Regulation of lung neutrophil recruitment by VE-cadherin

Janie Orrington-Myers, Xiaopei Gao, Panos Kouklis, Michael Broman, Arshad Rahman, Stephen M. Vogel, and Asrar B. Malik

Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois

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Orrington-Myers, Janie, Xiaopei Gao, Panos Kouklis, Michael Broman, Arshad Rahman, Stephen M. Vogel, and Asrar B. Malik. Regulation of lung neutrophil recruitment by VE-cadherin. Am J Physiol Lung Cell Mol Physiol 291: L764–L771, 2006. First published June 16, 2006; doi:10.1152/ajplung.00502.2005—Lung inflammatory disease is characterized by increased polymorphonuclear leukocyte (PMN) infiltration and vascular permeability. PMN infiltration into tissue involves signaling between endothelial cells and migrating PMNs, which leads to alterations in the organization of adherens junctions (AJs). We addressed the possible role of the protein constituents of AJs, endothelium-specific vascular-endothelial (VE)-cadherin, in the migration of PMNs. Studies were made using VE-cadherin mutant constructs lacking the extracellular domain (ΔEXD) or, additionally, lacking the COOH-terminus β-catenin-binding domain (ΔEXDΔβ). Either construct was transduced in pulmonary microvessel endothelium of mice using cationic liposome-encapsulated cDNA constructs injected intravenously. Optimal expression of constructs was seen by Western blot analysis within 24 h. Vessel wall liquid permeability measured as the lung microvessel capillary filtration coefficient increased threefold in ΔEXD-transduced lungs, indicating patency of interendothelial junctions, whereas the control ΔEXDαβ construct was ineffective. To study lung tissue PMN recruitment, we challenged mice intraperitoneally with LPS (3 mg/kg) for 6 h and measured PMN numbers by bronchoalveolar lavage and their accumulation morphometrically in lung tissue. ΔEXD expression markedly reduced the PMN sequestration and migration seen in nontransfected (control wild type) or ΔEXDΔβ-transfected (negative control) mice challenged with LPS. In addition, ΔEXD transfection suppressed LPS-induced activation of NF-κB and consequent ICAM-1 expression. These results suggest that disassembly of VE-cadherin junctions serves as a negative signal for limiting transendothelial PMN migration secondary to decreased ICAM-1 expression in the mouse model of LPS-induced sepsis.

vascular endothelial cadherin; intercellular adhesion molecule-1; polymorphonuclear leukocyte adhesion; vascular endothelial-cadherin mutants

THE MICROVASCULAR ENDOTHELIUM forms the principle barrier to passage of blood elements and plasma proteins from the circulation to tissues (32, 36). The barrier function of the endothelium depends primarily on adhesion between neighboring endothelial cells. Adherens junctions (AJs) are important determinants of interendothelial cell-cell adhesion and thus have central importance in regulating the macromolecular permeability of microvessels (11). Vascular endothelial (VE)-cadherin is an endothelium-specific transmembrane glycoprotein (2, 9, 29) that plays a major role in the organization of AJs. The extracellular domain of VE-cadherin is responsible for Ca2+-dependent homotypic VE-cadherin binding. Extracellular Ca2+ chelation (18) or the addition of antibodies against VE-cadherin’s extracellular domain increases endothelial permeability (9, 22, 25, 29), indicating the importance of this homotypic interaction in the regulation of endothelial barrier function. The cytoplasmic domain of VE-cadherin links the AJ to the actin cytoskeleton via a chain of intermediate proteins that includes β-catenin (directly bound to the cytoplasmic tail of VE-cadherin), plakoglobin, α-catenin, and α-actinin (directly bound to F-actin) (10, 11, 21, 28, 41). The association of VE-cadherin with the actin cytoskeleton is essential for the maximum strength and stability of AJs. The overexpression of VE-cadherin’s cytoplasmic tail results in the disruption of AJs in a dominant-negative manner (27, 35).

The microvascular endothelium maintains a nearly absolute barrier to leukocytes when endothelial junctions are intact. The manner in which polymorphonuclear leukocyte (PMN) interactions with activated endothelium signals the opening of AJs is largely unknown. Endothelial cell activation occurs when chemokines from cells in inflamed or infected tissue stimulate the expression of intercellular adhesion molecules such as ICAM-1. PMNs expressing β-integrins on their cell surface are rapidly and firmly recruited from the bloodstream to the site of inflammation (34). The process of PMN extravasation involves a well-defined series of PMN-endothelial cell interactions (34) that lead to firm adhesion of PMNs to the activated endothelium and migration into tissue across the vessel wall barrier. A requirement for firm adhesion of PMNs is the expression of ICAM-1, the endothelial counterreceptor for β-integrin CD11b/CD18. PMNs begin to transmigrate across the endothelial barrier within 2 min of firm adhesion (40). During this brief interval, PMN adhesion to the endothelium triggers profound modifications in the architecture of endothelial cell-to-cell junctions (45). VE-cadherin temporarily dissociates from β-catenin and other cytoplasmic binding partners and is internalized (3, 11, 43). Reannealing of AJs can occur within 5 min of PMN transmigration (40). VE-cadherin dissociation and internalization (1, 7, 33, 42, 48) may itself activate a signaling pathway required for the resealing of the junctions (41).

Loss of the VE-cadherin homotypic interaction during AJ separation could also have an impact on PMN transmigration. Evidence indicates that β-catenin, a protein associated with AJs, can physically complex with the transcription factor NF-κB (12). NF-κB in endothelial cells is an important regulator of ICAM-1 expression (15). In the present study, we investigated the possibility that homotypic association of VE-cadherin regulates the trafficking of PMNs into lung tissue. We used cationic liposomes to deliver mutant constructs of VE-cadherin to lung microvessel endothelia, thus causing disassembly of AJs. Our results demonstrate that separation of AJs...
in lung microvessels in vivo elicits a novel pathway to suppress PMN sequestration and migration. We postulate that this pathway represents an important negative feedback signal for limiting the degree of PMN infiltration into tissue in a time-dependent manner.

METHODS

Reagents. $^{32}$P was purchased from ICN Pharmaceuticals (Costa Mesa, CA). ICAM-1 polyclonal goat blocking antibody, anti-FLAG M2 mouse monoclonal antibody, goat anti-actin antibody, goat anti-mouse horseradish peroxidase (HRP)-linked IgG, mouse anti-goat HRP-linked IgG, and LPS Escherichia coli O111:B4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Experimental animals. Male CD1 mice weighing 25–35 g (The Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were housed in pathogen-free conditions with free access to food and water at the University of Illinois Animal Care Facility (Chicago, IL). All studies were made using approved institutional protocols conforming to National Institutes of Health guidelines. Unless otherwise indicated, anesthesia in the protocols described below was induced with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (2 mg/kg) in PBS.

DNA/liposome preparation and in vivo gene transfer. A mixture of dimethyldioctadecylammoniumbromide (DDAB; Sigma Chemical, St. Louis, MO) and cholesterol (Calbiochem, La Jolla, CA) in chloroform was used to prepare cationic liposomes as previously described by us (5). Briefly, the mixture consisting of DDAB and cholesterol in a 1:1 molar ratio was dried using an evaporator (model R-124 Rotavapor, Brinkmann, Westbury, NY) and resuspended in 5% glucose solution. The lipid suspension was sonicated for 30 min. DNA constructs of a VE-cadherin mutant lacking the extracellular domain ($\Delta$EXD) or, additionally, lacking the $\beta$-catenin-binding domain ($\Delta$EXD$\Delta$B) driven by the c-Myc tag promoter were made as previously described (5). The $\Delta$EXD or $\Delta$EXD$\Delta$B constructs (50 $\mu$g DNA/mouse) were combined with liposomes in a ratio of 1 $\mu$g of DNA to 8 nmol of lipid. For the injection of DNA-containing liposomes, mice were anesthetized (see above), the inner portion of the ear caruncle. Western blot analysis of expressed constructs. In these construct ($\Delta$EXD or $\Delta$EXD$\Delta$B) expression studies, animals survived for 0–48 h after the intravenous administration of DNA in liposomes. Mouse lungs were excised under anesthesia and homogenized using a tissue grinder in PBS (pH 7.4) containing protease inhibitor cocktail (60 $\mu$g/ml PBS, Sigma). Homogenates were centrifuged at 14,000 rpm for 10 min at 4°C. Supernatants were collected, and the protein concentration of each sample was measured with a bicinchoninic acid detector kit with BSA as the standard (Pierce, Rockford, IL). An equal amount of protein from each sample (~125–200 $\mu$g) was loaded onto lanes of a SDS-10% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA). After being incubated in blocking solution (5% dry milk in Tris-buffered saline with Tween 20) at room temperature for 1 h, membranes were immunoblotted (24-h period, 4°C) with anti-FLAG M2 monoclonal antibody (1:2,000) or anti-ICAM-1 polyclonal antibody (1:2,000). The secondary antibodies for detection were HRP-conjugated goat anti-mouse antibody (1:500) or anti-goat antibody (1:2,000), which was applied to the membranes for 1–2 h at room temperature. Peroxidase labeling was detected with the ECL Western Blotting Detection System (GE Healthcare, Piscataway, NJ).

Analysis of lung PMN sequestration and migration. In studies of PMN sequestration and migration, mice received construct or mock transfection and, 18–24 h later, received LPS (3 mg/kg) in PBS (pH 7.4) as an intraperitoneal injection. We made experimental observations 0, 1, 2, 4, or 6 h after LPS administration. For baseline studies without LPS, nontransfected control mice or transfected experimental mice received an equal volume of PBS for corresponding periods before the experimental period. For the analysis of lung PMN sequestration, we used a computer-based stereological method to quantify the numbers of sequestered PMNs in lung specimens as previously described by us (20). Briefly, tissue blocks were sectioned to a thickness of 5 $\mu$m and mounted on glass slides. Sections were stained for nuclear material using hematoxylin and eosin. PMNs were identified by their unique nuclear morphology. A known fraction of the sections (i.e., the area sampling fraction) was analyzed for PMNs. The optical counting system consisted of a microscope equipped with a $\times$65 oil-immersion objective with a 1.4 numerical aperture, a computer-controlled x-y-z motorized stage, a high-sensitivity video camera, computer-assisted image capture, and stereological software (MicroBrightField, Colchester, VT). The instrumentation was calibrated before each measurement. Using a two-dimensional raster of the microscope stage with known step lengths in the x and y directions, a series of 6,400 $\mu$m$^2$ squares (i.e., the sampling frames) were projected onto the section, starting from a random position outside of the section. A specific fraction of at least 5% of the sampling frame (i.e., the area sampling fraction) was counted for PMNs (19). The total number of PMNs in the lung specimen ($N$) was calculated using the formula $N = \frac{S}{Q^2} \times SSF$, where $Q^2$ is the total number of PMNs in the lung specimen counted by optical evaluation, SSF is the area sampling fraction, and SSF is the slice sampling fraction.

We evaluated transalveolar PMN migration by bronchoalveolar lavage (BAL). Mice were anesthetized as described above, and the trachea was rapidly cannulated with a blunt 21-gauge needle. We instilled 0.5 ml of sterile PBS containing 1 mM EDTA into the pulmonary air spaces. The fluid was immediately collected by gentle aspiration, and BAL was repeated with an identical 0.5-ml instillation. The fluid from both BAL cycles was pooled and centrifuged at 6,000 g for 4 min. The supernatant was removed, and the pellet was resuspended in 1 ml of PBS-EDTA. A 350-$\mu$l volume of resuspended cells was spun at 300 rpm for 5 min in a cytopsin apparatus (Shandon, Pittsburgh, PA). The cells were stained with HEMA (Biochemical Sciences, Swedesboro, NJ). Total cell counts were determined on a hemocytometer. Differential cell counts were enumerated on cytopsin-prepared slides. A total of 500 cells was counted in cross section per sample, and numbers of neutrophils were calculated as the total cell count times the percentage of neutrophils in the BAL sample.

Mouse lung isolation and perfusion. CD-1 male mice weighing 25–35 g were anesthetized with 3% halothane in room air. The trachea was cannulated with a 19-gauge stainless steel tube for constant positive pressure ventilation (rate of 120 breaths/min) with the anesthetic gas mixture. Heparin (50 units) was injected into the jugular vein as an anticoagulant. The abdominal cavity was opened to expose the diaphragm, which was ventrally punctured and cut free from the rib cage. A thoracotomy was then performed, and the two halves of the rib cage were retracted to expose the heart and lungs. To make the pulmonary artery accessible for cannulation, the heart was caudally retracted with a silk suture (5-0; Ethicon, Somerville, NJ) through the apical musculature of the right ventricle. An incision was made in the right ventricle at the base of the pulmonary artery for the introduction of the catheter. A flared polyethylene cannula (polyethylene-60) was maneuvered into the pulmonary artery via the pulmonic valve and secured by means of a suture around the pulmonary artery that included the aorta. An incision was made in the left atrium to insert a venous catheter. The lungs were perfused in situ using a peristaltic pump. The heart and the exsanguinated lungs were rapidly excised and transferred en bloc to a perfusion apparatus. For the monitoring of lung wet weight, preparations were suspended from a balanced lever arm coupled to a calibrated displacement transducer. The isolated
lungs were ventilated (120 breaths/min and end-expiratory pressure of 2.0 cmH₂O) and perfused at constant flow (2 ml/min), temperature (37°C), and venous pressure (+3 cmH₂O) with bicarbonate-buffered RPMI 1640 medium supplemented with 3 g/100 ml BSA (fraction V, 99% pure and endotoxin free). For the control of solution pH, the lungs were ventilated with a gas mixture containing 5% CO₂ (balance 20% O₂-75% N₂). Pulmonary arterial pressure was monitored throughout experiments using a Gould pressure transducer (model P23ID). Lung wet weight was electronically nullified when the preparation was mounted, and subsequent weight changes due to gain or loss of fluid from the lung were recorded. Lung weight and arterial and venous pressure recordings were displayed on a computer video monitor with the aid of amplifiers (Grass CP122 strain-gauge amplifier; Astro-Med, West Warwick, RI), an analog-to-digital converter, and commercial software that permitted the acquisition and logging of data (Labtech Notebook Pro for Windows; Labtech, Andover, MA).

Measurement of the capillary filtration coefficient in mouse lungs. For comparison with filtration coefficient (Kᵥc) measurements, the venous outflow pressure was elevated by means of a computer-controlled, two-way electronic pinch valve (P/N 98301-22; Cole-Parmer Instruments, Vernon Hills, IL) that channeled venous fluid into silicon tubing (1/16 in. inner diameter) that ended 3 or 9 cm above the level of the heart. The weight change resulting from the venous fluid change (i.e., 6 cmH₂O) was recorded during a 5-min period. The weight recordings thus obtained have two exponential components that reflect the rapid expansion of vascular volume and the slower phase of fluid filtration. The amount of fluid filtered in the 5-min period was determined by logarithmic extrapolation of the slower component to time 0. Kᵥc, a measure of liquid permeability, was computed in units of milliliters per minute per centimeter of water per gram dry weight by normalizing the estimate of filtered fluid by the time period, venous pressure change, and lung’s dry weight.

Nuclear extract preparation and EMSA. Lungs extracted from mice under anesthesia were minced on ice in 0.5 ml of ice-cold buffer A, which was composed of 10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 0.1% IGEPA30-630, and 0.5 mM PMSF (all ingredients purchased from Sigma Chemical). The minced tissue was homogenized using a Dounce homogenizer. The crude nuclear pellet was incubated for 30 min on ice at 28°C before being centrifuged at 14,000 rpm and 4°C for 10 min. The pellet was resuspended in 0.5 ml of buffer B [20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and 4 μM leupeptin] using a pestle before being incubated at 28°C for 30 min. The suspension was centrifuged at 14,000 rpm and 4°C for 30 min. The supernatant (nuclear proteins) was collected and kept at −80°C for use. The protein concentration was determined using a bicinchoninic acid assay kit with BSA as the standard (Pierce). EMSAs were performed as previously described (38).

Statistical analysis. Data are expressed as means ± SE. Comparisons between experimental groups were made by unpaired t-tests or ANOVA (as required) with the significance value set at P < 0.05.

RESULTS

These studies were designed to address the effects of AJ disaggregation, as induced by dissociation of homotypic VE-cadherin complexes, on PMN migration in the lung. We used a mutated form of VE-cadherin to disrupt homotypic VE-cadherin association. The mutant construct encodes VE-cadherin with its entire extracellular domain (∆EXD) deleted and replaced by a FLAG epitope; the remaining domains in ∆EXD have the wild-type amino acid sequence (27). ∆EXD is capable of associating with VE-cadherin’s normal cytoplasmic binding partners but incapable of homotypic intercellular adhesion with wild-type cadherin (27).

Expression of ∆EXD and ∆EXDΔβ in vivo. We transiently transfected intact lung microvessel endothelia with ∆EXD using cationic liposomes and conducted functional studies on PMN migration after verifying the expression of the construct. For comparison with ∆EXD, we transduced lung microvessels in separate experiments with ∆EXDΔβ, a mutated VE-cadherin lacking both the extracellular domain and cytoplasmic β-catenin binding domain. We determined the time course of expression of each cDNA construct. Whole lung homogenates were assayed for expressed protein by Western blot analysis. In ∆EXD-transfected lungs, immunoblots showed a reactive 34- to 36-kDa band predominantly at 16 and 24 h, indicating the expression of ∆EXD at these times (Fig. 1A). ∆EXDΔβ-transfected lungs likewise expressed a gene product of the expected molecular mass, i.e., 30–32 kDa after 16 h (Fig. 1B). Control immunoblots (using nontransfected lung preparations)

Fig. 1. Expression of mutant vascular endothelial (VE)-cadherin lacking the extracellular domain (∆EXD) and, additionally, lacking a β-catenin-binding domain (∆EXDΔβ) in vivo. A and B: Western blots of lung homogenates using anti-FLAG Ab show expression levels of ∆EXD (A) or ∆EXDΔβ (B) at 0, 16, 24, and 48 h after an intravenous injection of the corresponding cDNA constructs in cationic liposomes. Immunoblots for actin are provided for reference (top). Graphs in A and B, bottom, plot mean levels of expressed protein relative to actin versus time after LPS challenge; transduced proteins and actin bands in immunoblots were quantified by densitometry and plotted as densitometry ratios (i.e., ∆EXD to actin or ∆EXDΔβ to actin). *Significant difference from the unstimulated (time 0) value (P < 0.05). Bars are means ± SE; n = 3 per bar.
gave no reaction with detection antibodies (Fig. 1, A and B, 0-h blots). We quantified the expressed protein from lung homogenates relative to actin by densitometry (Fig. 1, A and B). We observed a significant increase in \( \Delta \text{EXD} \) expression between 16 and 24 h after an intravenous injection of cationic liposomes containing the cDNA construct; \( \Delta \text{EXD} \Delta \beta \) expression remained constant for up to 48 h postinjection. Therefore, in all subsequent studies on \( \Delta \text{EXD} \) or \( \Delta \text{EXD} \Delta \beta \), we adhered to a 24-h postinjection period for optimal expression of the constructs.

**Pulmonary microvascular permeability alterations induced by expression of \( \Delta \text{EXD} \) and \( \Delta \text{EXD} \Delta \beta \).** Because these studies used a mutated form of VE-cadherin to disrupt normal VE-cadherin junctional interactions, we next quantified the changes in pulmonary microvascular permeability resulting from construct expression. We measured \( K_{f,c} \) in the isolated perfused mouse lung preparation at the end of the 30-min equilibration period. The data were obtained by elevating venous pressure by 6 cm H2O and measuring the resultant increase in lung wet weight. Two phases of the wet weight change could be distinguished (see Fig. 2A): a rapid phase of volume expansion and a slower filtration phase; reference lines are provided in Fig. 2A to show the slope of the filtration phase. \( K_{f,c} \) values, based on the observed filtration rate (see METHODS), were increased only after \( \Delta \text{EXD} \) transduction. The summary graph shown in Fig. 2B shows \( K_{f,c} \) values obtained in nontransfected control preparations and those transfected with \( \Delta \text{EXD} \) or \( \Delta \text{EXD} \Delta \beta \) constructs. \( \Delta \text{EXD} \)-transduced lungs had a two- to threefold higher liquid permeability than nontransfected control or \( \Delta \text{EXD} \Delta \beta \) lungs (Fig. 2B). These data indicate that the transfection efficiency for \( \Delta \text{EXD} \) was sufficient to produce a marked increase in endothelial barrier permeability. The results also showed that the \( \beta \)-catenin-binding domain in \( \Delta \text{EXD} \) was required for the production of a significant permeability increase in lung microvessels, because \( \Delta \text{EXD} \Delta \beta \)-transduced lungs behaved in a manner similar to the control.

**Influence of AJ disruption on lung microvascular PMN transmigration and sequestration induced by LPS.** We next quantified PMN transmigration and sequestration in the mouse lung by analysis of BAL fluid and morphometric analysis, respectively. Mice were transfected with \( \Delta \text{EXD} \) or \( \Delta \text{EXD} \Delta \beta \) constructs or received no treatment (vector control) for a 24-h period. All mice then received LPS as an intraperitoneal injection. Between the 0 and 6 h postinjection, the lungs were lavaged as described (see METHODS). PMN migration in the nontransfected control showed a sevenfold increase (Fig. 3A) between time 0 and 6 h post-LPS; the data show that PMN migration increased monotonically during the 6-h stimulation period. Unstimulated PMN migration (time 0 values) was comparable in normal and transfected mice regardless of the construct used (Fig. 3A). In the \( \Delta \text{EXD} \)-transduced lungs, PMN migration was markedly reduced at time points from 1 to 6 h after LPS challenge compared with the corresponding values in the nontransfected group. The percent inhibition of migration was 89% of the control in the 6-h group (Fig. 3A). Transfection of \( \Delta \text{EXD} \Delta \beta \) also interfered with PMN migration, but the percent inhibition was only 21% of the control at the 6-h time point (Fig. 3A). These results indicate that \( \Delta \text{EXD} \) transfection is significantly more effective than \( \Delta \text{EXD} \Delta \beta \) transfection in inhibiting LPS-evoked PMN transmigration, particularly after LPS stimulation. To see whether the LPS-stimulated PMN transmigration reflected increased PMN capture and sequestration in lung microvessels, we determined tissue PMN counts by means of a morphometric analysis. In nontransfected lungs, LPS increased the sequestration of PMNs by 13- or 25-fold above the basal (no LPS) level after 1 and 6 h, respectively (Fig. 3B). \( \Delta \text{EXD} \) transfection gave a pronounced inhibition of PMN sequestration by 58% and 70% at 1 and 6 h post-LPS (Fig. 3B). Importantly, compared with the nontransfected control, \( \Delta \text{EXD} \Delta \beta \) transfection had no significant effect on PMN counts at either 1 or 6 h post-LPS (Fig. 3B). The results show the distinction between the two mutated forms of VE-cadherin used in terms of their effects on PMN sequestration. Specifically, the \( \beta \)-catenin-binding domain of \( \Delta \text{EXD} \) was required for marked inhibition of PMN sequestration in tissue.

**Fig. 2.** Pulmonary microvessel permeability as measured by the capillary filtration coefficient \( (K_{f,c}) \). A: continuously monitored lung wet weight \( (\Delta Wt, g) \) tracings after the step increase in pulmonary venous pressure \( (\Delta p_{pv}) \); slopes of the slower (filtration) phase are drawn for reference. B: results show that the expression of \( \Delta \text{EXD} \) increases the microvessel permeability in the lung as measured by \( K_{f,c} \) values. \( \Delta \text{EXD} \Delta \beta \) expression produced no significant increase in \( K_{f,c} \) compared with the vector control. *Significance \( (P < 0.05) \) with respect to the control. Bars are means \( \pm \) SE; \( n = 4 \) per bar.
Reduced NF-κB activation and ICAM-1 expression induced by disruption of AJs. To address the basis for the decreased PMN sequestration and migration observed after VE-cadherin disruption, we assessed LPS-induced NF-κB activation using EMSA. NF-κB activation was at a peak level 2 h after LPS in control lungs with mock transfection and was similar to the control in EXD-expressed lungs (Fig. 4A). By contrast, EXD-expressed lungs showed essentially no NF-κB activation (Fig. 4A); activation of NF-κB was not observed at any earlier time point (data not shown). Because NF-κB is known to regulate the transcription of ICAM-1, we tested the prediction that EXD expression would inhibit ICAM-1 protein expression. We determined ICAM-1 levels in lung homogenates by analysis of Western immunoblots (Fig. 4B). There was a time-dependent increase in ICAM-1 expression 1 and 6 h after LPS challenge in both control and ΔEXDΔβ-transfected mice (Fig. 4B and C). ΔEXD transfection fully prevented the induction of ICAM-1 expression by LPS (Fig. 4C).

Fig. 3. Effects of mutated forms of VE-cadherin on recruitment of polymorphonuclear neutrophils (PMNs) in lungs. A: for the assessment of PMN migration, mice were challenged with LPS for 0, 1, 2, 4, or 6 h, and bronchoalveolar lavage (BAL) was performed. Note that the recovery of alveolar PMNs at 6 h was less in ΔEXD-transduced lungs than the vector control or ΔEXDΔβ-transduced lungs. B: morphometric analysis of sequestered PMNs after LPS challenge showed no significant effect of ΔEXDΔβ expression compared with the vector control, i.e., mock-transfected liposomes. By contrast, marked inhibition of LPS-induced PMN infiltration was seen to result from ΔEXD expression. *Significance compared with the corresponding control (P < 0.05). Bars are means ± SE; n = 5 per bar in A and 3 per bar in B.

Fig. 4. A: suppression of LPS-induced NF-κB activation in ΔEXD-transduced mouse lungs. EMSA results showed peak activation of NF-κB 2 h after LPS challenge in mock-transfected and ΔEXDΔβ-transduced lungs but not ΔEXD-transduced lungs. Note the transient nature of NF-κB activation. B: Western blot analysis of ICAM-1 expression in ΔEXD- and ΔEXDΔβ-transduced lungs. Immunoblots show ICAM-1 and the reference protein actin in ΔEXD- and ΔEXDΔβ-transduced lungs at various time intervals (0, 1, or 6 h) after LPS challenge. C: bar graph showing mean levels of ICAM-1 (relative to actin) versus time after the LPS challenge; ICAM-1 and actin bands in immunoblots were quantified by densitometry and expressed as the ICAM-1-to-actin ratio. Results show that the expression of ΔEXD prevented the LPS induction of ICAM-1. *Significance with respect to the corresponding time 0 value. Bars are means ± SE; n = 3 per bar.
DISCUSSION

We observed that AJ disassembly itself did not stimulate PMN transmigration in vivo, but, to the contrary, it suppressed the recruitment of PMNs induced by LPS. This finding implies that the overriding effect of AJ disassembly is to inhibit the interactions between PMNs and the endothelium required for the PMN transmigration response.

The studies were based on inducing AJ disassembly using ΔEXD, a mutated form of VE-cadherin lacking the extracellular domain (27). We observed that the expression of this mutant in lung microvessel endothelial cells leads to the loss of junctional integrity. The mutant construct encodes VE-cadherin with its entire extracellular domain replaced by a FLAG epitope; the remaining domains in ΔEXD have the normal amino acid sequence. ΔEXD is capable of associating with VE-cadherin’s normal cytoplasmic binding partners but incapable of homotypic intercellular adhesion with wild-type VE-cadherin (27). We (27) have previously shown that ΔEXD localizes at endothelial AJs. This is expected because the sequences encoding the signal peptide, pre-peptide, and transmembrane domain of VE-cadherin were not deleted so that the mutant could be properly inserted in the plasma membrane. Previous findings in endothelial cells have shown that ΔEXD transfection induced the formation of interendothelial gaps in confluent cultures (27) and intact vessels (24) indicative of the loss of homotypic VE-cadherin interactions. The expression of the cytoplasmic domains of VE-cadherin has been shown consistently to block cell-cell adhesion in a variety of excitable and nonexcitable cell types both in vitro and in vivo (13, 17, 26, 37, 47). In endothelial cells, there is evidence that expression of VE-cadherin cytoplasmic domains titrates β-catenin from its downstream effectors and suppresses the expression of endogenous VE-cadherin (44). Studies (14, 39) have also shown that the overexpression of cadherin cytoplasmic domains modifies signal transduction via β-catenin. These observations suggested that AJ proteins may function as components of an “adhesion signaling system” that regulates vascular barrier function. In the present study, we present novel data showing that junctional proteins can also regulate transvessel neutrophil migration into tissue as activated by LPS.

Our experimental approach for examining the relationship between PMN migration and patency of endothelial AJs was to express ΔEXD or ΔEXDΔβ in lung vessels in vivo. ΔEXDΔβ, which lacks the β-catenin-binding domain but is otherwise identical to ΔEXD, did not disrupt endothelial AJs (27). We verified the expression of these constructs in lungs by Western blot analysis. Evidence that transfection of β-catenin-binding domains disrupted AJs in vivo was the finding of significantly increased vascular liquid permeability ($K_{fc}$) in the mouse lung after the transfection of ΔEXD but not after ΔEXDΔβ transfection. Expression of ΔEXD surprisingly (and unexpectedly) caused inhibition of PMN infiltration and migration. In mice, vascular PMN emigration after LPS challenge is CD18 dependent, and it is the result of CD18 interactions with ICAM-1 expressed on the endothelial plasma membrane (for a review, see Ref. 6). Therefore, we surmised that the inhibition of PMN migration in ΔEXD-transduced lungs may be the result of reduced ICAM-1 expression, which thereby mediates a reduction of firm PMN adhesion and consequently PMN migration.

In support of this hypothesis, we showed that ΔEXD transfection in fact reduced ICAM-1 expression. This evidently resulted from inhibition of ICAM-1 transcription, because expression of the ΔEXD mutant prevented the LPS-induced activation of NF-κB, the key transcription factor responsible for regulating ICAM-1 expression (15, 16). Our results also indicated that the β-catenin-binding domain of ΔEXD played an important role in inhibition of PMN sequestration in lungs, because ΔEXDΔβ transfection produced far less inhibition than ΔEXD transfection. Furthermore, ΔEXDΔβ transfection had no effect on either LPS-stimulated NF-κB activation or the subsequent increase in ICAM-1 protein expression.

NF-κB is thought to play an important role in PMN recruitment to inflammatory foci in the lung during sepsis by regulating endothelial ICAM-1 expression (31). In control endothelial cells, NF-κB exists in the cytoplasm in an inhibitory complex with IκB that prevents nuclear translocation of the transcription factor. On binding of LPS to Toll-like receptor 4, a cell surface receptor, IκB protein is rapidly phosphorylated by IκB kinase, ubiquinated, and degraded in proteasomes, thereby releasing NF-κB from IκB and allowing NF-κB to translocate to the nucleus for activation of gene transcription (16). Cytokines such as TNF-α, which is released primarily from resident macrophages responding to LPS (8), also activate NF-κB. A single NF-κB site within a 92-bp responsive region was essential for the ICAM-1 promoter to respond to TNF-α, IL-1β, PMA, and LPS in endothelial cells (30). A clinical study (46) has shown that NF-κB activation is associated with inflammatory disease, including acute lung injury.

Our findings of suppressed LPS-induced PMN infiltration and migration in ΔEXD-transfected lungs imply an important function of components of AJs in signaling of PMN migration across activated endothelium into lung tissue. Upon stimulation of cells with proinflammatory agents such as LPS or TNF-α, sequestered β-catenin may be released and stabilized by binding partners in the cytosol before translocating to the nucleus for the modulation of gene transcription (4, 23). Interestingly, free β-catenin has been shown to physically complex with NF-κB in cells (12). Thus interactions between released AJ proteins and pathways regulating ICAM-1 transcription in endothelial cells may be important mechanisms regulating the infiltration of PMNs into the lungs.

Our data are the first to suggest the role of protein components of AJs in regulating the expression of ICAM-1. This pathway may function in a negative feedback manner to limit PMN sequestration and migration under conditions in which endothelial adherens junctions are open. Such a mechanism could serve to minimize PMN migration and thereby tissue injury produced by accumulation of PMNs in the lung. AJs thereby may modulate the inflammatory response to proinflammatory mediators.

In conclusion, disassembly of AJs leads to marked reduction of PMN infiltration and migration through the downregulation of ICAM-1 expression by a NF-κB-dependent mechanism. This finding points to the possible role of VE-cadherin junctions in controlling the transcription of ICAM-1. Hence, AJ disassembly and the resultant decreased expression of ICAM-1 through the modulation of NF-κB activation may represent a critical negative feedback mechanism for limiting PMN transmigration and tissue infiltration.
REFERENCES


