ENDOTHELIAL CELLS AS EARLY SENSORS OF PULMONARY INTERSTITIAL EDEMA

By

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Running title: Response of lung endothelial cells to interstitial edema

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ABSTRACT

We studied the endothelial and epithelial cells response in the thin portion of the air-blood barrier to a rise in interstitial pressure caused by an increase in extravascular water (interstitial edema) obtained in anesthetized rabbits receiving saline infusion (0.5 ml/kg x min for 3h). We carried a morphometric analysis of the cells and of their microenvironment (electron microscopy); furthermore, we also studied in lung tissue extracts the biochemical alterations of proteins responsible for signal transduction (PKC, caveolin-1), for cell-cell adhesion (CD31), and of proteins involved in membrane to cytoskeleton linkage (alfa and beta tubulin). In endothelial cells we observed a folding of the plasma membrane with an increase in cell surface area, a doubling of plasmalemma vesicular density and an increase in cell volume. Minor morphological changes were observed in epithelial cells. Edema did not affect the total plasmalemma amount of PKC, betatubulin and caveolin-1, while alfa-tubulin and CD-31 increased. In edema, the distribution of these proteins changed between the detergent resistant fraction of the plasma membrane (DRF, lipid microdomains) and the rest of the plasma membrane (high density fractions, HDFs). PKC and tubulin isoforms shifted from DRF to HDFs in edema while caveolin-1 increased in DRF at the expense of a decrease in phosphorylated caveolin-1. The changes in cellular morphology and in plasma membrane composition suggest an early endothelial response to mechanical stimuli arising at interstitial level following a modest (about 5%) increase in extravascular water.

Keywords: pulmonary interstitial pressure, morphometry, air blood barrier, mechanotransduction, plasma membrane proteins
Introduction

Tissue edema is a common manifestation of most of the pathological processes. Compared to other organs, the pulmonary parenchyma is physiologically well protected against interstitial fluid accumulation due to a very low interstitial tissue compliance provided by matrix proteoglycans (14). Indeed, an increased microvascular filtration leads to an increase in hydraulic interstitial pressure that opposes further filtration (12, 13) and severe edema develops when microvascular filtration leads to a progressive fragmentation of proteoglycans (14). A biochemical study carried on plasma membranes isolated from lung tissue showed considerable modifications of their composition resulting in an increase in membrane fluidity (20). We could also document compositional changes in lipid microdomains that represent specialized regions of the plasma membranes enriched in signal transduction molecules (21). Finally, in pulmonary interstitial edema, we could also demonstrate an early activation of mRNA of pro-inflammatory cytokines involved in matrix remodelling (23). Altogether, the above data suggested an early activation of pulmonary cells when interstitial edema develops although we had no indications on the degree of response of the various types of lung cells. We now hypothesize that tissue factors relating to initial water accumulation in the thin portion of the air-blood barrier (TABB) (4), may induce cellular response and we tested this hypothesis through a parallel morphometric and biochemical study to detect signs of cellular activation. A signaling response can be attributable to either alteration in interstitial protein composition and/or to mechanical stresses elicited by the increased interstitial pressure. The morphometric study was done at high magnification on endothelial and epithelial cells of TABB and of their microenvironment. On biochemical ground we evaluated the changes in plasma membrane PKC and caveolin-1, important signal transduction proteins, and other proteins that can affect cell shape such as, adhesion molecules (CD 31), and proteins involved in membrane to cytoskeleton linkage (tubulin isoforms).

Materials and methods
The experiments were carried out on adult New Zealand rabbits [2.5 ± 0.5 (SD) kg body wt], anesthetized with a cocktail of 2.5 ml/kg of 50% (wt/vol) urethane and 40 mg/kg body wt of ketamine injected into an ear vein. Subsequent doses of anesthetic were administered during the experiments judging from the arousal of ocular reflexes. The trachea was cannulated to allow spontaneous breathing. The study was based a protocol accepted by D.L. 116/1992, art.3, 4, 5 and performed according to the established rules of animal care.

*Pulmonary interstitial edema formation*

The right superior jugular vein was cannulated and pulmonary interstitial edema was induced by infusing saline at a rate of 0.5 ml·kg⁻¹·min⁻¹ for 3h. The experimental protocol was shown to cause a slow development of interstitial edema (13) due to a decrease in plasma colloid osmotic pressure that leads to a greater microvascular filtration rate; in addition, the increase in plasma volume (∼15%) also causes a greater lung perfusion and therefore a greater filtration area. These animals will be simply referred to in the text as edematous or treated.

*In situ lung perfusion- fixation for electron microscopy*

Three groups of animals were used for morphological and morphometric analysis: 1) animals sacrificed immediately after anesthesia and tracheotomy (control; n=2); 2) animals kept under anesthesia for 3 hours (sham; n=3); 3) animals with mild interstitial edema (treated; n=4). The chest was opened through a midsternal splitting incision to expose the pericardium; in rabbits this allows to keep pleural sacs intact, therefore preserving the physiological lung expansion. The pericardium was opened and the pulmonary artery cannulated; the left atrium was sectioned to allow the drainage of blood and perfusate. Two reservoirs arranged in parallel and connected to the pulmonary artery were used for lung perfusion. They contained, respectively, saline (11.06g NaCl/l plus 3% dextran T-70 and 1,000U heparin/dl, 350mOsm) and fixative (phosphate buffered 2.5% glutaraldehyde plus 3% dextran T-70, 500 mOsm, pH 7.4). The upper level of the liquids in the reservoirs was adjusted at a height of 15 cm H₂O relative to left atrium level and maintained constant during perfusion. Animals were killed by an overdose of anesthetic just prior to the
perfusion procedure. The perfusion circuit was first primed with saline for about 3 min till the outflow appeared cleared of blood cells. Then the circuit was switched to fixative that was allowed to flow for 25-30 minutes.

Transmission electron microscopy preparation and tissue sampling

The fixed control, sham and treated right lungs were cut into five slices of equal thickness according to a stratified random sampling procedure (31). Five-seven small blocks were systematically obtained from each slice, immersed in 2.5% glutaraldehyde for 4 hours at 4°C and then processed for electron microscopy as previously described (4, 20). Two blocks were randomly selected from each slice and processed for morphometric analysis. A single ultrathin section, 60 nm thick, was obtained from each tissue block, mounted on uncoated 200-mesh copper grids, stained with uranyl acetate and lead citrate and observed in a Zeiss EM900 electron microscope. Six electron micrographs were systematically obtained from each section at two sequential primary magnifications (x15,000 and x28,000, respectively) on photographic paper as positive reversals from 70-mm negative films. The highest magnification was used to better investigate the morphometry of TABB; to this aim we examined a total of 75 fields from edematous lungs and 25 fields from control lungs, electronically acquired as positive reversals, and brought to a final magnification of x66,000 on the computer video screen.

Morphology of the thin portion of the air-blood barrier (TABB)

TABB corresponds to regions where only a fused basement membrane separates endothelium and epithelium with no intervening cells and fibrillar matrix.

We used a multipurpose M168 grid (length of test line d_{grid} = 0.174 \mu m). We counted the number of test points over endothelial and epithelial cells (Pen and Pep, respectively) and interstitium (Pint); point counting over a given compartment, relative to total point counting over the image, is proportional to the volume of the compartment (volume density, Vv) (31). Other techniques are available to estimate cell volume changes with a good time resolution however they can only be used for studying cell populations or single cells (25). Using the same M168 grid we also counted
the number of intersections of test lines with luminal and interstitial surface of endothelial cells (Ien lum and Ien int, respectively) and of interstitial front of epithelial cells (Iep int). Intersection counting, for a given profile separating compartments, is proportional to the surface development of the profile (31). The mean arithmetic thickness of endothelial and interstitial compartments was obtained as $\tau_{\text{en}} = (d \cdot \text{Pen}) / [2 \cdot (Ien \text{ lum} + Ien \text{ int})]$ for endothelial cells and $\tau_{\text{int}} = (d \cdot \text{Pint}) / [2 \cdot (Ien \text{ int} + Iep \text{ int})]$ for interstitial layer, respectively.

For a detailed morphometric study of the complex changes in shape of cells and their microenvironment in interstitial edema, we used a cycloidal C2 grid (8) that enables a more precise analysis.

**Distribution of plasmalemma vescicles (PVs)**

Plasmalemmal vesicles in endothelial and epithelial cells were identified by their morphology as being non-coated, 50-90nm in diameter. We computed the numerical density of PVs on micrographs obtained at x15,000 brought to a final magnification of x36,000, in endothelial and epithelial cells ($N_v, \mu m^{-3}$), i.e. number of PVs per unit cell volume, using a multipurpose test grid M168 (in this case $d_{\text{grid}} = 0.337 \mu m$). Numerical density was obtained as $N_v = \text{number of PVs/unit volume} \times \text{a correction factor given by } (\bar{D} + T - 2h)$ where: $\bar{D}$ is the true mean diameter of the PVs (considered to average 70 nm, as commonly accepted in literature (6)); $T$ is the thickness of the ultrathin sections (60 nm); $h$ is the depth by which a vesicle must penetrate the section before it is detected (5, 6, 31). We also computed the vesicular load ($N/S, \mu m^{-2}$), i.e. number of PVs per unit cell surface profile. In this case the micrographs were enlarged to a final magnification of x66000 and data were acquired using the cycloidal C2 grid (8).

**Chemicals**

The antibodies we used in this study were: mouse monoclonal anti-caveolin-1 C2297 (1:1000), anti-caveolin-1phosphorilated C-91520 (1:1000), from Transduction Laboratories (Lexington, KY, USA), mouse anti-PKC (1:200), from Santa Cruz Biotecnology (Santa Cruz, CA), mouse
monoclonal anti-CD31 (1:4000) from DAKO (Denmark), mouse monoclonal anti- α- and β- tubulin (1:500) from Sigma (St. Louis, USA).

Biochemical methods

Samples were obtained from control animals (sacrificed shortly after anesthesia and tracheotomy; n=5) and from animals that received slow saline infusion through the right superior jugular vein (0.5 ml/kg min) for 3 hrs to induce interstitial edema (n=5). Then we prepared the samples as previously described: briefly, we perfused the lungs for about 5 min at room temperature with mammalian Ringer’s solution (without calcium) containing nitroprusside (20 mg/ml). Nitroprusside is a donor of nitric oxide; however this effect should be present both in control and treated animal samples; therefore the observed differences in membrane protein response when comparing control to treated animals should be due to the specific conditions caused by interstitial edema. After this, the lungs were flushed with 50 ml of solution 1 (0.25 M sucrose, 20 mM Tricine pH 7.4 and 40 μg/ml of the protease inhibitors aprotinin, chymostatin, leupeptin and antipapain), excised from the chest and immersed in ice cold solution 1. The lung tissue was finely minced at 4°C and homogenated in solution 1, then filtered sequentially through 53 and 30 μm filters. The homogenate was subjected to centrifugation (1,000 g for 10 min) at 4°C, and the supernatants were saved. The resulting pellet was resuspended in 3 ml of buffer and subjected again to centrifugation as above. The pooled supernatants were overlaid over 25 ml of 30% Percoll in buffer. After centrifugation using a SW28 rotor at 84,000 g for 45 min at 4°C, we collected a single membranous band (about 1 ml) readily visible at about 2/3 from bottom of the tube. To reduce the volumes and concentrate the membranes, the bands were pelleted by first diluting the suspension 3 fold with PBS before centrifugation at 100,000 g for 10 min at 4°C. This membrane fraction was collected and called PMC and PME (plasma membranes control and plasma membranes edema, respectively).

Isolation of detergent-resistant fraction

The plasma membrane pellet was resuspended in 1 ml of MBS buffer (25 mM of MES buffer, pH 6.5, containing 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride and 75 units/ml aprotinin) and
we determined its protein content (BCA methods). Next, we took a volume containing 4.5 mg of protein, a quantity required for each gradient procedure. In order to maintain a constant protein/detergent ratio in all experiments, we added MBS buffer containing Triton X-100 up to a volume of 2 ml to reach a final Triton concentration of 1%. All the procedure was carried on ice for 20 min to maintain the integrity of lipid rafts. Finally, the 2ml were diluted with an equal volume of 80% (w/v) sucrose in MBS lacking Triton X-100 and placed at the bottom of a tube where a discontinuous sucrose concentration gradient was created (40, 30, 5 % sucrose, from bottom up) in MBS lacking Triton X-100. After centrifugation at 250,000 g for 18 hrs at 4°C with a TW-41 rotor (Beckman Instruments), 1 ml fractions were collected from the top of the gradient and submitted to further analysis. From now on, fraction 5 from the top will be referred as DRF (detergent resistant fraction) and fractions from 9 to 12 were pooled and indicated as HDFs (high density fractions).

Protein analysis

Aliquots of PMC, PME and all fractions collected from the gradient, were submitted to trichloroacetic acid precipitation. The pellets, washed with acetone, were suspended in water and protein quantity determined by BCA method (SIGMA, USA). Thereafter, 50 µg of PMC and PME and 10 µg of proteins collected from DRF and HDFs pellets, respectively, were loaded on SDS-PAGE; 10% polyacrylamide gel, and submitted to electrophoresis. Subsequently, the proteins were transferred to membranes that were stained with Ponceau S to assess protein loading by densitometry (BIORAD Densitometry 710, program Quantity one) (15, 24). To assess the adequacy of our loading-transfer technique, we performed densitometry of albumin loading (in the range 5-15 micrograms) transferred to nitrocellulose stained with Ponceau S; the regression between densitometry (Y) and protein loading was Y = 0.5385X + 4.14, R² =0.97. Furthermore, no significant difference in densitometry values was found on paired albumin loadings in the range 5-15 micrograms. We compared on our samples the densitometry of the whole lane for protein loading obtained from total plasma membranes, DRF and HDFs from control and treated animals.
Subsequently the membranes were submitted to Western blotting. After blocking, blots were incubated for 2 hr with the primary antibody diluted in PBS-T/milk. In the case of phosphorylated caveolin-1, the primary antibody was diluted in Tris 10mM, pH 8.0, NaCl 50mM, Tween-20 0.2%, Milk 2% and BSA 1%. Then, blots were incubated for 2 hr with horseradish peroxidase-conjugated anti-mouse/goat IgG (5,000-10,000-fold diluted in PBS-T/milk). The protein samples were obtained from 5 controls and 5 treated animals. Proteins were detected by ECL using the SuperSignal detection kit (Pierce, Rockford, IL). We performed in parallel immunoblot analysis of samples from one control and one treated animal for total plasma membrane, DRF and HDFs proteins. Immunoblot bands were analyzed by BIORAD Densitometry 710.

*Plasma protein concentration*

In all groups of animals, blood samples were drawn to determine plasma protein concentration by optical refractometry (SPR-Atago, precision within 3%).

*Statistical analysis*

For morphometric analysis, primary data (point, line intersection and vesicle counts) were summed over all the micrographs derived from each section and the parameters were computed as the ratio of sums. The parameters were then averaged over the various section samples. Data were expressed as means ± SE. The significance of the differences among groups was determined using one-way ANOVA and t-test. In order to evaluate the relationships between morphometric characteristics, linear models were fitted on data in the natural or logarithmic and exponential transformation where appropriate. The linear regression parameters were estimated using the least square method. This approach was also used for the estimate of the iso-shape curve.

**Results**

*Plasma protein concentration*
Total plasma protein concentration was $5.7 \pm 1$ (SD) g/dl in control condition (obtained by averaging data from both control and sham animals) and $3.3 \pm 0.2$ after 3 hours of saline infusion.

*Morphometry of the TABB*

The low magnification (x60) light microscopy images of Fig. 1 show the morphology of the lung in control and in interstitial edema (left and right, respectively). One can appreciate that in interstitial edema there is a fairly diffuse state of water imbibition spreading from the alveolar septa to the TABB, as indicated by the arrows.

Fig 2, panel A, presents a high magnification (x66,000) electron micrograph of the TABB separating the capillary lumen from the alveolar space (CL and AS, respectively). TABB is made of smooth endothelial and epithelial cells (en and ep, respectively) and their fused extremely thin basement membrane (bm). In interstitial edema the morphology of the TABB was quite variable. In some cases, a fairly normal morphology could still be observed for endothelial cells (B); in other cases an interstitial and luminal folding of endothelial cells was observed with a moderate increase in basement membrane thickness (C). Finally (D), in other endothelial cells, together with a membrane folding process, the cytoplasm volume appeared augmented and the number of non-coated vesicles (PV) was greatly increased. Apart from interstitial folding, no consistent morphological changes were observed in epithelial cells. The total volume density of TABB (carried at x 66000) on the average, doubled in edema (Table 1); endothelial cells contributed mostly to the enlargement of total volume as their volume density increased significantly by 2.4. The least increase was provided by epithelial cells (1.8, significant), while the increase in interstitial space was equal to the overall change in total volume of TABB.

Fig 3 shows the correlation between cell and interstitial volume for endothelial and epithelial cells of the TABB (A and B, respectively). The closed squares refer to the variability of data observed in control conditions, while open circles refer to interstitial edema. No significant differences between control and sham animals were found, therefore all values were pooled in the control group.

Recalling the definition given in the methods, the units on the ordinate represent the fraction of
volume of the cellular compartment relative to the total volume (air plus tissue) in each lung image. The $R^2$ value obtained of 0.66 indicates that the correlation between endothelial cell and interstitial volume (panel A) is adequately described by a linear regression model. Conversely, no such correlation could be detected for epithelial cell volume vs interstitial volume (panel B, $R^2 = 0.36$).

In Fig. 4 we plotted the data of interstitial surface profile vs basement membrane thickness. In this, as in the following figures, control values are represented by closed symbols while interstitial edema values (open symbols) were grouped to show the various degree of departure of the parameters considered relative to control. On comparing the control to the first edema group, we found a significant increase in interstitial surface profile ($P = 0.001$) but no significant increase in basement membrane thickness ($P=0.7$). Fitting the data of the edema groups with a linear regression (logarithmic transform), yields the $R^2$ value of 0.83 that indicates a significant correlation between the increase in endothelial surface profile on the interstitial side and basement membrane thickness.

Fig. 5 shows that the ratio of luminal to surface area profile, on the average, increases with increasing cell volume, indicating that the luminal bulging accounts for a larger increase in cell surface area.

Fig. 6A shows that the surface to volume ratio of the endothelial cells is highest in control conditions while it decreases progressively with increasing endothelial cell volume in edema. Fig. 6B shows that on increasing cell volume, the cell surface increases less than expected on the basis of a constant shape (line labelled iso-shape). The iso-shape surface value was obtained from $(V/V_c)^{2/3}$, where $V$ is the cell volume relative to control ($V_c$). The departure from the iso-shape line reflects a larger increase in cell volume with respect to the increase in cell surface: therefore, the increase in cell thickness exceeds the increase in surface area due to plasma membrane folding. The scatter of the data also suggests that the shape assumed by the cell on increasing lung volume is quite variable.
Morphometric distribution of plasmalemma vesicles (PVs) in endothelial and epithelial cells of the TABB

Table 2 shows the numerical density of PVs (Nv, number of vesicles per unit volume) estimated at x36,000: it appears that in interstitial edema Nv is doubled (P<0.001) in the endothelial cells of TABB, while it is only slightly increased in the epithelial cells and in the endothelial cells of the thick portion of the air-blood barrier. It has been suggested that glutaraldheyde fixation may induce vesicle formation, compared to frozen specimens (11, 32). Even though we can’t get rid of this possible artefact, we may at least assume that the time course of the fixation procedure is similar in control and treated lungs as tissue mass is substantially unchanged. Furthermore, in situ vascular perfusion fixation has been proposed as the most suitable method to yield optimal lung tissue preservation and minimal alterations to the lung architecture suitable for morphometry (31).

Analyzing the images at high magnification (x66,000) allows a correlation between vesicles number and morphology of the endothelial cells of TABB. Fig. 7A shows that the vesicular load of PVs (N/S, number of PVs per unit surface) increases significantly with increasing total surface profile (luminal plus interstitial). Fig. 7B shows that the numerical density of PVs (Nv, number of vesicle per unit cell volume) increased stepwise and significantly (P<0.001) for a negligible initial increase in cell volume; subsequently, the numerical density of PVs tended to decrease reflecting the fact that cytoplasm volume is increasing more than total vesicle number.

Biochemical results

Fig. 8 shows a representative example of Ponceau S staining of nitrocellulose membranes for plasma membrane, DRF and HDFs proteins. We compared the densitometry readings of the whole lane between control and treated samples loaded in parallel: the average % difference in the paired observations was not significant (0.11 ± 0.8 SE); therefore, a change in densitometry reading for a given protein after western blotting suggests a change in the amount of this protein relative to the total amount loaded.
Fig. 9 reports representative immunoblotting experiments for plasma membranes, DRF and HDFs proteins while Table 3 reports the statistical analysis relative to the observed differences in protein distribution. PKC remained unchanged in plasma membranes on comparing control to edema (Table 3, PM_EDEMA /PM_CONTROL). However, PKC was present in DRF and in little amount also in HDFs; in interstitial edema, PKC appeared to migrate from DRF towards HDFs, as the DRF/HDFs ratio decreased from 5.57 to about zero.

α-tubulin increased significantly in PM_EDEMA relative to PM_CONTROL. Furthermore, it appeared to migrate from DRF to HDFs in edema as the DRF/HDFs ratio decreased from 2.39 to zero.

β-tubulin did not change on comparing PM_EDEMA to PM_CONTROL, but shifted from DRF to HDFs as the DRF/HDFs ratio decreased from 0.9 to 0.18.

Caveolin-1 did not change on comparing PM_EDEMA/PM_CONTROL; it was only present in DRF and it increased significantly in this fraction in edema (Table 3, DRF_EDEMA/DRF_CONTROL).

Phosphorilated caveolin-1 was not detectable in plasma membrane, was present in DRF_CONTROL and it decreased in this fraction in edema.

CD31 (also known as PECAM) increased significantly in plasma membranes in edema, reflecting its increase in the HDFs.

**Discussion**

The present study is an attempt to investigate in an in-vivo model the early lung cellular response to a moderate increase in interstitial volume. In pulmonary interstitial edema, light microscopy reveals a fairly homogeneous distribution of the extravascular water in the interstitial space of the air-blood barrier (Fig. 1), at variance with severe edema where a high inhomogeneity of extravascular fluid accumulation has been observed (2, 28).

Recent data have clarified subcellular events in pulmonary cells in response to vascular stimuli induced by considerable elevations in capillary pressure (10, 30). In our experimental approach, the hydrostatic capillary pressure is essentially unchanged relative to normal (17) and the increase in
shear stress does not exceed 30% (20). Conversely, since interstitial fluid pressure increases markedly due to matrix imbibition from $\approx -10$ up to $\approx 5$ cm H$_2$O (13), we may hypothesize that stimuli triggering a cellular response originate mainly on the interstitial, rather than on the luminal front. This cellular response can be stimulated by either increased tissue pressure and/or the fragmentation of proteoglycans that are known to regulate a number of dynamic cellular processes, including cell adhesion and cell-matrix interaction (18).

At large magnification, there are considerable differences in morphology of the thin portion of the air blood barrier that are mostly restricted to the endothelial cells and the basement membrane (Fig. 3A). Indeed, on considering the relationships between the geometry of endothelial cells and their interstitial microenvironment, various degrees of alterations could be described possibly indicating a sequence of functional phases in the cellular response. Some endothelial cells appeared still similar to control conditions, namely flat and thin (Fig. 2B), while for others, considerable changes in morphology were observed (Fig. 2 C-D), suggesting a difference in the temporal sequence for the development of cellular functional adaptations.

The increase in interstitial cell surface, due to folding of the plasma membrane, appears to be an early cellular response to developing interstitial edema as it occurs for a negligible increase in basement membrane thickness (Fig. 4). The increase in the luminal to interstitial surface ratio of endothelial cells correlates to the increase in cytoplasm volume (Fig. 5) and reflects the formation of bulging processes towards the capillary lumen.

The increase in surface area in endothelial cells can only occur by creating new plasma membrane because these cells do not possess surface elements providing unfolding; this process requires lipid translocation from cytoplasm to cell surface and is fostered by increased fluidity of the plasma membrane reflecting, in particular, the modifications of the phosphatidylcholine/phosphatidylethanolamine and cholesterol/phospholipid ratios (20).

$\alpha$-and $\beta$- tubulin are major subunits of microtubules whose association with plasma membrane occurs through hydrophobic interactions that are established by palmitoylation of a tubulin cystein
residue (3). Palmitoylated-tubulin was also found in lipid microdomains (22) and the shift of this molecule away from DRF removes a membrane to cytoskeleton linkage and this might allow greater mobility of lipid microdomains and therefore greater deformation in these portions of plasma membrane.

CD31 is a mechanoresponsive cell-surface receptor, involved in control of microvascular permeability and its increase was shown to occur in response to shear induced perturbation of the endothelial plasma membrane at luminal level (19). The present data demonstrate that an increase in CD31 may also occur in response to interstitial edema. The observed changes in PKC and in CD31 (both increase in HDF in edema) may contribute to modulate the CD31/catenin association and therefore the physical link between cell surface and the nuclear envelope (19).

The increase in PVs per unit surface profile (vesicular load) was mostly observed in endothelial cells and correlates to the increase in cell surface profile (Fig. 7A). Conversely, no real correlation does exist when plotting the numerical density of PVs vs cell volume (Fig. 7B) as it doubles for a negligible increase in volume, remaining thereafter essentially steady. It is known that caveolin-1, either in phosphorylated or dephosphorylated form, affects the subcellular traffic of vesicles, corresponding, on morphological and biochemical ground, to caveolae (1). Reducing caveolin-1 phosphorylation prevents the endoplasmic reticulum targeting of caveolin, favoring instead the shift of lipid microdomains to cellular surface to form vesicles (27). Therefore, our finding of an increase in the ratio of caveolin-1/phosphorilated caveolin-1 from about 2.5 to about 11 in edema is in line with the increased vesicle formation occurring in the endothelial cells. PVs, besides representing a site for signalling proteins, are also involved in transcytosis. The increase in cytoplasm volume in endothelial cells could derive from the activation of plasma membrane mechanosensitive ion channels (25) and could also correlate to the previously observed increase in AQP-1 in DRF (21). In fact, the increase in endothelial cell volume, and to a minor extent, of epithelial cells, contributes to a remarkable re-shuffling effect of extravascular water. We might attempt a functional interpretation of this fact considering that preventing water accumulation in the interstitial space...
allows to protect its integrity, and in turn to avoid the development of severe edema: indeed, as long as the interstitial matrix maintains its integrity, the hydraulic interstitial pressure is positive enough to counteract microvascular filtration (14).

Mechanotransduction

Since endothelial cells mostly respond to interstitial edema with modifications in volume and shape, this suggests a functional link between mechanical stress and protein changes. On the contrary, the morphology of the epithelial cells was less affected, possibly indicating that these cells are less sensitive to parenchymal stimuli. Endothelial cells are equipped with numerous receptors, such as CD31, that allow them to detect and respond to the mechanical forces. The cytoskeleton and other structural components have an established role in mechanotransduction being able to transmit and modulate tension within the cell via focal adhesion sites, cellular junctions and the extracellular matrix (7, 16). Endothelial cells display the highest surface to volume ratio in control conditions (Fig. 6A) because of their attachments to the extracellular matrix and to the neighbouring cells and through the mechanical action of the cytoskeleton that keeps the cell in a highly deformed state. The tensional behaviour of a “hard-wired” cytoskeleton (9) might put endothelial cells in a good position to respond promptly to mechanotransduction. Moreover, the rigidity of the interstitial tissue (13) adds efficiency to the cellular response because a small increase in interstitial volume results in a considerable increase in interstitial pressure. A cellular response could also depend upon modifications of interstitial protein composition. Regardless of the mechanism, this paper provides the evidence for a prompt endothelial cellular response to stimuli arising at interstitial level when microvascular filtration is increased. The cellular response does not imply a considerable change in total plasma membrane contents for membrane proteins that we analyzed but, instead, in a modification of their distribution between lipid microdomains and the rest of the plasma membrane. Cell swelling stimulates protein synthesis and gene expression (29) and furthermore many intracellular pathways, including the MAPK cascade, are triggered by mechanical stimuli via sequential phosphorylation, the activation of transcription factors and gene expression. In summary,
the results of this study suggest that signs of cellular activation develop to a various degree mostly in the endothelial cells; these cells may act as early sensors of interstitial fluid accumulation, as they respond to minor increases in extravascular water (not exceeding 5%), possibly to trigger a reparative process.
Table footnote

Table 1. Values are means ± SE. * P<0.05 vs control.

Table 2. Values are means ± SE. * P<0.001 vs control.

Table 3. Values are means (± SE) of the average difference relative to 1, of the densitometry ratios indicated in the table; N=15; two tailed t test.
Acknowledgments

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References


Figure Legends

Fig. 1

Light microscopy at x60 of the lung in control and in interstitial edema.

AS: alveolar space; AD: alveolar duct; TABB: thin portion of the air-blood barrier indicated by arrow; C: capillaries.

Fig. 2

Ultrastructural appearance of the thin portion of the air-blood barrier in control lungs (A) and in mild interstitial edema at high magnification (x66,000) (B, C and D showing different degrees of alteration relative to control) Bar = 0.5 µm.

In all micrographs, capillary lumen (CL) and alveolar space (AS) are respectively above and below the thin portion of the air-blood barrier. Other structures are defined as follows: en, endothelium; ep, epithelium; bm, basement membrane; PV, plasmalemma vesicle.

Fig. 3

Relationship between cell volume and interstitial volume for endothelial and epithelial cells (A and B, respectively). Filled symbols refer to data points in control; open symbols refer to interstitial edema.

Units of volume are given by total number of points (Ptot) falling in each compartment.

Fig. 4

Relationship between endothelial cell interstitial surface profile vs basement membrane thickness for grouped data. For the first edema group the interstitial surface profile was significantly increased (P=0.001) relative to control (filled square). Surface area units are given by number of intersections with the endothelial cell interstitial profile using a cycloidal grid (Ien lum).
Fig. 5

Ratio of luminal to interstitial endothelial cell surface profile vs endothelial cell volume. Units of surface profile are given by number of intersections with each front using a cycloidal grid. Units of volume are given by total number of points (Ptot) falling on the endothelial cells.

Fig. 6

A. Total surface to volume ratio for endothelial cells vs cell volume.
B. Total surface of endothelial cells plotted vs cell volume. The line labeled iso-shape corresponds to the relationship for a cellular shape remaining equal to that in control conditions on increasing volume. For the calculations see the text. The actual increase in cell volume based on experimental data was fitted with a power regression. Filled symbols refer to data points in control; open symbols refer to interstitial edema.

Fig. 7

A. Vesicular load (number of non-coated vesicles per unit of cell profile) as a function of the increase in total cell profile.
B. Numerical density of vesicles (number of non-coated vesicles per unit cell volume) as a function of cell volume. Filled symbols refer to data points in control; open symbols refer to interstitial edema.

Fig. 8

Representative example of Ponceau S blots after protein loading on 10% SDS PAGE (50 μg for PMC and PME; 10 μg for DRF and HDF) and subsequent transfer to nitrocellulose membranes for control and edema protein samples.

Fig. 9

Representative immunoblotting of total plasma membrane proteins in control and edema (PMC and PME, 50 μg for lane, respectively) and of proteins found in detergent-resistant and high density fractions (DRF and HDFs, 10 μg for lane, respectively) in control and edema.
Table 1
Volume density ($V_V$) of the three compartments of the thin portion of the air blood barrier (TABB) estimated at x66000.

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<th>$V_V$, ENDOTHELIAL</th>
<th>$V_V$, INTERSTITIAL</th>
<th>$V_V$, EPITHELIAL</th>
<th>$V_V$,TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.075 ± 0.004</td>
<td>0.022 ± 0.001</td>
<td>0.061 ± 0.002</td>
<td>0.158 ± 0.006</td>
</tr>
<tr>
<td>edema</td>
<td>0.175 ± 0.005*</td>
<td>0.046 ± 0.001*</td>
<td>0.114 ± 0.002*</td>
<td>0.335 ± 0.007*</td>
</tr>
</tbody>
</table>

Table 2
Density of plasmalemma vesicles ($N_v$; number of PVs per $\mu m^{-3}$ cell volume) in epithelial and endothelial cells of the air-blood barrier estimated at x36,000. For endothelial cells density is given relative to thin (TABB) and thick portion of the air-blood barrier (ABB).

<table>
<thead>
<tr>
<th></th>
<th>Epithelium</th>
<th>Endothelium TABB portion</th>
<th>Endothelium Thick ABB portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>191 ± 17</td>
<td>243 ± 17</td>
<td>126 ± 10</td>
</tr>
<tr>
<td>Interstitial edema</td>
<td>227 ± 12</td>
<td>494 ± 35*</td>
<td>144 ± 12</td>
</tr>
</tbody>
</table>
Table 3

Statistical analysis of immunoblots for total plasma membrane proteins (PM) and for proteins found in DRF and HDFs in control and edema.

<table>
<thead>
<tr>
<th></th>
<th>( \text{PM}<em>{\text{EDEMA}}/\text{PM}</em>{\text{CONTROL}} )</th>
<th>( \text{DRF}/\text{HDFs} )</th>
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<tbody>
<tr>
<td></td>
<td>significance of the ratio relative to 1</td>
<td>control</td>
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<tr>
<td>PKC</td>
<td>mean</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>( t )</td>
<td>1.68</td>
</tr>
<tr>
<td>( \alpha )- tubulin</td>
<td>mean</td>
<td>2.60</td>
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<tr>
<td></td>
<td>SE</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>( t )</td>
<td>19.25</td>
</tr>
<tr>
<td></td>
<td>( P&lt;0.05 )</td>
<td></td>
</tr>
<tr>
<td>( \beta )- tubulin</td>
<td>mean</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>( t )</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>( NS )</td>
<td></td>
</tr>
<tr>
<td>Caveolin-1</td>
<td>mean</td>
<td>-0.01</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>( t )</td>
<td>0.10</td>
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<tr>
<td></td>
<td>( P&lt;0.05 )</td>
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<tr>
<td>Caveolin-1</td>
<td>mean</td>
<td>Not detectable</td>
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<tr>
<td>phosphorilated</td>
<td>SE</td>
<td></td>
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<tr>
<td></td>
<td>( t )</td>
<td></td>
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<tr>
<td></td>
<td>( P&lt;0.05 )</td>
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<tr>
<td>CD31</td>
<td>mean</td>
<td>3.72</td>
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<tr>
<td></td>
<td>SE</td>
<td>0.83</td>
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<tr>
<td></td>
<td>( t )</td>
<td>4.49</td>
</tr>
<tr>
<td></td>
<td>( P&lt;0.05 )</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1

CONTROL

INTERSTITIAL EDEMA
Fig. 3

A

Endothelial cell volume vs. Interstitial volume

\[ y = 3.7x - 0.28 \]

\[ R^2 = 0.66 \]

B

Epithelial cell volume vs. Interstitial volume

\[ y = 1.3x + 7.4 \]

\[ R^2 = 0.36 \]
Fig. 4

$y = 20.47 + 2.24 \ln(x)$

$R^2 = 0.83$
Fig. 5

\[ y = 0.011x + 0.88 \]

\[ R^2 = 0.76 \]
Fig. 6

A

Endothelial Surface/volume

\[ y = 2.5e^{-0.0251x} \]

\[ R^2 = 0.86 \]

Endothelial cell volume

B

Lum/Int. Surface profile

(\(V/V_0\))^{0.3521}\n
Iso-shape

Endothelial cell volume
Fig. 7
Fig. 8
<table>
<thead>
<tr>
<th></th>
<th>PMC</th>
<th>PME</th>
<th>CONTROL</th>
<th>DRF</th>
<th>HDFs</th>
<th>EDEMA</th>
<th>DRF</th>
<th>HDFs</th>
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<tr>
<td>80 KDa</td>
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<tr>
<td>55 KDa</td>
<td>alfa-TUB</td>
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<tr>
<td>55 KDa</td>
<td>beta-TUB</td>
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<tr>
<td>21 KDa</td>
<td>CAV-1</td>
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<tr>
<td>21 KDa</td>
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<td>N.D.</td>
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<tr>
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</tbody>
</table>

**Fig. 9**