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SPECIFIC ADHESION MOLECULES BIND ANCHORING FILAMENTS AND ENDOTHELIAL CELLS IN HUMAN SKIN INITIAL LYMPHATICS

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ABSTRACT

Anchoring filaments are a characteristic feature of initial lymphatic vessels. They connect the abluminal membrane of endothelial cells to the surrounding elastic fibers. The main molecular component of anchoring filaments is fibrillin. Initial lymphatic vessels of human skin were stained with monoclonal antibodies to fibrillin, integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$, vinculin, talin, ß-actin and focal adhesion kinase (FAK). A double-labeling immunofluorescence method was used to simultaneously stain fibrillin and $\alpha_3\beta_1$ integrin or FAK. Close contiguities between integrins and anchoring filaments were observed. These results suggest that the anchoring filaments connect the extracellular matrix and the endothelial cell cytoskeleton through the transmembrane integrin and FAK molecule. The results also demonstrate the presence of focal adhesions in the wall of initial lymphatic vessels. These connections possibly enable transmission of chemical and/or mechanical stimuli from the extracellular matrix to the endothelial cells. Here, they are transformed in cytoskeleton rearrangements and intracellular signaling events, some of which may contribute to the initial formation of lymph.

The initial or absorbing lymphatic vessels of human skin are characterized by an irregular lumen (20-120µm). They are often collapsed and contain intraluminal valves (1,2). Fine extensions of lymphatic endothelium project from the abluminal membrane into the extracellular matrix. The basement membrane is tenuous, discontinuous, or even absent (3). The initial lymphatics are also characterized by the presence of bundles of microfibrils that radiate from the abluminal surface of endothelial cells into the extracellular matrix. These bundles of microfibrils have been termed "anchoring filaments" (4).

Using immunohistochemistry, we have previously shown in the initial lymphatic vessels of the human skin that microfibrils of anchoring filaments contain fibrillin (5). Fibrillin is a filamentous molecule that was initially isolated and described in microfibrils associated with elastic fibers (6-8). As we previously demonstrated the microfibrils of anchoring filaments are similar to the microfibrils associated with elastic fibers (9). Hence a structural link between the lymphatic endothelium and elastic fibers may be hypothesized. This link, named the "fibrillar elastic apparatus" (10) is a morphofunctional "device," characteristic of the absorbing section of the lymphatic system. Little is known, however, as to the molecules which account for the adhesion of anchoring filaments to lymphatic endothelium. Even the exact functional significance of anchoring filaments is still unclear although it has been proposed, but not experimentally documented, that in the presence of edema the filaments

TABLE 1
Primary Antibodies

Antibody	IgG type	Antigen	Dilution
*MAB1919	Murine IgG1k	Human fibrillin	1:50
†V9131	Murine IgG	Human vinculin	1:20
*MAB1676	Murine IgG1	Human Talin	1:15
†A-5441	Murine IgG1	ß-Actin	1:15
*MAB1967	Murine IgG	Human $\alpha_2\beta_1$ integrin	1:20
*MAB1992	Murine IgG1	Human $\alpha_3\beta_1$ integrin	1:20
*MAB1976	Murine IgG1	Human $\alpha_v\beta_3$ Integrin	1:15
*MAB2156	Murine IgG1	FAK	1:10

*Chemicon Int. Inc. CA
†Sigma Chem. Co., MO

pull apart the endothelial junctions, thereby favoring lymph uptake with lowering of interstitial fluid pressure (11,12).

The aim of the present study was to determine how these filaments attach to the abluminal membrane of endothelial cells of human skin initial lymphatics and to elucidate further how this arrangement affects the function of initial lymphatics.

Because anchoring filaments connect endothelial cells to the matrix we hypothesized that integrin adhesive molecules are the means of joining them to the abluminal membrane. Integrins are a family of heterodimeric transmembrane proteins that act as receptors for extracellular matrix proteins (13-16). Moreover, in endothelial cells, these molecules are known to exert a strong connection between the extracellular matrix and the cytoskeleton (17). Without excluding other mechanisms we postulated that these connection points are also the sites of the cell-matrix junctions known as focal adhesions (18,19).

Demonstration of focal adhesions in the endothelium of initial lymphatics by immunolocalization of the focal adhesion kinase pp 125^{FAK} (FAK) is of structural and functional significance, because these adhesions are also sites of signal transduction. In fact activation of the integrin clusters of focal adhesions by the extracellular matrix leads to activation and autophosphorylation of FAK, which is an early step in a signal transduction cascade (20-25). Immunolocalization of the molecular components of focal adhesions in the lymphatic endothelium, as already demonstrated in blood endothelium (26-28), would also suggest that initial lymphatics are capable of reacting to the chemical binding and/or mechanical stimuli from the extracellular matrix. By means of such focal adhesions the anchoring filaments could activate a cascade of biochemical reactions directed toward the cytoskeleton or the nucleus. The endothelial cells may thereby adapt optimally to physiological conditions of the interstitium.

MATERIALS AND METHODS

Twenty-six skin biopsy specimens were

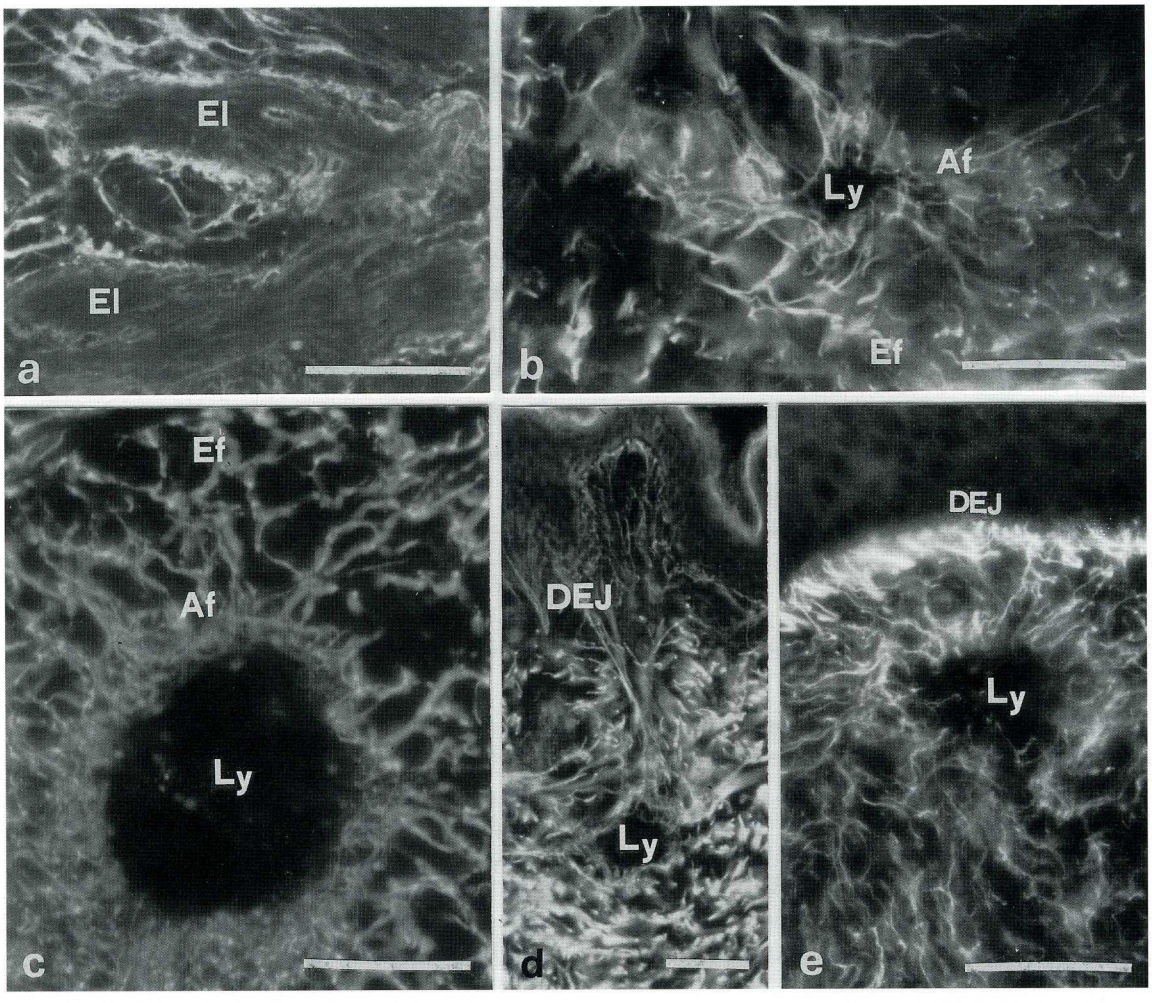

Fig. 1. Light microscopy. Fibrillin immunolocalization. (a): elastic fibers cut longitudinally. Fluorescent peripheral microfibrils and unreactive central elastin (El); (b) and (c): initial lymphatic vessels (Ly) with fluorescent anchoring filaments (Af) extending from the endothelium to the surrounding elastic fibers (Ef); (d) and (e): initial lymphatic vessels of the superficial papillary dermis. Anchoring filaments extend into the dermo-epidermal junction (DEJ); bar = 50 µm.

obtained from 15 male and 11 female patients (age 18-38 years). The specimens were obtained from the deltoid region of patients undergoing muscle biopsy or from the abdominal region of patients undergoing laparotomy. Informed consent was obtained from all subjects. Specimens showing skin abnormalities were discarded. The samples were divided into two groups: one group was snap frozen in liquid nitrogen and used for cryostat sectioning and immunolabeling, the second one was fixed in Karnovsky solution for 3h at 4°C and processed for transmission electron microscopy.

Immunohistochemistry

Cryostat sections (7µm) of skin were fixed in acetone at -20°C for 10 min and air dried. Non-specific binding sites were blocked by a 30 min incubation in phosphate buffered saline (PBS), pH 7.4, containing 3% bovine

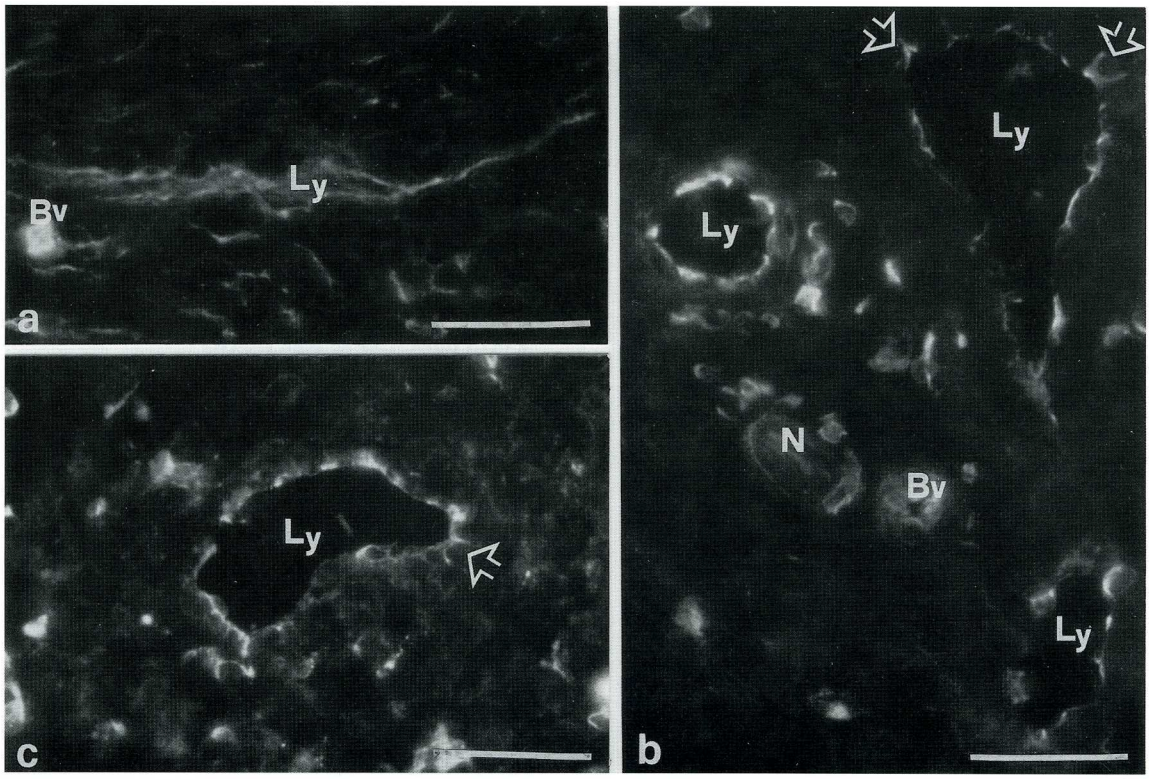

Fig. 2. Light microscopy. Immunostaining of initial lymphatic vessels (Ly) with (a) anti—vinculin, (b) anti-talin and (c) anti-ß actin antibodies. Sites and/or spike-like projections (arrows) of strongly fluorescent endothelium extend into the interstitium; Bv=blood vessel, N= nerve, bar=50 µm.

serum albumin (Sigma). Blocking solution was drained and replaced with primary antibodies (mouse monoclonal antibodies diluted in PBS pH 7.4, containing 0.1% BSA). For details of the primary antibodies see *Table 1.* After an overnight incubation at 4°C in a humid chamber, the sections were washed three times for 5 min in PBS. They were then incubated for 1 h at room temperature with a fluorescein isothiocyanate (FITC)/conjugated secondary antibody (goat anti-mouse IgG, Sigma) diluted 1:15. Anti FAK and anti-$\alpha_v ß_3$ integrin were applied into double immunofluorescence studies in combination with an anti-fibrillin monoclonal antibody. In both instances, the first antibody was visualized with fluorescein isothiocyanate (FITC)/conjugated goat antimouse IgG whereas the second labeling was visualized with tetramethylrhodamine isothiocyanate (TRITC)/conjugated goat anti-mouse IgG (Sigma). Negative controls were performed by omitting the primary antibody. Sections were washed in PBS, mounted in glycerol/PBS and observed with a Leitz DM-RB light microscope and under a bio-Rad MC600 laser scanning confocal microscope.

Ultrastructure

Skin fragments were fixed in Karnovsky solution, washed in 0.1M Na cacodylate buffer, pH 7.4, postfixed in 1% OsO_4 in 0.1M Na cacodylate buffer for 2 h at room temperature, dehydratated in ethanol and embedded in Epon 812. Ultrathin sections were cut with an LKB Nova ultramicrotome, stained in a LKB ultrastainer with lead

citrate and uranyl acetate and observed under a Philips CM 10 transmission electron microscope.

RESULTS

Immunohistochemistry

Fibrillin single-immunolocalization

Typical bright green immunofluorescence was observed where the anti-fibrillin antibody labeled the microfibrils associated with the elastic fibers of the dermis (*Fig. 1a*). Initial lymphatics were observed in the papillary and subpapillary dermis. They had a larger lumen than blood capillaries and an irregular profile. Fine fluorescent filaments extended from the external outline of lymphatics into the perivascular connective tissue (*Fig.1b,c*). As these filaments moved away from the lumen, they united with other larger filaments, which in turn merged with the vast complex network of the fluorescent elastic fibers of the dermis. Fine fluorescent filaments extended from the most superficial initial lymphatics towards the epidermis (*Fig. 1d*) and united with the dermo-epidermal junction (*Fig. 1e*).

Vinculin and talin

Vinculin immunostaining was observed along the endothelial profile of the initial lymphatics (*Fig.2a*). The fluorescence of talin was in some stretches punctate and in other stretches continuous (*Fig. 2b*). These fluorescent tracts sometimes looked like fine endothelial projections directed towards the outside of the vessel (*Fig. 2b*).

ß-actin

Fluorescence was sometimes condensed in those spots where fine extensions of endothelium project into the dermis (*Fig. 2c*).

Integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$

Reactivity to $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrin molecules (*Fig.3 a,b,c* respectively) was irregularly distributed throughout the initial lymphatic profile of the endothelium. It was stronger in the fine extensions of endothelium projecting into the dermis. Punctate fluorescence of endothelium was also observed in stretches. Endothelial cell shape was discernible; nuclei were unreactive.

Focal adhesion kinase (FAK)

Reactivity to the enzyme FAK was detected throughout the endothelial profile of initial lymphatic vessels. Stretches of stronger and weaker fluorescence alternate (*Fig.3d*). In some strongly fluorescent tracts of the vessel profile, the fluorescence increased towards the outside of the endothelium in a spike-like pattern. The nuclei of endothelial cells were unreactive.

Double immunolocalization of $\alpha_v\beta_3$-fibrillin and FAK-fibrillin

Fig. 4a-d and 4a'-d' were obtained by photographing histological sections on which double immunofluorescence had been performed, using two sets of filters, one for fluorescein isothiocyanate and the other for tetramethylrhodamine.

Bright red fluorescence of fibrillin (*Fig. 4a-d*), was observed in the fine filaments extending from the endothelium into the dermis. Bright green fluorescence of $\alpha_v\beta_3$ integrin (*Fig.4 a',b'*) and FAK (*Fig. 4c',d'*) was observed along the endothelial profile. There was often close contiguity between the two types of labeling (the fluorescence of the endothelium and that of filaments). The outwardly projecting fluorescent endothelial extensions were often continuous with fluorescent filaments.

Ultrastructure

Ultrastructural analysis of the wall of skin initial lymphatics confirmed the features

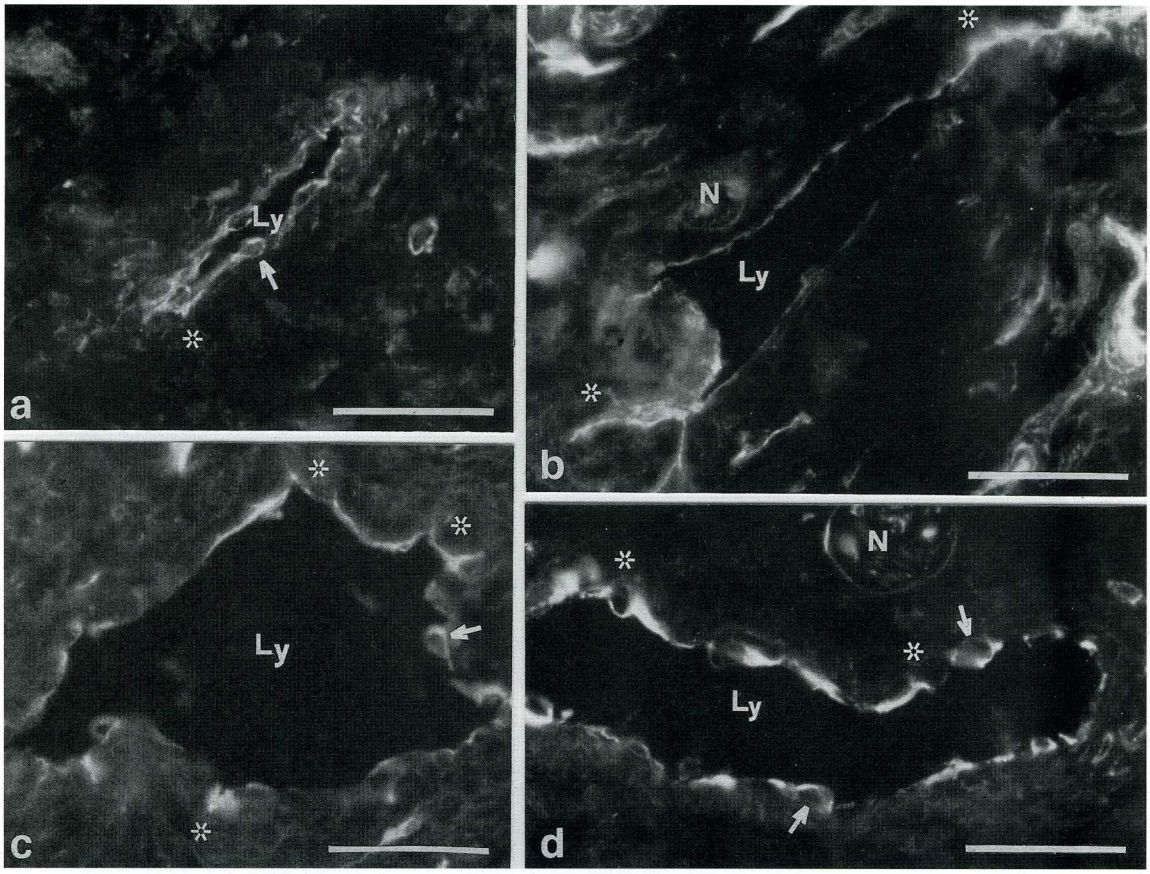

Fig. 3. Light microscopy. Initial lymphatic vessels (Ly) staining with anti integrin antibodies: (a) $\alpha_2\beta_1$, (b) $\alpha_3\beta_1$, (c) $\alpha_v\beta_3$ and FAK (d) antibody. Spike-like projections (*) of strongly fluorescent endothelium extending into the interstitium. Nuclei are unstained (arrows); N=nerve; bar = 50 µm.

previously described in human and laboratory animal organs (*Fig. 5*). In those areas where anchoring filaments microfibrils (10-12 nm in diameter) attached to the abluminal membrane of the endothelium, the clear demarcation between extracellular microfibrils and the cytoplasm disappeared (*Fig. 6a,c*). Electron dense areas were observed on the cytoplasmic side of the endothelial membrane (*Fig. 6b,d*). Cytoskeletal filaments, 5 nm in diameter, were observed in the putative attachment sites between the anchoring filaments and the lymphatic endothelium (*Fig. 6d*).

DISCUSSION AND CONCLUSION

Based on antibody specificity, these results demonstrated the presence of integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$, the enzyme FAK, and the binding molecules vinculin and talin in the wall of initial lymphatics of human skin. ß-actin filaments were found in the cytoplasm. As we have previously shown in an immunohistochemical study (5) using another antibody (HB8-Immunotech. S.A.) the anchoring filaments of initial lymphatics immunoreact to antifibrillin antibody. The evident close contiguities, in the lymphatic vascular wall, between the labeling of $\alpha_v\beta_3$ integrin or FAK and fibrillin of anchoring filaments point out the structural and putative functional correlation of these molecules.

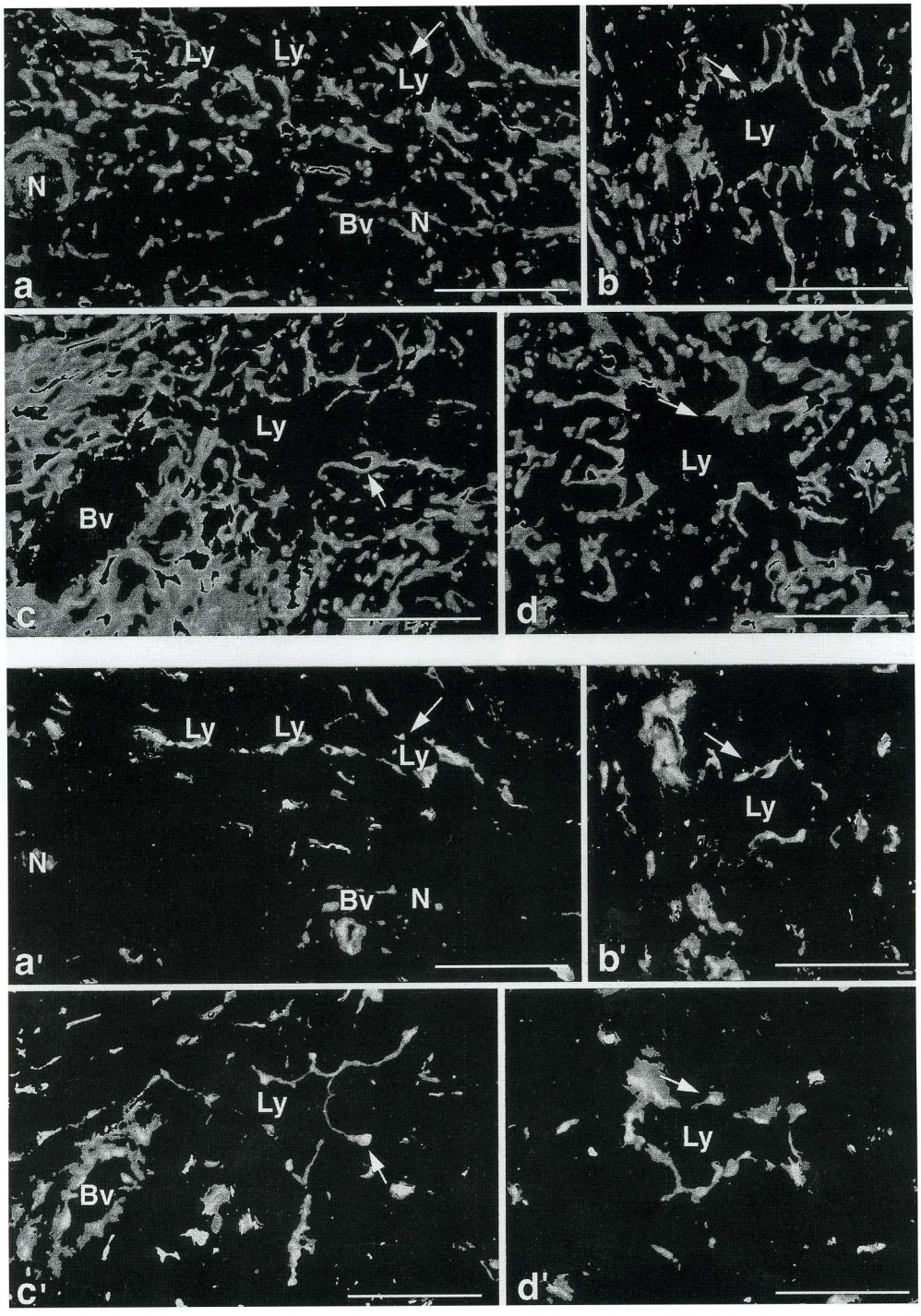

Fig. 4. Confocal microscopy. Double immunostaining: (a-d) TRITC staining of fibrillin in anchoring filaments; FITC staining of (a' and b') $\alpha_v\beta_3$ integrin, (c' and d') FAK in the endothelium of lymphatic vessels. Ly=lymphatic vessel; Bv=blood vessel; N=nerve; bar = 50 µm. Arrows show the close contiguity between the two types of fluorescence.

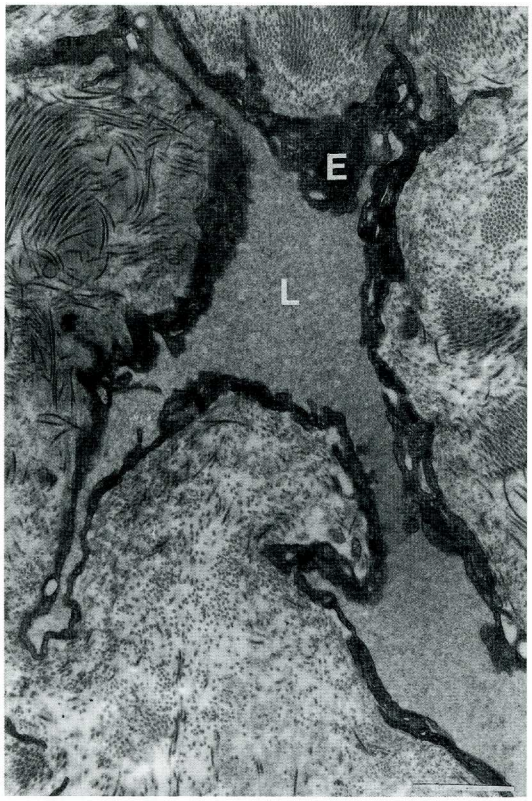

Fig. 5. Transmission electron microscopy. An initial lymphatic vessel with irregular lumen (L); bar=1 µm.

These results show that the endothelium of initial lymphatics possess molecules whose biological characteristics are capable of creating strong structural links between the anchoring filaments outside the lymphatics and cytoskeleton ß-actin filaments. These links are formed through the abluminal membrane, in agreement with those of others (29).

The extracellular side of integrin $\alpha_v ß_3$ has a receptor site for the RGD sequence of fibrillin (30,31), which, as we previously described (10), is the main structural molecule of the microfibrils of anchoring filaments. Consequently, we propose that the connection between these microfibrils and the abluminal membrane of the initial lymphatics is direct, without intermediate molecules such as fibronectin or vitronectin. This viewpoint, however, requires further investigation.

The immunohistochemical data obtained using the antibody antiFAK suggest that the abluminal membrane of the lymphatic endothelium has focal adhesions, possibly containing the integrin molecules $\alpha_2 ß_1$, $\alpha_3 ß_1$ and $\alpha_v ß_3$ or other types of molecules to be yet investigated. The histological sections showed diffuse fluorescence of the lymphatic endothelium for all the integrins studied, probably due to the thickness of the cryostat sections (7µm). In some segments or specific sites of the endothelium, the fluorescence is stronger suggesting that the molecules examined tend to cluster in spots. Of interest is the strong immunoreactivity of the fine endothelial extensions into the extracellular matrix of the dermis. Transmission electron microscopy showed that these projections are often connected to anchoring filaments, which in turn merge into the vast elastic network of the dermis. This stretching is particularly evident when the initial lymphatics are partially or completely collapsed. Hence, it emerges that the density of integrin molecules in focal adhesions, like that of the other molecules, is particularly high in the very place where these molecules would be expected to organize the union between the extracellular anchoring filaments and the cytoskeleton of lymphatic endothelium.

Focal adhesions have been described under transmission electron microscopy (32) in lymphatic vessels of the human heart. Our immunohistochemical demonstration of FAK opens new perspectives on the activity of initial lymphatics of human skin and hence on the function of the absorbing section of the lymphatic system. In fact it has been shown (33) that focal adhesions are not simple mechanical devices, but are also transmembrane bridges that transduce signals by means of groups of integrins and enzymes. They convert chemical binding stimuli and/or mechanical stimuli from outside the cell into metabolic stimuli for an optimum cell response. Through a cascade of biochemical signals to the cytoskeleton and/or the nucleus, the lymphatic endothelium may react to

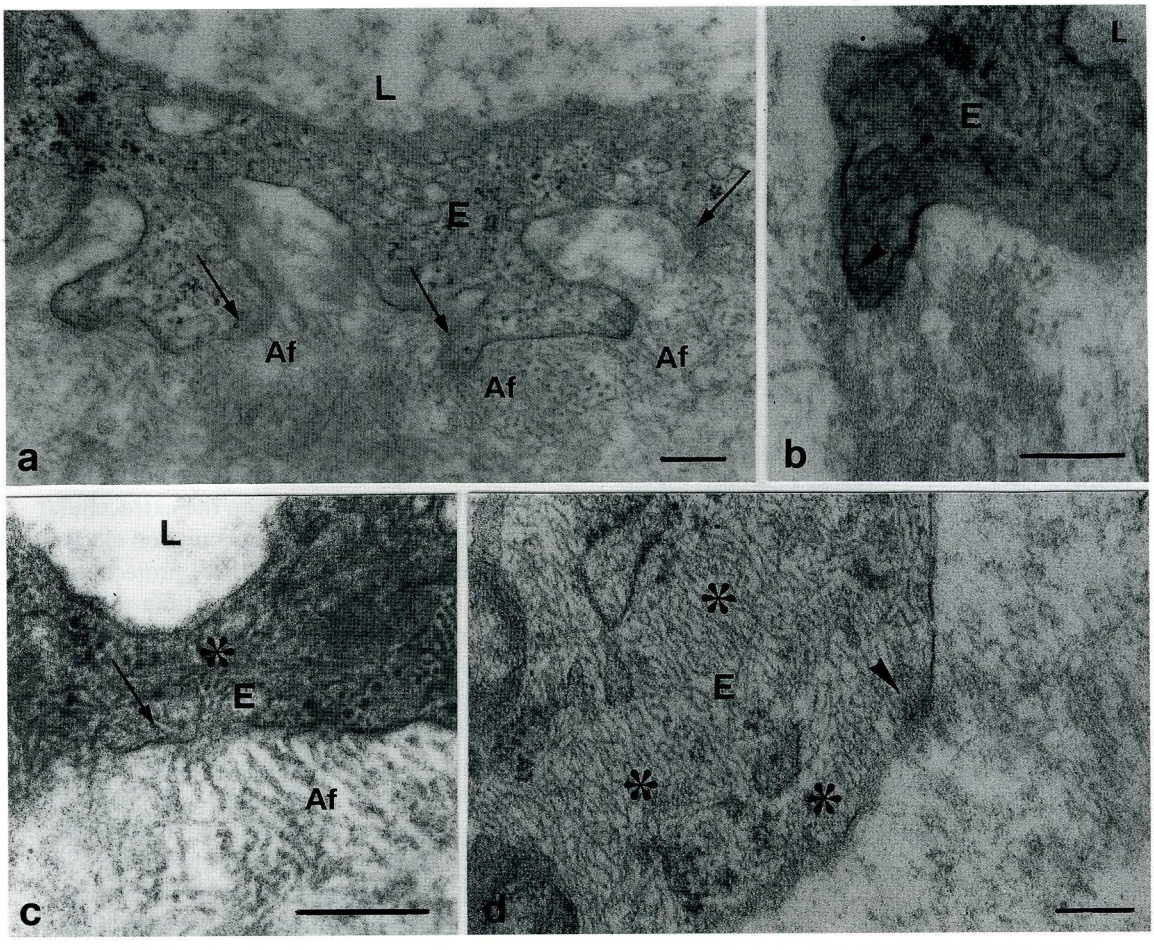

Fig. 6. Transmission electron microscopy. Initial lymphatic vessel endothelium (E); (a) and (c) modified membrane ultrastructure where anchoring filaments (Af) are attached to abluminal membrane(arrows);(b) and (d) electron-dense areas (arrowheads) on abluminal membrane; (c) and (d) microfilaments of endothelial cell cytoskeleton (*); L=lumen; bar=0.1 µm.

various physiological conditions of the extracellular matrix with changes in shape, polarity, metabolism, etc. These alterations could be relevant for orientating lymphatic endothelium cells and thereby for modulating its capacity for fluid filtration and reabsorption.

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