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AN IMMUNOLOGICAL CORRELATION BETWEEN THE ANCHORING FILAMENTS OF INITIAL LYMPH VESSELS AND THE NEIGHBORING ELASTIC FIBERS: A UNIFIED MORPHOFUNCTIONAL CONCEPT

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ABSTRACT

Little has been published on the histochemical and cytochemical properties of anchoring filaments of initial lymph vessels. Previous research suggests that the microfibrils of the anchoring filaments have ultrastructural, histochemical and cytochemical characteristics similar to those of the microfibrils associated with elastic fibers. With the aim of further investigating the histological identity of anchoring filaments, we performed an immunohistochemical study with human skin lymphatics, using antibody HB8, specific for elastic fiber microfibrils. The findings suggested strong molecular similarities between elastic fibers and the fibrils of anchoring filaments of the initial lymph vessels. A comparison of these fibrils showed both constitutional homogeneity and structural continuity from the abluminal surface of the initial lymph vessel to the perivascular elastic fibers and to the adjacent elastic network of connective tissue. In conjunction with previous findings, we propose a unified hypothesis that the elastic fiber system composed of anchoring filaments, perilymphatic sheath and adjacent connective tissue acts by alternating stretching and relaxation to propel lymph towards lymph collectors and draining lymph nodes.

One of the ultrastructural characteristics of initial lymph vessels are the "anchoring filaments" (1). These filaments have been studied by electron microscopy, especially in organs of laboratory animals (2-5). They appear as bundles of microfibrils arising from sites on the abluminal membrane of endothelial cells and projecting into the perivascular interstitial tissue. The microfibrils have a diameter of about 12 nm and a beaded appearance. In transverse sections, a central electron transparent core and a peripheral part with 3-5 electron dense subunits are evident. Little information is available on the histochemical and cytochemical characteristics of the microfibrils in human tissue, and reports that they resemble other categories of microfibrils are uncertain. Moreover, although the anchoring filaments are known to be joined to the abluminal or basal membrane of the endothelial cell (the manner in which they do so is unknown), the tissue structure to which they connect on the extravascular side has not been clearly identified.

Previous research indicates that initial lymph vessels of the skin are surrounded by elastic fibers (6-8). More recently, we showed by means of morphometric studies in various organs, especially in the skin, that human initial lymph vessels, unlike blood capillaries, are uniformly surrounded by specific elastic fibers (9). Earlier studies also suggest that the microfibrils of the anchoring filaments have ultrastructural, histochemical and cytochemical characteristics similar to those of

the elastic-fiber microfibrils (10,11). When these microfibrils are not associated with the amorphous component of elastin, they are referred to by the histochemical term, oxytalan microfibrils (12). A bundle of such microfibrils make up oxytalan fibers. A third type of fiber, intermediate between elastic and oxytalan fibers, is known as the elaunin fibers (13). According to some workers, they are young or developing elastic fibers (14). Accordingly, these microfibrillar elements, irrespective of their histological collocation and whether they are associated or not with elastin, may have similar, if not identical, structural and molecular organization.

Previously we demonstrated a structural connection between the wall of initial lymph vessels and the elastic network of the perivascular connective tissue, and termed this connection the "fibrillar elastic apparatus" (10). Starting from the lymphatic endothelial wall, it consists, according to classical terminology, of anchoring filaments (or in histochemical terms, oxytalan fibers or "pure" microfibrils), then elaunin fibers, and finally elastic fibers (microfibrils associated with elastin) which in turn are continuous with the vast elastic network of the connective tissue.

In order to investigate the histological identity of the microfibrils of the fibrillar elastic apparatus per se and its relation to the other interstitial microfibrils, we performed an immunohistochemical study using an antibody specific for the microfibrils of the elastic fibers. We compared the immunoreactive pattern of all microfibrils including those associated with elastic fibers, and those that resemble elastic fibers associated with initial lymph vessels.

MATERIAL AND METHODS

Skin specimens were excised from the deltoid region in seven patients of both sexes ranging in age from 12 to 34 years undergoing muscle biopsy but without cutaneous abnormality. Each specimen was divided into two parts. One was immediately frozen in isopentane at the temperature of liquid nitrogen until processing for histochemical studies. The other was reduced to tiny fragments, immersed for 3 h in Karnowsky fixative solution (15) and prepared by standard procedures for electron microscopy. Histological (semithin sections) and ultrastructural (thin sections) controls determined morphological preservation of the specimens.

Immunohistochemical Methods

Optical microscopy

Sections of 10µ thickness were cut in a cryostat, air dried and fixed with acetone at -20°C for 10 min. They were washed promptly in PBS and incubated for 60 min at 37°C under gentle shaking with HB8 antibody (Immunotech S.A., Marseille) diluted 1:50. This monoclonal antibody was synthesized using a technique of cellular hybridization after immunizing Balb/c mice with a suspension of lymphoid cells obtained from the human thoracic duct. One cell line secreted the IgG HB8 which labels elastic tissue. Cross-reactivity occurs only for a few lymphocyte subsets. The sections were then washed with PBS at pH 7.2 (3 x 15 min) and incubated with a second antibody, conjugated with fluorochrome (goat antimouse IgG labeled with FITC) and diluted 1:20, for 30 min at 37°C. The sections were washed with PBS as above, mounted in buffered glycerol and a total of 42 initial lymphatic vessels was analyzed with Leitz DMRB microscope fitted with a fluorescence accessory.

Other sections were stained with hematoxylin-eosin or with histochemical stain for elastic fibers. These sections acted as histological controls for the specimens prepared for immunohistochemistry. As a control of the specificity of the HB8 antibody reaction for microfibrils of elastic fibers, several sections were incubated as above but without the antibody HB8 in the first incubation.

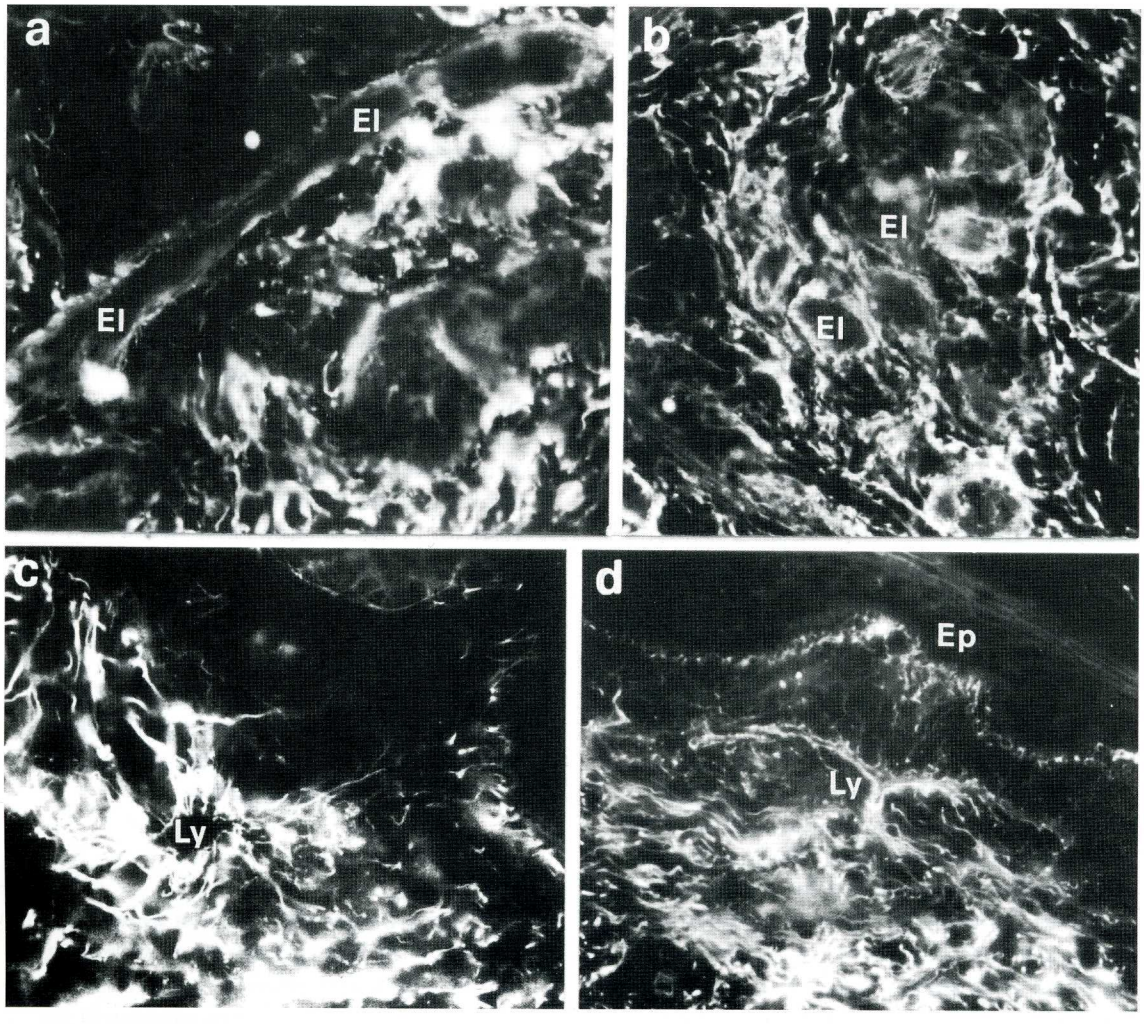

Fig. 1. Light immunomicroscopy. a) Longitudinal and b) transverse section of elastic fibers showing fluorescent profile of microfibrils surrounding non-fluorescent elastin center (El). c) Initial lymph vessels (Ly) showing fine fluorescent filaments extending from endothelium into surrounding elastic fibers, or d) directed towards epidermis (Ep). a-c x430, d x270.

Laser scanning confocal microscopy

The sections immunolabeled with FITC as described above were subjected to optical serial sectioning in the x-y plane. Specimens were examined with a Bio-Rad MC 600 laser scanning confocal imaging system equipped with krypton laser beam.

Electron microscopy

Sections of 15µ thickness were cut in the cryostat and fixed with acetone at -20°C for 10 min, washed with PBS at pH 7.2 (3 x 15 min) and treated for 45 min at 37°C with HB8 antibody diluted 1:40 in PBS, under gentle shaking. After further washing with

PBS (2 x 15 min) the sections were treated with the second antibody conjugated with gold particles (goat antimouse IgG bound with 10 nm diameter gold particles, Sigma Chemical Co.) diluted 1:2, for 45 min at 37°C. They were then washed in PBS as above, fixed with 2% glutaraldehyde for 20 min, treated with OsO_4 for 10 min and dried quickly (20 min), after which they were embedded in Epon-Araldite, with the section plane parallel to the support plane. Ultrathin sections obtained with an LKB NOVA ultramicrotome were stained with lead citrate and uranyl acetate and observed with a Philips CM 10 transmission electron microscope. To check the specificity of the immunocytochemical reaction, several cryostat sections were treated as above but without incubation with HB8.

RESULTS

Light Microscopy

Histologic sections showed the typical bright green immunofluorescent staining that, in relation to antibody specificity, labeled the microfibrillar component of the elastic fibers of the skin. The elastic fibers in the papillary region of the dermis were thin; further down, the fibers were thicker, more abundant and intricately interwoven. In these fibers, fine, strongly fluorescent peripheral filaments and an amorphous, non-immunoreactive elastin center, were observed. This latter feature was evident in longitudinal and transverse sections of elastic fibers *(Fig. 1a,b)*.

The initial lymph vessels were observed in the papillary and subpapillary dermis, together with blood capillaries. The former had a larger lumen than the latter, and a highly irregular endothelial profile. This feature was confirmed in control sections. From the lymph vessel profile, minute fluorescent filaments originated and extended into the interstitial tissue. Frequently, these fine filaments united with other larger perivascular filaments, which in turn joined with the complex fluorescent elastic network of the dermis *(Fig. 1c)*. The most superficial initial lymph vessels showed fine filaments directed towards the epidermis that united at the dermo-epidermal junction *(Fig. 1d)*.

Laser Scanning Confocal Microscopy

Laser scanning confocal microscopy observations confirmed those made by light microscopy. In addition, they revealed in a complete and detailed manner, the continuity of the fluorescent fibers of the dermis *(Fig. 2a)*. The structural connection between the wall of the most superficial initial lymphatic vessels and the dermo-epidermal junction was evident. Long branches emanated from the lymphatic wall and merged into fibers arranged parallel to the epidermis *(Fig. 2b)*. The latter fibers gave rise to many fine fibers that joined the dermo-epidermal junction perpendicularly *(Fig. 2c,d)*. There were no fluorescent fibrous elements near blood capillaries. In the control specimens the reaction was uniformly absent.

Electron Microscopy

The cell elements were not as well preserved as the extracellular fibrous elements. Elastic fibers were abundant and of large caliber in the reticular layer whereas in the papillary and subpapillary layers, they were scarce and smaller.

The elastic-fiber microfibrils were labeled with many single gold particles, unlike the amorphous elastin component. In longitudinal sections, it was possible to follow microfibrils labeled with gold particles for comparatively long distances *(Fig. 3a)*. In transverse sections, the elastic fibers showed an amorphous, unlabeled, central area of elastin, surrounded by microfibrils labeled with gold particles *(Fig. 3b)*. Collagen fibers of the dermis, in contrast, did not show immunoreactivity.

The wall of lymph vessels was observed to be very fine, with a tenuous, discontinuous

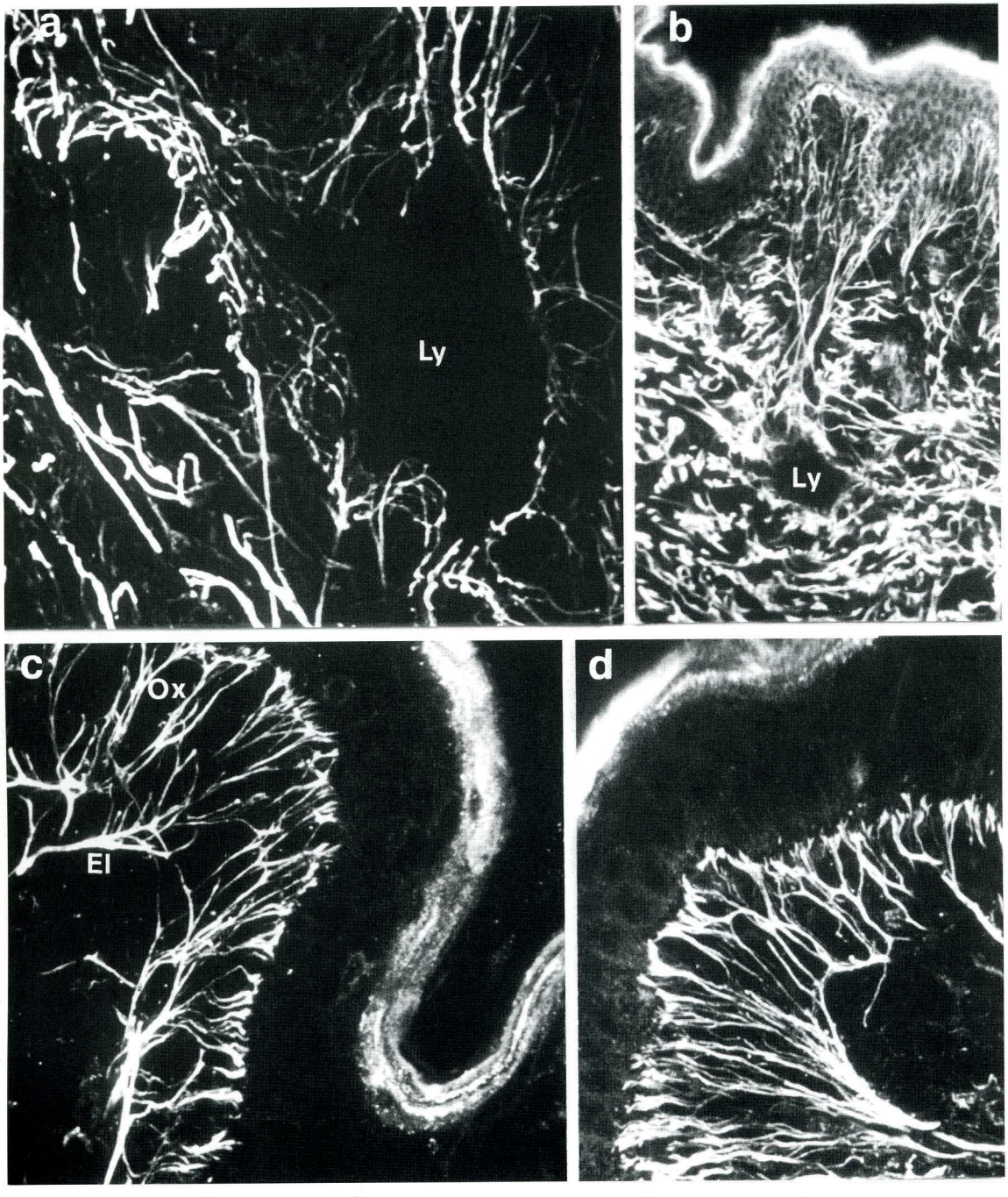

Fig. 2. Confocal microscopy. a) Initial lymph vessel with fluorescent filaments join up the surrounding elastic fibers of dermis x630. b) Initial lymph vessel whose fluorescent filaments are connected with the epidermis x100. c) Immunoreactive fibers of dermo-epidermal junction: oxytalan fibers (ox) and Elaunin fibers (El) x630. d) Oxytalan fibers joining to dermo-epidermal junction x700.

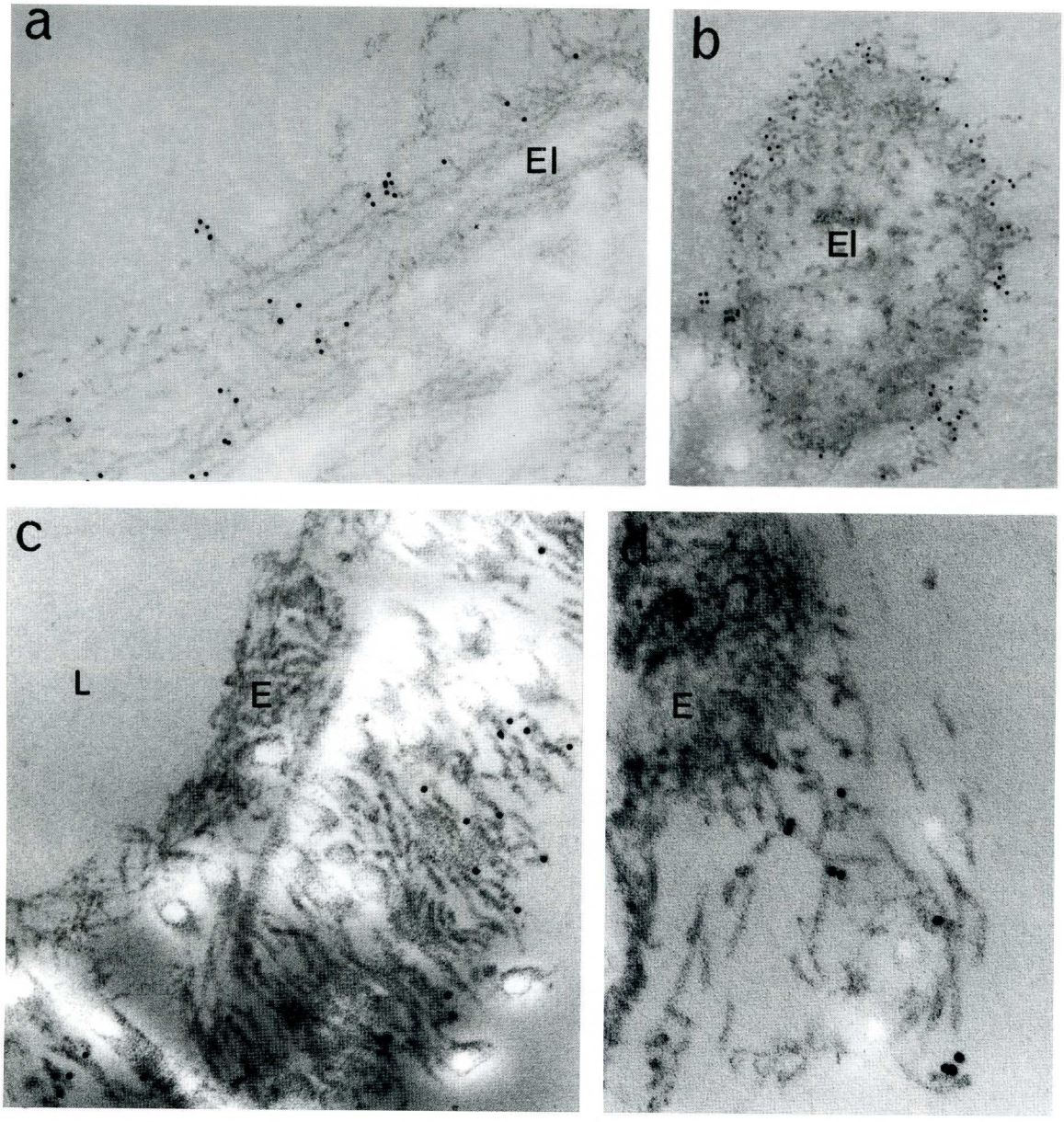

Fig. 3. Electron microscopy. a) Longitudinal and b) transverse fibers showing gold particles and unlabeled elastin (El). a x39000, b x27000. c,d: initial lymph vessel showing gold particles bound to anchoring filament microfibrils; L=lumen, E=endothelium. cx2000, d x64000.

basal membrane. The microfibrils of the anchoring filaments had the same ultrastructure as the microfibrils described above. Some gold particles were bound to single microfibrils *(Fig. 3c,d)*. Groups or clusters of gold particles were not observed on anchoring filament microfibrils.

Small elastic fibers were observed near the walls of initial lymph vessels. The amorphous elastin component was scanty and

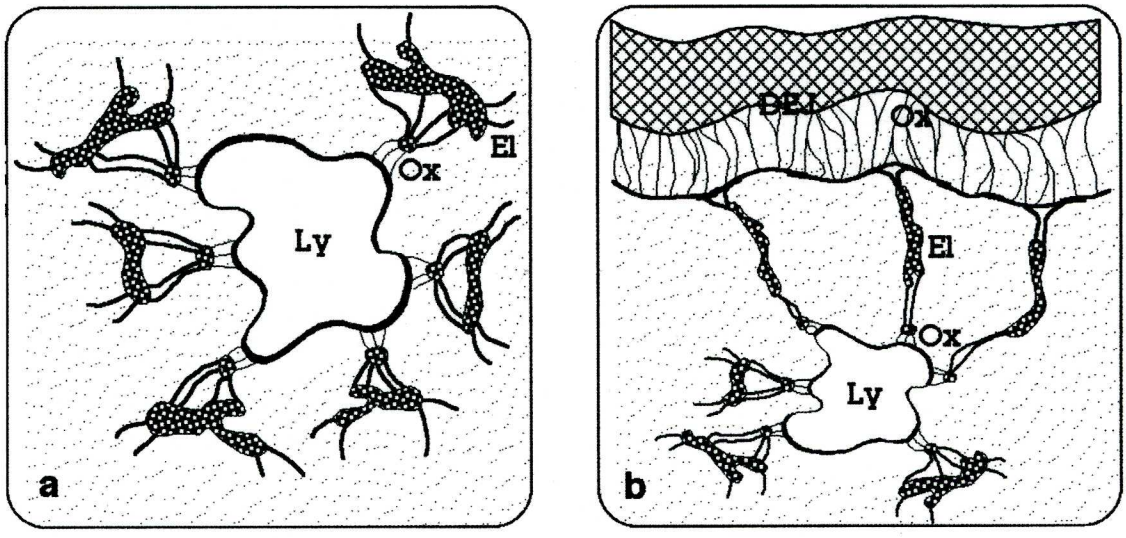

Fig. 4. Schematic diagram of initial lymphatic vessels (Ly) of dermis and their correlations with the elastic fiber system. a) The anchoring filaments (oxytalan fibers: Ox) of the initial lymph vessel joining elastic fibers (El). b) The anchoring filaments of the superficial lymph vessel also join with the dermal-epidermal junction (DEJ).

unlabeled. The microfibrillar component, however, was labeled with gold particles and often made contact with the endothelial surface of lymphatics.

Blood capillaries were not immunoreactive. The control preparations were negative to the reaction.

DISCUSSION

Observation of the samples and comparison with controls showed clearly that the antibody HB8 had specific immunoreactivity for elastic-fiber microfibrils of human skin. This finding conforms to other studies in which the same antibody was incubated with human skin preparations (16-18). Reactivity was also evident near the walls of initial lymph vessels. Immunoreactivity was expressed as strong fluorescence by the anchoring filaments of the initial lymphatic vessels. The results also confirm that the initial lymphatic vessels are consistently surrounded by a sheath of elastic fibers. The body of data indicates that the positive-reacting anchoring filaments are in continuity with the positive-reacting microfibrils of the perilymphatic elastic fibers and the vast elastic network of the dermis and therefore, more external with respect to the lymphatic vessel *(Fig. 4 a)*. The superficial lymphatic vessels were observed to be connected not only with the elastic fibers but also with the immunoreactive fibers of the dermo-epidermal junction. On the basis of previously published data, electron microscope observations and immunohistochemical studies, this connecting apparatus seems to be formed by the system of elastic fibers. Specifically, the oxytalan fibers are the immunoreactive fibers arranged perpendicular to the dermo-epidermal junction. The elaunin fibers form the thicker fibers parallel to the dermo-epidermal junction, which are connected with the elastic fibers of the subpapillary and reticular dermis. Some elastic fibers or their branches attenuate and become oxytalan fibers (or anchoring filaments) that connect with the wall of the initial lymph vessels. Hence the adhesion component is secured by oxytalan fibers, both on the epidermal and endothelial sides of the lymphatic *(Fig. 4b)*.

The immunoreactive electron microscopic images demonstrate and confirm with precision that labeling with gold particles is selective for the microfibril component of elastic fibers and for the microfibrils of the anchoring filaments of the initial lymph vessels. In general, we observed that all microfibrils with the same morphological features as those associated with elastic fibers were immunoreactive. Irrespective of their arrangement and function or whether they were associated or not with elastin, these microfibrils all showed similar immunoreactivity. This finding further supports that the microfibrils of the anchoring filaments have a similar, if not identical, molecular organization to the other antibody-labeled microfibrils in the dermis.

Although the molecular composition of these microfibrils is not completely known, it appears to be complex and modular. According to various authors (19-22), a family of molecules known as fibrillins, make up the molecular framework of the elastic-fiber microfibrils. Our results favor that the microfibrils of the anchoring filaments consist of fibrillins, which, together with other less rigorously defined molecules (23), modulate the molecular framework of all these microfibrils. The modulation seems to correlate with their connection with elastin and/or their specific collocation and tissue function. Thus, the epitope of the various types of microfibrils may react to different degrees with the antibody HB8, which is specific for the epitope of microfibrils associated with elastic fibers. The fluorescence of the anchoring filament microfibrils of the initial lymph vessels is of the same intensity as that of the elastic-fiber microfibrils. Although not specific, the two types of microfibrils seem to have a very similar molecular arrangement. Even the putative molecular differences are minimal and do not seem to significantly affect microfibril morphology. In fact, the control preparations for electron microscopy did not show appreciable differences in ultrastructure.

In summary, our observations suggest strong molecular similarities between elastic-fiber microfibrils and those of the anchoring filaments of the initial lymph vessels. In comparing these two types of microfibrils there is both constitutional homogeneity and structural continuity from the abluminal surface of the lymph vessel to the perivascular elastic fibers, and then to the vast elastic network of the connective tissue.

The present observations, and our previous findings (10,11), lead us to conclude that: the initial lymph vessels of the dermis are uniformly associated with a perivascular sheath of elastic fibers. In the framework of the lymphatic system, a new morphofunctional concept is introduced. This concept unifies the initial lymph vessel, the anchoring filament and the satellite elastic fibers into a single, integral formulation, which when considered in the context of the vast elastic network of the connective tissue, facilitates the necessary motility to perform the absorbing function of the lymphatic system. Lymphatics lack a central propelling organ like the heart of the blood circulatory system. We propose that this elastic fiber network in response to internal and external body movements on the initial lymph vessel wall contributes by means of alternating stretching and relaxation to the formation of lymph, and by virtue of the lymphatic valves, helps propel lymph towards the lymphatic collectors and lymph nodes. Perhaps derangements in elastic fiber function in conjunction with depressed immune responses plays a role in the cutaneous manifestations of such skin conditions as acne rosacea, leprosy, and mycosis fungoides.

REFERENCES

1. Leak, LV, JF Burke: Ultrastructural studies on the lymphatic anchoring filaments. J. Cell Biol. 36 (1968), 129-149.
2. Casley-Smith, JR: Are the initial lymphatics normally pulled open by anchoring filaments? Lymphology 20 (1980), 120-129.
3. Castenholz, A: Structural and functional

properties of initial lymphatics in the rat tongue: Scanning electron microscopic findings. Lymphology 20 (1987), 112-125.
4. Berens von Rautenfeld, D, D Luback, B Wensel-Hora, et al: New techniques of demonstrating lymph vessels in skin biopsy specimens and intact skin with the scanning electron microscope. Arch. Dermatol. Res. 279 (1987), 327-334.
5. Daroczy, J: *The Dermal Lymphatic Capillaries*, Chap. 4, Springer, Berlin Heidelberg New York, 1988.
6. Mortimer, PS, GW Cherry, RL Jones, et al: The importance of elastic fibres in skin lymphatics. Br. J. Dermatol. 108 (1983), 561-566.
7. Mortimer, PS, RL Jones, TJ Ryan: Human skin lymphatics: Regional variation and relationship to elastic. In: *Progress in Lymphology; Diagnostic, Therapeutic and Research Approaches to Lymphatic System Structure and Function*. 10 (1985), 59-64.
8. Ryan, TJ, PS Mortimer, RL Jones: Lymphatics of the skin. Int. J. Dermatol. 25 (1986), 411-419.
9. Gerli, R, L Ibba, C Fruschelli: Morphometric analysis of elastic fibres in human skin lymphatic capillaries. Lymphology 22 (1989), 167-172.
10. Gerli, R, L Ibba, C Fruschelli: A fibrillar elastic apparatus around human lymph capillaries. Anat. Embryol. 181 (1990), 281-286.
11. Gerli, R, L Ibba, C Fruschelli: Ultrastructural cytochemistry of anchoring filaments of human lymphatic capillaries and their relation to elastic fibers. Lymphology 24 (1991), 105-112.
12. Fulmer, HM, RD Lillie: The oxytalan fiber: A previously undescribed connective tissue fiber. J. Histochem.-Cytochem. 6 (1958), 425-430.
13. Gawlik, Z: Morphological and morpho-chemical properties of the elastic system in the motor organ of man. Folia Histochem. Cytochem. 3 (1965), 233-251.
14. Cotta-Pereira, G, F Guerra-Rodrigo, JF David-Ferreira: The elastic system fibers. In: *Advances in Experimental Medicine and Biology*. 79 (1977), 19-30.
15. Karnovsky, MG: A formaldehyde-glutaral-dehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27 (1965), 137a.
16. Brochier, J, S Saeland, G Cordier, et al: An antilymphocyte monoclonal antibody (HB8) which cross reacts with human dermal elastic fibers. Immunology Letters 7 (1984), 279.
17. Dawson, JF, J Brochier, D Schmitt, et al: Elastic fibres: Histological correlation with orcein and a new monoclonal antibody HB8. British J. Dermatol. 110 (1984), 539.
18. Schmitt, D, MC Chignol, J Brochier, et al: HB8: A monoclonal antibody recognizing elastic fibre microbrils. British J. Dermatol. 114 (1986), 661-665.
19. Sakay, LY, DR Keene, E Engvall: Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils. Cell Biol. 103 (1986), 2499-2509.
20. Sakay, LY, DR Keene, RW Glanwille, et al: Purification and partial characterization of fibrillin, a cysteine-rich structural component of connective tissue microfibrils. J. Biol. Chem. 266 (1991), 14763-14770.
21. Lee, B, M Godfrey, E Vitale, et al: Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. Nature (London) 352 (1991), 330-334.
22. Maslen, DL, GM Corson, BK Maddox, et al: Partial sequence of a candidate gene for the Marfan syndrome. Nature (London) 352 (1991), 334-337.
23. Rosenbloom, J, WR Abrams, R Mecham: Extracellular matrix 4: The elastic fiber. The FASEB Journal 7 (1993), 1208-1218.

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