph formation (27,28). Receptors on the plasma membrane of such endothelial cells could selectively determine "excess" extravasated ionic and protein components of the intracellular matrix, and internalize them, to control the homeostatic water and protein content of the intercellular substance. Protein concentration in lymph greater than that of the "tissue fluid" of the interstitial space, and of proximal greater than distal lymphatic content, has been observed (23,34,35), but is difficult to conceptualize as a result of osmotic Starling forces, or downstream contractions of the larger lymph vessels (36) selectively filing the lymphatic lumen with "excess" materials from the intercellular matrix. However, higher concentrations of protein in lymph than in interstitial fluid could be accumulated by active transport processes analogous to those of renal tubules, providing for selective accumulation of moieties on the "proper" side of membrane barriers (37). Migratory leukocytes, the disrupted interstitial tissue milieu produced by inflammation and the injection of exogenous substances into the tissue may be three entities that produce *in vivo* transport into the lymphatic lumen by other routes, i.e., interendothelial junction disruption, or by pinocytosis through the endothelial lining of lymph collecting ducts

TABLE 1
Mitochondrial Volume Percent (Mean±SE) of Blood and Lymphatic Capillary Endothelial Cells in Rat Skin

Capillary Type	Lumen Diameter (μ)	Mitochondrial Volume (% cytoplasm)
Blood	5-10	4.2±2.0%
All lymphatic	1-25	11.8±5.1%
Larger lymphatic	11-25	9.9±3.6%
Smallest lymphatic	1-10	14.5±5.7%

N=31 to 35 capillary profiles of each type; Student's t test: difference of mean of blood and lymph capillaries p<.0001; difference of mean of larger and smallest lymph capillaries p<.001.

(lymph "initial sinuses" and more proximal segments of the lymphatic system) (7,15,22,38,39).

Lymph capillaries smaller than 10 microns in lumen diameter have been identified before (see *Fig. 5* and 15,33) but without recognition of the ultrastructural features suggestive of more specialized endothelial transport capabilities that differ from larger lymph collection ducts more thoroughly studied by electron microscopy. Some organization of, or preferred pathways of, fluid/dye flow toward light microscopically definable lymph vessels has been recognized for tissues other than skin, and these patterns designated "prelymphatics" in the absence of definitive recognition of the structures in ultrastructural correlations (2,22-26). Partial endothelial lining of relatively large "spaces" adjoining lymph "initials" have been described, but under experimental conditions of intraluminal injections of the lymphatics, when smaller lymphatic branches could have been disrupted by internal pressure (2,26).

In this study, the fortuitous combination of stereotypical orientation of the small lymphatic capillaries in minute dermal papilla, good lymphatic luminal expansion in the buffer-prerinsed, perfusion fixed preparation, and interest in quantification of mitochondrial content of such vessels, has enabled separation of the ultrastructural features of these small lymphatic capillaries and their specialized endothelial termini from the background of larger but ultrastructurally similar, and perhaps less specialized lymph capillaries of the more proximal collecting system. Similar small, endothelial-lined lymphatic capillaries are likely to be found as the "prelymphatic channels" in other tissues of other mammalian species. We have observed similar, small-lumen diameter lymph capillaries, with focal endothelial aggregations of mitochondria, in rat ileal microvilli (unpublished observations).

Experimental and clinically observed lymphedema formation is associated with continued accumulation of lymph within distended collecting lymphatics, subsequent to interruption or luminal blockage of proximal lymph collecting ducts (40), or anoxia (35). This derangement may be the result of continued active transport of lymph constituents into small lymphatic terminal capillaries, responding to interstitial overload of extravasated protein and water. Classical theories of osmotic or hydrostatic gradient mechanisms of lymph formation resulting from protein/ionic concentration gradients or lymph collecting vessel contraction-suction (24-28,34-36) forcing water and protein into passive lymph conduits all fail to explain continued lymph formation after massive, static, lymphatic and tissue accumulations of protein-rich fluid have occurred. The concept of active transport processes producing accumulation of lymph constituents within the lymph collection system should provide incentive for a productive reconsideration of information accumulated on causation of lymphedema and its treatment with benzopyrones (41,42), as well as acceleration of the understanding of the normal functioning of the complex lymph tissue system in interstitial homeostasis and in immune surveillance.

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