Published in final edited form as:

Microvasc Res. 2008 April ; 75(3): 391–402. doi:10.1016/j.mvr.2007.10.006.

Heterogeneity of Barrier Function in the Lung Reflects Diversity in Endothelial Cell Junctions

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Abstract

Endothelial cells assemble unique barriers that confer specific permeability requirements at different vascular segments. We examined lung microvascular and artery endothelial cells to gain insight into mechanisms for segment-specific barrier functions. Transendothelial electrical resistance was significantly higher in microvascular barriers, and a 50% reduction in barrier function required 5-fold higher concentration of cytochalasin D in the microvascular compared to the arterial barrier. Transcriptional profiling studies identified N-cadherin and activated leukocyte cell adhesion molecule (ALCAM) to be most highly expressed in microvascular than in pulmonary artery endothelial cells. ALCAM was detected in microvascular endothelial cells in the alveolar septum but not in endothelial cells in larger pulmonary vessels in situ. This pattern was retained in culture as ALCAM was recruited to cell junctions in pulmonary microvascular endothelial cells but remained predominantly cytosolic in pulmonary artery endothelial cells. Confocal analysis revealed ALCAM in the lateral plasma membrane domain where it co-localized with N- and VE-cadherin. This finding was supported by co-immunoprecipitation studies demonstrating the presence of ALCAM in multiple adherens junction protein complexes. These functional, biophysical and molecular findings suggest specialization of the adherens junction as a basis for a highly restrictive endothelial barrier to control fluid flux into the alveolar airspace.

Keywords

Adherens junction; ALCAM; barrier function; N-cadherin

Introduction

The vascular system is a complex network of conduit and microvascular vessels exposed to different organ segments with unique local requirements for plasma protein, fluid, and leukocytes.(Butcher et al., 1980; Cavender, 1990; Leach, 2002; Leach et al., 2002; Thurston et al., 2000) Endothelial cells lining the lumen of these vessels assemble barriers that control the passage of circulating blood constituents into the interstitium.(Patterson and Lum, 2001) This suggests endothelial barriers are likely specialized to confer segment-specific phenotypes. The pulmonary microcirculation receives the entire blood volume as a requirement for saturating blood with oxygen. It possesses a relatively large surface area equal to the capillary

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surface area of the rest of the body ($\sim 70 \text{ m}^2$), which facilitates this process. Fluid homeostasis in this vascular bed and the adjoining alveolar airspace is therefore critical for perfusion of oxygen into the local circulation and supply of oxygen to all tissues and organs in the body. (Crandall et al., 1983) It is clear that the microanatomy of the blood-air barrier measuring only 0.1 micron thick along most of its border is adapted for this unique function, and part of this specialization is most likely the intercellular junctions that maintain barrier integrity.

Indeed, there is a preponderance of functional data indicating lung microvascular endothelial cells possess tight permeability barriers. (Chetham et al., 1999; Kelly et al., 1998; Moore et al., 1998; Parker and Yoshikawa, 2002) Protein and fluid conductance per unit surface area is significantly lower in the lung's microcirculation than in the pulmonary artery. (Parker and Yoshikawa, 2002) Studies in isolated rat lung preparations and monolayers of cultured lung endothelial cells indicate a more restrictive permeability barrier in pulmonary microvascular endothelial cells (PMVECs) than in pulmonary artery endothelial cells (PAECs). (Chetham et al., 1999; Kelly et al., 1998; Moore et al., 1998) However to date the molecular basis for the unique barrier phenotype in PMVECs has not been elucidated.

Protein and fluid flux across endothelial barriers occurs through paracellular channels between apposed endothelial cells or by a transcellular route involving vesicular transport. (Lum and Malik, 1994; Stevens et al., 2000) Several multi-protein complexes play an important role in regulating paracellular transport. (Lum and Malik, 1994; Stevens et al., 2000) Adherens and tight junctions promote cell-cell adhesion, integrin receptors mediate cell adhesion to intracellular matrix proteins and cytoskeletal structures exert an intracellular outward tension. (Dudek and Garcia, 2001; Gumbiner, 1996; Lum and Malik, 1994; Schnittler, 1998; Stevens et al., 2000) The adherens junction contains vascular endothelial cadherin (VE-cadherin), which is constitutively expressed in all endothelial cells. (Schnittler, 1998) There is hemorrhagic pulmonary edema and death likely due to respiratory distress in mice injected with monoclonal VE-cadherin antibody indicating a dominant role for VE-cadherin and multi-protein complexes containing VE-cadherin in lung permeability. (Corada et al., 1999) It will be important to identify the profile of this multi-protein complex in PMVECs.

In this study, we used biophysical assays to demonstrate unique intercellular interactions in PMVECs and PAECs. Microarray analysis demonstrated PMVECs and PAECs possessed characteristic gene expression profiles for several adhesion molecules. ALCAM, N-cadherin and VE-cadherin were enriched at cell junctions in PMVECs but were either sparsely distributed or lacking in the junctions in PAECs. ALCAM was linked to β -catenin and Dlg confirming its localization in the adherens junction. These findings highlight unique specialization of the adherens junction as a potential mechanism for tightly controlling vascular permeability at the blood-air barrier.

Materials and methods

Antibodies

Generation of primary anti-rabbit ALCAM antibody has previously been described. (Matsumoto et al., 1997) Primary non-conjugated monoclonal antibodies used were anti-ALCAM clone ND4 (a gift from Dr. Sviridov), -ALCAM clone MOG/07 (Novacastra, Newcastle, UK), -VE-cadherin clones ab7047 (Abcam Limited, Cambridge, UK) and F-8 (Santa-Cruz Biotech, Santa Cruz, CA), -beta catenin, -alpha-catenin, -p120 catenin (BD Bioscience Pharmingen, San Diego, CA), -ZO-1 and -N-cadherin (Zymed Laboratories Inc. San Francisco, CA). FITC-conjugated antibodies included anti-ALCAM (Antigenix America, Huntington Station, NY.) and von Willebrand factor (vWF) (Serotec, Raleigh, NC). Horse radish peroxidase (HRP) conjugated antibodies used included anti-mouse and -rabbit IgG

(Santa Cruz Biotech and Jackson Laboratories, West Grove, PA). Alexaflour 488 and 555 and Oregon green 488 phalloidin were from Molecular Probes, (Eugene, OR).

Isolation and culture of rat main pulmonary artery endothelial cells (PAECs)

Main pulmonary arteries were isolated as previously described. (Creighton et al., 2003; Stevens et al., 1999) Briefly, 300-400 g Sprague-Dawley rats were euthanized by an intraperitoneal injection of 50 mg of pentobarbital sodium (Nembutal, Abbott Laboratories, Chicago, IL). The heart and lungs were excised en bloc after sternotomy and the mainstem pulmonary artery and two vessel generations were isolated and removed. The artery was inverted and the intimal lining was carefully scraped using a scalpel. Harvested cells were then placed into T25 flasks (Corning Inc., Corning, NY) containing F12 Nutrient Mixture and Dulbecco's modified eagle medium (DMEM) (Dulbecco's Modified Eagle Medium, Gibco BRL) mixture (1:1) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL, Grand Island, NY) and passed up to 15 times. The endothelial cell phenotype was confirmed by acetylated LDL uptake, Factor VIII-Rag immunocytochemical staining, and the absence of immunostaining with smooth muscle cell α -actin antibodies.

Isolation of pulmonary microvascular endothelial cells (PMVECs)

PMVECs were isolated and cultured using a modification of a previously described method. (Creighton et al., 2003; Stevens et al., 1999) Male Sprague-Dawley rats (300-400 g) were euthanized by intraperitoneal injection of 50 mg of pentobarbital sodium (Nembutal, Abbott Laboratories). After sternotomy, the heart and lungs were removed en bloc and placed in a DMEM bath containing 90 µg/ml penicillin and streptomycin. Thin strips were removed from the lung periphery adjacent to the pleural surface, finely minced, and transferred with 2-3 ml DMEM to a 15-ml conical tube containing 3-ml digestion solution. [0.5 g BSA, 10,000 units type 2 collagenase (Worthington Biochemical Co, Lakewood, NJ), and cmf-PBS (Gibco BRL) to make 10 ml total volume]. The digestion mixture was allowed to incubate at 37°C for 15 min before pouring through an 80-mesh sieve into a sterile 200-ml beaker. An additional 5 ml of normal medium [10% FBS (Fetal Bovine Serum, Hyclone, Logan, UT) with 30 µg/ml penicillin and streptomycin in DMEM] was used to wash the sieve. The isolation mixture was transferred to a 15 ml conical tube and centrifuged at $300 \times g$ for 5 min, the medium aspirated, and the cells resuspended with 5 ml complete medium [1 part microvascular conditioned medium: three parts incomplete medium (80% RPMI 1640, 20% FBS, 12.3 units/ml Heparin (Elkins-Sinn, Cherry Hill, NJ), and 6.7 µg/ml Endogro (Vec Technologies, Rensselaer, NY) with 30 µg/ml penicillin and streptomycin]. Centrifugation/aspiration was repeated, the cells resuspended in 2-3 ml complete medium and allowed to incubate at 37°C for 30 min before being placed drop wise onto 35-mm culture dishes. After 1 h at 37°C with 5% CO₂, 3 ml of complete medium was added. The dishes were checked daily for contaminating cells that were removed by scraping and aspiration. Endothelial cell colonies were isolated with cloning rings, trypsinized, re-suspended in 100 µl complete medium and placed as a drop in the center of a T-25 flask. The cells were allowed to attach (1 h at 37°C with 5% CO₂) before the addition of 5 ml complete medium. Cultures were characterized using, uptake of 1,1'-dioctadecyl-3, 3,3', 3'-tetramethylindocarbocyanine-labeled low-density lipoprotein (DiI-acetylated LDL), a lectin-binding panel and were routinely passaged by scraping.

Transendothelial electrical resistance

Endothelial cells were seeded on polycarbonate wells containing small evaporated gold microelectrodes (10^{-3} cm²) in series with a large gold counter-electrode and electrical resistance measured utilizing an electrical cell-substrate impedance sensing (ECIS) system (Applied Biophysics Inc., Troy, NY). Current was applied across the electrodes by a 4000-Hz AC voltage source with amplitude of 1 V in series with a 1-MΩ resistance to approximate a

constant current source ($\sim 1 \mu\text{A}$). Electrical resistance was monitored for 30 min to establish a baseline resistance followed by real time transcellular resistance measurements after applying cytochalasin D. TER was recorded for an additional 4 hour period and data from each microelectrode normalized as fractional resistance and plotted versus time. Differences in TER were measured with the GraphPad software using two-tailed student's t-test. Differences were considered to be significant when a p value less than 0.05 was obtained.

Transmission Electron microscopy (TEM)

PAECs and PMVECs were seeded (PMVEC density 2.7×10^5 ; PAEC density 6.7×10^5) onto $0.4 \mu\text{m}$ polycarbonate membranes (Nunc, Naperville, IL) and grown for 4 days to confluence. Cultures were fixed in 3% glutaraldehyde in cacodylate buffer, rinsed in cacodylate buffer, and post-fixed for 30 min with 1% osmium tetroxide. The cells were dehydrated using a graded alcohol series. Portions of the filters were embedded in PolyBed 812 Resin (Polysciences Inc., Warrington, PA). Thick sections ($1 \mu\text{m}$) were cut with glass knives and stained with 1% toluidine blue. Thin sections (80 nm) were cut with a diamond knife and then stained with uranyl acetate and Reynold's lead citrate. Cultures were examined and photographed using a Philips CM 100 transmission electron microscope (FEI Company, Hillsboro, OR).

Scanning electron microscopy (SEM)

Endothelial cell cultures for SEM were fixed in 3% glutaraldehyde in cacodylate buffer, rinsed in cacodylate buffer, and post-fixed for 30 min with 1% osmium tetroxide. The cells were dehydrated using a graded ethanol series and dried using hexamethyldisilazane (Ted Pella, Inc. Redding, CA). The coverslips with cells were attached to aluminum stubs using double stick carbon tabs and sputter coated with gold-palladium. Evaluation and photographs were made using a Phillips XL20 scanning electron microscope (FEI Company, Hillsboro, OR).

DNA microarray

Analysis of mRNA expression levels was performed using Affymetrix DNA microarray filters (Affymetrix, Santa Clara, CA). Total RNA was extracted from cultured PMVECs and PAECs using the RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). Fifteen micrograms of total RNA was collected from each sample and evaluated individually. Twenty micrograms of the labeled cRNA mixture was applied to the GeneChip microarray analysis as described previously. (Geraci et al., 2001) Samples were hybridized to the Affymetrix Rat Genome U34A arrays. Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described. (Lockhart et al., 1996)

Quantitative PCR

cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) and oligothymidine primers at 42°C for 60 min. For real-time PCR reactions, one hundredth volume of the cDNA product was combined with $12.5 \mu\text{l}$ of Quantitect Syber Green I buffer (Qiagen, Valencia, CA) and 10 pmol of gene-specific oligonucleotide primer set. The reaction volume was raised to $25 \mu\text{l}$ with sterile distilled water and PCR amplifications performed using the iCycler system (Bio-Rad, Hercules, CA) as we have previously described. (Pace et al., 2004) The reaction mixture was heated at 95°C for 10 min followed by 40 cycles of denaturation at 95°C and annealing at 56°C . Data was acquired at the end of each annealing step and melt-curve analysis performed to ensure each PCR reactions yielded a single DNA product. Serially diluted cDNA samples were used to generate a standard curve for each reaction, which was linear within 5-log scales.

Cell fractionation and Immunoblotting

Endothelial cells seeded in 35-mm culture dishes were rinsed three times with ice cold phosphate buffered saline supplemented with 1% complete protease inhibitor cocktail (PIC) (Roche, Indianapolis, IN) and 1mM PMSF, covered with 250 µl of ice-cold Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) containing 1% triton X-100 (v/v) supplemented with fresh 1mM PMSF and 1% PIC and incubated on ice for 30 min. Homogenate was clarified by centrifugation at 13,000 rpm for 15 min at 4°C, and soluble proteins quantified using a Lowry protein assay (Sigma, St Louis, MO). Thirty micrograms of cellular protein was combined with Laemmli buffer (Sigma, St Louis), boiled for 2 min and resolved by electrophoresis on a 10% polyacrylamide gel. Samples were blotted onto nitrocellulose membranes, probed with antibodies, and protein bands identified by chemiluminescence (Fujifilm LAS-1000 plus quantitative imaging system, FujiFilm, Valhalla, NY).

Immunocytochemistry

Endothelial cells seeded on glass coverslips were fixed with 2% paraformaldehyde or methanol and blocked with 2% normal goat serum for 10 min and stained for surface adhesion molecules. Where indicated the same slides were permeabilized with 0.1% triton X-100 (v/v), and stained for cytoplasmic plaque proteins. Cells were then mounted with Dako Fluorescent mounting media (Dako, Carpinteria, CA). For negative control, the step with primary antibody was omitted, and no specific immunoreactivity was detected in those slides. Stained cells were visualized using a laser confocal scanning microscope (Leica TCS SP2, Leica, Exton, PA) and by epifluorescence (Nikon TE2000, Nikon Instruments Inc., Melville, NY).

Immunohistochemistry

Sprague Dawley rats were anesthetized and lungs perfusion fixed with 70% ethanol. Lungs were processed and embedded in paraffin. Sections (4-5 µm) were rehydrated with sequential alcohol washes and blocked with 3% normal goat serum for 30 min, followed by staining initially for ALCAM and then vWF. Slides were mounted with Dako Fluorescent mounting media (Dako, Carpinteria, CA). For negative controls, the step with primary antibody was omitted, and no specific immunoreactivity was detected in these tissue sections.

Immunoprecipitations

Total cellular protein (500µg) was diluted to a concentration of 1mg/ml with lysis/wash buffer containing 10% NP-40 and 2.5% deoxycholic acid (Upstate, Lake Placid, NY) treated with 50 µl of pansorbin (Calbiochem, San Diego, CA) for 2 hours by gentle rotation at room temperature. The sample was centrifuged and the pre-cleared lysates combined with primary antibody, normal rabbit serum, mouse or rabbit IgG (4µg). Antibody Capture Affinity Ligand (1µg; Upstate Lake Placid, NY) was added to lysate/antibody mix in a spin column, and immunoprecipitation performed by gentle rotation of the spin column at room temperature for 1 hr. The immunoprecipitate was pelleted, washed five times with lysis/wash buffer, and released from the ligand with 60 µl of elution buffer (Upstate). 5-10 µl of the immunoprecipitate was combined with an equal volume of Laemmli buffer (Sigma, St Louis, MO) and blotted as described above.

Results

Lung microvascular endothelial cells assemble a more restrictive barrier than their macrovascular counterparts

PMVECs and PAECs were seeded in electrical cell impedance system (ECIS) chambers containing ten evaporated gold electrodes, and cultured for 4-6 days. After confirmation by phase contrast microscopy that the culture was confluent and that each electrode was covered

with a monolayer of endothelial cells, the chamber was inserted into ECIS blocks and TER recorded at 37°C, 5% CO₂ in a humidified chamber. TER was steady for over 20 hours in confluent cultures indicating attainment of stable endothelial barriers. Monolayer TER was significantly higher in PMVECs (1807 ± 28.3 Ohms) than PAECs (1278 ± 18.58 Ohms) ($p < 0.0001$) (Figure 1A). The data shown in Figure 1A represent greater than fifty individual TER measurements each from wells containing ten gold electrodes. It is clear from this data that PMVECs form significantly tighter endothelial barriers than PAECs.

We found no difference in the amount of actin in cell lysates or circumferential actin located at cell junctions in PMVECs and PAECs that would have influenced barrier function (data not shown). This suggested the higher TER in PMVECs was likely due to increased recruitment in the amount of junctional proteins (extracellular or plaque). To investigate this further, confluent endothelial cells seeded on evaporated gold electrodes were treated with a dose range (5 ng/ml to 10 µg/ml) of Cytochalasin D, which is widely used to disrupt barrier function. High concentrations of cytochalasin D (10 µg/ml) reduced monolayer TER by 60% to 70% in both PMVECs and PAECs, however, at doses of 25 ng/ml to 300 ng/ml, cytochalasin D had a differential effect on barrier function. At 50 ng/ml cytochalasin D reduced TER in monolayers consisting of PMVECs by $12.8\% \pm 2.6\%$ compared to $29\% \pm 7.2$ when the monolayer consisted of PAECs (Figure 1B). This difference was significant ($p = 0.002$, $n = 6$) and persisted at higher doses (Figure 1B). A fifty percent decrease in TER relative to the maximum attainable required a 5-fold lower concentration of cytochalasin D for PAECs (48.05 ng/ml) compared to PMVECs (220.7 ng/ml). This suggested a major difference in cell adhesion mechanisms in PMVECs and PAECs.

Morphology of cell junctions in PMVECs and PAEC

By transmission electron microscopy, both PMVECs and PAECs formed characteristic electron dense structures at sites of cell-cell contact (Figure 2A). The adherens junction is constitutive along the length of the endothelial junction (Schnittler, 1998) and was identified in the lateral plasma membrane domain in both cell types as a thin contiguous electron dense band. We found no variation in the thickness or density of this structure in the two cell types. Tight junctions were clearly identified as regions of closest contact in the lateral plasma membrane domain, and were inserted at 2-4 sites in both cell types (Figure 2A). Interestingly, when PMVECs and PAECs were co-cultured and analyzed by SEM, the two populations formed distinct cell populations, and clearly distinguishable sites of heterotypic cell-cell contacts (Figure 2B). PAECs grew in circular colonies which enabled clear identification of the two cell types in co-cultures (Figure 2B left panel). Segregation of PMVECs and PAECs in these co-culture experiments suggested either one or both lung endothelial cells possessed unique recognition molecules.

Transcriptional profile of adhesion molecules in lung endothelial cells

Gene microarray analysis was used to identify molecules involved in the unique cell-cell recognition mechanisms in PMVECs and PAECs. Using an Affymetrix Rat Genome U34A chips we profiled mRNA expression of 7,000 genes in PMVECs and PAECs and screened the results for variability in expression of cadherins, immunoglobulins and integrins. N-cadherin mRNA was 16.5 fold higher in PMVECs than in PAECs (Figure 3). No other classical cadherin including VE-cadherin was differentially expressed, although the amount of mRNA for several protocadherins and occludin, a tight junction transmembrane protein, differed in the two cell types (Figure 3). Among the immunoglobulin superfamily of cell adhesion molecules, activated leukocyte cell adhesion molecule (ALCAM) mRNA was 8.5-fold higher in PMVECs than PAECs. ALCAM was originally cloned from activated leucocytes (Bowen et al., 1995) but has recently been implicated in capillary tube formation (Ohneda et al., 2001) and vessel invasion (Arai et al., 2002). Higher ALCAM mRNA expression in PMVECs was confirmed by

quantitative PCR analyses (data not shown). Integrin α -1 was 2.3-fold more abundant in PMVECs, however, there is no evidence this molecule is involved in cell-cell adhesion. Thus, N-cadherin and ALCAM were the most highly expressed cell adhesion molecules in PMVECs compared to PAECs, however neither molecule had previously been associated with the endothelial cell junction.

Differential expression of junctional proteins in lung endothelial cells

The gene microarray data indicated ALCAM was significantly more abundant in PMVECs than in PAECs and this was confirmed by western blot analysis (Figure 4A). An anti-ALCAM serum reactive band of ~110 kDa was identified in lysates from both PMVECs and PAECs, consistent with expression of the fully mature protein in both lung endothelial cells. Minor bands of lower molecular weight were identified including ~65 kDa band, which is likely the native non-glycosylated species. We expected significantly larger amount of N-cadherin in PMVECs as shown in Figure 4A, based on the microarray data, however, we were surprised to find complete absence of N-cadherin in PAECs. The result shown in Figure 4A is a representative of multiple experiments that consistently showed no evidence for N-cadherin expression in PAECs. Surface expression of VE-cadherin is routinely examined in PMVECs and PAECs, and we have thus far found no difference in the amount of this cadherin in the two cell types, while PMVECs expressed E-cadherin at a marginally higher level than PAECs (data not shown). We next evaluated the amount of cytoplasmic plaque proteins in the adherens junction. Monoclonal antibodies identified ~90 kDa and ~120 kDa bands in cell lysates indicating the expression of β -catenin and p120 catenin respectively in both PMVECs and PAECs. Interestingly, there was relatively small difference in the amount of these junctional cytoplasmic molecules in PMVECs and PAECs, contrary to the marked variation observed for ALCAM and N-cadherin.

Differential recruitment of junctional proteins in PMVECs and PAECs

In confluent PMVECs, ALCAM was concentrated at cell-cell contacts, however, cell junctions in PAECs were virtually devoid of ALCAM (Figure 4B). N-cadherin was clustered at cell junctions in PMVECs but there was no immunoreactivity for N-cadherin in confluent PAECs (data not shown) consistent with the immunoblotting data (Figure 4A). The above data suggested differential recruitment of junctional proteins in PMVECs and PAECs was likely determined by the level of protein expression. To examine this hypothesis more extensively we evaluated sub-cellular localization of p120 and β -catenin, which were only marginally higher in PMVECs. We found no difference in junctional recruitment of p120 in the two cell types, however, β -catenin was more abundant at intercellular junctions in PMVECs than in the junctions in PAECs (Figure 4B). Junctional localization of β -catenin in PAECs was clearly demonstrated in co-cultures doubly stained for β -catenin and ALCAM in which expression of β -catenin and ALCAM at PMVECs junctions appeared comparable (Figure 4B, viii). By contrast, PAECs appeared to exclusively stain for β -catenin due to the significantly low level expression of ALCAM in this cell type (Figure 4B, viii). These data indicated PMVECs possess a unique ability to recruit molecules to the cell junctions, and in addition it demonstrated the level of protein expression *per se* was not a reliable indicator of junctional recruitment in PMVECs and PAECs.

Co-localization between ALCAM and VE-cadherin in PMVECs

As indicated earlier, we routinely screen PMVECs and PAECs for surface expression of VE-cadherin as part of endothelial profiling of cells isolated from the rat lung, and have consistently found no difference in the amount of VE-cadherin in the two cell types. However, the above data, for ALCAM and β -catenin indicated the level of expression was an unreliable indicator of how much protein was recruited to cell junctions. Therefore we evaluated the recruitment

of VE-cadherin in co-cultures of PMVECs and PAECs. As expected VE-cadherin was recruited to cell junctions in both lung endothelial cells, however, VE-cadherin was clearly more visible in PMVECs than PAECs (Figure 5). Both ALCAM and VE-cadherin were readily co-recruited to sites of cell-cell contact in sparse and confluent PMVEC cultures (data not shown). Therefore, differential localization of these and other junctional proteins in PMVECs and PAECs was not due to potential variability in the maturity of the monolayers analyzed. Co-recruitment of VE-cadherin and ALCAM was confirmed on multiple occasions in mono as well as co-cultures, therefore, we evaluated the spatial relationship between the two cell adhesion molecules by analyzing multiple 0.3 μm confocal lateral photo sections of PMVECs. We found both ALCAM and VE-cadherin stained most brightly in the apico- and baso-lateral membrane domains consistent with their localization in regions of cell-cell contact (Figure 6A). In addition, in every 0.3 μm cell section that was analyzed ALCAM and VE-cadherin co-localized. It is worth noting that analysis of significantly thicker (0.4 μm) cell sections have successfully been used to resolve VE-cadherin from other molecules in the endothelial cell junction. (Ayalon et al., 1994) Therefore, we interpreted these confocal results to mean close proximity between ALCAM and VE-cadherin in endothelial cell junctions.

ALCAM is present in a multi-protein adherens junction complex

In light of the above data, we sought to demonstrate physical interaction between ALCAM and VE-cadherin by co-immunoprecipitations. PMVEC lysates were prepared using rabbit anti-ALCAM serum and the immune complexes probed with mouse anti-VE-cadherin and mouse anti- β -catenin monoclonal antibodies by immunoblotting. Single bands of ~130 kDa and ~90 kDa were recognized by the anti-VE-cadherin and anti- β -catenin antibodies, respectively, in the ALCAM immune complex (Figure 6B). These bands were identified as VE-cadherin and β -catenin, respectively, since they were also identified in the VE-cadherin and β -catenin immunocomplexes, but not identified in immunocomplexes prepared using non-immune rabbit serum (data not shown). Intensity of the VE-cadherin band was consistently stronger than the β -catenin band. In the inverse immunoprecipitation experiments we probed for ALCAM in VE-cadherin immune complexes, and identified a ~110 kDa band with anti-ALCAM serum in the VE-cadherin immune complex, and as expected a 90 kDa band for β -catenin (Figure 6B). Next we probed for ALCAM in immune complexes prepared with mouse anti-N-cadherin antibody, using a mouse monoclonal antibody, and found four immunoreactive ALCAM proteins including the mature (~110 kDa) and native (~65 kDa) species. Screening the N-cadherin immune complexes with rabbit anti-ALCAM serum identified only the mature ALCAM species (data not shown). These results indicate ALCAM is part of a multi-protein adherens junction complex in PMVECs containing N- and VE-cadherin.

To examine the extent of ALCAM's association with other validated adherens junction molecules, we probed immune complexes of β -catenin, p120 and Dlg for ALCAM. Ironically, we discovered stronger signals for ALCAM than VE-cadherin in the β -catenin immune complexes (Figure 6C). ALCAM was not identified in immune complexes of p120 catenin, which as expected contained VE-cadherin and β -catenin (Figure 6C). This result indicated ALCAM was most likely recruited to the adherens junction by a trafficking molecule other than p120 catenin. Dlg is well known for trafficking proteins to neuronal cell junctions and has more recently been identified as a critical component of the adherens junctions in several epithelial tissues. (Firestein and Rongo, 2001; Laprise et al., 2004; Peng et al., 2000; Wu et al., 1998) We identified ALCAM in immune complexes prepared with anti-human Dlg antibodies in multiple experiments using rabbit anti-ALCAM serum and mouse monoclonal ALCAM antibody reagents (Figure 6C, and data not shown). Interestingly, junction-associated Dlg redistributes into cytoplasmic vesicles in PMVECs treated with anti-ALCAM serum, which suggest physical coupling between ALCAM and Dlg (data not shown). Collectively, the co-

localization and co-immunoprecipitation data shown here support the conclusion that ALCAM is a component of a multi-protein adherens junction complex in PMVECs.

ALCAM is segmentally regulated in the pulmonary endothelium

Our *in vitro* data identified ALCAM as a novel component of the adherens junction in PMVECs, therefore, it was important to verify its expression in the lung microvascular bed. We stained several tissue sections of rat lungs perfusion fixed with ethanol for ALCAM and vWF. Confocal microscopic imaging revealed multiple ALCAM-positive cell types in the alveolar septum including immunoreactive vWF cells that are likely capillary endothelial cells (Figure 7). Contrary to the positive staining of endothelial cells in the microvascular lung segment, we found no ALCAM immunoreactivity in the pulmonary artery and in endothelial cells in medium-sized vessels (Figure 7,i). This negative staining may be due to relatively low level of ALCAM expression in PAECs *in vivo* as was observed in cultured PAECs *in vitro* (Figure 3 and Figure 4A). Alternatively, it is possible that only the ALCAM species in plasma membrane domains and not the cytoplasmic species found in PAECs were accessible for staining. We identified membranous staining of ALCAM in other cells in the alveolar septum, including macrophages and type I and type II pneumocytes, and have since discovered that expression of ALCAM by these various cells in the alveolar septum is conserved in the human lung (King et al., unpublished data).

Discussion

This study was devised on the principle that endothelial barriers are highly specialized to confer segment-specific permeability phenotypes in different vascular beds. This idea is particularly important in the lung microvascular bed, which contains relatively flat endothelial cells anatomically structured to facilitate efficient alveolar-gas exchange, and likely possess specialized adhesive mechanisms to maintain barrier integrity. To unravel the unique attributes of this barrier we compared morphological, molecular and functional properties of PMVECs with endothelial cells from the pulmonary artery.

The TER results shown in Figure 1A clearly indicated an enhanced barrier phenotype in PMVECs in agreement with a previous report, which demonstrated significantly slower rate of permeability of PMVEC monolayers with dextran solutes, when compared to PAECs. (Kelly et al., 1998) Although TER provides a highly sensitive biophysical assay for assessing the state of cell shape and focal adhesion, there is general agreement that in confluent cell monolayers, TER is predominantly a measure of cell-cell adhesion. (Giaever and Keese, 1993; Schaphorst et al., 2003) Indeed, epithelial cells, which form significantly tighter cell-cell adhesions usually have higher TERs than endothelial cells. (Liu et al., 2002) We therefore interpreted the higher TER in monolayers of PMVECs as confirmation of a tighter cell-cell adhesion mechanism in this cell type compared to PAECs. To address this hypothesis further, we treated lung endothelial cell monolayers with cytochalasin D, a non-receptor agonist that reduces endothelial barrier function (Shasby et al., 1982) by directly affecting VE-cadherin-mediated adhesion. (Baumgartner et al., 2003) Cytochalasin D reduced TER 5-fold more in PAECs than in PMVECs (Figure 1B,C). This finding highlighted the adherens junction as a potential source for the unique barrier phenotypes in the two cell types. However, we have consistently found no difference in surface expression of VE-cadherin in PMVECs and PAECs (data not shown). To reconcile these apparent discordant observations we proposed a model whereby the PMVEC barrier was dominantly controlled by a multi-protein complex containing both VE-cadherin and hitherto unknown adhesion molecules variably expressed in the two cell types.

The concept of unilateral expression of major cell surface adhesion molecule by either one or both lung endothelial cells was supported by the SEM data, which showed clear segregation of PMVECs and PAECs in co-culture experiments (Figure 2B). Transcriptional profiling

studies identified N-cadherin and ALCAM as the most differentially expressed cell adhesion molecule although neither molecule had previously been associated with the endothelial junction. Junctional localization of N-cadherin in PMVECs was contrary to what has previously been observed in other endothelial cells, where N-cadherin is found all over the cell surface, and is believed to be relegated to this position by VE-cadherin.(Dejana, 2004; Navarro et al., 1998; Salomon et al., 1992) Clearly, our data indicate PMVECs perhaps representing a subset of endothelial cells recruit N-cadherin for homotypic cell recognition. Indeed, sub-cellular localization of N-cadherin may be determined by endothelial cell lineage given that the non-junctional N-cadherin paradigm is based on observations made in macrovascular endothelial cells *in vitro* and *in vivo*. (Navarro et al., 1998; Salomon et al., 1992) Future studies using a diversity of endothelial cells will be required to fully resolve this issue. In the context of this study however, it is reasonable to presume that junctional recruitment of N-cadherin contributes to the tighter barrier phenotype in PMVECs.

ALCAM mediates tight adhesion between myeloid progenitors and stromal cells in the bone marrow microenvironment.(Pourquie et al., 1992; Uchida et al., 1997) and has more recently been implicated in tight adhesion of blastocysts to endometrial epithelial cells during implantation. (Fujiwara et al., 2003) Our data indicates ALCAM is located at cell-cell contacts in PMVECs and is therefore likely to increase the strength of homotypic endothelial cell adhesion. Previous studies have demonstrated an essential role for ALCAM in capillary tube formation(Ohneda et al., 2001) and invasion of blood vessels into cartilage,(Arai et al., 2002) which combined with our findings highlighted cellular and functional similarities between ALCAM and VE-cadherin. Using confocal microscopy and co-immunoprecipitation assays we demonstrated co-localization and co-occupancy between ALCAM and VE-cadherin. ALCAM has previously been reported to be co-recruited with E-cadherin to epithelial cell junctions in prostate cell lines transfected with α -catenin.(Tomita et al., 2000) However, to our knowledge this report is the first to demonstrate co-localization between ALCAM and cadherins in endothelial cells.

Once considered a structural domain for cadherins, the adherens junction is now recognized to express immunoglobulins,(Dejana, 2004; Fukuhara et al., 2003; Hoshino et al., 2004; Takai and Nakanishi, 2003) notably nectin,(Fukuhara et al., 2003; Hoshino et al., 2004; Takai and Nakanishi, 2003) which ironically regulates homotypic adhesion of E-cadherin in epithelial cells.(Fukuhara et al., 2003; Hoshino et al., 2004) The implication from our observations was that ALCAM is a component of the adherens junction. This idea was confirmed by demonstrating the presence of ALCAM in immune complexes of VE-cadherin, N-cadherin, and β -catenin. ALCAM was, however, not associated with p120 catenin which is responsible for recruitment and stabilization of N- and VE-cadherin at the plasma membrane.(Chen et al., 2003; Davis et al., 2003; Xiao et al., 2003) There is currently no published data on accessory proteins that regulate membrane localization of ALCAM although the cytoplasmic ALCAM tail contains clusters of positively charged amino acid residues that typically interacts with members of the 4.1/ezrin/radixin/moesin/ (FERM) family.(Hanada et al., 2003; Lue et al., 1994) We found no association between ALCAM and moesin or ezrin by co-immunoprecipitation assays (data not shown). However, ALCAM was present in immune complexes of Dlg, a highly conserved scaffolding molecule in the adherens junction with consensus binding domains for FERM proteins.(Firestein and Rongo, 2001; Laprise et al., 2004; Peng et al., 2000; Wu et al., 1998; Yonemura et al., 1998) This result supports the conclusion that ALCAM is located in the adherens junction and for the first time identifies Dlg in the endothelial adherens junction.

In summary, we have identified a multi-protein complex consisting of ALCAM, N- and VE-cadherin at the adherens junction in lung microvascular endothelial cells. The importance of this complex is evident from the development of hemorrhagic pulmonary edema in mice

administered neutralizing antibodies against VE-cadherin, (Corada et al., 1999) which presumably, triggers disassembly of this multi-protein complex. The need to tightly control fluid flux into the alveolar airspace is critical to maintaining efficient diffusion of gasses across the blood-air barrier. Our data suggests this physiological requirement is met by specialization of the adherens junction in PMVECs, which contains a wide diversity of adhesion molecules precisely at sites of cell-cell apposition, than has previously been appreciated. Future studies will examine how inflammatory agonists that increase pulmonary edema at capillary leak sites compromise ALCAM and N-cadherin.

References

- Arai F, et al. Mesenchymal stem cells in perichondrium express activated leukocyte cell adhesion molecule and participate in bone marrow formation. *J Exp Med* 2002;195:1549–63. [PubMed: 12070283]
- Ayalon O, et al. Spatial and temporal relationships between cadherins and PECAM-1 in cell-cell junctions of human endothelial cells. *J Cell Biol* 1994;126:247–58. [PubMed: 8027182]
- Baumgartner W, et al. Cadherin function probed by laser tweezer and single molecule fluorescence in vascular endothelial cells. *J Cell Sci* 2003;116:1001–11. [PubMed: 12584244]
- Bowen MA, et al. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. *J Exp Med* 1995;181:2213–20. [PubMed: 7760007]
- Butcher EC, et al. Organ specificity of lymphocyte migration: mediation by highly selective lymphocyte interaction with organ-specific determinants on high endothelial venules. *Eur J Immunol* 1980;10:556–61. [PubMed: 6157544]
- Cavender DE. Organ-specific and non-organ-specific lymphocyte receptors for vascular endothelium. *J Invest Dermatol* 1990;94:41S–48S. [PubMed: 2191054]
- Chen X, et al. p120 catenin associates with kinesin and facilitates the transport of cadherin-catenin complexes to intercellular junctions. *J Cell Biol* 2003;163:547–57. [PubMed: 14610057]
- Chetham PM, et al. Segmental regulation of pulmonary vascular permeability by store-operated Ca²⁺ entry. *Am J Physiol* 1999;276:L41–50. [PubMed: 9887054]
- Corada M, et al. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc Natl Acad Sci U S A* 1999;96:9815–20. [PubMed: 10449777]
- Crandall ED, et al. Recent developments in pulmonary edema. *Ann Intern Med* 1983;99:808–22. [PubMed: 6360001]
- Creighton JR, et al. Coordinate regulation of membrane cAMP by Ca²⁺-inhibited adenylyl cyclase and phosphodiesterase activities. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L100–7. [PubMed: 12471013]
- Davis MA, et al. A core function for p120-catenin in cadherin turnover. *J Cell Biol* 2003;163:525–34. [PubMed: 14610055]
- Dejana E. Endothelial cell-cell junctions: happy together. *Nat Rev Mol Cell Biol* 2004;5:261–70. [PubMed: 15071551]
- Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* 2001;91:1487–500. [PubMed: 11568129]
- Firestein BL, Rongo C. DLG-1 is a MAGUK similar to SAP97 and is required for adherens junction formation. *Mol Biol Cell* 2001;12:3465–75. [PubMed: 11694581]
- Fujiwara H, et al. Human blastocysts and endometrial epithelial cells express activated leukocyte cell adhesion molecule (ALCAM/CD166). *J Clin Endocrinol Metab* 2003;88:3437–43. [PubMed: 12843199]
- Fukuhara A, et al. Involvement of nectin-activated Cdc42 small G protein in organization of adherens and tight junctions in Madin-Darby canine kidney cells. *J Biol Chem* 2003;278:51885–93. [PubMed: 14530286]
- Geraci MW, et al. Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis. *Circ Res* 2001;88:555–62. [PubMed: 11282888]

- Giaever I, Keese CR. A morphological biosensor for mammalian cells. *Nature* 1993;366:591–2. [PubMed: 8255299]
- Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 1996;84:345–57. [PubMed: 8608588]
- Hanada T, et al. Protein 4.1-mediated membrane targeting of human discs large in epithelial cells. *J Biol Chem* 2003;278:34445–50. [PubMed: 12807908]
- Hoshino T, et al. A novel role of nectins in inhibition of the E-cadherin-induced activation of Rac and formation of cell-cell adherens junctions. *Mol Biol Cell* 2004;15:1077–88. [PubMed: 14699074]
- Kelly JJ, et al. Pulmonary microvascular and macrovascular endothelial cells: differential regulation of Ca²⁺ and permeability. *Am J Physiol* 1998;274:L810–9. [PubMed: 9612297]
- Laprise P, et al. Human homolog of disc-large is required for adherens junction assembly and differentiation of human intestinal epithelial cells. *J Biol Chem* 2004;279:10157–66. [PubMed: 14699157]
- Leach L. The phenotype of the human materno-fetal endothelial barrier: molecular occupancy of paracellular junctions dictate permeability and angiogenic plasticity. *J Anat* 2002;200:599–606. [PubMed: 12162727]
- Leach L, et al. Vasculogenesis, angiogenesis and the molecular organisation of endothelial junctions in the early human placenta. *J Vasc Res* 2002;39:246–59. [PubMed: 12097823]
- Liu F, et al. Hepatocyte growth factor enhances endothelial cell barrier function and cortical cytoskeletal rearrangement: potential role of glycogen synthase kinase-3beta. *Faseb J* 2002;16:950–62. [PubMed: 12087056]
- Lockhart DJ, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 1996;14:1675–80. [PubMed: 9634850]
- Lue RA, et al. Cloning and characterization of hdlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1. *Proc Natl Acad Sci U S A* 1994;91:9818–22. [PubMed: 7937897]
- Lum H, Malik AB. Regulation of vascular endothelial barrier function. *Am J Physiol* 1994;267:L223–41. [PubMed: 7943249]
- Matsumoto A, et al. Cloning and characterization of HB2, a candidate high density lipoprotein receptor. Sequence homology with members of the immunoglobulin superfamily of membrane proteins. *J Biol Chem* 1997;272:16778–82. [PubMed: 9201982]
- Moore TM, et al. Signal transduction and regulation of lung endothelial cell permeability. Interaction between calcium and cAMP. *Am J Physiol* 1998;275:L203–22. [PubMed: 9700080]
- Navarro P, et al. Differential localization of VE- and N-cadherins in human endothelial cells: VE-cadherin competes with N-cadherin for junctional localization. *J Cell Biol* 1998;140:1475–84. [PubMed: 9508779]
- Ohneda O, et al. ALCAM (CD166): its role in hematopoietic and endothelial development. *Blood* 2001;98:2134–42. [PubMed: 11568000]
- Pace BS, et al. Selective inhibition of beta-globin RNA transcripts by antisense RNA molecules. *Cell Mol Biol (Noisy-le-grand)* 2004;50:43–51. [PubMed: 15040426]
- Parker JC, Yoshikawa S. Vascular segmental permeabilities at high peak inflation pressure in isolated rat lungs. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1203–9. [PubMed: 12388351]
- Patterson CE, Lum H. Update on pulmonary edema: the role and regulation of endothelial barrier function. *Endothelium* 2001;8:75–105. [PubMed: 11572478]
- Peng CY, et al. The tumour-suppressor genes *lgl* and *dlg* regulate basal protein targeting in Drosophila neuroblasts. *Nature* 2000;408:596–600. [PubMed: 11117748]
- Pourquie O, et al. BEN, a surface glycoprotein of the immunoglobulin superfamily, is expressed in a variety of developing systems. *Proc Natl Acad Sci U S A* 1992;89:5261–5. [PubMed: 1608932]
- Salomon D, et al. Extrajunctional distribution of N-cadherin in cultured human endothelial cells. *J Cell Sci* 1992;102(Pt 1):7–17. [PubMed: 1500442]
- Schaphorst KL, et al. Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L258–67. [PubMed: 12626332]

- Schnittler HJ. Structural and functional aspects of intercellular junctions in vascular endothelium. *Basic Res Cardiol* 1998;93(Suppl 3):30–9. [PubMed: 9879442]
- Shasby DM, et al. Role of endothelial cell cytoskeleton in control of endothelial permeability. *Circ Res* 1982;51:657–61. [PubMed: 6890416]
- Stevens T, et al. Control of cAMP in lung endothelial cell phenotypes. Implications for control of barrier function. *Am J Physiol* 1999;277:L119–26. [PubMed: 10409238]
- Stevens T, et al. Mechanisms regulating endothelial cell barrier function. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L419–22. [PubMed: 10956614]
- Takai Y, Nakanishi H. Nectin and afadin: novel organizers of intercellular junctions. *J Cell Sci* 2003;116:17–27. [PubMed: 12456712]
- Thurston G, et al. Determinants of endothelial cell phenotype in venules. *Microcirculation* 2000;7:67–80. [PubMed: 10708338]
- Tomita K, et al. Coordinate recruitment of E-cadherin and ALCAM to cell-cell contacts by alpha-catenin. *Biochem Biophys Res Commun* 2000;267:870–4. [PubMed: 10673383]
- Uchida N, et al. The characterization, molecular cloning, and expression of a novel hematopoietic cell antigen from CD34+ human bone marrow cells. *Blood* 1997;89:2706–16. [PubMed: 9108388]
- Wu H, et al. Subcellular targeting and cytoskeletal attachment of SAP97 to the epithelial lateral membrane. *J Cell Sci* 1998;111(Pt 16):2365–76. [PubMed: 9683631]
- Xiao K, et al. Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. *J Cell Biol* 2003;163:535–45. [PubMed: 14610056]
- Yonemura S, et al. Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. *J Cell Biol* 1998;140:885–95. [PubMed: 9472040]

A

B

C

** $p < 0.005$

* $p < 0.05$

Figure 1. Transendothelial electrical resistance in PMVECs and PAECs

(A) PMVECs and PAECs were seeded at densities of 7,000 and 20,000 respectively in gold evaporated electrodes and cultured to confluency, and TER measured as detailed in the method section. The data shown represent fifty-three measurements from eight independent experiments. (B) PMVECs and PAECs were cultured to confluency on evaporated gold

electrodes as described in methods. After recording basal TER for 30 min, monolayers were challenged with cytochalasin D or DMSO (vehicle) and monitored for 4 hours. No change in TER was recorded in vehicle treated monolayers (n=10), and the mean TER from these control experiments was assigned a reference value of 1.0. Treatment of monolayers with 50 ng/ml cytochalasin D reduced TER to 0.87 ± 0.02 in PMVECs and 0.71 ± 0.07 in PAECs compared to the control, while At 200 ng/ml cytochalasin D, TER was reduced to 0.75 ± 0.01 in PMVECs and 0.54 ± 0.02 in PAECs. The data shown are from six independent experiments. Reductions in TER were significantly higher in PAECs than in PMVECs ($p=0.002$; 50 ng/ml, $p=0.03$; 200 ng/ml). (C) Dose-response curve showing loss of cell-cell adhesion in lung endothelial cells treated with cytochalasin D. High doses of cytochalasin D (2.5 $\mu\text{g/ml}$ to 5 $\mu\text{g/ml}$) reduced TER by 70% in both cell types and this was assigned a loss of cell-cell adhesion reference value of 1.0. Note the EC_{50} is 5-fold higher for PMVECs compared to PAECs.

A **B**

Figure 2. Morphology of lung endothelial cell junctions

(A) TEM analysis revealed PMVECs and PAECs formed typical junctional contacts including an electron dense adherens junction (along most of the cell contact) and tight junctions (arrowheads). (B) Co-culture demonstrating clear segregation of PAECs and PMVECs. Left panel shows three clusters of PAECs separated from a continuous centrally located PMVEC population. Note the circular pattern of the PAEC clusters, which is illustrated in the bottom cluster. (Middle panel) PMVECs on the right show more prominent cell-cell contacts compared

to the less pronounced contacts in PAECs. (Right panel) High magnification image showing homotypic (H) and heterotypic (T) cell contacts between PMVECs and PAECs. All heterotypic cell-cell contacts are indicated with a broken white line.

Figure 3. Transcriptional profile of cell adhesion molecules in PMVECs and PAECs
mRNA species differentially expressed by PMVECs and PAECs. Total RNA samples were hybridized to the Affymetrix Rat Genome U34A arrays, and hybridization signals quantified as described.²² Open and filled bars represent the mean fold increase and decrease, respectively, of mRNA expression in PMVECs compared to PAECs.

Figure 4. Differential expression of junctional proteins in lung endothelial cells

(A) Immunoblotting demonstrates variable amount of junctional proteins in PMVECs and PAECs. Total cell lysate (30 μ g) prepared from confluent cultures were probed for ALCAM, N-cadherin, β -catenin and p120. Molecular weight (MW) markers were identified by chemiluminescence detection of spotted fluorescent dyes. Note relatively low level of ALCAM and lack of N-cadherin expression in PAECs. Equal protein loading was verified by staining gels with Coomassie blue and immunoblotting membranes for eEfl α (data not shown). (B) Differential recruitment of junctional molecules in lung endothelial cells. Confluent monolayers of PMVECs (i, iii, iv) and PAECs (ii, v) were fixed with methanol prior to staining with primary and TRITC (red) or FITC (green) conjugated secondary antibodies, and visualized by epifluorescence. ALCAM was visibly clustered at cell junctions in PMVECs (i), but was predominant in the cytoplasm in PAECs permeabilized with triton X-100 (ii). N-cadherin was clustered at cell junctions in PMVECs (iii), but was not detected in PAECs (data not shown). p120 catenin was found at cell junctions in both PMVECs (iv) and PAECs (v). Co-cultures of PMVECs and PAECs (vi-viii) were doubly stained for β -catenin (vi, green) and

ALCAM (vii, red). Note the heavy concentration of β -catenin at cell junctions in PMVECs (left side of co-culture) and the relatively sparse distribution in PAECs (right side of co-culture). The merged image (viii) shows co-localization of ALCAM and β -catenin in PMVECs (yellow) adjacent to PAECs showing essentially only a β -catenin stain due to the low level expression of ALCAM at the junctions in this cell type (green). The arrow and arrow-head (vi-viii) point to PMVEC-PMVEC and PMVEC-PAEC borders, respectively, in the same cell (PMVEC) and highlights the unique cell-cell interactions in the two lung endothelial cells.

Figure 5. Heterogeneity of adherens junctions in PMVECs and PAECs

PMVECs and PAECs were seeded on the same glass coverslips to establish co-cultures, the monolayer doubly stained for VE-cadherin (green) and ALCAM (red) and analyzed by confocal microscopy. (i) Normarski optics confirming confluency of both PMVECs and PAECs which are shown separated by the broken white line. The same culture is shown in i-iv. Both VE-cadherin (ii) and ALCAM (iii) were heavily clustered at cell junctions in PMVECs but were relatively sparse at junctions in PAECs. (iv) Co-localization of ALCAM and VE-cadherin superimposed on the normarski image.

A

B

Figure 6. Localization of ALCAM in the adherens junction

(A) Confocal microscopic photosections of PMVECs doubly stained for ALCAM (red) and VE-cadherin (green). Photo-sections of 0.3 μ m thick (3 of 16 shown here) were taken from top to bottom of PMVECs, and the position of ALCAM and VE-cadherin compared. Sections shown are top (apical), center (basolateral) and bottom (basal). ALCAM and VE-cadherin co-localized in every section analyzed. (B) PMVEC lysates prepared with 1% triton X-100 buffer were immunoprecipitated and immunoblotted for the indicated molecules. A minimum of three experiments was performed for each reaction. Anti-ALCAM serum or normal rabbit serum was used for immunoprecipitation and the immune complex probed for VE-cadherin, β -catenin and ALCAM. The ALCAM immune complex contained a strong signal for VE-cadherin at the expected size ($\sim$ 130 kDa) and a relatively weak signal for β -catenin ($\sim$ 90 kDa, arrowhead). As expected ALCAM was identified by immunoblotting in the ALCAM immunocomplex but not in the immunocomplex prepared with rabbit serum (data not shown). Immunoprecipitate of VE-cadherin contained ALCAM ($\sim$ 110 kDa) and as expected β -catenin ($\sim$ 90 kDa) and VE-cadherin (data not shown). Non-specific heavy ($\sim$ 55 kDa) and light ($\sim$ 30 kDa) chain immunoglobulins indicated (*) were identified in reactions in which precipitation and blotting assays were performed using antibodies raised in the same species (mouse). Immunoprecipitate of N-cadherin probed with mouse monoclonal anti-ALCAM (MOG/07) identified the $\sim$ 110 kDa mature (arrowhead), $\sim$ 65 kDa native (arrowhead) and two intermediate-sized (arrow) ALCAM species. (C) Demonstration of the $\sim$ 110 kDa mature ALCAM in immunoprecipitates of β -catenin and Dlg but not p120 catenin. VE-cadherin and β -catenin were found in the expected immune complex. Specific protein bands are indicated by arrowheads and non-specific heavy and light chain immunoglobulins marked (*). Multiple non-specific heavy chain immunoglobulins were identified in the p120 immune complex. MW= molecular weight markers.

Figure 7. ALCAM expression in pulmonary endothelium

Isolated lungs from Sprague Dawley rats were perfusion fixed with 70% ethanol and embedded in paraffin. Sections were doubly stained for ALCAM (red) and von Willebrand factor (vWF; green), and tissue sections scanned using confocal microscopy. The endothelium of large pulmonary vessels (arrowheads, i, ii) stained negative for ALCAM (i) but were positive for vWF (ii). The alveolar septum contained microvascular endothelial cells doubly positive for ALCAM and vWF (arrows). ALCAM^{pos}/vWF^{neg} cells (*) were most likely interstitial alveolar macrophages based on morphology determined from co-staining lung sections for ALCAM and nuclear DNA (data not shown). Co-expression of ALCAM and vWF in endothelial cells in the alveolar septum is shown in an enlarged image (iii, iv).