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**References**

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Soluble Fas Ligand Induces Epithelial Cell Apoptosis in Humans with Acute Lung Injury (ARDS)1

Gustavo Matute-Bello,*‡ W. Conrad Liles,§ Kenneth P. Steinberg,†‡ Peter A. Kiener,¶ Stephen Mongovin,*† Emil Y. Chi,‖ Mechthild Jonas,‖ and Thomas R. Martin2*‡

The goals of this study were to determine whether the Fas-dependent apoptosis pathway is active in the lungs of patients with the acute respiratory distress syndrome (ARDS), and whether this pathway can contribute to lung epithelial injury. We found that soluble Fas ligand (sFasL) is present in bronchoalveolar lavage (BAL) fluid of patients before and after the onset of ARDS. The BAL concentration of sFasL at the onset of ARDS was significantly higher in patients who died. BAL from patients with ARDS induced apoptosis of distal lung epithelial cells, which express Fas, and this effect was inhibited by blocking the Fas/FasL system using three different strategies: anti-FasL mAb, anti-Fas mAb, and a Fas-Ig fusion protein. In contrast, BAL from patients at risk for ARDS had no effect on distal lung epithelial cell apoptosis. These data indicate that sFasL is released in the airspaces of patients with acute lung injury and suggest that activation of the Fas/FasL system contributes to the severe epithelial damage that occurs in ARDS. These data provide the first evidence that FasL can be released as a biologically active, death-inducing mediator capable of inducing apoptosis of cells of the distal pulmonary epithelium during acute lung injury. The Journal of Immunology, 1999, 163: 2217–2225.

The acute respiratory distress syndrome (ARDS) is characterized by a major increase in the permeability of the alveolar-capillary membrane, with morphological and physiological evidence of severe epithelial injury in distal airways and alveolar walls (1, 2). In contrast to the endothelium, which changes permeability reversibly in response to stimuli, the epithelium forms a tight barrier that restricts the passage of solutes and proteins into the alveoli (reviewed in Ref. 3). The mechanisms responsible for epithelial damage in humans with acute lung injury remain uncertain.

We investigated the hypothesis that Fas ligand (FasL) shed into the alveolar fluid of patients with ARDS induces Fas-dependent apoptosis of epithelial cells in the alveoli and distal airways. The Fas/FasL system plays an important role in the regulation of cell life and death. This system is comprised of the cell membrane surface receptor Fas (CD95) and its natural ligand (FasL) (4). Fas is a 45-kDa type I membrane protein member of the TNF family of surface receptors (5). The natural ligand of Fas is Fas ligand (FasL), a 37-kDa type II protein (6). FasL exists as membrane-bound and soluble forms (7). Membrane-bound FasL mediates lymphocyte-dependent cytotoxicity, clonal deletion of alloreactive T cells, and activation-induced suicide of T cells (8–10). The soluble form (sFasL) results from cleavage of membrane FasL by metalloproteinases (11) and induces apoptosis in susceptible cells (7).

Several lines of evidence suggest that sFasL may be involved in the pathogenesis of tissue injury. Circulating sFasL is elevated in the serum of patients with leukemia (12), lymphoma (12, 13), and inflammatory diseases (14). Blockade of Fas (CD95) in humans with toxic epidermal necrolysis stops the progression of disease (15). Administration of sFasL to mice pretreated with bacteria produces death from hepatic failure (16), and mutant mice deficient in either Fasl (gld mice) or Fas (lpr mice) show decreased endothelial damage in response to the administration of IL-2 (17). Recent studies, however, suggest that sFasL is relatively ineffective in inducing apoptosis, and that the release of sFasL may actually down-regulate the apoptotic activity of membrane-bound Fas (11, 18). Thus, the role of sFasL in vivo is controversial.

The role of the Fas/FasL system in humans with acute lung injury has not been studied. Subsets of alveolar epithelial cells express Fas on their surface and undergo apoptosis in response to Fas ligation (19). Monocytes release sFasL in vitro when activated by phytohemagglutinin, immune complexes, or superantigen (20), raising the possibility that activated macrophages in the lungs might also release sFasL. Thus, FasL, on the cell surface or released as sFasL by activated macrophages might induce apoptosis of lung epithelial cells, resulting in the epithelial injury and permeability changes characteristic of ARDS.

We investigated whether sFasL accumulates in alveolar fluids during the course of ARDS, whether sFasL can be released by cells in the airspaces, and whether lung fluids from patients with ARDS can cause Fas-dependent apoptosis of lung epithelial cells in vitro. The results suggest that the Fas/FasL system is likely to be of fundamental importance in mediating injury to the lung epithelium in humans with ARDS.
Materials and Methods

Abs and proteins

The Abs used for sFasL detection by ELISA were monoclonal anti-human sFasL, mAb, as capture Ab (Clone 4H9; MBL, Nagoya, Japan), and biotinylated anti-human sFasL, mAb for detection (Clone 4A5; MBL). For flow cytometric detection of Fas expression, we used anti-Fas R-PE-conjugated mouse monoclonal IgG1 (clone DX2), and a PE-conjugated monoclonal mouse IgG1 as an isotype control Ab (clone MOPC-21) (both Abs from PharMingen, San Diego CA). The sFasL and the fusion protein Fas-Ig were prepared and purified as described previously (21). Anti-Fas mAb (IgG1, mouse, clone ZB4) and anti-FasL mAb (IgG, hamster, clone 4H9) were purchased from Coulter/Immuneon (Miami, FL). Anti HCG IgG1 (Pierce Chemical, Rockford IL) was used as an irrelevant control.

Distal lung epithelial cell culture

Frozen primary cultures of human distal lung epithelial cells (DLEC) were purchased from Clonetics (San Diego, CA) and cultured in complete growth media (SAGM; Clonetics) according to the manufacturer’s protocol. These cells were isolated from distal small airways (less than 1 mm in diameter) immediately postmortem from humans who were free of known respiratory diseases. These cells grow in monolayers, and by electron microscopy show evidence of lamellar-like bodies and absence of cilia and neurosecretory granules.

The cells were seeded in 75-cm² flasks at a density of 1 × 10⁶ cells/cm² and incubated at 37°C and 5% CO₂. The cells were subcultured when they reached 60–80% confluency. Cells were used for the experiments after the first or second subculture. In these culture conditions, the cells form monolayers and do not differentiate into ciliated cells.

Patient population and bronchoalveolar lavage protocol

All patients admitted to the intensive care units of Harborview Medical Center (Seattle, WA) between 2/14/94 and 3/12/97 were prospectively evaluated and enrolled if they met predetermined criteria either for being at risk for ARDS following sepsis or acute trauma, or for having established ARDS. Specific criteria for sepsis and trauma risks and for ARDS have been described (22). All patients with ARDS met the American European Consensus Conference definition of ARDS (23). The patients at risk for ARDS underwent fiberoptic bronchoscopy and bronchoalveolar lavage (BAL) within 24 h of the onset of risk for ARDS, then again 48 h later if they had not developed ARDS. Patients with established ARDS underwent fiberoptic bronchoscopy and BAL within 24 h of the onset of ARDS (day 1), and then again on days 3, 7, 14, and 21. Fiberoptic bronchoscopy and BAL were also performed on healthy volunteers who were free of lung disease. The BAL were performed by instilling five separate 30 ml aliquots of 0.9% NaCl to remove mucus. The lavage fluid was spun at 200 g for 30 min, and the supernatant was removed aseptically and stored at −70°C as individual aliquots in polypropylene tubes (22). Informed consent was obtained by the Human Subjects Review Committee of the University of Washington.

sFasL release by BAL cells

The ARDS BAL cell pellet was resuspended at 1 × 10⁶ cells/ml in RPMI 1640 (Life Technologies, Grand Island, NY) containing L-glutamine (292 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml), and incubated for 24 h at 37°C and 5% CO₂ in the following conditions: media only; medium + 10% heat-inactivated human AB serum; medium + 10% LPS, 100 ng/ml; and medium + LPS (100 ng/ml) + 10% heat-inactivated human AB serum. Data represent means ± SE.

FasL measurements

sFasL was measured with a modification of a sandwich immunoassay (24). Briefly, 96-well plates (Costar, Cambridge, MA) were coated overnight at 4°C with the capture Ab at 1.0 μg/ml. After incubation, the wells were washed three times with PBS containing 0.05% Tween-20 (Sigma; PBS-T) and blocked for 1 h at 37°C with 10% nonfat milk in PBS. After washing three times with PBS-T, the standards and samples were added to the wells, and the plate was incubated 1 h at 37°C. After washing three times with PBS-T, the detection Ab was added at 0.1 μg/ml, and the plate was incubated for 1 h at 37°C. The plate was washed three times with PBS-T, and AP-streptavidin (Zymed, San Francisco, CA) was added at a 1:2000 dilution, followed by 1 h incubation at 37°C. After incubation, the wells were washed three times with PBS-T and twice with 0.9% NaCl, then the ATTOphos fluorescence reagent (JBL Scientific, San Luis Obispo, CA), 200 μL/well was added to the wells, and the plate was incubated for 1 h at room temperature. FIGURE 2. sFasL concentrations in supernatants of BAL cells isolated from seven patients studied on day 3 of ARDS. The cells were incubated for 24 h at 37°C and 5% CO₂, under the following conditions: medium only; medium + 10% heat-inactivated human AB serum; medium + 10% LPS, 100 ng/ml; and medium + LPS (100 ng/ml) + 10% heat-inactivated human AB serum. Data represent means ± SE.

Table I. Demographic characteristics

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*Means ± SD at ICU admission.
temperature in the dark. After incubation, fluorescence was read on a Cytoscan II microtiter plate fluorometer (PerSeptive Biosystems, Framingham MA) using 430-nm excitation and 560-nm emission wavelengths. The assay was sensitive to an alamar Blue concentration of 4.0 pg/ml.

**Determination of Fas expression by flow cytometry**

Briefly, DLEC were cultured in complete media (SAGM; Clonetics) until reaching 70–90% confluence, detached with 0.025% trypsin containing 0.26 mM EDTA (Life Technologies), and washed with PBS. The cell pellet was resuspended at 4 × 10⁶ cells/ml in PBS with 10% FCS (HyClone, Logan UT), and incubated 45 min at 4°C in the dark with 10 µL of either anti-Fas PE-conjugated mAb or control mAb (0.5 mg/ml) for each 10⁶ cells. After incubation, the cells were washed twice and resuspended in 250 µL PBS, then analyzed by flow cytometry using a FACScan instrument (Becton Dickinson, San Jose, CA).

**Determination of apoptosis**

Apoptotic target cells (DLEC) were identified by three different methods: 1) alamar Blue reduction, 2) acridine orange staining, and 3) DNA end nick labeling assay. The alamar Blue assay is an assay of cell viability; the acridine orange stain and the DNA end nick labeling assay were used to show that the mechanism of cell death was apoptosis.

**Alamar Blue assay.** Alamar Blue (Biosource, Camarillo CA) is an oxidation/reduction indicator that fluoresces red when it accepts electrons generated during cellular metabolism. The DLEC were grown on 96-well tissue culture plates (Costar). After reaching 60–80% confluence, the experimental media were added to a total volume of 200 µl/well, and the cells were incubated for 24 h at 37°C, 5% CO₂. Following incubation, alamar Blue (25 µL) was added to the wells, and the plate was incubated for 1 h at 37°C, 5% CO₂. Fluorescence was measured on a Cytofluor II fluorometer using a 530-nm excitation filter and 590-nm emission filter.

**Acridine orange staining.** The DLEC were grown on 1.8-cm² chamber slides (Lab-Tek-8, Nunc, Naperville, IL). After reaching 60–80% confluence, the experimental media were added, and the cells were incubated for 24 h at 37°C, 5% CO₂. Following incubation, the chambers were removed, and the cell monolayers were labeled with 0.27-M acridine orange in PBS (Sigma) and examined by fluorescence microscopy. Cells were considered apoptotic if they showed condensation of the cytoplasm, and condensation of nuclear chromatin and/or nuclear fragmentation. The total number of cells and the number of apoptotic cells were counted in two low power fields (×160). The results are expressed as the percentage of total cells that were apoptotic.

**DNA end nick labeling assay.** For the DNA end nick labeling assays, the DLEC were grown in chamber slides as described above. The slides were submerged in 10% neutral buffer formalin for 10 min, followed by 70% ethanol for 5 min. The slides were rehydrated for 10 min in PBS and treated with 0.002% proteinase K (Sigma) in distilled water for 5 to 15 min at room temperature. Endogenous peroxidase was removed by placing the slides in 2% hydrogen peroxide in distilled water for 5 min. For equilibration, the slides were treated in 1× Klenow labeling buffer (TACS In situ Apoptosis Detection Kit; Trevigen, Gaithersburg, MD) for at least 1 min and then incubated for 60 min at 37°C with Klenow enzyme and Klenow dNTP mix in 1× Klenow labeling buffer (all reagents from Trevigen) prepared according to instructions from the manufacturer. As a negative control, slides were incubated with the labeling mixture without the Klenow enzyme. After incubation, the slides were completely submerged in 1× Klenow Stop buffer (Trevigen) for 5 min at room temperature and rinsed in 1× PBS for 2 min. The samples were then treated for 15 min with streptavidin-HRP detection solution (Trevigen), washed twice for 2 min in PBS, and incubated in diaminobenzidine (DAB) (Trevigen) for 7 min at room temperature. The samples were then rinsed twice in distilled water and stained with 1% methyl green in 0.1 M sodium acetate (pH 4.0) for 5 min, quickly dehydrated in 95% and 100% ethanol, cleared in xylene and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

**Statistical Analysis**

For normally distributed data, the two-tailed Student t test was used for comparisons between two groups. One-way ANOVA was used to compare multiple groups. When data were not normally distributed, the Wilcoxon rank sums test was used for comparisons between two groups. For comparisons between multiple groups, a Kruskal-Wallis one-way analysis of variance was used. If the Kruskal-Wallis reached statistical significance, ANOVA with Fischer’s post hoc analysis was performed on log₂-transformed data.

**Results**

**sFasL is released into the lung airspaces**

sFasL was measured by immunoassay in the BAL fluid of 20 patients at risk for ARDS and 45 patients with ARDS who were intubated and mechanically ventilated. Patients at risk for ARDS underwent BAL as soon as the risk factor for ARDS was defined. Patients with ARDS were studied on days 1, 3, 7, 14, and 21 after onset of ARDS. The main characteristics of the patient population are shown in Table I.

sFasL was detected in BAL fluid from patients at risk for ARDS and from patients with ARDS studied throughout the course of the disease (Fig. 1). In contrast, in BAL fluid from normal volunteers (n = 4), sFasL was undetectable in three individuals and present at 18 pg/ml in one individual. The median concentration of sFasL was elevated in the patients at risk for ARDS (128 pg/ml). In patients with ARDS, the median concentration of sFasL was elevated at the onset of ARDS (135 pg/ml) and varied with time during the course of the disease. At each time, more than 50% of the patients had detectable concentrations of sFasL in their BAL fluid.
FIGURE 4. Effect of recombinant sFasL on DLEC. The DLEC were incubated for 18 h at 37°C, 5% CO₂ in medium supplemented with either: (a and b) isotype-matched control mAb, 1 μg/ml (a, ×160; b, ×400); (c and d) sFasL, 500 ng/ml + control mAb, 1 μg/ml (c, ×160; d, ×400); (e) sFasL + anti-FasL mAb (4H9), 1 μg/ml (×160); (f) sFasL + anti-Fas mAb (ZB4), 1 μg/ml (×160), or (g) sFasL + Fas-Ig fusion protein, 100 μg/ml (×160). After incubation, the cells were stained with acridine orange and examined by fluorescence microscopy. The apoptotic cells are characterized by their smaller size and bright yellow-green nucleus. The red perinuclear staining is RNA staining unrelated to apoptosis. In c and d, most of the visible cells are apoptotic (the arrows show typical apoptotic cells), and the monolayer is disrupted; e, f, and g show few apoptotic cells and intact monolayers. The figure shows representative photomicrographs from three experiments with similar results.
Airspace cells release sFasL

To determine whether sFasL can be released by cells present in the airspaces of patients with ARDS, cells were isolated by centrifugation from the BAL fluid of seven patients with early ARDS and incubated overnight in the following conditions: media only, media with 10% serum, media with 100 ng/ml LPS, and media with 100 ng/ml LPS and 10% serum. After 24 h, the supernatants were collected and assayed for sFasL (Fig. 2). Cells incubated in media released small amounts of sFasL (466 ± 162 pg/ml). The supernatants from cells stimulated with LPS contained increased amounts of sFasL (1096 ± 474 pg/ml). Serum did not enhance the effect of LPS.

Fas ligation induces apoptotic death in primary human DLEC

Primary human DLEC expressed membrane Fas (CD95) as detected by flow cytometry (Fig. 3A). To determine sensitivity to sFasL, the DLEC were grown in 96-well tissue culture plates until reaching 80% confluency. The cells were exposed to serial dilutions of sFasL ranging from 15.62 ng/ml to 500 ng/ml for 18 h at 37°C, 5% CO₂, and the cells were analyzed with alamar Blue. Exposure of DLEC to sFasL caused a linear decrease in fluorescence over sFasL concentrations ranging from 62.5 to 500 ng/ml (Fig. 3B).

To demonstrate the specificity of these effects for sFasL, the DLEC were incubated in medium containing an irrelevant Ab (Fig. 4, a and b) or medium containing 500 ng/ml of sFasL, plus either an irrelevant mAb (Fig. 4, c and d), inhibitory anti-FasL mAb (4H9) (Fig. 4e), inhibitory anti-Fas mAb (ZB4) (Fig. 4f), or Fas-Ig fusion protein, which acts as a soluble receptor for FasL (Fig. 4g) (21). The cells exposed to sFasL, and the control Ab became apoptotic, whereas the cells exposed to sFasL with either anti-FasL mAb, anti-Fas mAb, or Fas-Ig did not develop morphologic features of apoptosis. The total number of cells, the number of apoptotic cells, and the percentage of apoptotic cells that appear in each panel of Fig. 4 are shown in Table II.

Effect of BAL fluid from patients at risk for ARDS or with ARDS on DLEC apoptosis

To determine whether the lung fluids from patients with ARDS caused Fas-dependent apoptosis of these target cells, we first incubated DLEC in medium supplemented at a 50% concentration with either BAL fluid from normal healthy volunteers, with BAL fluid from five patients on the first day of meeting criteria for risk of ARDS, or with BAL fluid from five patients on day 1 of ARDS. All of these lavage fluids had detectable concentrations of sFasL. The BAL fluids were tested under the following conditions: isotype-matched mAb control, inhibitory anti-FasL mAb (4H9), anti-Fas mAb (ZB4), or Fas-Ig fusion protein.

After 18 h incubation, the percentage of apoptotic cells in the wells exposed to BAL fluid from patients with ARDS was significantly higher than in the wells exposed to BAL from normal volunteers (p < 0.05) (Fig. 5). Inhibition of the Fas pathway with either anti-Fas mAb, anti-FasL mAb, or Fas-Ig fusion protein blocked the proapoptotic effect of every ARDS BAL fluid. In comparison, the BAL fluid from patients at risk for ARDS did not cause a significant increase in apoptosis of the target cells, as compared with normal BAL fluid. The morphologic characteristics of apoptosis and the disruption of the monolayer following incubation with ARDS BAL fluid are shown in Fig. 6. The DNA end nick labeling assays supported the interpretation that the mechanism of death was apoptosis (Fig. 7).

sFasL and survival

There was a significant relationship at the onset of ARDS between sFasL in BAL fluid and survival (Fig. 8). The patients who subsequently died had significantly higher concentrations of sFasL (median 344.2 ng/ml, range 4–1328.5 pg/ml) in the BAL fluid on day 1 of ARDS, as compared with those who lived (median 70.4 ng/ml, range 4–883.6 pg/ml) (p = 0.04). There was no significant association between the concentration of sFasL in BAL fluid and either the risk factor for ARDS, total BAL protein, total neutrophils (PMN), or the number of days on mechanical ventilation.

Discussion

The main goal of this study was to determine whether the mechanism of epithelial damage in acute lung injury could be related to epithelial cell apoptosis mediated by sFasL present in the airspaces. We found that sFasL is present in the lung fluids of humans before and after the onset of clinically defined ARDS. The lung fluids of patients with ARDS, but not the patients at risk, induced apoptosis of primary human lung epithelial cells by a Fas/FasL-dependent mechanism. The finding that sFasL concentrations were significantly higher in patients with ARDS who died suggests that this pathway is likely to be important in determining the fate of the lung epithelium in humans with acute lung injury.

The alveolar epithelium forms a tight barrier between the airspaces and the vascular compartment (25). In addition, alveolar
FIGURE 6. Morphologic characteristics of DLEC incubated with ARDS BAL fluid. DLEC were incubated in medium supplemented at a 50% concentration with either BAL fluid from a normal volunteer or BAL fluid from three patients studied on day 1 of ARDS. The cells were incubated for 18 h at 37°C, 5% CO₂, then stained with acridine orange and examined by fluorescence microscopy. Apoptotic cells are small and have yellow-green nuclear staining (arrows). The red perinuclear staining is RNA staining and unrelated to apoptosis. The figure shows representative photomicrographs (×160) from: (a) normal BAL fluid; (b) ARDS BAL fluid (sFasL concentration 5208 pg/ml); (c) ARDS BAL fluid (sFasL 5884 pg/ml); and (d) ARDS BAL fluid (sFasL 51329 pg/ml). Incubation with ARDS BAL fluid caused apoptosis and disruption of the monolayer (b, c, and d). Panels e–g show results of incubating cells in ARDS BAL fluid (shown in c) in the presence of: (e) anti-FasL mAb (4H9), 1 μg/ml; (f) anti-Fas mAb (ZB4), 1 μg/ml; and (g) Fas-Ig fusion protein, 100 μg/ml. Each of these different anti-Fas system strategies resulted in fewer apoptotic cells and healthy monolayers in the presence of ARDS BAL fluid (e–g). The figure shows representative photomicrographs from three experiments with similar results.
epithelial cells actively regulate the transport of fluid and electrolytes between the airspaces and the interstitium (3, 26). A characteristic feature of the acute respiratory distress syndrome is widespread destruction of the alveolar epithelium (27) leading to noncardiogenic pulmonary edema. The mechanism of destruction of the alveolar epithelium is unclear. We report here that human DLEC express Fas and that sFasL can induce apoptosis of these target cells by a Fas-dependent mechanism. Furthermore, BAL fluid from patients with ARDS contains sFasL at concentrations sufficient to induce Fas-dependent apoptosis of human DLEC. The findings demonstrate that sFasL is released in vivo during human disease as a biologically active, death-inducing effector molecule capable of inducing apoptosis in Fas-susceptible target cells of the lungs, and suggest that the Fas/FasL system could play a role in the pathogenesis of the epithelial injury seen in ARDS.

The role of sFasL in the pathogenesis of tissue injury has been controversial. Some evidence indicates that sFasL can cause injury in the lungs and other tissues. For example, subsets of murine type II pneumocytes express Fas Ag, and the intratracheal instillation of an activating anti-Fas Ab induces apoptosis of type II cells in vivo (28). However, two separate groups have recently found that sFasL exerted an antiapoptotic effect by competing with membrane-bound FasL for binding to Fas (11, 18). Tanaka et al. found that sFasL in solution was less effective than membrane FasL in inducing apoptosis, and that sFasL inhibited the cytotoxicity mediated by membrane FasL (11). Thus, it was proposed that sFasL might function to protect healthy bystander cells from cells bearing FasL, such as cytotoxic T cells. Schneider et al. found that sFasL is 1000-fold less active than membrane-bound FasL, and that inoculating high doses of sFasL in mice did not cause hepatic failure, providing support for the idea that sFasL is less efficient than membrane FasL in mediating cell death (18).

The data from this study may help clarify the role of sFasL in the pathogenesis of tissue injury in general and of acute lung injury in particular. We found that human DLEC are sensitive to Fas ligation, but that relatively high concentrations of sFasL are required for the induction of apoptosis in vitro (e.g., 100 ng/ml). These results appear to confirm those of Tanaka et al. (11). However, when the cells were incubated in BAL fluid from patients at the onset of ARDS, they became apoptotic, even though the concentrations of sFasL in the BAL fluid were less than those required to induce apoptosis of DLEC in vitro (e.g., 1.0 ng/ml; Fig. 1). The BAL fluid from patients at risk for ARDS had no effect on DLEC apoptosis, despite containing similar concentrations of sFasL as the BAL fluid from patients at the onset of ARDS. This suggests that a cofactor may be required for the development of the apoptotic response to sFasL. The need for other factors that potentiate the effects of sFasL would also explain the fact that, in the at risk population, the BAL fluid concentrations of sFasL were unrelated to subsequent development of ARDS and to mortality, while during early ARDS the BAL fluid concentration of sFasL was significantly higher in the patients who died. These factors could include oxidative stress (29, 30), which induces apoptosis in cell lines (31–33), inflammatory cytokines, or a previously undescribed mediator. In preliminary studies, however, we have found that the DLEC used in these experiments are not sensitive to TNF-α or IL-1β, either alone or in conjunction with sFasL (data not shown). The requirement of a cofactor for the development of a full apoptotic response to sFasL may explain the apparent discrepancies between our finding that sFasL can induce apoptosis of target cells and those of Tanaka et al. (11) and Schneider et al. (18), which suggest that sFasL is a poor inducer of apoptosis.

![FIGURE 7. DNA end nick labeling assays performed on distal small airway epithelial cells incubated in either BAL fluid from a normal volunteer or BAL fluid from five patients with ARDS. The figure shows representative photomicrographs of cells incubated in BAL fluid from a normal volunteer, showing no signal (a, ×160; b, ×400), or cells incubated in ARDS BAL fluid, showing apoptotic cells with nuclear staining from the DAB reactive product (c, ×160; d, ×400) (arrows) (×160). Similar results were obtained with all five ARDS BAL fluids.](image1)

![FIGURE 8. FasL concentration measured by immunoassay in BAL fluid from patients on day 1 of ARDS, according to survival. Bars represent median values. p < 0.05 by Student’s t test (two-tailed).](image2)
There are several potential sources for the sFasL detected in the lung fluids. The data show that cells recovered from the airspace of patients with ARDS release sFasL, and that the release increases after stimulation with LPS, which is detectable in the BAL fluid of many patients with ARDS (34). Because the cells recovered by BAL are a mixed population of alveolar macrophages and neutrophils, the data do not indicate which cell type is responsible for sFasL release. The alveolar macrophage is a possible source, since cells of the human monocyte/macrophage lineage contain FasL and release it when activated by phytohemagglutinin, immune complexes, or superantigen (20). A second possibility is that sFasL increases in the circulation as part of the systemic inflammatory response, and then moves into the airspaces when endothelial and epithelial permeability increase. Indeed, plasma sFasL concentrations are elevated in the serum of patients with inflammatory diseases (14), and we have detected high circulating concentrations of sFasL in the serum of patients with sepsis (data not shown). Thus, it is possible that locally shed sFasL and systemic influx of circulating sFasL account for the increased concentrations that we detected in BAL fluid from patients at risk for and with ARDS.

In earlier studies, we found that BAL fluid from patients with early ARDS (days 1 and 3) inhibits neutrophil apoptosis, and that this effect is mediated primarily by the cytokines G-CSF and GM-CSF (22). We now report that BAL fluid obtained at the same times during the course of ARDS contains sFasL and has the ability to induce apoptosis in primary human lung epithelial cells. These findings indicate that, during ARDS, proapoptotic and antiapoptotic mediators are present in the airspaces and that the biologic effects depend on the specific mediators and cell types. Thus, the inflammatory milieu may be proapoptotic for some cells (e.g., epithelial cells) and antiapoptotic for others (e.g., neutrophils).

The target cells that we used to establish a role for sFasL are nonciliated pulmonary epithelial cells that are derived from distal small airways (1–2 mm diameter) of normal lungs. In the culture conditions that we used, they form monolayers and do not differentiate into ciliated cells (Fig. 4). Cytokeratin staining indicates that these cells are of epithelial origin. By electron microscopy, the cells lack neurosecretory granules and form lamellar-like bodies. We have also found that they stain positive with two different murine mAb raised against human surfactant protein A (data not shown). However, they are not primary alveolar Type I or Type II cells, which are difficult to isolate from human lungs. Traditional surrogates of alveolar epithelial cells, such as A549 cells, are not necessarily good targets for studies of normal apoptosis pathways because they are immortal cells. The DLEC provide an approximation of the responses of alveolar epithelial cells, but studies with authentic Type I or Type II alveolar epithelial cells will be required to confirm these findings. In recent studies, we have found that the activation of the Fas/FasL system in the lungs of mice results in apoptosis of cells in the alveolar epithelium and development of alveolar injury (35). Further studies in animal models of lung injury, particularly with mice deficient in Fas (lpr mice) or Fasl (gld mice), could help clarify the role of the Fas/Fasl system in the pathogenesis of the epithelial destruction seen during acute lung injury.

The results of this study have important implications. The data show that sFasL can induce apoptosis of human lung epithelial cells during acute lung injury. In addition, the data suggest that the permeability changes seen in ARDS could be due at least in part to Fas/Fasl-dependent apoptosis of lung epithelial cells. Strategies designed to inhibit the Fas/Fasl system might prevent or modify the lung epithelial damage that occurs in humans with acute lung injury.

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References


