

Apical rigidity of an epithelial cell monolayer evaluated by magnetic twisting cytometry: ICAM-1 *versus* integrin linkages to F-actin structure

Emmanuelle Planus, Stéphane Galiacy*, Sophie Féréol, Redouane Fodil,
Valérie M. Laurent**, Marie-Pia d'Ortho and Daniel Isabey***

INSERM UMR 651 (ex 492), Fonctions Cellulaires et Moléculaires de l'Appareil Respiratoire et des Vaisseaux, Faculté de Médecine et des Sciences, Université Paris XII - 8, rue du Général Sarraill, 94010 Créteil cedex, France

Abstract. Using Magnetic Twisting Cytometry (MTC) technique, we attempted to characterize *in vitro* the rigidity of the lining tissue covering the lung alveolar wall from its apical face. We purposely used a cellular model constituted by a monolayer of human alveolar epithelial cell (A549) over which microbeads, fixed to InterCellular Adhesion Molecule (ICAM-1), exert a controlled mechanical stress. ICAM-1 expression was induced by Tumor Necrosis Factor- α (TNF- α). Rigidity measurements, performed in the course of cytochalasin D depolymerization, reveal the force transmitter role of the transmembrane receptor ICAM-1 and demonstrate that ICAM-1 and F-actin linkages confers mechanical rigidity to the apical face of the epithelial cell monolayer resembling that provided by integrins. These results confirm the ability of MTC in identifying transmembrane mechanoreceptors in relation with F-actin. Molecular linkages between ICAM-1 and F-actin were observed by spatial visualisations of the structure after double staining of F-actin and anti ICAM-1 antibody through confocal microscopy.

Keywords: Alveolar epithelial cell line, cell mechanics, F-actin structure, ICAM-1, transient magnetic bead twisting cytometry, migration, adhesion, 3D visualisation, cytoskeleton

Abbreviations

Dulbecco's modified Eagles medium, DMEM; human alveolar epithelial cell line, A549; human fibronectine, FN; tumor necrosis factor α , TNF- α ; intercellular adhesion molecule 1, ICAM-1; arginin-glycin-aspartic acid peptide, RGD (in the single-letter amino-acid code); magnetic twisting cytometry device, MTC; bovine serum albumin, BSA; filamentous actin, F-actin; cytochalasin D, Cyto-D; extracellular matrix, ECM; monoclonal antibody, mAb; antibody, Ab.

* Present address: Duke University Medical Center, 368 Nanaline Duke Building, Research DR, Durham, NC 27710, USA.

** Present address: Laboratoire de Neuro-Physique Cellulaire, LNPC, UFR Biomédicale, 75 270 Paris cedex 06, France.

*** Corresponding author: Daniel Isabey, Inserm UMR 651, Equipe Biomécanique Cellulaire et Respiratoire, Faculté de Médecine, 8 rue du Général Sarraill, 94010, Creteil cedex, France. Tel.: +33 1 49 81 37 00; Fax number: +33 1 48 98 17 77; E-mail: daniel.isabey@creteil.inserm.fr.

1. Introduction

The lung ensures a variety of functions as different as gas exchange and scavenging inhaled foreign particles. Among the numerous biochemical factors that control the function, the mechanical factors, i.e., the interactive forces between cells and neighboring tissues (involving the extracellular matrix) and/or the mechanical properties of the cells (including the internal tension), are supposed to play a key role – although not totally understood – in the control of the cellular function. It is recognized for instance that lung ventilation which requires fresh air penetration, generates cyclic physical forces that result in mechanical stretch at the level of alveolar epithelium, which in turn influences some specific lung cell functions such as surfactant secretion by pneumocytes [1–4]. Similarly, during mechanical ventilation of an injured lung, it is thought that cyclic efforts lead to a stress-induced upregulation of the inflammatory response [5] mediated for instance by interleukin (IL-8) produced by both type II pneumocytes [4] and/or macrophages [6]. In general, the elimination of particles and pathogens resident in inspired air implicates mobilization of inflammatory cells which thus participate to the cleaning and sterilization of alveolar lung surface. Noteworthy, the underlying epithelium – which mechanically resists to cyclic deformation – also constitutes the support of migration of mobile inflammatory cells a mechanism of prior interest in terms of lung defense. The role of molecular bridges ensuring the physical linkage between mobile cells and a lining epithelium as well as the mechanical properties of the latter, mostly remains to be understood and evaluated. Clearly identified molecular links between cells are called intercellular adhesion molecule (ICAM) which allow mobile cells to firmly adhere to lining alveolar epithelium and organize their cyto-architecture [7–10]. ICAM's are also known to control the migration of inflammatory cells toward the sites of inflammation [11]. A particular member of this ICAM family: the ICAM-1 (CD54) which is a single-chain 80–114 kDa protein composed of (i) extracellular immunoglobulin-like domains, (ii) a transmembrane spanning region, and (iii) a cytoplasmic tail [12], could thus play an important role in the interactions of mobile cells with their cellular support. Surprisingly, the mechanical properties of the underlying epithelium supporting the immunological synapse in the inflammatory process have never been evaluated through such molecular link. Based on previous studies showing the association of ICAM-1 with actin cytoskeleton of type I pneumocyte, ascertained by the colocalization of ICAM-1 with actin binding proteins, e.g., α -actinin and ezrin [13,14], it can be postulated that the apical face of epithelial cells could offer appropriate mechanical sites for mobile cell interaction thanks to ICAM-1 receptors. Hence, the basic idea of the present study is to evaluate the linkage between ICAM-1 receptors and CSK and the mechanical resistance to deformation conferred by the molecular links available at the surface of the epithelium. This requires to use a cell micromanipulation method which allows a receptor-specific approach from the apical cell surface. The Magnetic Twisting Cytometry (MTC) technique offers such a possibility by applying a controlled magnetic torque through magnetic microbeads coated to specific transmembrane receptors [15–18]. Noteworthy, MTC technique measures cell rigidity through the link between a given receptor and the underlying CSK. To do so, we defined a biological model, i.e., a confluent cell monolayer of human AEC (A549) in culture, over which microbeads, coated with anti-ICAM-1 mAb, mimic the mechanical interactions with mobile cells and exert a controlled mechanical stress specifically through ICAM-1 receptors. The expression of ICAM-1 receptors was induced by TNF- α . Rigidity measurements in the course of cytochalasin D depolymerization revealed the force transmitter role of the transmembrane receptor ICAM-1 and demonstrated that ICAM-1 and F-actin linkages confer a mechanical rigidity to the apical face of epithelial cell monolayer resembling that provided by integrins also linked to F-actin [19]. These results confirm the ability

of MTC in identifying properties of specific transmembrane mechanoreceptors. Molecular linkages between ICAM-1 and F-actin were separately observed by spatial visualisations after double staining of F-actin structure and anti-ICAM-1 antibody through confocal microscopy, confirming MTC results.

2. Materials and methods

2.1. Materials

RGD peptide was obtained from Telios Pharmaceuticals Inc. (CA, USA), monoclonal mouse anti-Human ICAM-1 antibody clone 6.5B5 was obtained from DAKO (Denmark). Recombinant human tumor necrosis factor- α was obtained from Valbiotech, Paris, France. Goat anti-mouse IgG (Fc) Ferro-Magnetic Particles (#FMFc-40-5) and Carboxyl Ferro-Magnetic Particles (#CFM-40-10) were purchased from Spherotech Inc. (IL, USA). Trypsin, EDTA, DMEM, Fetal Calf Serum (FCS), L-glutamine, penicillin, streptomycin, HEPES were purchased from Gibco BRL (Cergy-Pontoise, France). Bovine Serum Albumin (BSA), Cytochalasin D, rhodaminated Phalloidin, Protease Inhibitor Cocktail (#P8340) were purchased from Sigma Chemicals (l'Ile d'Abeau Chêne, France).

2.2. A549 alveolar epithelial cell culture

A549 are human Alveolar Epithelial Cells (AEC) (American Type Culture Collection, Rockville, MD, #CCL-185) from a human lung carcinoma with properties of AEC [20]. Cells were grown to confluence in DMEM containing 10% FCS, 2 mM L-glutamine, 50 IU/ml penicillin, 50 μ g/ml streptomycin, and incubated in a 5% CO₂–95% air atmosphere. Routine subcultures (passages 89 to 92) were done at one-twentieth split ratios by incubation with 0.025 g% trypsin–0.02 g% EDTA in calcium-and-magnesium-free PBS for 10 minutes at 37°C.

2.3. ICAM-1 kinetic expression under TNF α -treated A549 cell monolayer

Cells were grown for 24 h in complete medium and an additional 24 h in serum free–1% BSA medium, on human Fibronectin (FN) coated bacteriologic dishes (96-well and 35 mm Petri dish). Then, 10 ng/ml of TNF- α was added for 24 h. At times 0 h, 4 h, 6 h, 8 h, and 24 h, wells were incubated with microbeads (coated with either RGD peptide or anti-ICAM-1 antibody) as described forward in the text and the remanent magnetic fields of magnetized-attached microbeads were measured. In parallel, proteins from cell monolayers grown on 35 mm Petri dishes coated with FN were extracted, and then separated by SDS-polyacrylamide gel electrophoresis (PAGE; 4–15% acrylamide). The separated proteins were electrophoretically transferred to PVD membrane and after saturation with 1% BSA–5% dry light milk in TBST buffer (0.05 M Tris, 0.15 M NaCl, Tween-20[®] 0.05%, pH 7.6) incubated with the same antibody against ICAM-1 than used to coat microbeads diluted at 1 : 200 in TBS supplemented with 1% BSA–5% dry light milk for 1 hour and was removed by several washing in TBST. Then the developing antibody (goat anti-mouse antibody horse radish peroxidase conjugate) at 1 : 2500 dilution in TBS supplemented with 1% BSA–5% dry light milk was incubated for 30 min. Blot was developed with chemiluminescence system (Amersham) and exposed to film (Kodak).

2.4. Double-staining of F-actin and anti-ICAM-1 antibody coated microbeads in Triton X-100 extracted cell monolayers

A549 cells were plated and grown for 24 hours in the same conditions than described above but on round glass coverslides limited by a plastic tube stick with silicon joint (interior diameter 1.5 cm) and coated with FN at $5 \mu\text{g}/\text{cm}^2$ and then placed in Petri dishes. Then RGD or anti ICAM-1 antibody coated microbeads were added for 20 min at 37°C in presence or not of cyto-D ($1 \mu\text{g}/\text{ml}$). Then, cells were rinsed twice with warm cytoskeleton (CSK) buffer (50 mM NaCl, 300 mM sucrose, 3 mM MgCl_2 , 10 mM EGTA, 10 mM PIPES, pH 6.8) which maintains the integrity of the CSK, as previously described [21] and incubated with or without 0.5% Triton X-100 for 10 min on ice in CSK buffer supplemented with protease inhibitor cocktail diluted at 1/100 and rhodaminated phalloidin ($4 \mu\text{M}$) which stabilized actin fibers systems. After two washes in CSK buffer, cells were fixed immediately in methanol at -20°C for 6 min and rinsed again twice in CSK buffer. Follow 30 min incubation in PBS-1% BSA at RT to saturated unspecific sites. Anti-ICAM-1 mAb coated microbeads were stained with FITC-sheep anti-mouse antibody 1/120 diluted and incubated for 30 min in PBS-1% BSA in humid dark chamber at RT. Then cells were rinsed 3 times in PBS and incubated with $1.5 \mu\text{M}$ rhodaminated phalloidin for additional 15 min. Samples were rinsed three fold in PBS for 15 min, and a last rinse with ddH_2O . To finish, cell monolayers were rinsed and then covered with mounting medium (10% Mowiol 4-88, 25% glycerol in Tris buffer 0.2 M pH 8.5). Samples were stored at 4°C over night before observation by laser confocal microscopy.

2.5. Confocal microscopy and 3D visualisations of the actin CSK

Stained cell monolayers were observed using the LSM 410 invert confocal microscope (Zeiss, Rueil-Malmaison, France). The latter has two internal helium–neon laser and one external argon ion laser. Image processing was performed using LSM 410 software. Fields of cells were randomly selected, brought into focus using either $\times 63/1.25$ numeric aperture Plan Neofluor objective or $\times 100/1.3$ numeric aperture Plan. Epifluorescence was detected for 488 nm excitation and 515–520 nm emission (green) and 543 nm excitation and 570 nm emission (red). A cross sectional image was recorded under confocal conditions and used to establish a plane of focus above the glass surface. Optical cross-sections were recorded at $0.25\text{-}\mu\text{m}$ intervals to reveal intracellular fluorescence. Before 3D visualisation, the stack of gray-level images (8 bits) was subjected to a deconvolution procedure for each wave length, i.e., red and green colors, to correct the distortion caused by the optical system of the confocal microscope. Then, 3D visualisation (as in Fig. 6) was performed using Amira software (Version 3.1.1., TGS Inc., CA, USA).

2.6. Isolation of actin and α -actinin from ICAM-1 bound microbeads

This method was previously described by Plopper and Ingber [22] with some modifications. Cells were plated at the density of 125×10^4 cells/dish in complete medium for 24 hours and then incubated for an additional 24 hours in presence of 10 ng/ml of human $\text{TNF-}\alpha$, cells formed a confluent monolayer expressing ICAM-1. Goat anti-mouse pre-coated magnetic microbeads were coated with anti-human ICAM-1 mAb (clone 6.5B5) following the company procedure (Spherotech). Microbeads were then added to the cells (1 mg per dish) for 20 minutes at 37°C in a 5% CO_2 –95% air incubator. Unbound microbeads were washed away 3 times with serum free medium–1% BSA. Cells with attached microbeads were incubated with a pretreatment of phalloïdine ($10 \mu\text{g}/\text{ml}$) for 30 min in serum free medium–1% BSA to stabilize F-actin structure. Then, cells were removed from the dish with a nonenzymatic cell

dissociation solution (Sigma), and washed 3 times in CSK buffer supplemented with protease inhibitor cocktail diluted at 1/100 by pelleting cells bound to magnetic microbeads with a magnet. On ice, Triton X100 (0.5% in CSK buffer) was added to the cells before to be sonicated and homogenized, then cells were extensively washed to remove cellular structure not intimately associated with microbeads. Lastly, proteins bound to microbeads were extracted, on ice in Rippa buffer and kept at -20°C . Proteins were then separated by SDS-polyacrylamide gel electrophoresis (PAGE; 4–15% acrylamide). The separated proteins were electrophoretically transferred to PVD membrane. Membrane was saturated with 1% BSA–5% dry light milk in TBST buffer (0.05 M Tris, 0.15 M NaCl, Tween-20[®] 0.05%, pH 7.6) and incubated with rabbit antibody against α -actinin (Sigma) or monoclonal anti-actin (Sigma) diluted both at 1 : 500 in TBS supplemented with 1% BSA–5% dry light milk. Then the developing antibody (goat anti-mouse or anti-rabbit antibody horse radish peroxidase conjugate) at 1 : 2500 dilution in TBS supplemented with 1% BSA–5% dry light milk was incubated for 30 min. Blot was developed with chemiluminescence system (Amersham) and exposed to film (Kodak).

2.7. Cellular stiffness measured by magnetic twisting cytometry

Cytoskeleton (CSK) stiffness was assessed by Magnetic Twisting Cytometry (MTC) using a laboratory-made device already described in previous papers [17,18,23–25] mainly similar to the one initially described by Wang et al. [26]. Basically, the change in the projected moment of magnetic beads is continuously measured by a magnetometer while a magnetic torque is applied to a set of about 10^5 microbeads attached to a population of about 5×10^4 adherent living cells. Magnetic microbeads (presently carboxyl chromium-dioxide beads of 4.0–4.5 μm in diameter, Spherotech) are usually coated with arginine–glycine–aspartic acid (RGD in the single-letter amino-acid code) peptide allowing attachment to integrins while we used a coating with monoclonal anti-human ICAM-1 antibody (clone 6.5B5) for comparison with RGD. Cells were plated at the density of 50×10^3 cells/well in complete medium for 24 hours and then incubate for an additional 24 hours in presence of 10 ng/ml of human TNF- α . Before used, coated-beads were incubated in serum free medium supplemented with 1% BSA for at least 30 minutes at 37°C to block non-specific binding. Microbeads were then added to the cells (40 μg per well) for 20 minutes at 37°C in a 5% CO_2 –95% air incubator. Unbound microbeads were washed away with serum free medium–1% BSA supplemented with 25 mM HEPES by means of three successive lavages. A brief 1500 gauss magnetic pulse was applied to magnetize all surface bound microbeads in the same horizontal direction, then a magnetic torque was generated by applying an orthogonal uniform magnetic field (4.2 mT). Associated changes in angular strain of the microbeads were measured by an on-line magnetometer and subsequently transformed into analogic data with an acquisition system (AcqknowledgeIII[®], BIOPAC Inc., CA, USA). Apparent stiffness was defined as the ratio of stress (proportional to torque divided by microbead volume) to angular strain. This ratio measures the cell capability to resist to a local deformation, i.e., the CSK stiffness. Cytochalasin D (Cyto-D) was added for 20 minutes and cell stiffness measured again, then Cyto-D was flushed away and replaced by fresh serum free medium–1% BSA, and after 20 min additional measures were performed.

A problem with PBM-MTC shown by Fabry et al. [27] is that unattached microbeads rotating freely in the culture medium tend to have a higher weight than firmly attached microbeads especially at low torque value where the microbead rotation is minimal. This is due to the nonlinearity of the arc cosine function required to convert the change in mean projected magnetic moment into the average bead deviation (e.g., $\theta = \arccos(B_0/B_{\text{eq}})$, see also [18]). The standard procedure used to attach the microbeads to cultured cells involves adding the microbeads to the cells for 30 min at 37°C in a 5% CO_2 –95% air incubator

then washing away the unbound microbeads with serum-free culture medium containing 1% bovine serum albumin. In agreement with the standard protocol, we used three successive washings to ensure that unbound microbeads were eliminated from culture wells. Experiments on 20 wells with cultured epithelial cells showed that neither three repeated washing procedures nor a given microbead twisting significantly affected the remnant field B_0 or the rigidity value [49].

3. Results

3.1. *In vitro* characterization of the human alveolar epithelial cell monolayer

The *in vitro* model of human alveolar epithelium surface over which mobile cells adhere and migrate was a monolayer culture of human AEC (A549) at confluence. The substrate was a glass coated with human fibronectin (FN) over which cells could adhere (see Materials and methods). Under these model conditions, cell layer was tight and flat and the cells were cubical as it can be seen on fluorescence images presented below (Fig. 3). To mimic the alveolar space in which the adhesion of inflammatory cells on the alveolar epithelial cell layer would occur through adhesion receptors ICAM-1 largely expressed on the epithelium apical surface [7], we induced ICAM-1 expression at the apical cell surface of cultured cells by a TNF- α treatment, i.e., 10 ml^{-1} . This allowed anti-human ICAM-1 mAb coated microbeads to be firmly attached to epithelial cells thus mimicking the attachment of inflammatory cells to the alveolar epithelial cell surface. We found an increase in ICAM-1 expression as duration of TNF- α treatment was increased (Fig. 1). The found time-dependence of ICAM-1 expression was assessed at 0, 4, 6, 8 and 24 hours of TNF- α treatment from (i) measurements of magnitude of the remanent magnetic field of microbeads coated with anti-human ICAM-1 mAb and specifically fixed to ICAM-1 receptors (Fig. 1A), (ii) evaluation by western-blot analysis (Fig. 1B). The remanent magnetic field shown in Fig. 1A actually reflects the amount of anti-ICAM-1 mAb-coated microbeads bound to specific ICAM-1 receptors at the apical cell surface. We preliminary verified from competition experiments that using soluble anti-ICAM-1 mAb showed inhibition of anti-ICAM-1 coated beads fixation over the cell surface and TNF- α untreated confluent A549 cell cultures were unable to fix such specifically coated microbeads. This is similar to Thornhill et al. [28] who have previously shown an inhibition of T-cell adhesion to human endothelial cells treated by TNF- α when using this specific monoclonal antibody. These experiments revealed that between 6 to 8 h of TNF- α treatment, cell cultures efficiently expressed a relatively stable amount of ICAM-1 receptors, while, at 24 h, the expression of ICAM-1 became higher. This is because the increased quantity of ICAM-1 at this longer time mainly resulted from cell proliferation secondary to TNF- α treatment. The cell culture after 24 h TNF- α treatment condition (reference condition) was chosen to study the specific alveolar epithelial cell monolayer response through apical ICAM-1 membrane receptors.

3.2. *Apical rigidity of the alveolar epithelial cell monolayer*

Microbeads bound to ICAM-1 receptors at the apical face of the alveolar epithelial cell monolayer were twisted with a known magnetic torque revealing the relative change in apparent rigidity of the underlying CSK (at equilibrium, i.e., after about a minute of torque application) (Fig. 2A). The relative change in apparent rigidity of the same epithelial cell monolayer has also be measured through integrins for comparison (Fig. 2B). The effect of depolymerizing filamentous actin with cytochalasin D resulted in a marked decrease in cellular rigidity for both receptors. However, the cyto-D decrease in rigidity

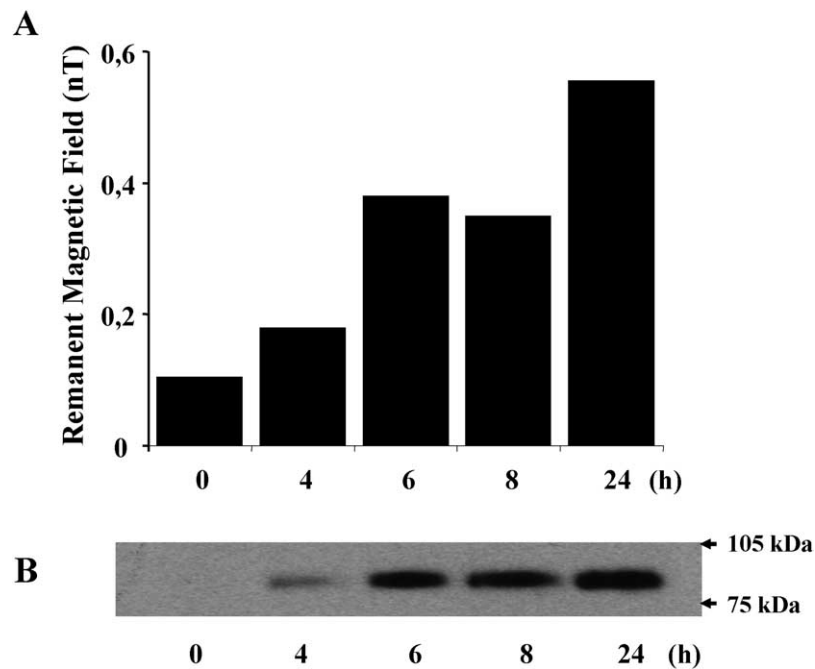


Fig. 1. Amount of anti-ICAM-1 mAb-coated microbeads bound to specific receptors at the apical cell surface, and ICAM-1 kinetic expression induced under $\text{TNF}\alpha$ -treated AEC monolayer. At times 0, 4, 6, 8, and 24 h, AEC monolayer was incubated with anti ICAM-1 antibody coated microbeads. (A) The remanent magnetic fields of magnetized-attached microbeads on ICAM-1 receptors were measured with a magnetometer: increased signal when time exposure of $\text{TNF}\alpha$ increased reveal an increasing number of microbeads capable to bind ICAM-1 receptors at the cell surface. In parallel, (B) proteins from AEC monolayers were extracted at the same times of $\text{TNF}\alpha$ exposure, and analyzed by western blotting for ICAM-1 expression and reveal the identical time dependent expression of ICAM-1 under $\text{TNF}\alpha$ treatment.

appeared less important through ICAM-1 receptors (30%) (Fig. 2A) than through integrin receptors (45%) (Fig. 2B). Moreover, when filamentous actin returned to a polymerized state, e.g., 20 min after Cyto-D lavage, CSK rigidity went up above initial values, i.e., before cyto D treatment (Fig. 2A), likely due to the additional recruitment of filamentous actin (CSK remodeling), at the sites where ICAM-1 receptors were clustered under the microbeads. Because ICAM-1 bound magnetic microbeads resulted in a measurable CSK deformations and resistance, it can be thought that alike integrins, ICAM-1 were able to transmit mechanical forces across the membrane in response to the magnetic torque imposed during MTC.

3.3. Structural attachment between the microbead and the cytoskeleton structure in the apical region

To reveal the F-actin CSK structure involved in the mechanical resistance to deformation at the sites where apical cell surface of AEC was probed, colocalization of F-actin and bead binding was performed using double fluorescence staining of filamentous actin and specific bead binding to ICAM-1 (Figs 3 and 4). In Figs 3A and 3B, the AEC were treated with a non-ionic detergent treatment (0.5% Triton X-100 in buffer thought to reproduce intracellular osmolarity and stabilizing actin filaments) which solubilized cell membrane and cytosolic proteins, leaving intact cytoskeletal structure and its associated proteins. Note that in Fig. 3A, ICAM-1 bound microbeads remains present in spite of membrane-detergent treatment because the integrity of the molecular linkage between the apical surface receptors ICAM-1 and

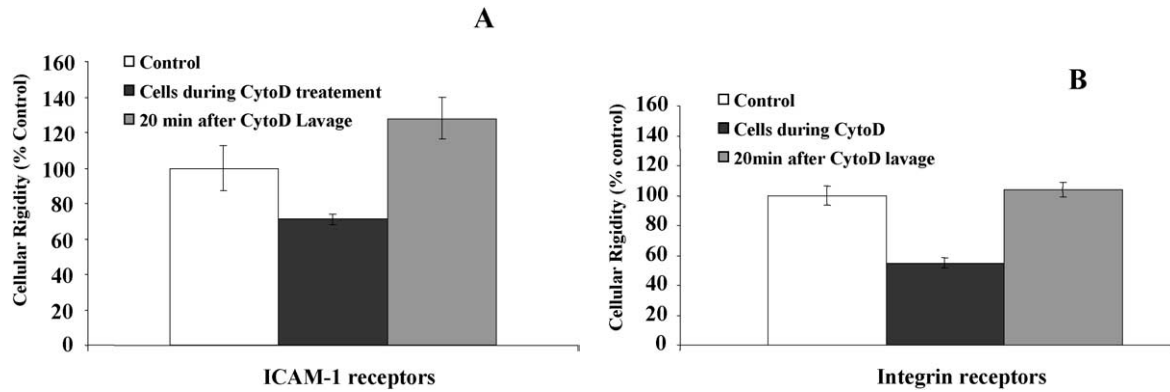


Fig. 2. Relative change in cytoskeleton stiffness of alveolar epithelial cells at confluence was measured through ICAM-1 and integrins using magnetic twisting cytometry using microbeads fixed to (A) ICAM-1 receptors and (B) integrins receptors. Control condition is before cytochalasin D treatment (white column). Addition of F-actin depolymerizing drug (Cyto-D) for 20 minutes (black column) lead to a marked decrease in rigidity for measurements through ICAM-1 receptors and to an even more marked decrease for measurements through integrin receptors, meaning that mechanical forces are transmitted from the microbead to the F-actin cytoskeleton. Replacing cyto-D by fresh serum free medium–1% BSA (gray column) restores after 20 minutes the cellular rigidity which means a re-polymerization of filamentous actin polymers in the cases (ICAM-1 and integrins receptors). Values are means $\pm$ SD ($n = 3$). Similar results were obtained in six others experiments.

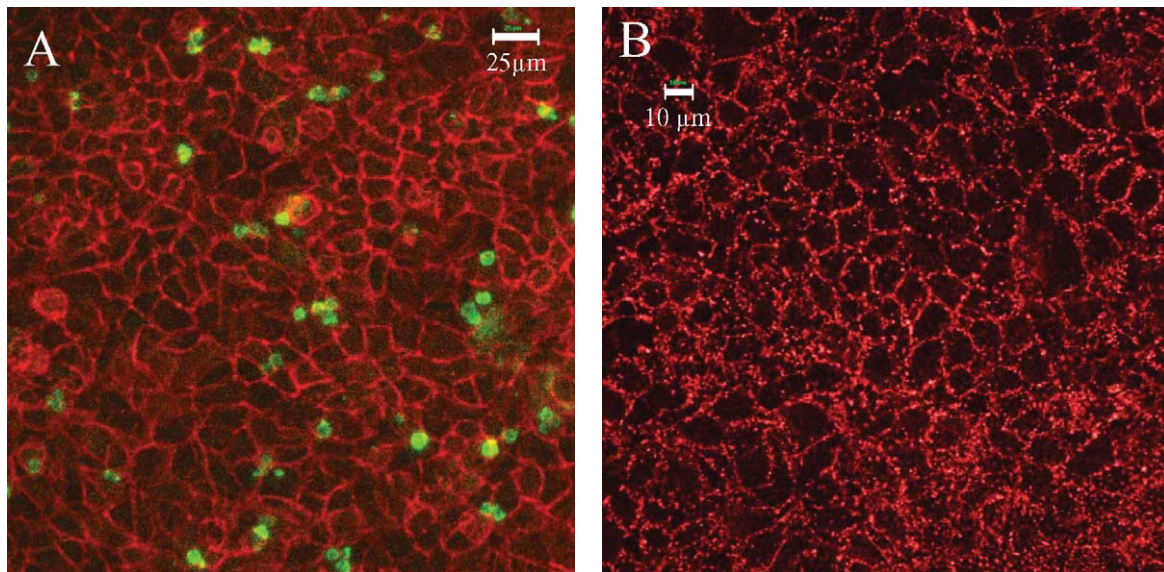


Fig. 3. Double staining of F-actine (in red) and Anti-ICAM-1 mAb coated microbeads (in green) bound to the apical surface of confluent alveolar epithelial cell monolayer in culture. (A) shows a representative microscopic field of detergent treated cell culture (bar represent 25 μ m) revealing attachment of anti-ICAM-1 antibody coated microbeads (green beads) over the AEC monolayer after non ionic membrane-detergent extraction. (B) shows AEC monolayer without bound microbeads revealing that the double treatment with Cyto-D and detergent leaved the cell surfaces free of all anti-ICAM-1 mAb coated microbeads (bars represent 10 μ m), see the loss in polymerized F-actin structure (in B). Images were generated by confocal microscopy in double fluorescence.

F-actin was not spoiled by the treatment. By contrast, Fig. 3B shows that if actin filaments assembly under the ICAM-1 receptors bound microbeads were disconnected by a cyto-D preliminary treatment, at the detergent step, all microbeads were removed from the cell surface. Similar results were found for integrin bound microbeads (data not shown). Finally, the magnetic properties of the ICAM-1 bound microbeads was used to physically isolate the microbeads-associated proteins in the detergent extracted buffer that maintains CSK integrity (see details in Materials and methods) and Western blot analysis of ICAM-1 bound microbeads-extracted protein clearly revealed the presence of actin and actin-associated protein α -actinin (Fig. 5).

The detailed observation of F-actin structure in the neighborhood of bead binding is provided plane by plane in Fig. 4 through a gallery of confocal images shown every 1 μm from the basal (A) to the apical (H) horizontal planes. Images (A–H) show throughout the entire cell height, the intimate linkage between architectural assembly of F-actin CSK and the anti-ICAM-1 mAb ligand coating the beads (Fig. 4). Note that a physical link was found between the F-actin structure and the microbead even above the AEC apical face visible in plane E.

Altogether, these data confirmed that anti-ICAM-1 coated microbeads were mechanically related to sub-membrane F-actin CSK network of AEC monolayer.

4. Discussion

Cell rigidity measurements performed by the magnetic twisting cytometry (MTC) technique reveal that (i) the apical face of an alveolar epithelial cell (AEC) monolayer provides a mechanical resistant support to imposed deformations, (ii) the intercellular adhesion molecule ICAM-1 can mediate the mechanical force transfer across the apical membrane. This is due to the physical link – evidenced by MTC – between the ICAM-1 receptors and F-actin structure of the AEC. This ICAM-1/F actin physical link is also assessed by confocal images and 3D visualisations showing the physical continuity existing between the F-actin structure of the AEC and the anti-ICAM-1 mAb (presently used as bead coating) which specifically binds to ICAM-1 receptors. Thus, beyond the well recognized role of ICAM-1 as an adhesion receptor involved in the lung immune response, we presently assess the mechanical transmitter role of ICAM-1. This is possible because ICAM-1 is (i) on one side, associated to the intracellular F-actin structure and (ii) on the other side, exposed to the extracellular alveolar space. Thus, the F-actin/ICAM-1 molecular mechanical linkages revealed by the present study confer to alveolar epithelium the ability of providing a suitable environment available for, e.g., inflammatory cell interactions. To our knowledge, the mechanical function of ICAM-1/F-actin linkages at the apical face of AEC has never been precisely documented before, although it is a critically important factor in the context of lung immune response. In that sense, the way of understanding the role of ICAM-1 differs from the previous studies which exhibited mainly the role of biochemical transducer for ICAM-1 such as a receptor for cellular recognition, adhesivity and signaling in the immunological synapse. Indeed, the present mechanical approach appears mainly complementary from previous approaches since adhesive function of ICAM-1 likely involves both mechanical and chemical interactions. ICAM-1 and their counterpart integrin receptors LFA-1 on lymphocytes and Mac-1 on macrophages have been described in several cell types, principally in response to inflammatory mediators [29–31]. Moreover, a number of chemical factors, e.g., cytokines and steroid hormones [11,32], mechanical factors, e.g., shear stresses [33] and cell shape factors [8] are known to regulate ICAM-1 expression. Noteworthy, ICAM-1 has been previously recognized for specific and reversible intercellular communication [11,30,34], and a signaling function which

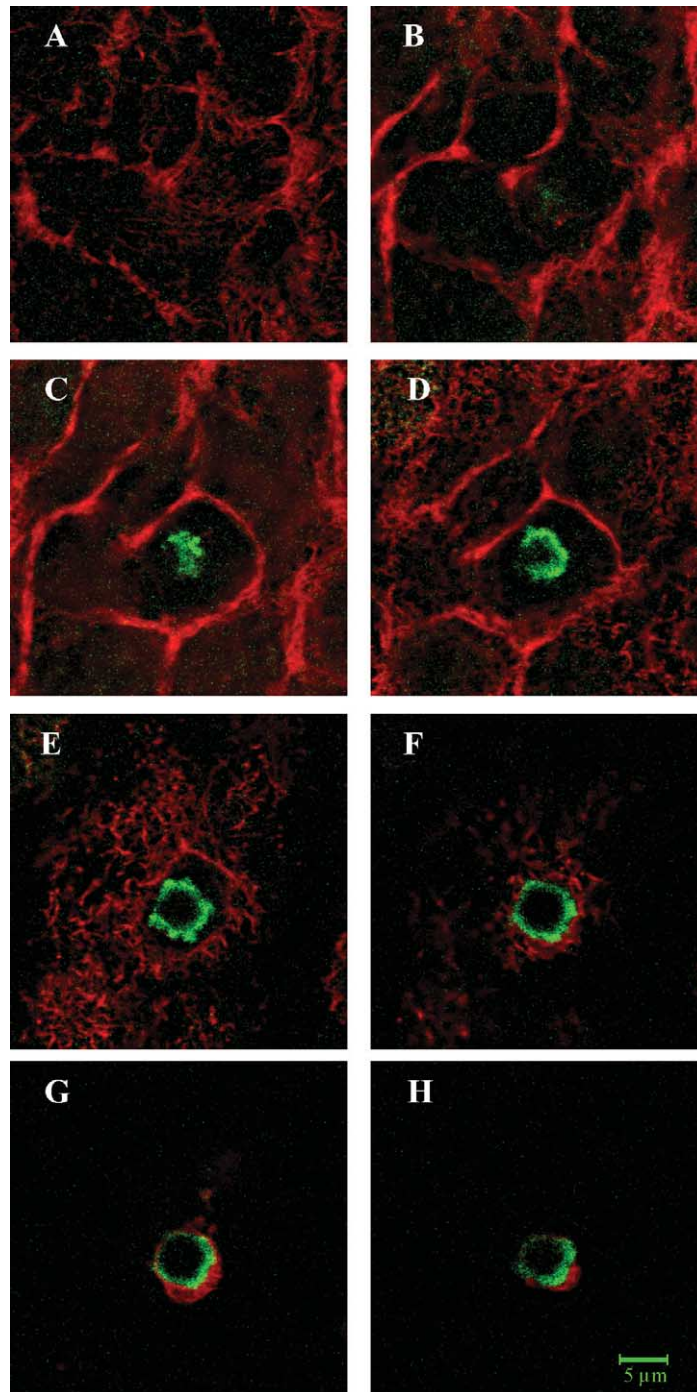


Fig. 4. Optical sections of a representative anti-ICAM-1 mAb coated microbeads bound to AEC. Pictures processing was performed using LSM 410 software and field of cell were randomly selected, brought into focus using a $\times 63/1.25$ numeric aperture Plan Neofluor objective. The gallery of confocal images were generated every $2\ \mu\text{m}$, to reveal intracellular fluorescence, from the basal (A) to the apical cell surface (H) in double fluorescence, revealing the microarchitecture of F-actin network (in red) in the vicinity of bound anti-ICAM-1 mAb coated microbead (in green) over AEC in culture. Bar represents $5\ \mu\text{m}$.

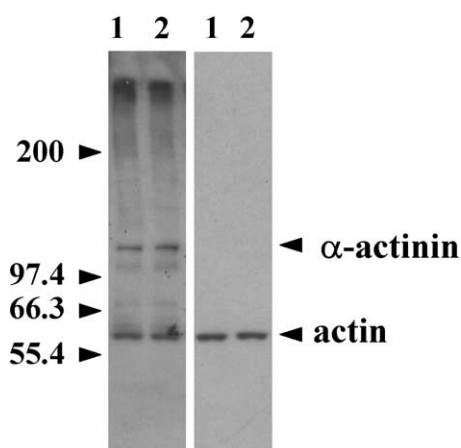


Fig. 5. ICAM-1 bound microbeads-associated proteins. Western blot analysis of proteins bound to the anti-ICAM-1 coated microbeads reveal the presence of actin and α -actinin. Proteins have been revealed here by specific antibodies: (1) anti- α -actinin Ab, (2) anti-actin mAb, were from the components that are insoluble when AEC monolayers with ICAM-1 bound microbeads were delipidated with detergent in buffer stabilizing F-actin network and that mimic cytoplasmic ionic conditions.

consists in transmitting signals from the outer to the inner of the cell [35,36]. Concerning the immune process, interactions between leukocyte integrins and ICAM-1 activated cells have already been shown to play a key role for binding, spreading and locomotion of adhering leukocytes over endothelial cells [34,37–39]. It remains true however that the leukocyte–AEC interactions through ICAM-1 were much less studied than the leukocyte–endothelial cell interactions [40], hence the present study. Accordingly, there are only scarce investigations about cell locomotion process over alveolar epithelium. Nevertheless, it is interesting to note that a broad range of experiments showing leukocyte locomotion has been performed *in vitro* on purified ICAM-1 absorbed to substrates as plastic or glass culture dishes [34]. These substrates necessarily exhibit a rigid mechanical behavior which favors the development of the required CSK tension which also controls cell locomotion. In fact, cellular locomotion is known to depend on micro-environmental interactions between the cell–surface adhesion receptors and the components of the strengthened extracellular support [41]. These interactions result from traction forces generated by the actin–myosin CSK motors mainly located at the sites of adhesion of migrating cells [42]. It is now well established that cellular forces are generated secondary to a double binding mechanism occurring (i) through the extracellular domain of adhesion receptors, to ensure the basolateral attachment of the cell to the matrix components, and (ii) through the cytoplasmic domain of adhesion receptors, to ensure the linkage to the actin CSK elements via focal-adhesion-complex proteins. This process has been extensively described *in vitro* for integrins [43]. Moreover, it has been shown that the net cellular movement on ECM, not only results from forces generated through bounded integrins as described above, but also requires ECM rigidity to strengthen integrin cytoskeletal linkage [44]. The results of the present study suggest that the AEC surface would be sufficiently stiff to permit, through similar binding mechanisms, the migration of inflammatory cells at the sites of adhesion on the apical face of the alveolar epithelium. Present results show that the origin of the mechanical resistance to deformation of the apical face of AEC monolayer resides in the cytoskeletal structure of AEC. This assertion is consistent with the results obtained in endothelial cells, showing that interaction between leukocyte integrins and ICAM-1 on activated cells consolidates binding and promotes spreading and locomotion of adhering cells [34,45].

Magnetic twisting experiments performed through microbeads treated by ligands for transmembrane receptors is confirmed by the present study to be an appropriate technique to reveal the mechanical

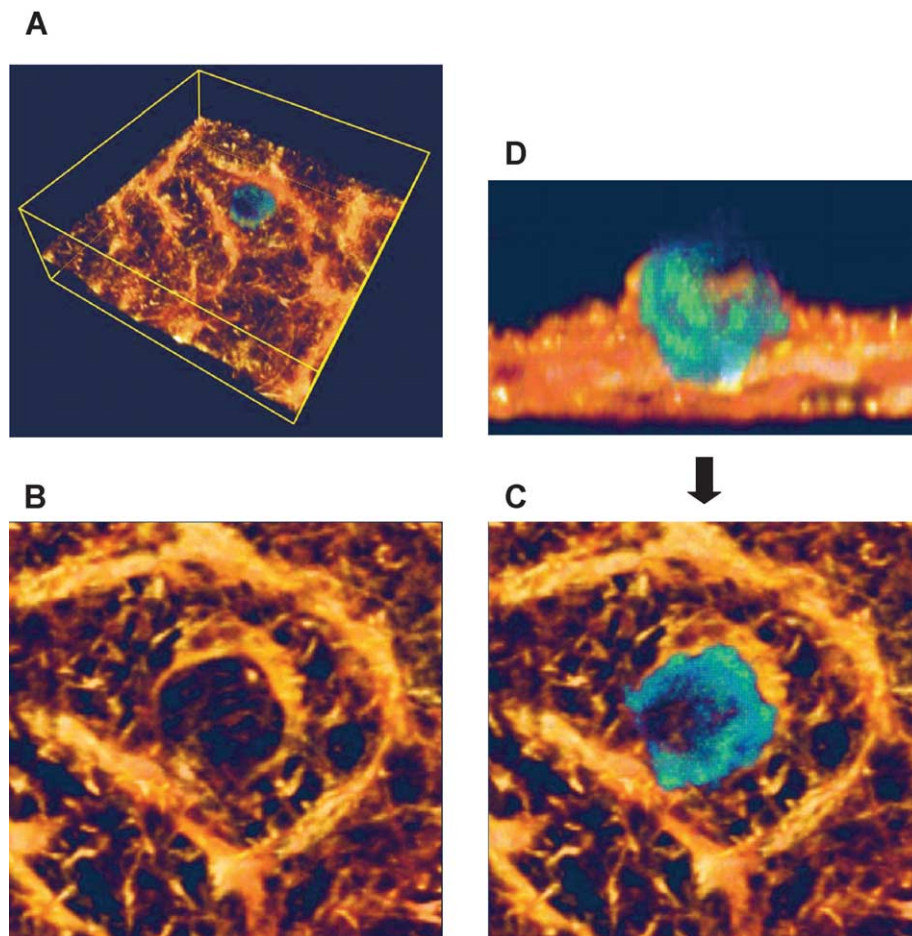


Fig. 6. 3D visualization of F-actin (in yellow) and microbead coating (in green) in AEC monolayer performed from optical planes partially shown in Fig. 4. The microbead size (about $4.5\ \mu\text{m}$) gives an idea of the length scale. These spatial visualizations represent the entire cumulated intensity for the two fluorescence wave length used. (A) Oblique 3D view of the microbead partially immersed in the F-actin structure of a given adherent AEC. (B) Top view of the F-actin reconstructed structure (excluding the microbead coating) and showing the local remodeling of F-actin in response to the microbead attachment to the ICAM-1 receptors. (C) Same as in B but with the microbead coating showing the microbead insertion in the F-actin structure. (D) Vertical cross-section (viewed from the side indicated by arrow in (C)) showing how the microbead was inserted in the F-actin structure.

nature of the cell-to-cell interactions as well as to study the specificity of these interactions. This is because the twisted microbeads sense the mechanical CSK properties of AEC, through the specific terminations that adhesion points constitute. Note that, previous studies in highly adherent/not highly mobile cells, e.g., adherent endothelial cells or fibroblasts [44] or AECs [25] have shown that the amount of cellular rigidity depended closely on the strength of cell-matrix interaction as well as on cytoskeletal tone. We postulate however that these factors might be less important in the less tensed/rapidly moving inflammatory cells, meaning that properties of inflammatory cells would be less important than those of highly adherent cells for the migration function. This means that previous models used to describe the mechanical behavior of attached/tensed cells such as adherent epithelial cells could not be adequate to describe the behavior of mobile cells [17,46,47]. It is noteworthy that most mobile cell types, notably

leukocytes, exert the weakest traction, while the contractile strength of fibroblasts is very much larger than that needed for cell locomotion [48]. Say differently, mobile cells require only weak traction forces and thus low forces applied on the support. Since the magnetic twisting experiments is known to produce an intermediate amount of cellular deformation, i.e., deformation is 0.6–1.4 μm for a range of bead rotation of 15–35° and imposed torques in the range 400–1200 pN at bead equilibrium [18,49], we can postulate that mobile cells would generate much smaller deformations than MTC. Noteworthy, the similar magnitude of cellular rigidity found through integrins on the one hand and ICAM-1 on the other suggest that the CSK structure that sustains the apical face of the AEC monolayer is about the same whatever the molecular bridge used for migration.

In conclusion, we have characterized the physical link existing between ICAM-1 and the underlying epithelial actin CSK meshwork of an AEC monolayer, and postulate that this ICAM-1/F-actin molecular link confers rigidity and mechanical support for inflammatory cells interaction and locomotion over epithelial cells in the alveolar space. Noteworthy, the magnetic twisting cytometry is confirmed to be a pertinent method to assess the mechanical interactions between specific transmembrane receptors and the underlying cytoskeleton and to quantify the resistance to deformation of the complex cellular substrate (epithelium, endothelium) used for inflammatory cell migration.

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