

MECHANOTRANSDUCTION IN LYMPHATIC ENDOTHELIAL CELLS

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ABSTRACT

Initial lymphatic vessel endothelial cells are connected to the surrounding elastic fibers by fibrillin anchoring filaments that have been hypothesized to favor interstitial fluid drainage in edema pulling apart interendothelial junctions. We hypothesized a biochemical mechanism involving mechanotransduction. This study was designed to verify whether a relation exists between focal adhesion molecules and anchoring filaments and whether they may transduce extracellular forces to the nucleus. We first performed an immunohistochemical study on human skin cryostat sections to evaluate whether fibrillin and αv - $\beta 3$ integrins, FAK and fibrillin, or αv - $\beta 3$ integrins and FAK co-localize in lymphatic endothelium. We observed that integrins and FAK co-localize and that fibrillin filament attachment sites to endothelial cells merge with these molecules. These data may suggest that fibrillin anchoring filaments are connected to endothelial cells through focal adhesions. Mechanotransduction was investigated applying static stretching to bovine thoracic duct segments and lymphatic endothelial cells cultured on elastic membranes and immunohistochemically evaluating the expression of ERK1/2. Under stretching conditions, ERK1/2 labels the nucleus. Western blotting on cultured cells confirmed the presence of ERK1/2 in stretched cells. Based on our data we speculate that

anchoring filaments may trigger a focal adhesion-mediated cascade of mechanotransduction toward the nucleus for genetic modulation and thus contribute to endothelial adaptation to interstitial requirements.

Keywords: lymphatic endothelium, anchoring filaments, focal adhesions, mechanotransduction, ERK1/2

The lymphatic vascular system comprises initial or absorbing lymphatic vessels (also referred to as lymphatic capillaries) and larger drainage vessels that convey lymph to lymph nodes and eventually to veins. Initial lymphatic vessels are larger than blood capillaries, with an irregular profile and an extremely thin endothelium. They lack pericytes (1). Their distinctive ultrastructural features are a discontinuous basement membrane and the presence of anchoring filaments. The latter are bundles of microfibrils that detach from some sites of the abluminal plasma membrane of endothelial cells and radiate into the surrounding extracellular matrix (2,3).

It has been hypothesized that anchoring filaments in edema and also in physiologic conditions, may pull apart lymphatic endothelium dilating the vessel lumen and opening intercellular junctions. This would favor fluid drainage and relieve interstitial swelling and pressure (4).

It has been previously demonstrated by transmission electron microscopy and

immunohistochemistry that anchoring filaments have a diameter of 10-12nm and are made of fibrillin (5,6). Fibrillin is the main molecule of 10-12nm microfibrils of the extracellular matrix (7,8) which may be found alone or in association with amorphous elastin in elastic fibers (9). Anchoring filaments merge with perivascular elastic fibers and, more in general, with the vast elastic network of connective tissue (10).

Anchoring filaments of initial lymphatic vessels can be assumed to be identical to microfibrils of elastic fibers. In many human organs, initial lymphatic vessels have been shown to possess a typical "Fibrillo-elastic apparatus" that connects endothelial cells with the extracellular matrix (5,6). Based on these data, it appears evident that the endothelium of initial lymphatic vessels is always connected to the elastic network of connective tissue. Initial lymphatic vessels are hence continuously exposed to external alternating physiologic forces like muscle contraction, respiratory movements, arterial pulse etc.

What is still missing is information on the molecular adhesion device of anchoring filaments with the endothelium of initial lymphatic vessels. We previously reported that some molecules, like integrins that connect the extracellular matrix and the cytoskeleton, are expressed by the abluminal plasma membrane of skin initial lymphatic vessels (11).

Integrins are a large family of proteins made of two transmembrane subunits known as α and β chain. On the external domain they have an attachment site for the RGD sequence (arginine-glycine-aspartic acid) which is present in many extracellular matrix proteins including fibrillin (12). The smaller cytoplasmic domain of integrins binds, through intermediate proteins, cytoskeletal actin filaments and other molecules that activate a signal cascade directed towards the nucleus (13,14). Lymphatic endothelial cells express α v- β 3 integrins (11), which are considered specific for endothelial cells (15)

and contain β -actin filaments and other molecules of focal adhesions (11,16).

Focal adhesions are the molecular devices responsible for the transduction of mechanical signals from the extracellular matrix into the cytoplasm (17,18). They are formed by clustering of integrins at various sites of the cell membrane. Since integrins have no enzymatic activity, they associate with enzymatic molecules. Many of the signaling functions of focal adhesions rely on a cytoplasmic tyrosine kinase known as "focal adhesion kinase" (FAK) (19). When integrins cluster at the cell-matrix junction sites, FAK molecules are transported and concentrated in focal adhesions by anchoring cytoplasmic proteins like talin, that binds the β subunit of integrins or paxillin, that binds the α subunit (20).

The molecules of FAK, grouped in focal adhesions, cross-phosphorylate with each other on a specific tyrosine residue. This creates a phosphotyrosinic anchoring site for the members of other tyrosine-kinase families. These kinases, phosphorylating FAK in further tyrosines, create anchoring sites for a series of adapting intracellular proteins. The signal is thus released in the cell (21-23). Tyrosine phosphorylation is a short term event. To exert a significant effect on the cell, it must be transformed in a longer lasting one. A series of phosphorylations in serine/threonine (TEY motif), that have a much longer life, are so activated and support the cytoplasmic biochemical cascade signal to the cytoskeleton to control cell shape, and to the nucleus to modulate gene expression (24). Several serine/threonine kinases participate in this phosphorylation cascade. The best known in mammalian cells is Mitogen Activated Protein Kinase (MAPK) formed by five distinct protein cascades. Following an external stimulus its isoforms ERK1-44kDa and ERK2-42kDa are phosphorylated in threonine/tyrosine by a MAPKK (MEK1/2), that in turn is phosphorylated by MAPKKK (Raf) (25,26). Upon phosphorylation, approximately 75% of ERK1/2 leave the

cytoplasm and enter the nucleus (23), where, together with the intermediate molecules Jun and Fos, act on the promoter of genes for transcriptional modulation (27). In synthesis, MAP kinases convert the short duration membrane signals of tyrosine-phosphorylation in long lasting serine-threonine phosphorylations inside the cell toward the nucleus. Recent data indicate that ERKs dimerize *in vivo* and activity peaks are associated to biphosphodimers and not with monophosphodimers or phosphorylated monomers which maintain the basal activity of ERKs (28). MAP-kinases are deactivated by cellular phosphatases.

FAK phosphorylation is a critical event also in stretching of cells (29,30). It induces the activation of ERK1/2 and p38 suggesting that mechanotransduction occurs at focal adhesions. Traditionally mechanotransduction differs from other types of signal transduction in that it is thought to occur independently of the receptor-ligand interaction at cell surface (31). It rather implies the conversion of an extracellular mechanical stimulus in a chemical intracellular event via membrane devices, including focal adhesions.

It has been reported that shear stress (the tangential component of hemodynamic forces of blood flow) and cyclic tension induced by arterial pulse, respectively induce an activation of ERK1/2, JUNK and p38 (32,33). Lymph progresses slowly with low pressure, and no rhythmic modifications comparable to those of arteries occur in lymphatic vessels. Other types of mechanical stimuli, like expansion and outward stretching, which both act through anchoring filaments, are probably more likely to apply to lymphatic endothelium under physiologic and pathologic conditions.

This study was designed to evaluate whether focal adhesion key molecules, particularly αv - $\beta 3$ integrins and FAK, co-localize in initial lymphatic vessel endothelium and whether there is contiguity between focal adhesions and fibrillin

microfibrils of anchoring filaments. An *in vitro* model of mechanotransduction in lymphatic endothelial cells is also proposed to evaluate whether these cells respond to the mechanical stimulus of stretching expressing key molecules of signal transduction like MAPK, in particular its phosphorylated ERK1/2 isoforms.

MATERIALS AND METHODS

Immunohistochemistry

Double labeling and focal adhesion molecules in skin sections

Samples of human foreskin of 8 children were obtained, with the informed consent of the parents, from the Pediatric Surgery of the University of Siena, snap frozen in isopentane precooled in liquid nitrogen and kept at -80°C until use. Cryostat sections of 5-7 μm thickness were fixed 10 min in cold acetone at -20°C and incubated 30 min in PBS containing 3% BSA to block unspecific binding sites. The following double labelings were performed: FAK and fibrillin, αv - $\beta 3$ integrins and FAK, and αv - $\beta 3$ integrins and fibrillin. All primary antibodies were obtained from Chemicon Int., Inc., (Temecula, CA, USA), secondary antibodies were from Sigma Aldrich (St. Louis, USA). First labeling was performed overnight at 4°C in a humidified chamber with primary polyclonal antibodies diluted in PBS with 0.5% BSA (buffer) as follows: rabbit polyclonal antibody to FAK 1:10, rabbit polyclonal antibody to αv - $\beta 3$ integrin 1:15. Sections were washed three times for 5 min with buffer, incubated for 2h with a goat anti-rabbit FITC conjugated secondary antibody diluted 1:100, washed with buffer and incubated 40 min with 5% goat serum and 3% BSA in PBS to block unspecific binding sites. For the second labeling, sections were incubated 2h with primary monoclonal antibodies (mouse monoclonal antibody to FAK 1:20 in buffer, mouse monoclonal antibody to fibrillin 1:100

in buffer), washed and incubated 2h with a goat-anti mouse TRITC conjugated secondary antibody diluted 1:100 in buffer. Sections were mounted with DABCO (Sigma) and observed under a Nikon Eclipse E600 microscope. Negative controls were obtained by omission of primary antibodies.

Stretching of bovine thoracic duct

Five bovine thoracic ducts visualized by injection of 0.1% Evans Blue in the caudal end, were isolated from fat and cannulated at both extremities (34). After flushing with PBS to remove the dye, the proximal end of the duct was ligated and the duct filled with Dulbecco's Modified Eagle's Medium (DMEM) at the pressure of 50cm of water. A wall dilatation of approximately 30% was obtained under these conditions. After 10 min at 37°C, the duct was cut into segments that were snap frozen in liquid nitrogen pre-cooled isopentane, mounted with OCT and kept at -80°C. Controls were segments of the same vessels taken before distension.

Immunohistochemical labeling of ERK1/2 in thoracic duct sections

Cryostat sections of 5-7µm were fixed 10 min with 4% paraformaldehyde, washed with PBS containing 0.1% Tween 20 (buffer) and treated 10 min with 3% H₂O₂ to quench endogenous peroxidase activity. After washing and blocking unspecific binding sites for 1h with blocking solution (PBS containing 5% Horse Serum and 0.1% Tween 20), sections were incubated overnight with a rabbit monoclonal antibody to ERK1/2 (Phospho p44/42 MAPK, Thr202/Tyr204, Cell Signaling Technology, Danvers, MA, USA), diluted 1:100 in blocking solution. Sections were then washed and incubated 30min at r.t. with a biotinylated goat anti-rabbit monoclonal antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:50 in blocking solution, washed, incubated for 1h with VECTASTAIN ABC reagent (Vector) followed by diamino-

benzidine tetrahydrochloride (DAB, Sigma), washed, dehydrated in ethanol and mounted with Eukitt (Bio Optica, Milano). Negative controls were obtained by using blocking solution instead of the primary antibody.

In Vitro Models

Stretching of lymphatic endothelial cell cultures

Lymphatic endothelial cells were isolated from bovine thoracic duct by enzymatic treatment with trypsin-EDTA solution (Cambrex, NJ, USA trypsin 0.5mg/ml, EDTA 0.2mg/ml in PBS, 15 min at 37°C). The isolated LEC, resuspended in complete tissue culture medium [DMEM with 20% Fetal Bovine Serum (FBS), 100mg/ml Endothelial Cell Growth Supplement (ECGS), and 50mg/ml gentamycin] were seeded on ethanol sterilized elastic vinyl membranes mounted on a plastic support and coated with 0.1% gelatin at the density of 2x10⁶ cells/membrane of 3.8mm diameter. Cells cultured on extensible membranes were randomly assigned to the following experimental conditions: A) stretching of cells kept in complete medium (20% FBS and ECGS), B) stretching of starved cells (0.1% FBS for 16h), C) controls (unstretched cells grown in the presence of 20% FBS and ECGS), D) starvation without stretching (0.1% FBS). We used two different kinds of media with 20% or 0.1% serum because serum and ECGS may trigger the biochemical activation of ERK1/2. Cultures to be starved, beginning two days after seeding, received daily changes of fresh medium containing decreasing concentrations of FBS: 10, 5, 2% and eventually, 16 h before stretching, 0.1%. On day 7 after seeding, the membranes were stretched for 10 min at 37°C by the aid of a dome-shaped plastic support to obtain a 30% dilatation. Controls were left unstretched.

Immunocytochemical evaluation of ERK1/2 in cells cultured on elastic membranes

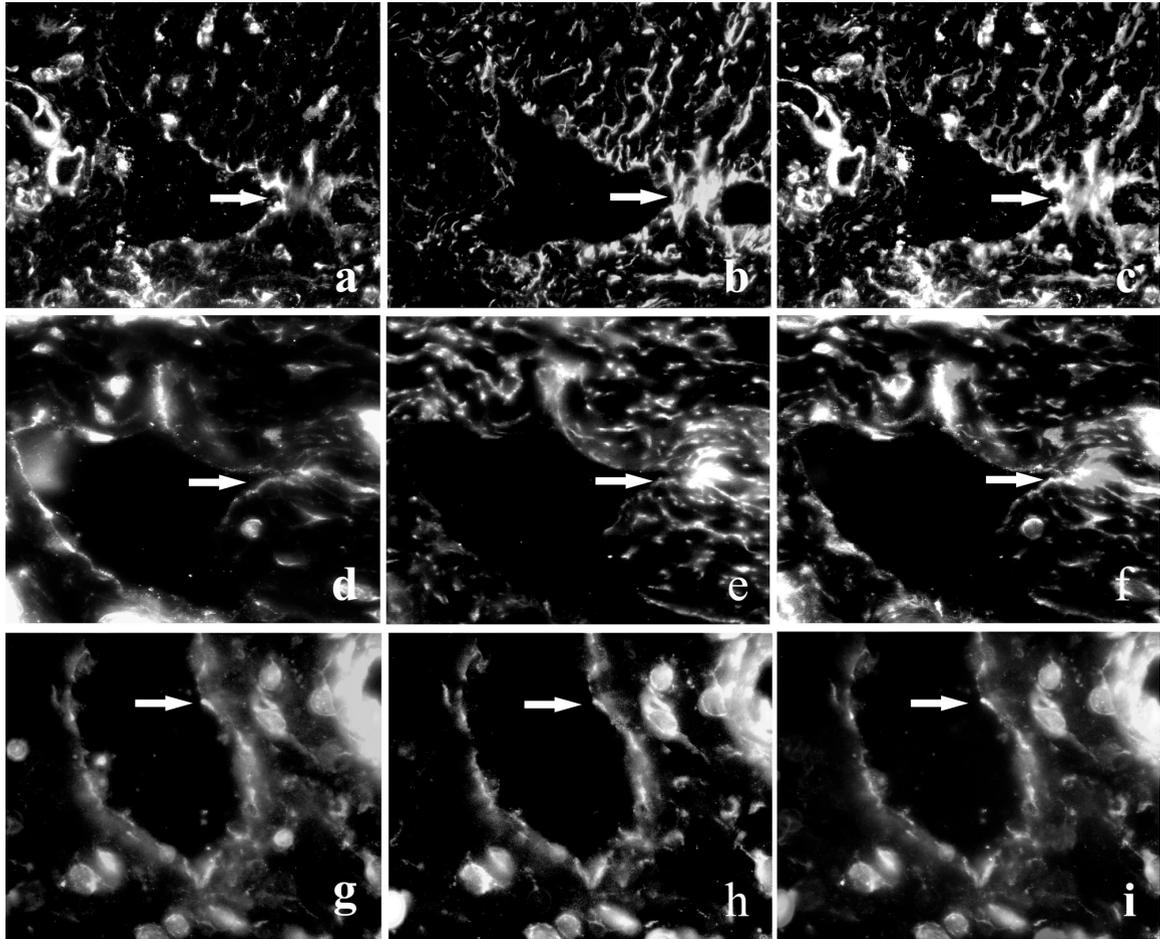


Fig. 1. Double labeling of human skin lymphatic vessels with αv - $\beta 3$ integrins (a) and fibrillin (b); FAK (d) and fibrillin (e); and αv - $\beta 3$ integrins (g) and FAK (h). Right column: respective merged images. On the whole, crossed double labelings suggest that the three molecules co-localize in lymphatic endothelium. In particular, fibrillin anchoring filaments co-localize with αv - $\beta 3$ integrins and FAK at focal adhesions. Original magnification $\times 40$.

Medium was removed and cells washed and fixed with 4% paraformaldehyde in PBS for 10 min, washed 5 min with Tris Buffered Saline (TBS) pH 7.4 and permeabilized with 0.2% Triton in PBS for 5 min. Unspecific binding sites were blocked for 1h with 5.5% goat serum in TBS containing 0.1% Triton. Cells were then incubated overnight with the same antibody as above to ERK1/2 diluted 1:500 in TBS containing 3% bovine serum albumin (BSA), washed, and incubated with the secondary antibody above. The reaction detected by Vectastain ABC reagent following the manufacturer's instructions for cells.

Western blotting

Western blotting was performed on cells grown on stretched and unstretched membranes. Culture medium was aspirated and 100 μ l lysis buffer were added to each membrane. Cells were gently removed by a cell scraper, broken by repeated freezing-thawing in liquid nitrogen and centrifuged 20 min at 10,000 rpm. The supernatant was used for protein determination by the method of Lowry and for western blotting. Western blotting was performed using SDS-PAGE electrophoresis on 12% gels. Gels were then

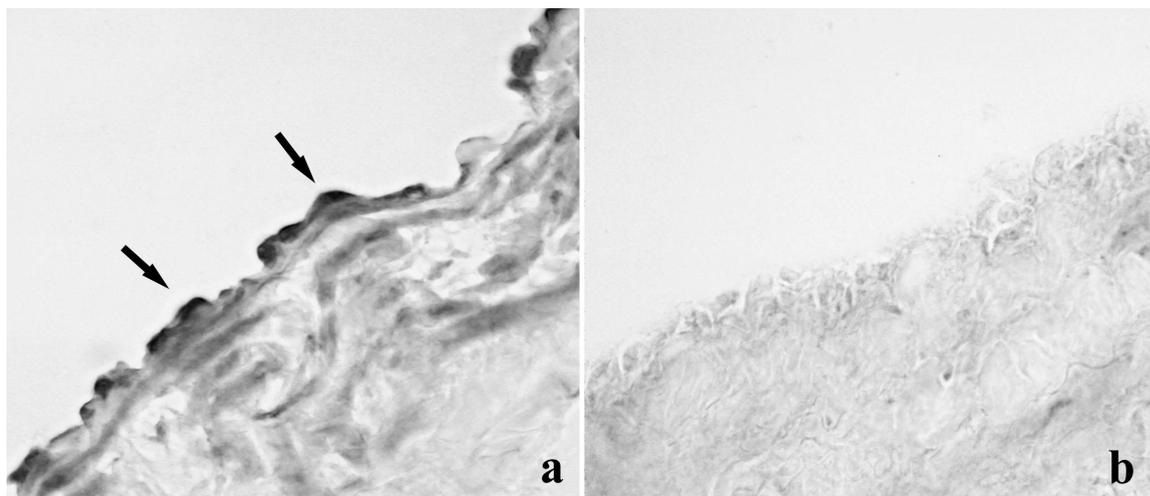


Fig. 2. Immunohistochemical localization of ERK1/2 in cryostat sections of bovine thoracic duct, a) stretched segments: the reaction is mainly positive in the nuclei of endothelial cells (arrows); b) unstretched segments: no reaction. Original magnification $\times 40$.

transferred to nitrocellulose membranes and incubated overnight at 4°C with the antibody to ERK1/2 diluted 1:2000 in PBS containing 0.5% BSA. After washing, nitrocellulose membranes were incubated for 1h with a horseradish peroxidase conjugated secondary antibody (Amersham Biosciences Corp, Piscataway, New Jersey, USA) diluted 1:3000 in PBS containing 0.5% BSA. The specific bands were detected by ECL (Amersham).

RESULTS

Immunohistochemistry

Double labeling of human skin sections

α v- β 3 integrins and fibrillin

Skin initial lymphatic vessels were mainly present in the papillary and subpapillary region of the dermis where they were easily recognized from blood capillaries for their larger dimensions and irregular, tortuous profile. α v- β 3 integrins were recognized as short FITC-labeled dashes (Fig. 1a). The fluorescence was brighter in the thin endothelial protrusions that extended into

the perivascular connective tissue. The red fluorescence of TRITC was visible in the extracellular matrix, externally to the endothelial profile, where the antibody to fibrillin labeled anchoring filament microfibrils (Fig. 1b). Long and thin fibrillin filaments detached from endothelial cells and radiated into the dermal connective tissue, often getting thinner as they moved away from the endothelium thus assuming the aspect of “flames.” These filaments almost always detached from the endothelial sites that were more intensely labeled by α v- β 3 integrins. In some regions the attachment sites of fibrillin filaments to the abluminal plasma membrane of endothelial cells co-localized with integrins (Fig. 1c).

FAK and fibrillin

The lymphatic endothelial profile was brightly labeled by FITC indicating immunoreactivity to FAK (Fig. 1d). The reaction was variably distributed in the endothelium and was particularly intense on the abluminal side and in endothelial protrusions. The nuclei were not labeled.

Bundles of fibrillin filaments were evident

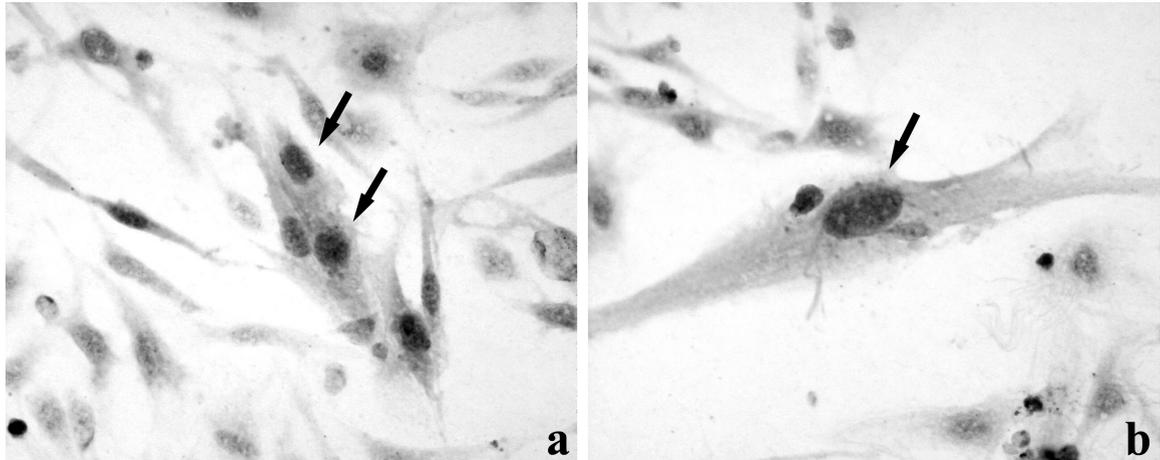


Fig. 3. Immunocytochemical localization of ERK1/2 in stretched lymphatic endothelial cells in culture. Labeling mainly seen in the nuclei (a,b arrows). Original magnification x40.

on the outside of the endothelium (Fig. 1e). Areas of contiguity between the green fluorescence of FAK molecules and the red one of fibrillin filaments could be detected particularly in endothelial protrusions (Fig. 1f).

α v- β 3 integrins and FAK

Integrin dashes (Fig. 1g) and FAK (Fig. 1h) were visible at tracts in the endothelial profile. Co-localization of these two molecules, indicated by yellow fluorescence, was observed in endothelial protrusions (Fig. 1i).

Phosphorylated ERKs in bovine thoracic duct segments

Cryostat transverse sections of bovine thoracic duct, stretched for 10 min with a pressure of 50cm of H₂O, were easily recognized from unstretched ones because their wall was clearly distended. Only the intimal layer with endothelial cells and the underlying connective tissue, maintained a slightly corrugated profile. Smooth muscle cells were elongated.

Immunoreactivity was strong in the nuclei of endothelial cells, whereas the cytoplasm was unstained (Fig. 2a). We failed

to detect any labeling in unstretched thoracic ducts (Fig. 2b) and in negative controls, obtained by omission of the primary antibody.

In Vitro Studies

Phosphorylated ERKs in cultured cells

Endothelial cells exposed to static stretching for 10 min were variably elongated and partially retracted from each other. The nucleus was labeled by phosphorylated ERK1/2. The cytoplasm was at times slightly colored (Fig. 3a and b). Cells that were not exposed to static stretching had faintly stained nuclei and cytoplasm. No labeling could be detected in negative controls (data not shown), obtained by omission of the primary antibody.

Western blotting

Western blotting (Fig. 4) performed on lysed cells demonstrated two distinct bands of equal intensity at 37kDa and 50kDa molecular weights corresponding to the phosphorylated isoforms ERK1 44-kDa and ERK2 42-kDa. In cultures exposed to medium containing 20% FBS and ECGS these bands were of equal intensity irrespective of stretching whereas in cultures exposed

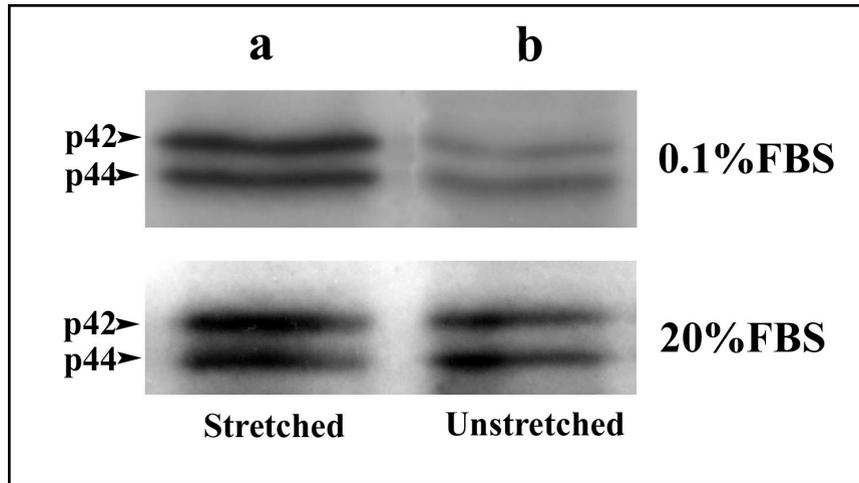


Fig. 4. Western blotting for ERK1/2 in cultured stretched (a) and unstretched (b) lymphatic endothelial cells with 0.1% (top) and 20% (bottom) FBS. When cells are metabolically activated by high serum and ECGS, ERK1/2 are similarly expressed in stretched and unstretched conditions whereas when they are starved for 16 h before stretching, ERK1/2 are more intensely expressed in stretched cultures.

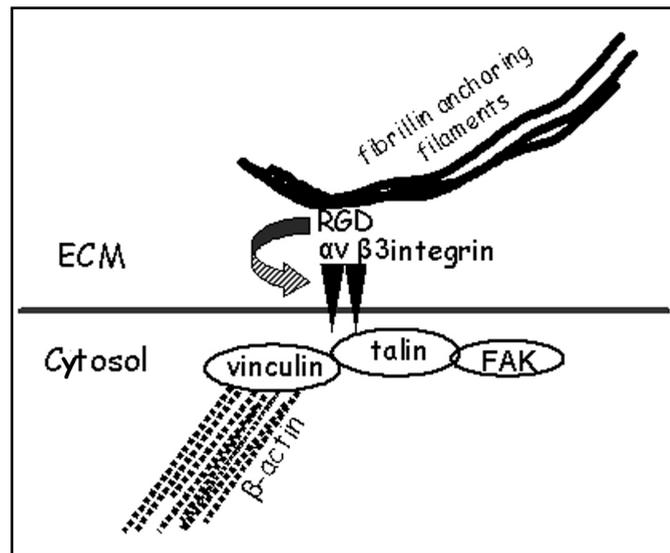


Fig. 5. Hypothetical connection between fibrillin anchoring filaments and focal adhesions of lymphatic endothelium.

to 0.1% FBS they were more intensely stained in stretched than in unstretched cultures.

DISCUSSION

Our data on human skin sections suggest that αv - $\beta 3$ integrins co-localize with FAK and participate in focal adhesion formation

in lymphatic endothelium and, additionally, that the attachment sites of fibrillin anchoring filaments to the abluminal plasma membrane of endothelial cells merge with αv - $\beta 3$ integrins at focal adhesions. The presence of β -actin filaments in lymphatic endothelial cells and of the connection molecules talin and vinculin (11), strongly

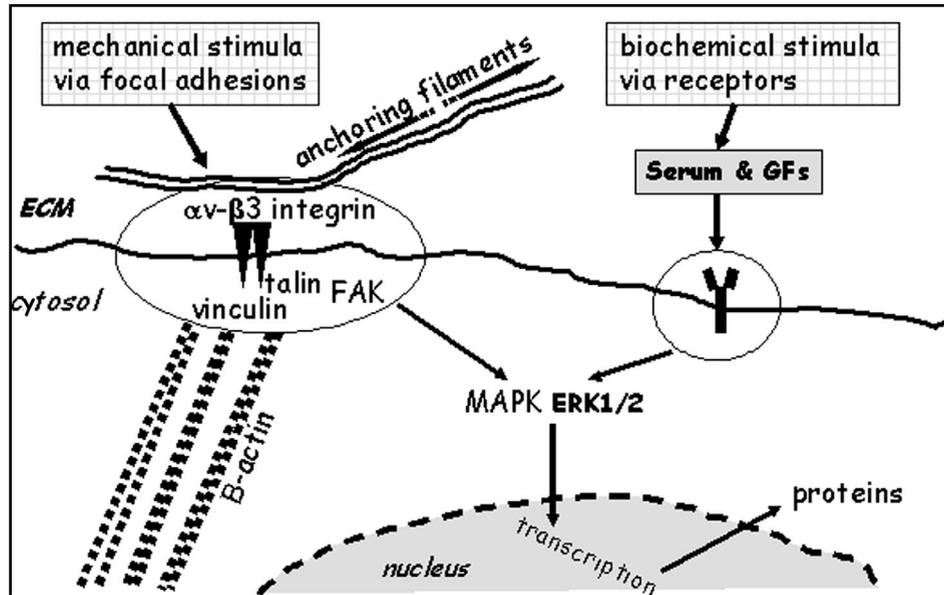


Fig. 6. Convergence of mechanical (via focal adhesions) and receptor stimulation on MAPK-ERK1/2.

suggest that anchoring filaments, through focal adhesions, may be connected to the cytoskeleton (Fig. 5). Of course, co-localization of two proteins does not necessarily imply a functional interaction between them.

We here report that fluorescence is stronger on endothelial protrusions into the perivascular connective tissue. Previous transmission electron microscopy studies on initial lymphatic vessels (11), have shown that endothelial protrusions are pulled outwards by anchoring filaments. This suggests that a connection between anchoring filaments and integrins exists. These molecules are in fact more concentrated (as indicated by the greater intensity of fluorescence) where focal adhesions, and hence the connection sites of anchoring filaments, are organized. Moreover, since fibrillin molecules have a RGD sequence and $\alpha v\text{-}\beta 3$ integrins an attaching site for this sequence, one may speculate that the connection may occur directly, without intermediate molecules like fibronectin (12,13).

The presence of focal adhesions in the endothelium of initial lymphatic vessels raises

the question of how they function. To our knowledge, no data are available in literature on this subject specifically referred to lymphatic endothelial cells. Blood endothelial cells have instead been the object of various studies that show that cyclic tension stimuli, at various frequencies and periods, or "shear stress" generated from blood flow on the endothelium, are transduced in biochemical cascades inside the cell, influencing cell shape and physiology (35,36). Focal adhesions may regulate barrier functions, in particular endothelial permeability (37-39). Given the morphologic and functional differences existing between blood and lymphatic endothelium, the data obtained in blood endothelium may not apply to the lymphatic one.

Keeping in mind that initial lymphatic vessel anchoring filaments pull and stretch endothelial cells, specially when the volume of interstitial fluid increases, we hypothesized that anchoring filaments may stimulate focal adhesions that, acting as mechanical signal transducers, activate a biochemical cascade in the cytoplasm. The resulting signal may control cell shape when directed to the

cytoskeleton, or influence transcriptional activity, regulating for instance endothelial permeability and hence lymph formation, when directed to the nucleus (*Fig. 6*).

Our present data bring support to this hypothesis: stretching of isolated thoracic duct segments and lymphatic endothelial cells cultured on elastic membranes, in fact activates the expression of MAPK ERK1/2. Western blotting confirmed that ERK1/2 are already activated after a 10 min stretching and that the two phosphorylated isoforms of ERK1 and ERK2 are equally expressed. Immunohistochemical data indicate that, after a 10 min stretching, MAPkinases have already left the cytoplasm and entered the nucleus. ERK1/2, in the organ model of thoracic duct segments as in cultured cells, are hence apparently phosphorylated and transferred to the nucleus in less than 10 min.

Our experimental conditions of static stretching have been designed in relation to the physiologic activity of lymphatic vessels. In lymphatic vessels, mechanical forces are mainly represented by slow expansions and contractions, with consequent alternation of filling and emptying periods, whose frequencies are not known. In the absence of precise parameters, we have arbitrarily chosen experimental times that might represent landmarks for future trials.

We are aware of the fact that bovine thoracic duct is a propulsion vessel, and that the behavior of its endothelium may differ from that of absorbing lymphatic vessels. Previous studies have however shown that anchoring filaments are also present in the wall of other large lymphatic vessels like precollectors and collecting vessels (40,41). Moreover, the wall of lymphatic vessels is not as well organized and compact compared to that of arteries. Smooth muscle cells do not form a continuous ring, but are rather arranged either helicoidally or in groups. The morphology of propulsion vessel is therefore not incompatible with absorbing mechanisms.

As to our cell culture model, it offers great advantages: the possibility to study the

behavior of cells taken from the same type of vessel used for organ experimentation, the availability of bovine thoracic ducts at the local slaughterhouse, the large number of cells that can be obtained from a single duct with enzymatic digestion (approximately 1×10^6), and the possibility to work on primary cells that are likely to retain the same characteristics as *in vivo*. When permeability is taken into account, as our conclusions imply, it is of outmost importance to work on freshly isolated cells since culture conditions have been demonstrated to profoundly affect this parameter (42).

Lymphatic endothelial cells have been cultured on gelatin coated membranes. The expression of MAPK in fibroblasts has been reported to be matrix-dependent and to occur on some components of basement membrane (laminin and fibronectin) and not on collagen (24). Lymphatic vessels, however, lack a continuous basement membrane and most of their endothelium is directly in contact with the surrounding connective tissue with its collagen fibers. This situation may be better mimicked by gelatin than by fibronectin or laminin.

We believe that our data may provide insights, even if indirect, on the physiologic behavior of absorbing lymphatic vessels. In particular, they indicate that isolated lymphatic endothelial cells have the intrinsic capacity to perform signal transduction in response to external stimuli. Signal transduction in lymphatic endothelial cells may be activated by biochemical (via receptors) and mechanical stimulation by anchoring filaments (via focal adhesions). The latter mechanism may occur independently of the former as shown by activation of ERK1/2, the principal molecule of the MAPK cascade, upon stretching in low serum conditions.

Based on our data, with the limitation that stretching was performed on a propulsion vessel and immunohistochemistry on initial lymphatics, we speculate that, when the interstitial pressure increases and pulls outwards anchoring filaments, lymphatic

endothelial cells would react in a much more sophisticated way than simply opening interendothelial junctions as previously thought. By signal transduction of mechanical stimuli, lymphatic endothelial cells would be able to precisely and continuously regulate their physiologic activity to produce lymph in response to the physiologic requirements of the interstitium.

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