FUNCTIONAL MICROANATOMY OF INITIAL LYMPHATICS WITH SPECIAL CONSIDERATION OF THE EXTRACELLULAR MATRIX

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ABSTRACT

In current conceptions on the functional morphology of initial lymphatics, the extracellular matrix (ECM) as an integral part of the lymphatic vascular wall has not been duly considered. In the present study based on scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) of tongue tissue in rats, new insights were obtained into both the architecture of the fibrous network of the ECM and its functional features. A digestion technique was applied, by which the endothelium of initial lymphatics was detached thereby allowing a direct view of the ECM from both sides. Fluorescent latex particles and liposomes were used as indicators of transmural permeability, whereas labeled macrophages served as a model for penetrating cells. The two layers of the lymph vascular wall were also examined under experimental edema conditions with tissue pressures ranging from 10 to 150 mmHg. A concept is proposed which considers the histomechanics of the initial lymphatics with the surrounding connective fiber tissue including the structural basis for the permeability of the lymphatic vascular wall. The role of the ECM as a supporting element and prefilter for the lymphatic endothelium is emphasized.

By 1960, a new era of morphological research was initiated in lymphology, when the first papers based on transmission electron microscopy (TEM) of lymphatics described the fine structural features of the initial lymphatics (1,2). With respect to their lymph forming function and the histomechanical mechanisms controlling it, however, several questions have remained open. Accordingly, in current hypotheses on lymphatic vascular properties, the extracellular matrix (ECM) of initial lymphatics is not usually considered as to its significance for vital processes such as tissue homeostasis and cell trafficking. Since the early eighties, scanning electron microscopy (SEM) has offered new insights into the surface morphology of both terminal blood vessels and initial lymphatics (3-6) while also providing the ability to examine the lymph-tissue interface from a microanatomical and functional perspective.

In 1935, Pullinger and Florey (7) already supposed that in the microvasculature, fine anchoring filaments kept initial lymphatics open while small blood vessels collapsed under conditions of edema. In their fundamental TEM studies, Leak and Burke (8) and Leak (9) confirmed these findings. They described an irregular meshwork of single filaments and fibers, which directly inserted at the endothelium of the initial lymphatics and became distended when the fluid volume in the tissue increased. Casley-Smith (10,11), who examined initial lymphatics in normal
tissue and in a state of edema by TEM, came
to a similar conclusion and stated further that
the anchoring filaments pull the vascular wall
of initial lymphatics outwards only at certain
sites whereas the intermediate sections of the
lymphatic vascular wall remain unaffected.
Today, this "single filament mechanism" is
commonly cited to explain the effect of tensile
strengths from the tissue on lymphatic vascu-
lar dilation and endothelial permeability.
Recently, we applied a digestion method,
by which the endothelium of initial lymphati-
cs can be removed from the underlying
ECM. We were thereby able to examine with
SEM the ECM also from the luminal side
providing a view impossible to achieve in
common tissue specimens. Thus, using TEM,
new information was obtainable on the
structural organization of the outer lymphatic
vascular wall comprising the whole area of
the basement membrane recognizable under
light microscopy. In addition, using this
approach further insights were gained as to
the special features of the vascular wall of
initial lymphatics including its barrier
properties against corpuscular elements and
migratory cells. In contrast to the endothe-
lum, the role of the ECM as a filtering
membrane has not as yet been examined
under experimental conditions. To provide
maximum insight into these issues, we opted
to study the behavior of the lymphatic
endothelium and ECM under different
interstitial pressure conditions as in previous
studies (12,13), and to study concomitantly
transmural passage of particles and cells
using CLSM. Different fluorescent markers,
laxt standard particles and liposomes were
used, which as indicators for lymphatic
vascular permeability and cell migration were
applicable to both CLSM and SEM.

MATERIALS AND METHODS

Twenty-eight rats (Han Wistar strain) of
both sexes, weighing 250-380 g, were used in
this study. The rats were anesthetized with
ther and, for long lasting experiments, with
Thiopental-sodium injected intraperitoneally
(100 mg/kg body weight). In 20 rats, 0.3 ml
fluorescent standard particles (Fluoresbrite®,
Polyscience, Oregon USA) ranging in size
between 0.15 and 4.5 μm were injected into
the tongue's body, in 8 rats liposomes with a
size of 5 to 10 μm stained with fluorescent
yellow were injected in a correspondent
quantity. The experiments were finished at
different time intervals: after 2 hours, 10 after
5 hours, 8 after 24 hours, 4 after 2 days and
4 after 5 days. Two rats were used for each
time interval.

Two hours before each experiment was
finished, a cannula was inserted into the body
of the tongue and connected by a small
plastic tube to a funnel fixed on a support.
The system was filled with Ringer's solution.
The infusion time of the tongue was in each
instance 1 hour. Thereafter vital fixation of
the tongue was introduced by replacing the
Ringer's solution against 2.5% glutardial-
dehyde in 0.2 M phosphate buffer (pH 7.4)
for 1 hour. The infusion pressure for both
fluids was adjusted to 10, 30, 50, 80, 120, and
150 mmHg. Three rats were investigated at
each pressure level.

After Ringer infusion and vital fixation
of the tongue, the rats were killed by
thoracotomy and a cut through the cardiac
ventricles. The blood circulatory system was
rinsed with a 38°C heparinized Ringer's
solution via the aortic arch until the efflux of
the right cardiac ventricle was clear. Then
perfusion followed with ice cold 2.5 glutardi-
dehyde in 0.2 phosphate buffer. Both
procedures were carried out with moderate
hand pressure. The tongue was dissected and
postfixed by submersion in 2.5% glutardial-
dehyde for 24 hours. The lymph nodes of
the anterior neck region were also removed
and postfixed.

Cross sections were made of the tongue's
body by hand using a razor blade under
binocular microscopy. In addition, sections
were made cut parallel to the ventral and
lateral surface of the tongue. For CLSM, wet
sections were mounted on slides and sealed
Fig. 1. High power SEM micrograph showing a view of the luminal surface of an initial lymphatic. The characteristic oak-leaf pattern of the endothelial boundaries with numerous interdigitating sections between facing cells is well seen. In this specimen, the lymphatic vessel exhibited only a moderately dilated state. Thus, the open junctions of the cellular boundaries are covered by overlapping cytoplasmic flaps and remain invisible. Arrows point to sites of overlapping flaps. N = nuclear portion of endothelial cells.

Fig. 2. A digested specimen of an initial lymphatic shows the ECM forming a continuous fibrous layer in the lymph vascular wall, which maintains its tube-like shape similar to the appearance in common tissue preparations.
under cover glasses with resin (Merckoglas®, Merck, Darmstadt, Germany). The tissue of some sections were stained before mounting with Azure II, Acridine orange and Eosin. These specimens were examined in the CLSM (Leitz, Bensheim, Germany) at excitation maxima of 488 nm and 514 nm using the two channel system of the device.

For SEM, sections of the tongue were dehydrated in ethanol at graded concentrations. Some sections of each experiment were critical point-dried in CO2. Others were brought for incomplete digestion of the tissue into a beaker filled with 2% osmium tetroxide in distilled water for 24 hours and then critical point dried as well. Previous investigations had shown that delamination of the endothelium from initial lymphatics occurs if the concentration of the osmium acid is higher than 1% and the time for incubation takes at least 24 hours. On the other hand, no or only minor effects had been seen in samples treated with 2% osmium acid in phosphate buffer for 24 hours. All specimens were mounted on stubs with conducting carbon, sputtered with platinum and examined in a Hitachi S 4000 field emission microscope at an accelerating voltage of 10 kV. Micrographs were taken on 6 x 7 cm Agfapan film (APX 100).

RESULTS

The Two Layers of the Lymphatic Vascular Wall.

SEM revealed that the fine lymphatics in specimens of the rat tongue pretreated with Ringer infusion and vital fixation were dilated, and their lumen in most cases no longer contained any residues of lymph. Hence, the specimens offered optimum conditions for SEM. At low magnifications, the initial lymphatics of the subepithelial plexus of the tongue showed the endothelial lining richly equipped with protruding crest-like structures, folds, trabeculae, and sometimes even true valves. Under these conditions the endothelial boundaries were highly visible and enclosed extraordinarily large cell territories. Single endothelial cells were connected to each other in the mode of an interdigitating system. At some sites individual cells overlapped the neighboring ones with long processes. Fusiform cells appeared at other places and some were arranged in groups to form primitive valve structures. High power SEM micrographs of common tissue specimens revealed in the course of the endothelium borderline structures of alternately open and tight junctions. In slightly dilated lymph vessels, the zones of open junctions were covered by cytoplasmic flaps of the related cells so that the openings were not visible (Fig. 1). With increased interstitial pressure, the overlapping structures became shortened or completely disappeared thereby allowing direct view into the hole-like open junctions.

In specimens subjected to the digestion method, the lymphatic endothelium was detached from the underlying ECM. Thus, that particular layer of the lymph vascular tube could be represented in SEM from both sides. The tube-like shape and dimensions of the initial lymphatics remained unchanged in these specimens (Fig. 2). At low magnification the luminal side of the ECM exhibited a profile which looked very similar to that of the endothelium. As shown in Fig. 3, in which two unequally treated specimens were compared, crest-like protrusions, trabeculae and valves of the endothelium were supported by corresponding fibers of the ECM. High power micrographs revealed that a network of fine filaments formed the main component of the ECM. On the luminal site this component at some sites was relatively dense and smooth; other sections exhibited a spongiform arrangement of filaments (Fig. 4). In many places this filamentous network was filled with a fine granular or homogenous material, which clearly belonged to sparse ground substance.

From the abluminal view, the ECM showed a distinctly different image
Fig. 3. A sectional area of an initial lymphatic with valve is represented in these SEM micrographs. Fig. 3a was taken from a common tissue preparation showing the lymphatic vessel with an intact endothelial lining. Fig. 3b was taken from a specimen subjected to incomplete tissue digestion. The endothelium has been removed, while the second layer of the lymphatic vascular wall represented by the ECM remains unchanged. Because the fibrous network of the ECM forms a supporting basis for valves and other protruding elements, the typical lumen profile created by these structures is also recognized in digested specimens.
characterized by a relatively loose arrangement of filaments and fibrils. A granular component could not be detected in this area. The bundles of filaments and fibrils were interwoven in many directions and formed a crisscrossed pattern. From the surrounding tissue, radial fibers and fibrils connected to the fibrous texture of the ECM. High power micrographs showed that single radial filaments changed their course, when entering the outer zone of the ECM and merging with the fibrous network (Fig. 5). But from the specimens it could not be demonstrated whether these fibers proceeded to the innermost zone and were in intimate contact with the endothelium. In all specimens examined, the ECM was complete (but only 1-3 mm thick layer) and without sections of large disconnection. Pore-like holes, however, were found at many places in its fibrous network. Under normal tissue conditions their width was 3.5 μm. About 200 pores were counted in an area of 100 mm². The average size of single filaments of the ECM ranged from 40 to 60 nm in diameter. In SEM it was not possible to differentiate between filaments of reticular or elastic nature.

The Initial Lymphatic under Increased Interstitial Pressure

Ringer’s infusion carried out at increased pressure levels uniformly caused an edema in the living tongue. Different degrees of tissue swelling were seen in relation to the pressure applied during infusion. Thus, only a slight swelling of the tongue occurred at a pressure of 10 mm Hg, whereas an extreme edematous state was produced by pressures of 120 and
Fig. 5. SEM micrographs of the abluminal side of the ECM. Fig. 5a shows at low magnification the dense fibrous layer of the outer zone along with a system of fine joining radial filaments. In Fig. 5b an enlarged region is represented (compare marked inset area in Fig. 5a). Note that the single filaments bend before they merge into the outer fibrous matrix.
Fig. 6. SEM micrograph of the endothelium of an initial lymphatic. The specimen was exposed to an interstitial pressure of 30 mmHg during Ringer infusion before vital fixation. Due to the pretreatment, a wide dilation of the lymph vascular tube can be detected. The overlapping structures, normally covering the apertures of the open junctions, have now retracted thereby creating space for interendothelial openings (arrows).

150 mm Hg. Intermediate stages of edema were observed between these extremes of pressure. Under edema conditions, the initial lymphatics consistently exhibited widely dilated vascular tubes. The effect was already recognized at low infusion pressures and intensified at higher pressures. In undigested specimens the endothelial lining of the dilated lymph vessels revealed an extremely smooth luminal surface. The overlapping endothelial flaps normally covering the open junctions retracted under those conditions thereby allowing view into the wide apertures of the open junctions with the underlying diaphragm of the ECM (Fig. 6). Signs of disintegration of the endothelium were not observed in experiments infused up to pressure levels of 80 mmHg. At the highest pressures of 120 and 150 mmHg, artifacts in the mode of endothelial disruptions were detected in some specimens, whereas others, even under these conditions, exhibited only extremely widened open junctions (Fig. 7).

Digested specimens with removed endothelium contained more pore-like holes in the fibrous layer of the ECM as compared with untreated specimens. In tissue infused at a pressure of 30 mmHg and higher, the width of the pores increased and reached values up to 8 μm. Radial fibers and fibrils joining the fibrous network of the ECM from the adjacent tissue were extremely “stressed.” In cross sections of an initial lymphatic, these fibers/fibrils appeared as a characteristic system of radial structures arranged around the lymph vascular tube like a halo of anchoring elements.

Permeation of Corpuscles and Cells Across the Lymphatic Vascular Wall

The permeability of initial lymphatics for
Fig. 7. High power SEM image showing an interendothelial open junction of a specimen that was infused and vitally fixed at a tissue pressure as high as 150 mm Hg. Under these circumstances, due to the extremely distended lymphatic vascular wall, the intercellular opening extends to an extraordinary wide aperture thereby allowing view onto the underlying fibrous diaphragm formed by the ECM.

different particles and cells was examined by both SEM and CLSM. As stated in previous CLSM studies (13), a large amount of the injected latex standard particles and liposomes were taken up by tissue macrophages by 1 hour after application. Many of these labeled macrophages migrate from the center of the tongue into the subepithelial area, where, 1 to 3 days after the injection, they enter the initial lymphatics. In later stages of the experiment, the labeled cells could be identified as elements in the neck lymph nodes by CLSM. In the present study, similar behavior of standard particles, liposomes and labeled macrophages was detected by CLSM of wet sections of tongue tissue carried out prior to the SEM of the dried specimens. Different time stages of the experiments from 2 hours up to 5 days after injection of the marker were evaluated using this approach.

From the CLSM examination the impression was gained that predominantly small standard particles with a size up to 2 μm and liposomes with a maximum size up to 5-6 μm could migrate into lymphatics, whereas bigger elements could not. CLSM showed that 2-5 days after injection labeled macrophages passed the vascular wall of initial lymphatics. SEM of corresponding specimens showed a similar result and revealed that both the ECM and the endothelium were permeable for corpuscular markers and labeled cells. At higher magnifications, the SEM image documented that small corpuscles up to a size of 2 μm penetrated the fibrous matrix of the ECM at any site, whereas bigger corpuscles had to use the pore-like openings to gain access to the endothelium (Fig. 8). In later experimental stages the ECM also contained cells identified in CLSM as labeled macrophages. These cells
Fig. 8. Luminol view of the ECM, in which, one day prior to the SEM investigation, fluorescent standard particles were applied. The image shows single or groups of these particles passing through the pore-like openings in the fibrous network of the ECM.

Fig. 9. The second day after application of the standard particles into the tongue tissue, cellular elements (C), represented by labeled macrophages, can be detected during their passage through wide openings of the ECM.
DISCUSSION

SEM clearly revealed that the endothelium of initial lymphatics is covered on the outside by a thin but continuous layer of delicate fibers forming the structural elements of the ECM. Interest and knowledge of that particular layer has increased enormously over the last years (for details on tissue functions, organ-specific differentiation and
molecular components of the ECM, see 14). The findings of the present study concerning the ECM, although contrary to some TEM observations (9), reconfirm previous observations based on SEM (15,16). In the interim, an “extravascular matrix” as a component of the outer vascular wall region of initial lymphatics has been described by others (17). With the present methodical approach using incomplete tissue digestion, it was possible to visualize the ECM separate from the endothelium thereby allowing a view from both sides using SEM.

Histotopographically the ECM basically coincides with that of the basement membrane as seen with light microscopy. In general, basement membranes underlie epithelial layers and the blood vascular endothelium (18-20). Among the basement membranes of the body there exists great variation concerning their structural differentiation as well as their functions. In the kidney, for instance, the basement membrane of the glomerular capillaries are fenestrated by a fine pore system that acts as an ultrafilter. The sinusoids in the liver, on the other hand, which have high vascular permeability are missing such a layer or have only an incomplete basement membrane. Basement membranes also form stabilizing elements of the vascular wall. Considering all these aspects, the ECM of initial lymphatics shows remarkable morphological and functional characteristics distinguishing it from other ECMs. Delicate fibers of the reticular and presumably also of the elastic type represent the main components of this layer, whereas a ground substance is only sparsely developed and limited to the innermost zone. The fibrous network of the ECM is constructed after a certain functional principle with filaments and fibrils arranged in a crisscrossed pattern interspersed with some pores. Towards the tissue this texture of the ECM desegregates to form a corona of radially oriented fibrils and fibers. This system corresponds in its position
Fig. 12. Two SEM micrographs taken from a specimen not completely washed out by Ringer infusion. Filamentous material, probably related to precipitated protein, is seen in the lumen of an initial lymphatic (Fig. 12a). At some sites the sponge-like structure is connected to the endothelium. In Fig. 12b such an area is represented at higher magnification (see marked inset area in Fig. 12a). Note that single protein filaments can be traced back to the open junctions of the cellular boundary.
to the lamina fibroreticularis of basement membranes.

**Histomechanics of the ECM**

Two functions mark the ECM with its fibrous texture. One is the special histomechanical property of this layer and the other is its capacity to act as a relative barrier for interstitial fluid and corpuscular elements. The special arrangement of the fibrous material provides optimal adaptive properties to change the dimensions of the lymphatic vascular tube within a great range. Thus, the initial lymphatic is capable of assuming wide dilatation or tight constriction. The fibrous nature of the ECM also acts as an effective mechanical protector for the tenuous, thin endothelial layer during a state of extreme dilatation. With alternating constriction and dilatation of the lymph vessel, the ECM regulates transendothelial permeability with changes of width of the open junctions. Each mechanical event occurring in the fibrous system of the ECM affects equally the mechanical properties of the endothelium. The ECM thereby becomes the ultimate determining factor for transmural permeability during lymph formation.

The histomechanics of the lymphatic ECM can only be understood properly if its relationship to the surrounding tissue is taken into account. The system of radial fibers featuring the outer zone of the ECM and described as “anchoring filaments” in current conceptions does not apply at the outer endothelial surface. Coming from different directions of the collagenous tissue these fibers bend into the outer fibrous matrix, where they merge into the fibrous network of the ECM. The radial fibers thereby convert the pulling forces from the tissue into circular stretching ones on the lymphatic vascular wall promoting a state of vascular dilatation. Correspondingly, release of the pulling forces results in a contraction of the lymph vessel. The particular structures and dynamics are depicted in Fig. 13.

The common “anchoring filament hypothesis” seems less convincing in the light of the SEM findings and should be replaced by a concept which considers the entire ECM as an integrated, functional element between the connective tissue and the lymphatic endothelium. In such a “functional system” of connective fiber tissue, as conceived by Benninghoff (21), all fibers, fibrils and filaments are connected with one another in the mode of a hierarchical principle. Hence, every histomechanical event influences each part down to the last reticular filament around each cell. Applied to the initial lymphatics, that concept contributes to the ECM’s role as the final component of the connective tissue fiber system, which integrates all physical events occurring in the related organ to the lymphatic endothelium (22). The “elastomotoric micromechanism” of the connective tissue as envisioned by Huzella (23, 24) is also based on the hierarchical order of the tissue fiber system and, concerning histomechanics, is similar to that discussed with respect to initial lymphatics. But active physical elasticity of the tissue as an impelling force as suggested by Huzella could not be documented for the lymphatic system till now. Another type of tissue organization is the concept of Rodbard and Feldman (25). It is based on the existence of tissue units (modules) each supplied with an arteriole with related capillaries and separated from the tissue by a fibrous capsule. This tissue model, which considers fluid dynamics rather than the histomechanics of fine lymphatics, contributes less, however, to an overall functional-morphological aspect.

**The Permeability of the Lymphatic Vascular Wall**

The permeability of initial lymphatics must be regarded as the result of both the permeation conditions of the lymphatic endothelium and those of the ECM. The role the lymphatic endothelium plays during lymph formation, primary lymph transport,
and lymphogenous cell trafficking was the subject of previous papers (26-28). Endothelial permeability can widely change from a state of virtual impenetrability for fluid to free transmural passage of cellular elements. This quality is due to the capability of the intercellular junctions to convert their shape from well sealed and overlapped structures into openings with wide apertures. The intercellular openings have been commonly recognized as preferably used passageways for fluid and corpuscular markers in TEM observations (29). As shown by SEM in the present study, cells from the tissue also use this system to enter the lymph vascular lumen. Bigger cellular elements, however, are forced to overcome small intercellular openings by deformation. The routes different substances such as fluid and particulate matter use during transmural passage are shown in Fig. 14. Azzali (30), who studied transendothelial cell migration in rat lacteals found that macrophages enter the endothelium via the cytoplasm, while lymphocytes used the intercellular passage. The present study also has shown that viscous material such as protein enters the initial lymphatics via the intercellular junctions. Protein as water-binding substance can thereby be quickly removed from the extravascular space and a high water load of the tissue thereby be minimized.

The ECM with its fibrous network provides a prefllter for the endothelium. This point of view has been little considered and based only on TEM investigations (31,32). In light of the new SEM findings, the permeability of the ECM, in contrast to the lymphatic endothelium, is remarkably consistent and unchanging and must be judged highly permeable. Thus, this layer of the lymph vascular wall only acts as a coarse filter with a relative barrier function even for foreign material and cells. Free passage of
Fig. 14. Diagram showing the permeation routes of fluid and corpuscular elements from the tissue through the ECM and the lymphatic endothelium. Motion of fluid from the tissue spaces (1 a) through the fibrous network of the ECM (1 b) and endothelial gaps of the endothelial boundaries (1 c). Passage of standard particles and liposomes from the tissue spaces (2 a) through small pores of the ECM (2 b) and endothelial open junctions (2 c). Migration of motile tissue cells (3 a) through broad openings of the ECM (3 b) and, by diapedesis, through endothelial open junctions (3 c).

Fluid through the ECM seems always possible independent of the functional state of the tissue. The pores of the fibrous network with widths up to 5 μm are extraordinarily large and similar to that of the basement membranes of other organs, calculated for instance in the kidney as being in the range of a few nm (33,34). This fact largely contributes to the high permeability of the ECM that exists already under normal tissue pressures. Thus, the ECM is a relative barrier primarily for immobile cells, whereas mobile cells can easily pass. It is conceivable that in the instance of a protein-rich edema, a restricted passage through the ECM also exists for tough viscous material.

Finally, it should be noted that both properties of the ECM, i.e., to function as a transmitting mechanical link and as a prefilter, require tissue conditions in which integrity within the connective fiber tissue is secured. Loss or disintegration of any of its structural elements necessarily leads to malfunctions in the whole system and interference with the function of the ECM. It may be, therefore, that the true final pathway or “Achilles heel” of fluid homeostasis is the ECM rather than other microvascular elements.
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