Disparate mechanisms of sICAM-1 production in the peripheral lung: contrast between alveolar epithelial cells and pulmonary microvascular endothelial cells

Michael P. Mendez,1,3 Susan B. Morris,1 Steven Wilcoxon,1 Ming Du,1 Yeni K. Monroy,1 Henriette Remmer,4 Hedwig Murphy,2,5 Paul J. Christensen,1,3 and Robert Paine III1,6

1Pulmonary Section and 4Pathology and Laboratory Medicine, Veterans Affairs Health System, Ann Arbor; 2Division of Pulmonary and Critical Care Medicine and Departments of 4Biological Chemistry and 5Pathology, University of Michigan, Ann Arbor, Michigan; and 6Division of Pulmonary and Critical Care Medicine, University of Utah, Salt Lake City, Utah

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INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) is an ~100-kDa molecule belonging to the immunoglobulin supergene family. The membrane-bound form of this protein (mICAM-1) serves as a counter receptor for the β2-integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), found on leukocytes. Interactions with mICAM-1 facilitate leukocyte transmigration across the endothelium (34) and over the surface of alveolar epithelial cells (AEC) in the lung (27). A soluble form of the molecule, soluble intercellular adhesion molecule-1 (sICAM-1), is found in serum and in the alveolar lining fluid (7, 19, 23). sICAM-1 may be generated by proteolytic cleavage and/or alternative splicing of mICAM-1 messenger RNA (4, 35, 37). Like mICAM-1, sICAM-1 interacts with LFA-1/Mac-1 to compete with leukocyte binding to mICAM-1 (36) and to stimulate leukocytes (31).

sICAM-1 is normally present in the alveolar lining fluid of humans and mice (7, 13, 14, 16, 17, 23). We have previously demonstrated that type I AEC are the likely source of sICAM-1 in the alveolar lining fluid and that sICAM-1 is constitutively produced by AEC in primary culture that expresses features of the type I cell phenotype (23). However, little is known regarding the significance of sICAM-1 in the alveolar lining fluid. The abundance of sICAM-1 in alveolar lining fluid, combined with its known effects on leukocyte adhesion and stimulation, suggests a modulatory role for sICAM-1 in AEC-leukocyte interactions in the setting of inflammation. The functional implications of sICAM-1 expression in AEC-leukocyte interactions in the setting of inflammation. The functional implications of sICAM-1 expression in AEC-leukocyte interactions in the setting of inflammation.

Disparate mechanisms of sICAM-1 production,

Disparate mechanisms of sICAM-1 production, and murine PVEC and compared experiments to contrast the regulation of sICAM-1 in two

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by oligopeptide protease inhibition assays. In addition, we assessed AEC and PVEC for the presence of alternative splicing of miCAM-1 mRNA in the juxtamembrane sequence.

**MATERIALS AND METHODS**

**Reagents.** TNFα was purchased from R&D Systems (Minneapolis, MN). LPS, apropin, pepstatin, bestatin, and E-64 were obtained from Sigma (St. Louis, MO). BB2516 was obtained from British Biotechnology (Oxford, UK). Antibodies (Ab) used included anti-surfactant protein C (SP-C) Ab (US Biologicals, Swampscott, MA), anti-vimentin Ab (Santa Cruz Biotechnology, Santa Cruz, CA), donkey anti-goat and anti-rabbit Texas Red-conjugated secondary antibodies (Santa Cruz), and anti-ICAM-1 Ab (Santa Cruz). The following 16-mer oligopeptides were used: inhibitory peptide 1 (IP1) (ICAM-1469–484 (NVTRNYLTLYHQS)); inhibitory peptide 2 (IP2) (ICAM-L401–416 (CHAFFSSHGNVTRNVYL)); control peptide (Ac-KKAAKKAKKAHKKKK-NH2). Inhibitory peptides were designed based on the sequence of murine ICAM-1 reported by Ballantyne et al. (2) and Horley et al. (15). Solid phase methodology using Fmoc chemistry was employed for synthesis of oligopeptides using an automated multiple peptide synthesizer (Symphony, PTI) followed by purification with HPLC (Varian, Beckman-Coulter) using reversed phase C18 columns (Machery-Nagel). Quality control was performed by electrospray mass spectrometry (LCQ Duo, Thermo Finnegan) and analytical HPLC (Beckman-Coulter).

**Animals.** Specific pathogen-free, 6- to 12-wk-old, wild-type C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in isolator cages within the Animal Care Facilities at the Veterans Affairs Research Laboratories. Mice received food and water ad libitum. The animal care committees at the Veterans Affairs Medical Center (Ann Arbor, MI) and the University of Michigan (Ann Arbor, MI) approved the experimental protocols.

**Isolation and culture of murine AEC.** Marine type II AEC were isolated based on the method of Corti et al. (8), as described previously (28). Briefly, after the pulmonary vasculature was perfused with PBS, type II AEC were freed from the lung by enzymatic digestion previously (28). Briefly, after the pulmonary vasculature was perfused with PBS, type II AEC were freed from the lung by enzymatic digestion with Dispase (Worthington Biochemical, Lakewood, NJ) infused via tissue culture plates in DMEM (GIBCO, Grand Island, NY) supplemented with 10% FCS (GIBCO). To test for purity, cells in suspension were centrifuged onto glass slides and stained for SP-C before culture on fibronectin-coated plates. Cells were >99% SP-C positive. After 3 days in culture, adherent cells were stained for vimentin (to identify cells of mesenchymal origin, including fibroblasts, endothelial cells, and alveolar macrophages) and found to be >95% vimentin negative. For cell culture experiments, cells were plated in 24-well plates at 0.5 × 10⁴ cells/well in DMEM supplemented with 10% FCS, penicillin, and streptomycin (GIBCO). Cells were allowed to adhere and become confluent over 72 h, at which time the cells exhibit certain features of the type I AEC phenotype, including abundant expression of miCAM-1 (23). All experiments were carried out under serum-free conditions. Assay for cellular injury was carried out using an ELISA for LDH (Sigma) in supernatants. LDH was not detectable in any condition.

**Isolation and culture of murine PVEC.** Endothelial cells were isolated from 3-wk-old C57BL/6 mice. PVEC were isolated from peripheral lung as previously described (5, 26). Briefly, strips of peripheral lung were removed, minced, and incubated in 1% gelatin-coated 25-cm² tissue culture flasks in growth media. All tissue was removed at 65 h. Cells were passaged once and seeded at 7.5 × 10⁴ cells/well in a gelatin-coated 24-well plate. For all experimental studies, cells were used at ~90% confluence at passage 1. Cells were grown in RPMI 1640 media supplemented with Endothelial Cell Growth Factor (BD Bioscience, Bedford, MA), 20% FCS (Invitrogen), and penicillin-streptomycin (GIBCO). Purity of endothelial cell culture was assessed using Di-Ac-LDL (acylated low-density lipoprotein labeled with 1,1’dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate; Biomedical Technologies, Stoughton, MA) in separate gelatin-coated Labtek culture slides. Cells were incubated for 6 h in the presence or absence of Di-Ac-LDL, followed by nuclear counterstain with DAPI (Santa Cruz), and were examined by fluorescence microscopy. Greater than 95% of cells were positive for Di-Ac-LDL. All experiments were carried out in serum-free conditions. As with AEC, there was no detectable injury in any condition by measuring LDH in the supernatants.

**siCAM-1 ELISA.** Analysis of total siCAM-1 levels in culture supernatants was performed using the EMICAM kit from Pierce Biotechnology (Rockford, IL). The lower limit of detection for this ELISA is <5 ng/ml. AEC and PVEC culture supernatants were clarified by high-speed centrifugation (100,000 g; with an ultracentrifuge (Beckman) to remove any contaminating membrane fragments. Although representative experiments are depicted in the figures, it should be noted that there is some variance in measured siCAM-1 from experiment to experiment. Over greater than eight separate measurements, we have observed a mean value of 158 ng/ml (SD 42) in AEC supernatant (24-h incubation in serum-free media). In PVEC supernatants (24-h incubation in serum-free media), mean values were 15 ng/ml (SD 8.4) under the same conditions.

**Alternative splicing.** RNA was prepared from primary culture of PVEC (stimulated with TNFα) and primary culture of AEC (nonstimulated) using RNA Nanoprep kits (Stratagene, LaJolla, CA). cDNA was prepared using Brilliant SYBR Green QRT-PCR mastermix kit, 1 step (Stratagene). Amplification and data analysis were performed using an Mx3000P real-time PCR system computerized cycler from Stratagene, as previously described (29). Primer sequences for murine ICAM-1 amplification included the following primer pairs: 51 (5'-CGG CTG GAC GAG ACG GAC TGC-3') and 31 (5'-AGA GGA AGT GGC TGA GGG TAA ATG-3'); 52 (5'-CAT CGG GTG GGT GAA xGTG TGT C-3') and 32 (5'-TGC GTG CTA CCA TTC GTG TCA AAA-3') and 52 paired with 33 (5'-CAG GAG GCC CAC AAT GAC CAG CAG TA-3') (see Fig. 6A). Control reactions excluding template or RT were also used to exclude the presence of contaminating template DNA. Primer sets were chosen using PrimerSelect (Lasergene Software) and synthesized and HPLC-purified by Invitrogen Life Technologies (Carlsbad, CA). The PCR products were analyzed on 4% NuSieve agarose gels (Lonza, Rockland, ME).

**Statistical analysis.** Data are expressed as means with SD represented by an error bar. The data were compared using a two-tailed Student's t-test or ANOVA, if more than two groups were compared, with the InStat software package from GraphPad Software (San Diego, CA). Differences were considered statistically significant if P values were <0.05.

**RESULTS**

**AEC, but not PVEC, in primary culture constitutively produce siCAM-1.** We have previously shown that siCAM-1 is present in abundance in the alveolar lining fluid of normal mice. In vitro, we have also shown that miCAM-1 expression is induced as type II AEC in primary culture spread and assumes characteristics associated with the type I cell pheno-
type (3, 6). Similarly, murine AEC in primary culture constitutively produce sICAM-1 without a specific stimulus or other cell types present (23). Extending our previous observations, we now find that stimulation of murine AEC in primary culture with TNFα (50 ng/ml) or LPS (10 μg/ml) results in a minimal increase (∼1.2-fold for both stimuli) in the amount of sICAM-1 found in culture supernatant after 24 h compared with serum-free control (Fig. 1A). We have observed little to no difference in sICAM-1 levels between control and TNFα-treated cells at 24 and 48 h (not shown). Together, these results suggest that, as in our previous work with mICAM-1, expression of sICAM-1 by AEC is closely tied to the state of epithelial cell differentiation, with very little influence from inflammatory mediators in the environment.

Having confirmed constitutive expression of sICAM-1 in AEC, we next examined the pattern of release of sICAM-1 in primary culture of peripheral PVEC. In contrast to AEC, PVEC produce minimal sICAM-1 at baseline. However, upon stimulation with TNFα (50 ng/ml) or LPS (10 μg/ml), sICAM-1 production increases by ∼3.4-fold (Fig. 1B). These results are consistent with observations in other cell types in which sICAM-1 release is only induced after stimulation (20, 32). Interestingly, over multiple cell isolations, production of sICAM-1 by confluent monolayers of unstimulated AEC was significantly greater than that of maximally stimulated (TNF 50 ng/ml) PVEC [158 ng/ml (SD 42) vs. 97 ng/ml (SD 57), in separate cell preparations, P < 0.001]. These data highlight the dichotomy in the peripheral lung between differentiation-related expression by AEC and induced microvascular endothelial cell expression of sICAM-1 despite the close physical proximity of these two cell types.

A serine protease mediates shedding of sICAM-1 in AEC, but not in PVEC. Differences in the circumstances of sICAM-1 expression between AEC and PVEC led us to determine whether there might also be significant differences in the mechanisms of sICAM-1 production. Ectodomain shedding by proteolytic cleavage is a common mechanism for release of cell-surface molecules (1, 9). To date, a matrix metalloprotease or a cysteine protease has been implicated in the proteolytic cleavage of mICAM-1 in various cell types (4, 11, 21, 35). In this experiment, we examined the effect of inhibitors of four major classes of proteases on sICAM-1 shedding from AEC. In contrast to results published for other cell types, our data demonstrate a significant 37% inhibition of sICAM-1 shedding with a serine protease inhibitor, without inhibition by other classes of protease inhibitors (Fig. 2A). In addition, no additive or synergistic inhibition was observed when cells were incubated with a cocktail of the inhibitors at the same concentrations. This suggests that cleavage-mediated constitutive release of sICAM-1 is regulated differently in AEC compared with expression induced by inflammatory signals described in other cell types.

Because others have demonstrated that sICAM-1 shedding in HUVEC is mediated through cleavage of mICAM-1 by ADAM17 (35), we suspected that a similar mechanism might exist in PVEC in culture. However, production of sICAM-1 by PVEC was not inhibited by protease inhibitors from the cysteine, MMP, serine, or aspartic protease class (Fig. 2B). In separate experiments, BB2516, a broad inhibitor of MMPs, which would be expected to inhibit ADAM class proteases, did not affect sICAM-1 production (data not shown). In contrast to our observations in AEC, aprotinin had no effect on sICAM-1 shedding in PVEC. This indicates that sICAM-1 production in PVEC and AEC involves disparate mechanisms.

An oligopeptide completely abrogates sICAM-1 production in AEC, not PVEC. Although protease inhibitors may be class specific (serine, cysteine, etc.), their ability to inhibit the enzymatic activity of specific proteases may vary depending on the interaction between the inhibitor and the active site of a given protease. In addition, use of some antiproteases is limited by cellular toxicity in in vitro systems. Having determined that production of sICAM-1, at least in part, is dependent on proteolytic cleavage, we hypothesized that oligopeptides corresponding to the juxtamembrane sequence would specifically block proteolytic cleavage. Because we have previously shown murine sICAM-1 from alveolar lining fluid to be ∼90 kDa (23), we focused our attention on the juxtamembrane sequence. This area would likely correlate to the expected site of cleavage based on the size of sICAM-1. Our strategy is shown in Fig. 3. We synthesized 16-mer peptides that would theoretically compete with the active site of the protease and/or protect the substrate site on mICAM-1, thus allowing us to better assess the mechanism of sICAM-1 production in AEC and PVEC.

Fig. 1A. Characterization of soluble ICAM-1 (sICAM-1) production by primary culture alveolar epithelial cells (AEC) and pulmonary microvascular endothelial cells (PVEC). Primary cultures of AEC and PVEC were established as described in MATERIALS AND METHODS. Cells were gently washed with PBS before addition of serum-free media with or without LPS or TNFα. Supernatants were collected after 24 h and clarified by high-speed centrifugation before measurement by ELISA. AEC cultures were stimulated with TNFα or LPS (A). PVEC cultures were stimulated with TNFα or LPS (B). N = 3. *P < 0.05 vs. all conditions. †P < 0.001 vs. all conditions.
of sICAM-1 by ELISA (data not shown). The dose (100 μM) was chosen based on the effective concentration of peptide used by Budnik et al. (4) in similarly designed experiments using human keratinocytes in culture. Interestingly, we found that IP1 completely inhibited shedding of sICAM-1 by AEC, whereas IP2 inhibited shedding by 33% (Fig. 4A). A dose-response experiment revealed that IP1 at a concentration of 25 μM was capable of completely inhibiting sICAM-1 shedding from AEC (Fig. 4B). An unrelated control 16-mer peptide did not alter shedding. These data suggest that IP1 inhibits an as yet unidentified protease, acting at the AEC surface to cleave mICAM-1, resulting in sICAM-1 shedding. They also support the hypothesis that cleavage of mICAM-1 at the epithelial cell surface is the exclusive source of AEC-derived sICAM-1.

Having demonstrated that IP1 is capable of completely abrogating sICAM-1 shedding in AEC, we next examined its effects on TNFα-induced sICAM-1 production in PVEC. Incubation of TNF-stimulated PVEC with IP1 at a concentration that completely suppressed sICAM-1 release from AEC (50 μM) resulted in a modest 20% decrease in sICAM-1 production compared with incubation with TNFα alone (Fig. 5A). In contrast to AEC, PVEC exhibit no decrease in sICAM-1 production in the presence of IP2 (50 μM). Dose-response experiments were performed for each peptide. IP1 did not influence shedding at lower doses (Fig. 5C), whereas IP2 did not inhibit shedding at any dose (Fig. 5B). As controls, incubation of PVEC with peptide alone (in the absence of TNFα) did not result in any change in measured sICAM-1 levels (Fig. 5, B and C). This suggests that sICAM-1 production by PVEC involves mechanisms other than cleavage of mICAM-1 at the juxtamembrane region.

Alternative splicing of ICAM-1 yields sICAM-1 in both AEC and PVEC. Previously, sICAM-1 production through alternative splicing of ICAM-1 transcript has been demonstrated in human tissues (32, 37). Using a similar strategy to assess for alternative splicing, we designed primers spanning the juxtamembrane sequence of ICAM-1 (Fig. 6A). RNA was isolated from TNFα-stimulated PVEC and AEC cultured in media alone. RT-PCR was performed using three primer sets spanning exons 6 and 7 (which would span the juxtamembrane sequence). Products were run on a 4% agarose gel. Figure 6B demonstrates a dominant fragment for each primer pair that is of the expected size based on the published murine ICAM-1 sequence (2). However, a minor band ~50 bp smaller than the dominant fragment was noted using primer sets 51–31 and 52–32, suggesting the presence of alternatively spliced fragments. Two possible explanations for the lack of a similar dominant fragment with primer set 52–33 are that 1) small size and quantity of fragment may have escaped detection or 2) splicing occurs outside of this sequence.

Fig. 2. Effects of protease inhibition on production of sICAM-1 in AEC and PVEC. Primary cultures of AEC and PVEC were established as described in MATERIALS AND METHODS. Cells were gently washed with PBS before addition of protease inhibitors and incubated for 24 h. Supernatants were collected and clarified as described in MATERIALS AND METHODS. Inhibitors used were class specific (aprotinin-serine, pepstatin-aspartic, bestatin-metalloprotease, E64-cysteine). Additionally, a cocktail of all inhibitors was used to assess for synergistic or additive effects. AEC were incubated with inhibitor for 24 h (A). PVEC were incubated with TNFα and inhibitor for 24 h (B). N = 3. ***p < 0.001 vs. control, pepstatin, bestatin, and E64. †p < 0.001 vs. all conditions. NS, no significant difference between treatment conditions.

Fig. 3. Design of oligopeptide inhibitors. Inhibitory peptide 1 (IP1) and inhibitory peptide 2 (IP2) were synthesized as described in MATERIALS AND METHODS. The 16 amino acid peptides correspond to the juxtamembrane sequence of murine ICAM-1 (A). IP2 (461–476) and IP1 (469–484) are aligned with the native ICAM-1 sequence (B). Transmembrane domain is underlined and in gray lettering.
This qualitative assessment of the presence of alternative splicing in these two cell types suggests that this mechanism is present in both cell types. However, given that AEC, and not PVEC, have near 100% suppression of sICAM-1 release in the presence of blocking peptide suggests that the relative contribution of proteolytic cleavage to sICAM-1 release is greater in AEC than in PVEC.

DISCUSSION

sICAM-1 is a soluble adhesion molecule that has been shown to reduce mICAM-1-mediated leukocyte adhesion to endothelium (18) and to enhance leukocyte stimulation (22). sICAM-1 can be produced as a result of proteolytic cleavage or alternative splicing (4, 21, 32, 36, 37). We have reported that sICAM-1 is normally present in the alveolar lining fluid and that AEC in culture spontaneously release significant quantities of sICAM-1 (23). In contrast, many other cell types, including aortic smooth muscle cells, dermal microvascular endothelial cells, bronchial epithelial cells, keratinocytes, and lung fibroblasts in culture, produce, at most, low amounts of sICAM-1 at baseline and require stimulation with inflammatory mediators to induce significant release of sICAM-1 (20, 21, 32). As a result of these observations, we hypothesized that, despite their close proximity in the alveolar wall, these cells in the distal lung might regulate shedding of sICAM-1 by different mechanisms. To test this hypothesis, we studied the mechanism of sICAM-1 release from AEC in primary culture and compared this mechanism with that in PVEC.

We now demonstrate that murine AEC in primary culture produce sICAM-1 via cleavage of mICAM-1 at the cell sur-

![Graph A](http://ajplung.physiology.org/)  
![Graph B](http://ajplung.physiology.org/)  
![Graph C](http://ajplung.physiology.org/)

Fig. 4. Effect of oligopeptides on sICAM-1 shedding in AEC. Primary cultures of AEC were established as described in MATERIALS AND METHODS. Cells were gently washed with PBS before incubation with oligopeptides in serum-free media. Supernatants were collected after 24 h and clarified as described in MATERIALS AND METHODS. AEC culture was incubated with IP1 or IP2 peptides at 100 μM (A). A dose response of IP1 to sICAM-1 shedding was performed in addition to incubation with control 16-mer peptide (no sequence homology to mICAM-1) (B). N = 3. *P < 0.001 for all comparisons. ***P < 0.001, all IP1 conditions vs. no peptide and peptide control. N = 3.

![Graph D](http://ajplung.physiology.org/)  
![Graph E](http://ajplung.physiology.org/)  
![Graph F](http://ajplung.physiology.org/)

Fig. 5. Effect of oligopeptides on sICAM-1 shedding in PVEC. Primary cultures of PVEC were established as described in MATERIALS AND METHODS. Cells were gently washed with PBS before incubation with oligopeptides and/or TNFα in serum-free media. Supernatants were collected after 24 h and clarified as described in MATERIALS AND METHODS. PVEC culture was incubated with IP2 or IP1 at 100 μM (A). A dose response of IP2 to sICAM-1 shedding by PVEC was performed (B). A dose response of IP1 to sICAM-1 shedding by PVEC was performed (C). N = 3. ***P < 0.001, serum-free vs. all conditions. *P < 0.05, TNF-IP1 vs. TNF-IP2. †P < 0.001 in B and C, serum-free vs. all TNF-IP2/IP1 conditions. *P < 0.05, TNF-IP1 (50 μM) vs. TNF-IP1 (25 μM) in C.
demonstrated that exogenous recombinant sICAM-1 re-

Pseudomonas pneumonia (30). The mechanism for decreased 

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tissues in the setting of inflammation through interaction with 

to play important roles in the recruitment of leukocytes to the 

In contrast, under serum-free conditions, PVEC produce min-

mechanism that results in either sICAM-1 shedding or release. 

additional stimulus, it is evident that AEC possess an intrinsic 

of AEC constitutively produce sICAM-1 in the absence of 

vation may provide insight into the role of sICAM-1 in 

alveolar and vascular compartments in the distal lung. 

mICAM-1, which is normally expressed on the surface of 

endothelium, interacts with the ligands, Mac-1 and LFA-1, on 

the surface of leukocytes. These β2-integrins have been shown 

to play important roles in the recruitment of leukocytes to the 

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endothelial cell mICAM-1 (24, 25, 30). For example, intrave-

uous administration of LFA-1-blocking antibodies can de-
CREASE recruitment of leukocytes to the lung in a murine model of 

Pseudomonas pneumonia (30). The mechanism for decreased 

recruitment is decreased binding to membrane ICAM-1. In 

a similar fashion, sICAM-1 is capable of competing with 

mICAM-1 for β2-integrin binding. For example, Kusterer et al. 

(18) demonstrated that exogenous recombinant sICAM-1 re-

duces leukocyte adhesion in gut reperfusion injury model, 

suggesting that sICAM-1 release provides a negative feedback 

loop for leukocyte recruitment from the vascular space. Thus, 

the constitutive release of sICAM-1 from AEC in the alveolar 

space may represent a mechanism for the modulation of adhe-

siveness of leukocytes to AEC, perhaps to facilitate migration of 

alveolar macrophages over the alveolar epithelial surface. Con-

sidering that injury to AEC can be propagated by activated 

neutrophils (33), expression of sICAM-1 may impede access of 

activated leukocytes to the epithelium thereby limiting “bystander 

injury.”

The literature suggests that sICAM-1 production may occur 

through proteolytic cleavage of mICAM-1 or alternative splic-

ing of mICAM-1 message. One or more matrix metallopro-

tases are likely involved in the cleavage of mICAM-1, result-

ing in sICAM-1 shedding (11, 21, 36). Because sICAM-1 

release from AEC is distinctive compared with most other cell 

types, we sought to compare the effect of class-specific pro-

tease inhibitors on levels of sICAM-1 in culture supernatant of 

primary culture AEC and PVEC. To explore the possibility of 

cleavage by different proteases, we assessed inhibitors of four 

major classes of proteases for their ability to prevent sICAM-1 

shedding: serine (aprotinin), MMP (bestatin), cysteine (E64), 

and aspartic (pepstatin). We now demonstrate that a serine pro-

tease inhibitor substantially decreases spontaneous sICAM-1 re-

lease by AEC. This suggests that, at least in part, sICAM-1 

production in AEC occurs through cleavage. Because primary 

culture of AEC produce sICAM-1 constitutively without a 

specific stimulus, it is likely that the protease responsible for 

shedding is either expressed on the surface of AEC or released 

by AEC. To our knowledge, this is the first report of serine 

protease-mediated cleavage of mICAM-1. In contrast, none of 

the inhibitors tested inhibited sICAM-1 production in PVEC at 

the same doses (Fig. 2B).

In other cell types, MMP have often been implicated in 

sICAM-1 shedding. For example, in primary culture of rat 

astrocytes, all classes of protease inhibitors were tested, and 

only MMP inhibitors were shown to inhibit sICAM-1 shedding (21). Tsakadze et al. (36) demonstrated varying levels of 

inhibition of sICAM-1 shedding (65–95%) from HUVEC with 

various inhibitors of MMPs. Budnik et al. (4) have shown that 

only cysteine protease inhibitors are able to inhibit sICAM-1 

shedding in human keratinocyte cultures. Of note, in each of the 

above examples, a stimulus was required to induce mICAM-1 

expression and sICAM-1 shedding. Together, these data suggest 

that the production of sICAM-1 occurs through proteolytic cleav-

age of mICAM-1 and that the protease responsible for sICAM-1 

shedding may be tissue specific.

On the basis of the known size of sICAM-1 originating from 

the alveolar lining fluid (~90 kDa) (27), it was likely that 

cleavage occurred near the membrane domain. This motivated 

our strategy to use oligopeptides corresponding to the jux-

tembrane domain to prevent the cleavage of mICAM-1 (Fig. 3). 

Using a similar strategy, Budnik et al. (4) synthesized 16-mer peptides corresponding to the amino acid sequence of 

ICAM-1 to demonstrate that a specific sequence was capable of 

inhibiting shedding of sICAM-1 in human keratinocyte cultures. The peptide with sequence homology to the juxta-

membrane extracellular region of mICAM-1 resulted in 68% inhibi-

tion of sICAM-1 shedding in the culture supernatant. Interest-

ingly, in our study, we demonstrate complete inhibition of
sICAM-1 shedding in murine AEC cultures using a 16-mer oligopeptide IP1 analogous to the amino acid sequence immediately juxtaposing the transmembrane domain. The fact that a more distal overlapping peptide IP2 only partially inhibited cleavage suggests that the overlapping eight amino acid sequence (NVTRNVYL) between IP1 and IP2 contains a portion of the substrate necessary for proteolytic cleavage. In contrast, when we incubated IP1 or IP2 with activated PVEC, no inhibition was demonstrated with IP2, and very modest inhibition was noted with IP1. Possible explanations for this disparity are that the mechanisms for sICAM-1 production in murine AEC and PVEC involve different proteases or involve varying degrees of induction of alternatively spliced ICAM-1 transcripts that yield sICAM-1.

In addition to the ample evidence demonstrating cleavage of mICAM-1 as a pathway for sICAM-1 shedding, alternative splicing of mICAM-1 transcript has been shown to yield sICAM-1 protein. Wakatsuki et al. (37) have demonstrated the presence of unique mRNA encoding sICAM-1 from normal human spleen, liver, and lymph node. RT-PCR analysis using primers spanning exon 6 and exon 7 (analogous to extracellular domain and transmembrane/cytoplasmic domains, respectively) yielded two distinct products. When sequenced, one product was equivalent to the conventional mICAM-1 molecule, whereas the other demonstrated a 19-bp deletion at the 3' region of exon 6. This resulted in a frame shift mutation causing premature termination, yielding a molecule without a hydrophobic transmembrane domain or cytoplasmic domain. Using 5' primers specific to the two unique transcripts for RT-PCR analysis, they were able to show disparate effects of cytokine stimulation on the levels of the two products in cultured cell lines (human hepatoma and colon adenocarcinoma). Using a similar strategy to detect alternatively spliced forms of ICAM-1, we demonstrated that both AEC and stimulated PVEC possess alternatively spliced fragments. In Fig. 6A, we demonstrate that two out of three of our primer sets reveal a minor splice-variant band. Two possible explanations for the lack of similar splice variant fragments with primer set 52–33 are that J) the small size and quantity of fragment may have escaped detection or 2) splicing occurs outside of this sequence. However, the observation that IP1 completely abrogated sICAM-1 shedding (Fig. 4A) suggests that alternative splicing is not a significant contributor to sICAM-1 production in AEC.

Two possible explanations for the differences in sICAM-1 regulation in AEC and PVEC may relate to the specific environment to which the AEC and PVEC are exposed or 2) to differentially regulated alternative splicing. Differences in the local microenvironment are likely to influence the specific manner in which sICAM-1 production has developed at different sites. Endothelial cells, including PVEC, are constantly exposed to serum and its complex mixture of proteases and antiproteases that might influence sICAM-1 release. Thus it is plausible that sICAM-1 expression by endothelial cells might involve interactions with serum proteins, intracellular cleavage, or alternative splicing of ICAM-1 transcripts. In contrast, the surface of the alveolar epithelium is exposed to a simpler environment with far fewer proteins than found in serum. Thus, it is also plausible that AEC might themselves harbor a protease responsible for sICAM-1 cleavage and that this cleavage might take place at the cell surface.

Different mechanisms of alternative splicing have been shown to produce various isoforms from a single gene. For example, Emeson et al. (10) have demonstrated that splicing can be tissue specific and can be regulated by cis-active elements at splice junction consensus sequences. They demonstrated that the production of calcitonin and calcitonin gene-related peptide were products of the same gene but were differentially spliced by cells originating from different tissues. There is evidence that the soluble form of ICAM-1 can be differentially spliced in response to inflammatory stimuli (12, 32, 37). Giorelli et al. (12) demonstrated both mICAM-1 and sICAM-1 splice variants in HUVEC and human blood mononuclear cells. The splice products are differentially upregulated in response to inflammatory mediators in both cell types. We speculate that although AEC and PVEC both possess the capability to alternatively splice the ICAM-1 gene transcript (Fig. 6B), the relative contribution of alternatively splicing to sICAM-1 protein expression is quite different in these cells.

In summary, our study demonstrates that there are likely disparate mechanisms of sICAM-1 production in PVEC and AEC. An important mechanism for sICAM-1 production in AEC appears to be dependent on proteolytic cleavage by a serine protease within a 16-residue sequence in the juxtapembrane region of mICAM-1. In contrast, the mechanism for sICAM-1 shedding in PVEC may depend on alternative splicing and proteolytic cleavage by a different protease. These studies point to a critically important difference in the mechanism of regulation ICAM-1 in the peripheral lung that may have implications on the importance of sICAM-1 shedding in the alveolar space in the setting of inflammation.

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REFERENCES
10. Emeson RB, Hedjran F, Yeakley JM, Guise JW, Rosenfeld MG.

11. Fiore E, Fusco C, Romero P, Stamenkovic I.

12. Grigg J, Riedler J, Robertson CF.


14. Ishii Y, Kitamura S.

15. Labarrere CA, Nelson DR, Miller SJ, Nieto JM, Conner JA, Pitts DE.

16. Lyons PD, Benveniste EN.


19. Kirlin PC, Halbrook HG.

20. Leung KH.


