SCANNING ELECTRON MICROSCOPY OF THE INITIAL LYMPHATICS OF THE SKIN AFTER USE OF THE INDIRECT APPLICATION TECHNIQUE WITH GLUTARALDEHYDE AND MERCOX® AS COMPARED TO CLINICAL FINDINGS

PART II: THE EFFECTS ON THE OPENING APPARATUS OF THE INITIAL LYMPHATICS

B.I. Wenzel-Hora, D. Berens von Rautenfeld, H. Partsch

Department of Radiology, Clinical Research, Schering AG, Berlin (BIW-H), Zentrum Anatomie, Medizinische Hochschule Hannover (DBvR), Federal Republic of Germany, Vascular Service of the Hanusch-Hospital, Vienna, Austria (HP)

For Abstract, Introduction, and Materials and Methods, see previous article (Part I.)

RESULTS

Effects on the opening apparatus of initial lymph sinuses

With the indirect glutaraldehyde (GA) method, conditions resemble those after indirect lymphography with the contrast medium IOTASUL at different rates of application at the wall of the initial lymph sinuses (ILS). Scanning electron microscopy (SEM) of the luminal and abluminal endothelial coating of the ILS are possible only in sections of tissue preparations which have been diluted and fixed in this state (Fig. 5a). Lymphtics are not usually demonstrable in the precontrast SEM preparation or after application of IOTASUL because of collapse after excision.

The “opening apparatus” (Fig. 5b-d) of the ILS consists of interendothelial openings (accessus interendotheliales, NT), abluminal stabilizing elements (Figs. 5e-f; also see Ila-e) in the form of anchoring filaments (fibrae fixationes, NH) and luminal stabilizing elements of endothelial bridges (ponticuli interendotheliales, NT) and connective tissue trabeculae (trabeculae fibroendotheliales, NT). A subendothelial mantle of filaments arranged in the shape of a net (fibrae basilares, NT) and about 5μm wide forms a wide-meshed filter network (Fig. 5e). The anchoring filaments (Fig. 5f) form a structural part of this fibrous network (see below), that only partially replaces the function of a missing basal lamina. Contrast medium molecules such as IOTASUL as well as larger particles of marker dyes (India ink, Berlin blue) are able to pass through this filter network of filaments. Apart from the anchoring filaments, these filaments probably play no role in fixing the ILS in the adjacent scaffold of connective tissue fibers. Bundles of collagen fibers (Fig. 5g) surround the fibrae basilares and should not be interpreted as a constituent part of the wall of the ILS.

Under physiological conditions, the interendothelial openings (accessus interendotheliales, NT) of the endothelial coating function as inlet valves for fluids from the interstitial space into the lumen of the ILS (Figs. 5-7). Only the endothelial cells of the ILS display the characteristic shape of an oak leaf. Under transmission electron micro-
Fig. 5. Structural elements of initial lymph sinuses (ILS) from the temporal skin of man (5a-e and 5g) and of the pig after indirect application of glutaraldehyde (injection rate 0.004 ml/min). Luminal views of two dilated ILS fixed in this state (5a), of several type I interendothelial openings (IEO) between two (A and B) endothelial cells (5b), of two type II IEO (5c) and of a type III IEO (5d). The endothelium of the ILS is flanked abuminally by a fibrous network of filaments (5e). Figure 5f shows the fibrous network (arrowheads) as in 5e and anchoring filaments (AF) connecting the endothelium of an ILS (LSL = lymph sinus lumen) with the skeleton of connective tissue fibers (elastic fibers). Bundles of collagen (5g) lie on top of the fibrous network.

scopy, the processes of each endothelial cell display overlaps as open junctions to the neighboring endothelial cell which characterize the interendothelial openings. The approximately 10-20 inlet valves of adjacent endothelial cells are secured in the intercellular regions lying between them by cell denticulations and closed junctions. The interendothelial openings display a maximum diameter of 5 μm (2.5 μm), so that cells the size of an erythrocyte can pass through the inlet opening. Since needle insertion often causes microhemorrhage at the injection site, erythrocytes enter the lumen of the ILS via the interendothelial openings as fluid is infused. Only rarely do these erythrocytes block fluid flow as for example, contrast media in the lymph sinuses of the tributary lymph...
nodes of these ILS. The phenomenon of intralymphovascular erythrocyte passage cannot, however, be prevented by indirect application.

We distinguish three types of interendothelial openings depending on the increasing filling pressure (Fig. 6). With a closed interendothelial channel (type I), the endothelial cell processes lie tightly on top of each other (Fig. 5b). As the filling pressure increases, the closed interendothelial channel (type I) is transformed into an open interendothelial channel (type II) (Fig. 5c), into which the luminal endothelial process of the interendothelial openings protrudes as fluid influxes into the lumen of the ILS (Fig. 7, arrow). Types I and II are virtually the only types demonstrable in completely collapsed ILS (Fig. 6). As the filling pressure increases further—in the state of mild edema formation—pore opening (type III) occurs in which the abluminal cellular process of the inlet valve is pressed back into its cell in the direction of the flow of fluid (Figs. 5d, 7, phase A-C). With local edema, pore openings with or without endothelial defects (types IV and V) occur. We define the staging (A-C) of this local edema formation (Fig. 6) only on the basis of endothelial defects and not on physiological parameters. In stage V, endothelial defects occur only around the interendothelial openings (type IV), while stage C is marked by inter- and intraendothelial changes in other areas of the endothelial coating.

At moderate (0.03 ml/min) and high (0.3 ml/min) rates of application, the interendothelial openings function not only as inlet, but also as outlet valves (Fig. 8). The phenomenon of contrast medium diffusion can be demonstrated not only by indirect lymphography, but also with the indirect MERCOX® method. If the filling pressure is too high, the fluid applied enters the ILS within the injection wheal via inlet openings and, from there, the ILS of adjacent lymphatic areas of skin via intercapillary or precollector reflux. In the ILS of the reflux area, the fluid diffuses via interendothelial openings—mainly type II, which function here as outlet valves—back into the interstitial connective tissue (Fig. 8). Analysis of the transformation of the interendothelial openings in relation to the different rates of application (0.003, 0.03, and 0.3 ml/min) discloses three types of opening next to one another in an ILS. This holds true for dilated but not for constricted ILS ex-

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Fig. 6. Types (I-V) of interendothelial openings in relation to the injection rate and with reference to the endothelial situations in transmission electron microscopy (TEM) (E = endothelial cell, L = lumen, IN = interstitium).

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Fig. 7. Transformation of an interendothelial opening (IEO) from type II to type III in three phases (A-C) from inside the lumen. The subendothelial fibrous network of filaments can be seen through the pore opening (B and C).

amined by transmission electron microscopy after immersion fixation. Type I and II interendothelial openings are virtually the only ones demonstrable in constricted ILS. Our studies show that, using the indirect injection technique, the filling conditions differ within the injection wheal and even at every ILS. Thus, the type of opening which occurs cannot be predicted from the injection rate chosen. The filling pressure at the ILS apparently changes within the range of a few tenths of a millimeter. Endothelial defects increase only at injection rates of 0.3 ml/min, and are largely preventable by choosing lower rates (0.003 and 0.03 ml/min).

The anchoring filaments (fibrae fixationes, NH) as a second structural element of the opening apparatus are adhesive structures in the form of filaments which connect the skeleton of connective tissue fibers to the outer endothelial wall of the ILS (Fig 5f). Structurally, the anchoring filaments are correctly classified as belonging to the subendothelial fibrous network of filaments.
Fig. 8. Intercapillary and precollector reflux with reference to the interendothelial openings in the initial lymph sinuses as inlet valves (arrows in the injection wheal) or as outlet valves (arrows in the intercapillary reflux area).

Fig. 9. Function of the anchoring filaments: the schematic drawing illustrates the synchronous dilatation of the connective tissue fibers and the initial lymph sinus via the anchoring filaments (AF) at an increasing injection rate (from A to B). At the same time, transformation of the interendothelial openings of the initial lymph sinuses occurs from type II (A) to type III (B). The anchoring filaments probably play no role in the transformation of type I and II interendothelial openings (see text).

(fibra basilaris, NT). The function of the anchoring filaments is illustrated in Fig. 9: as the interstitial pressure increases in an injection wheal, the skeleton of connective tissue fibers draws apart. Anchoring filaments transfer this force to the ILS. The ILS then become dilated, as a consequence of which type II interendothelial openings are transformed to type III openings. The increasing filling pressure in the ILS is probably primarily responsible for the transformation of type I interendothelial openings into type II openings.
openings, since the skeleton of connective tissue fibers and, hence, the anchoring filaments as well are subject to only slight, if any, traction forces during physiological filling processes.

In the skin, a high proportion of elastic fibers in association with anchoring filaments reduces the traction forces acting on the ILS (Fig. 5f). This arrangement creates better conditions for the use of the indirect injection technique than if connections existed to bundles of collagen. There have been no observations of anchoring filaments detaching from the endothelial coating with the indirect GA method even after use of high injection rates of 0.3 ml/min. If the anchoring filaments were to become detached from the endothelial coating, the ILS would be unable to dilate and the influx of fluids via interendothelial openings would cease.

Effects of the interendothelial openings and anchoring filaments in the precollectors

On a structural basis, the valvular precollectors (vasa lymphatica praecollectoria, NT) are vasa fibrotypica (NA) with their own connective tissue part of the wall (Fig. 10d) in which isolated, discontinuously arranged smooth-muscle cells may be present. In contrast to the ILS, the endothelial coating displays straighter intercellular borders (Fig. 10a). Our studies show that interendothelial openings also exist in precollectors and even in collectors (vasa lymphatica collectoria, NT) (Fig. 10b) which function as outlet openings (overflow valves) under conditions of high intralymphatic pressure. Open junctions are rare in postsinusoidal sections of the lymphatics in comparison to the ILS. Anchoring filaments can also be demonstrated in basal membrane-free sections of the wall. Because there is no continuous smooth-muscle media, dilatation of the precollectors is probably also regulated passively via the connective tissue apparatus. The valves (distance between valves 0.13-0.2 mm) of the precollectors (Figs. 10c-e) determine the direction of flow of fluids in these vessels. The valve mouth resembles more the shape of an unstable funnel valve than that of a stable pocket valve which may explain the frequent occurrence of valvular insufficiency using the indirect application technique. At higher injection rates, valves can easily be overwhelmed in a retrograde direction and accounts for precollector refluxes (Fig. 2). No morphological changes of the precollectors are demonstrable even at higher injection rates of 0.3 ml/min.

Effects on the luminal stabilizing elements in the ILS and precollectors

The luminal stabilizing elements (endothelial bridges and connective tissue trabeculae) must be regarded as a third component of the opening apparatus (see above). Under conditions of high lymphatic pressure, the endothelial coating of the initial lymphatics is protected not only abluminally by anchoring filaments, but also luminally by stabilizing elements (Fig. 11). Endothelial bridges (ponticulus interendotheliales, NT) occur in the ILS and precollectors of the skin of man (Figs. 11a-c) which connect the adjacent (Fig. 11a) or non-adjacent (Fig. 11b) cells to each other. In precollectors, they are characteristically in the region of valves (Fig. 11c).

Endothelial bridges are demonstrated more frequently in initial lymph sinuses than in precollectors and display a length of 5-10 μm. Another luminal stabilizing element consists of ramified or non-ramified connective tissue trabeculae (trabeculae fibroendotheliales, NT) in the precollectors which, because of their connective tissue core, stabilize these sections of large-caliber lymphatics more effectively than do endothelial bridges (Figs. 11d-e). No defects were demonstrable in the stabilizing elements even after high injection rates. However, the endothelial bridges in particular dilate even after low rate injection (0.003 ml/min).

DISCUSSION

Interendothelial openings have been demonstrated in the initial lymph sinuses (ILS) in the skin and other organs of various animal species, but not in man, whether the silver-plating technique (38), transmission
Fig 10. SEM precollector situations from the skin: luminal view of a precollector with three endothelial cells (A-C) and a few wavy endothelial cell margins (10a, pig). Intereendothelial opening (type 1) between two endothelial cells A and B (10b, pig). Precollector valve from the temporal skin of man (10c). Nuclei (thin arrows) are demonstrable on the endothelial coating only at a low injection rate (0.004ml/min). The hatched arrow marks the direction of lymph flow (10c). Precollector ramification (10d, pig) with a valve (VM = valve mouth, VP = valve pocket). Fig 10e shows a corresponding ramification situation of a precollector after manual injection of MERCOX®. Incomplete filling of precollectors (PC) with MERCOX® from the temporal skin of man (10f). Indirect injection of GA at a rate of 0.004ml/min.
electron microscopy (3940), or scanning electron microscopy (10,11,13) was used. Exact examination of structural changes in the lymphatic system and hence, also in the opening apparatus by TEM (41) and SEM (11) in relation to the interstitial pressure (10) or via injection rate (12,15) became possible only with the introduction of the indirect GA method. Since indirect lymphography also involves the measurement of injection rates, measurement of interstitial pressure was considered unnecessary. In the illustrations published by Castenholz (10), the physiological opening types I and II predominate at
interstitial pressures of 10-30mmHg, while almost only type III openings are present at 50 and 70mmHg. In our studies with injection rates of 0.003, 0.03, and 0.3ml/min, all opening types occur next to each other at the inner margin of an ILS, but type IV and V interendothelial openings are seen more frequently at injection rates of 0.3ml/min (see further discussion below).

Neighboring endothelial cells are secured by Macula occludentes directly around interendothelial openings and by tight and gap junctions between the openings (28,40 v. Rautenfeld unpublished observations); these securing elements are not demonstrable by scanning electron microscopy. However, there are also no indirect signs of destruction of these closed junctions even after high injection rate (0.3ml/min). The question of whether closed junctions occur at times in type I interendothelial openings or whether Macula occludentes always flank the edge of the openings (42) still appears unanswered. Our studies also show that, at high injection rates, open junctions can function as outlet openings not only in ILS but also in precollators and even in marginal sinuses of the lymph nodes (15). At excessive injection rates, extravasation in the collectors is seen during lymphography (43,44).

The lymph-vascular passage of erythrocytes into the ILS via the interendothelial openings has been reported (45,46), but its effects are probably of less consequence with the indirect application technique if the possibility of partial blockage of the lymph nodes is ignored.

The initial lymphatics contain luminal endothelial bridges and connective tissue trabeculae and abluminal anchoring filaments as stabilizing elements. The anchoring filaments discovered by Pullinger and Florey (47) and the subendothelial fibrous network is best interpreted as a morphological unit on the basis of TEM findings (42,48,49).

Elastic fibers are abundant particularly in the perilymph-vascular region of the skin of the pig (50) and man. The contact between the elastic fibers and the anchoring filaments (46,49) reduces the traction forces on the wall of the ILS and precollators with increased edema formation after indirect injection of fluids. Our SEM findings show that, even at high rates of injection (0.3ml/min), endothelial defects around interendothelial openings (type IV and V) are more likely to develop detachment of anchoring filaments from the endothelial coating or from the elastic fiber unit. The idea that, at high interstitial pressures, anchoring filaments in the injection region rupture and ILS collapse (46) is best abandoned in favor of excessive dilatation of the channels with endothelial defects. The changes mentioned for the capillary region are quickly reversible because of high regeneration potential of the lymphatic system (51), and thus permanent damage, as, for example, after a high injection rate (0.3ml/min) is unlikely.

In agreement with Castenholz (10), the endothelial bridges are cell processes which may form a syncytium between neighboring and more remote cells. These cells stabilize the endothelial coating not only of ILS, but also of precollators in various organs, and particularly around interendothelial openings and valves. Connective tissue trabeculae in precollators have already been demonstrated (46) in the skin on the bottom of the feet of birds and dogs. They are frequently present in the particularly large-caliber precollator networks of the organ capsules, which supports their function as stabilizers.

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B.I. Wenzel-Hora. M.D.
Department of Radiology
Clinical Research
Schering AG
Müllerstrasse 170-178
D-1000 Berlin 65