Demonstration of Initial Lymphatics in Excised Human Skin Using an Extension Technique and Dye Injection

Dietrich Lubach, Silke Nissen, and Dagmar Neukam
Department of Dermatology (Hautklinik Linden), Medizinische Hochschule Hannover, Hannover, F.R.G.

Using a newly developed technique (extension technique) we succeeded in presenting dermal lymph vessel networks by subepidermal injection of an aqueous Berlin blue solution in excised human skin. Of 94 skin specimens taken from the safety margin of excised melanomas it was possible to demonstrate 32 networks and 14 single dermal lymph vessels. The networks can be documented by macroscopic photographs.

Lymph is drained from the skin via a dermal lymph vessel network (DLVN). The network system consists of a flat subepidermal network and a wide-meshed network that drains the deeper dermis. The DLVN is formed of lymph capillaries and precollectors that comprise the initial lymphatics (IL). We prefer the term initial lymphatics for the smallest, most peripheral elements of the lymphatic system. They are often termed "lymphatic capillaries," "terminal lymphatics," "small lymphatics," and "peripheral lymphatics" [1,2]. The wall of IL is the continuous type of endothelium surrounded by a fibrous network of filaments embedded in a homogeneous matrix. Anchoring fibrils link the lymphatic endothelium to adjacent collagen bundles [3,4].

Only a few systematic investigations into IL have been carried out because of the lack of available histologic techniques for demonstrating them.

In this respect a change is taking place. Bollinger and co-workers have developed a new technique (fluorescence microlymphography) that allows the demonstration of the IL of the skin by intradermal injection of a fluorescent dye [5]. The vessels demonstrated with this technique are filmed subsequently. Very recently, indirect lymphography has been developed in Europe. In this technique a newly synthesized contrast medium (Iodasul) is injected intradermally [6]. Dermally situated lymph vessels become visible around the contrast medium depot. Both methods are employed in lymph vessels in situ.

Already in the last century intradermal dye injections into the DLVN were successfully demonstrated [1,7]. This technique was carried out on cadaver skin. It was rather difficult and only occasionally a lymphatic network could be made visible. Such preparations are unsuitable for ultrastructural investigations. This is why very few systematic examinations of the initial lymphatics could be performed using these methods. As far as we know there is as yet no method for making the DLVN visible in excised human skin.

We have developed a new method that we have termed the extension technique. This method allows us to present the DLVN in excised human skin. The methodical basis as well as the histologic and electron microscopic demonstration, that the networks have shown in this way are in fact the lymphatics, have been published elsewhere [8]. In the meantime we have been able to improve the preparation and documentation of the networks, so that it is now possible to give a survey of the ascertained results according to 32 network demonstrations.

MATERIALS AND METHODS

Skin Specimens
The skin specimens were derived from the safety margin of surgically removed malignant melanomas and were at a constant distance of 2-4 cm from the tumor. They were processed immediately after the operation. Skin specimens from 95 patients (35 female, 60 male, ages from 21 to 78 years) were examined. Table I gives details of the parts of the body from which the skin specimens were taken.

EXTENSION METHOD
Surgical sutures were tied on opposite sides of a 1-2-cm² skin sample and connected with loops to elastic bands. The skin specimen was expanded and thus attached to a specially designed holder (Fig 1). Vertical expansion was achieved using a 25-g weight that was sutured at one point to the subcutaneous fat.

Expansion Force
Preliminary tests with a defined spring tension showed that expansion with a determined traction force is unsuitable because skin samples require different traction forces for identical expansion due to their varying size and, specifically, due to their location on the patient's body. As we have seen, it is best to stretch the skin with elastic bands until the previously wrinkled surface looks smooth and shiny.

MACROSCOPIC PRESENTATION OF THE DLVN
Through a careful subepidermal injection of an aqueous Berlin blue solution (BBS), the network presentation was achieved on the extended preparation. The staining fluid was manually injected into...
the skin with a hypodermic needle (cannula No. 23), using very slight pressure. After successful network presentation, the preparation was fixed in the extended state.

**Acetic Acid Fixation** After filling the DLVN with BBS, the extended preparations were immersed in a 20% acetic solution and left there for approximately 12 h. Subsequently the epidermis was stripped off carefully, using a pair of forceps and a blunt scalpel. Rounded epidermal residues that remained associated with the follicles could be easily removed with a fine pair of forceps. Subsequently the preparations were air-dried for 2–3 d at room temperature. The subcutaneous layer was dissected off carefully with a razor blade. The dried preparations were viewed using immersion oil.

**DOCUMENTATION**

The networks were photographed first using a camera (Olympus OM4) with macro-equipment (Makrotubus, Faltenbalg, 50-mm lens). For this purpose a source of light from below the preparation was necessary. The photographs were made with a transparency film for artificial light (Kodak Ektachrome 160) or with a black and white film (Kodak Panatomic X, 32 ASA). The exposure time was 1–3 seconds. Later a stereo-microscope (SZH, Olympus) with photographic equipment (Olympus ON4) was used. Thus the preparation could be documented by lighting on or through and with enlargements of 1:7.5 to 1:65.

**RESULTS**

**Extension** Stretching the skin preparation is a prerequisite for demonstrating a DLVN. Repeated attempts to demonstrate a network in unextended skin only led to the formation of interstitial depots without any vessels being demonstrated. The skin had to be extended so much that the epidermis gave a superficial and shining appearance.

Freshly excised skin preparations were used. Network demonstrations could be achieved exclusively 2–3 h after the excision of skin biopsies. We did not examine whether filling the network at a later time would be possible.

**Injection Technique** The injection of dye was to be carried out using the finest possible cannula, exerting a very slight pressure at an extremely low angle to the surface of the skin (the cannula aperture points to the epidermis). Typical networks were then immediately filled (Fig 2). However, it often happened that the dye only filled the interstitial tissue, i.e., a depot was formed. Then the injection was repeated at another part of the extended preparation. After preliminary difficulties we were able to make networks or parts of networks visible. If we had already succeeded in filling a network with BBS at the first attempt in one preparation, then as a rule networks in other parts of the preparation could be filled easily. The success in filling a network depended considerably on the body area from which the excised skin specimen was taken (Table 1).

**Description of the Networks** When we succeeded in filling with dye, an approximately 3–8-mm network area was formed around the site of injection (drainage unit). When such a drainage unit was filled, it could not be further enlarged in spite of an additional injection. However, by stroking the surface of the skin cautiously, we were sometimes successful in causing the network to enlarge abruptly, filling up additional neighboring drainage units. An injection made just a few millimeters away from one network unit often led to the filling of a further drainage unit almost always of the same size (Fig 3). At the areas of contact the networks units merged into each other (Fig 4).

Observation through a stereo-microscope shows an approximately 0.2 to 1 mm polygonal network. The network structures show distinct differences according to the body areas from which the specimens were taken.

The filled vessels show wide and narrow segments running straight or curved, winding at some parts. At bifurcations the vessels always have a wider lumen. At a greater enlargement, hemispherical sacculations can be seen. Very typical for the DLVN are vessels of different lengths with mainly narrow lumen. These vessels do not participate in network formation but seem to have blind endings (Fig 5).

The vessels forming the subepidermal network lie on a horizontal level just under the epidermis. However, deeper-lying vessels filled with BBS can be seen (precollectors) that usually run obliquely (Fig 3). Network formation cannot be discerned here, although in this type of vessel ramifications can be seen.

**Acetic Acid Fixation** Through this method the preparations become transparent, resulting in a three-dimensional picture under stereo-microscope, thus enabling assessment of the characteristics of the vessels compared to each other. Unfortunately, the three-dimensional quality cannot be documented photographically. Such preparations are durable for months and can therefore be directly compared to each other. Oil immersion is necessary for viewing. In these preparations one-sided vascular dilatations can be seen well (Fig 5). These could be the sacculae depicted by Braverman and Yen [9].

**Vessels in the Subcutaneous Layer and the Cutis-Subcutis Border** By removing the fatty tissue layer by layer, rather large lymph vessels filled with BBS were discovered parallel to the surface. These vessels are from the dermal network and leave the dermis at an oblique angle. These lymphatics had a width varying from...
Table I. Survey of the Dye-Filled Dermal Lymph Vessel Networks in Excised Skin Specimens from Various Body Areas

<table>
<thead>
<tr>
<th>Number of Skin Specimens</th>
<th>Body Areas</th>
<th>Network Demonstrations</th>
<th>Single Lymph Vessels</th>
<th>Number of Vessel Demonstrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>Back</td>
<td>18</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>Breast, abdomen</td>
<td>1</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>13</td>
<td>Lower leg</td>
<td>2</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>11</td>
<td>Upper arm</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>Feet (sole, back, toe)</td>
<td>1 (sole)</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>Forearms</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>Lateral abdomen</td>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Thigh</td>
<td>2 (scalp)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Groin area</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Neck</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Hollow of the knee</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Back of the hand</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>95</td>
<td></td>
<td>32</td>
<td>15</td>
<td>48</td>
</tr>
</tbody>
</table>

23 µm (narrow parts) to 60 µm (Fig 6). Such vessels extended along the subcutis and then passed obliquely into the dermis where they were soon integrated into the dermal network. Characteristic of these vessels were restrictions followed by balloon-like dilatations, probably due to valves.

Histologic and Transmission Electron Microscopic Findings

By histologic (Epon embedded 0.5-µm sections) and transmission electron microscopic investigation of skin preparations, we were able to demonstrate that the networks filled with BBS were composed of initial lymphatics (Fig 7). Details of the microscopic findings have already been published [8]. Inspected by light microscope they can be recognized by size, by thin endothelial cells, marked endothelial nuclei, lack of pericytes, and a continuous basal lamina. Using the transmission electron microscope, an abluminal subendothelial lying fibrous network, as well as typical overlapping areas of neighboring epithelial cells, are found to be characteristic of the IL.

DISCUSSION

The method described here is based on the concept that the lumina of the initial lymphatics do not open due to increased intravascular pressure but rather due to traction via the anchoring filaments [2,3].

Figure 3. Initial lymphatics. Demonstration of two drainage units. The dye drains from the subepidermal network into deeper lying dermal lymph vessels (arrows). The diffuse patches of dye represent the puncture points. Dorsal skin of a man.

Figure 4. Initial lymphatics. Two neighboring draining units merging into each other. Diffuse patches of dye: puncture points. Dorsal skin of a woman.

Figure 5. Initial lymphatics. Acetic acid preparation. Subepidermal network. Several closed tubes (arrows) and hemispherical saculation (thin arrow) are discernible. Abdominal skin of a woman. Enlarged × 240.
Excised skin contracts and the initial lymphatics collapse. Stretching a skin specimen makes the initial lymphatics open again. In such stretched preparations histologic semi-thin sections reveal a large number of opened lymph vessels [8].

In about every third specimen we succeeded in filling a DLVN with BBS. BBS remains in the vessels and does not flow out into the tissue, as for example patent blue does. Considering that the dye has to be injected immediately under the epidermis and that we have become more familiar with this technique, we are now successful in almost every second excised skin specimen in demonstrating networks or single vessels. We hope that we can improve our results further by using cannulas with a very fine caliber. It is remarkable that in some body areas (mid-back, scalp) DLVN can be stained very easily, whereas in other skin specimens (lower leg, forearm) networks are considerably more difficult to demonstrate.

Usually a defined network area with a diameter of 3–8 mm is filled. It can be assumed that this is a lymphovascular drainage unit as has also been described by Kubik [10] as a lymphatic area, or by Braverman and Yen as defined functional areas [9]. These areas are probably drained by deeper lying precollectors. A drainage unit represents a functional state. Careful, tangential spreading, for instance with forceps, sometimes makes the dye run into adjacent networks and fills them. The dye spreads abruptly. It can be assumed that these drainage units cease to exist during the pathologic condition of lymphatic edema. Findings of fluorescence microlymphography seem to confirm this. In patients with lymphatic edema the fluorescent dye spreads over wider networks than in healthy persons [5].

The major part of the IL is organized as a network [1,2,4]. There do seem to be vessels in the skin, however, that possess a closed end, as has been described by other authors [9]. Blind ending vessels are frequently visible in our pictures. Nevertheless, it cannot be determined whether the parts of networks involved are those that have only been incompletely filled with BBS, or whether they are closed vessels. In 50 DLVN that have hitherto been investigated, we have not been able to prove the existence of closed vessels. Serial sections necessary for clarifying such questions have not been carried out. We have, however, repeatedly seen fine finger-shaped diverticulations that could quite possibly prove to be so-called closed tubes [9].

The extension technique we have described permits the demonstration of dermal lymph vessel networks in excised human skin. By improving the injection technique we hope to raise the frequency of network demonstration. This method thus appears to enable systematic investigations into structural variations of the DLVN in relation to body area, sex, and age. Exploring the exact topographical anatomy of the IL could be fundamental in comprehending the frequency of metastases forming from malignant skin tumors in various body areas. In addition, this technique permits specific histologic and electron microscopic investigations of the IL.

REFERENCES