

## TRANSLATIONAL PHYSIOLOGY | *Acute Lung Injury*

# Elevation of KL-6, a lung epithelial cell marker, in plasma and epithelial lining fluid in acute respiratory distress syndrome

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**Ishizaka, Akitoshi, Tomoyuki Matsuda, Kurt H. Albertine, Hidefumi Koh, Sadatomo Tasaka, Naoki Hasegawa, Nobuoki Kohno, Toru Kotani, Hiroshi Morisaki, Junzo Takeda, Morio Nakamura, Xiaohui Fang, Thomas R. Martin, Michael A. Matthay, and Satoru Hashimoto.** Elevation of KL-6, a lung epithelial cell marker, in plasma and epithelial lining fluid in acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 286: L1088–L1094, 2004. First published September 5, 2003; 10.1152/ajplung.00420.2002.—KL-6 is a pulmonary epithelial mucin more prominently expressed on the surface membrane of alveolar type II cells when these cells are proliferating, stimulated, and/or injured. We hypothesized that high levels of KL-6 in epithelial lining fluid and plasma would reflect the severity of lung injury in patients with acute lung injury (ALI). Epithelial lining fluid was obtained at onset (*day 0*) and *day 1* of acute respiratory distress syndrome (ARDS)/ALI by bronchoscopic microsampling procedure in 35 patients. On *day 0*, KL-6 and albumin concentrations in epithelial lining fluid were significantly higher than in normal controls ( $P < 0.001$ ), and the concentrations of KL-6 in epithelial lining fluid ( $P < 0.002$ ) and in plasma ( $P < 0.0001$ ) were higher in nonsurvivors than in survivors of ALI/ARDS. These observations were corroborated by the immunohistochemical localization of KL-6 protein expression in the lungs of nonsurvivors with ALI and KL-6 secretion from cultured human alveolar type II cells stimulated by proinflammatory cytokines. Because injury to distal lung epithelial cells, including alveolar type II cells, is important in the pathogenesis of ALI, the elevation of KL-6 concentrations in plasma and epithelial lining fluid could be valuable indicators for poor prognosis in clinical ALI.

alveolar type II cell; pulmonary edema; microsampling

THE PATHOPHYSIOLOGY of the acute phase of clinical acute lung injury (ALI) is characterized by accumulation of protein-rich edema fluid resulting from injury to both the endothelial and epithelial barriers of the lung (27, 30). The significance of lung endothelial injury and increased permeability in ALI is well documented (6). Because an intact alveolar epithelial barrier is necessary to prevent alveolar flooding and facilitate recovery

from acute respiratory distress syndrome (ARDS), lung epithelial integrity and function are important determinants of the clinical outcome in ALI (22, 26, 32). When alveolar type II cells are injured, normal alveolar epithelial fluid transport and removal of alveolar edema fluid are impaired (23). Furthermore, injury to type II cells reduces the production and turnover of surfactant (12) and may also cause intrapulmonary bacterial translocation, leading to sepsis (21).

Some biochemical markers have recently been described that predict the development of ALI/ARDS or the clinical outcome in patients with ALI/ARDS. For example, the appearance of type III procollagen peptide in edema fluid or bronchoalveolar lavage (BAL) fluid identifies patients with fatal outcomes, perhaps because it indicates that fibrosing alveolitis has begun early in the course of ALI (7, 8). Abnormalities of surfactant-associated proteins in BAL fluid and plasma have also been associated with poor clinical outcomes (3, 12). However, very few clinical studies have measured biochemical markers in ALI to assess pulmonary epithelial damage per se. The present study focused on the appearance of KL-6 in pulmonary epithelial lining fluid (ELF) and plasma. KL-6, a pulmonary epithelial mucin with low molecular weight, is an integral membrane glycoprotein classified as cluster 9 (MUC1) (14, 28), with an extracellular domain consisting mostly of tandem repeats of 20 amino acid sequences and a cytoplasmic tail (13). KL-6 splits off at the S-S bond near the epithelial membrane surface and becomes distributed in pulmonary ELF (13). This glycoprotein is mainly expressed on alveolar type II cells in the lung (20) and is expressed more prominently on proliferating, regenerating, or injured type II cells than on normal type II cells (19, 20). The presence of KL-6 has been used to monitor severity of disease in idiopathic pulmonary fibrosis (19).

The primary objectives of this study were 1) to determine the changes in KL-6 levels in plasma and ELF in patients with ALI to study injury to the lung epithelial barrier and 2) to test the

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hypothesis that elevated levels of KL-6 in plasma or ELF may be a useful biochemical predictor of outcome in ALI patients.

## METHODS

**Study populations and protocol.** The clinical study was conducted at the hospitals of Keio University and Kyoto Prefectural University of Medicine between July 1999 and September 2001. The Human Research committees of Keio University and Kyoto Prefectural University of Medicine approved this study, and informed consent was obtained from each study participant or from an immediate family member. Thirty-five patients were identified as having ALI or ARDS prospectively according to the definitions of the American European Consensus Conference on ARDS (2). They were included if they met consensus conference oxygenation and radiographic criteria for either ALI or ARDS and were followed until death or hospital discharge (the latter is defined as "survival"). The lung injury score and the bone criteria for sepsis syndrome were used (5, 24). Patients <15 yr old or admitted to the intensive care unit (ICU) >24 h after the diagnosis of ALI were excluded from the study.

Control data were obtained from 18 men and 3 women, 39–74 yr old, who underwent bronchoscopy so that causes of hemoptysis could be identified or small, solitary, peripheral pulmonary nodules could be examined. Chest computed tomography revealed no diffuse interstitial lung abnormality, and pulmonary function tests and  $\text{SaO}_2$  were normal. In addition, five patients with cardiogenic pulmonary edema (four men and one woman, 54–77 yr old) that was confirmed by pulmonary artery wedge pressure >18 mmHg or echocardiogram were included as reference patients.

Bronchoscopic microsampling (BMS) was not performed if the patient met any of the criteria of ALI severity described by Steinberg et al. (29): 1)  $\text{PaO}_2 < 80$  mmHg with  $\text{FiO}_2 = 1.0$ , 2) systolic blood pressure <90 mmHg, 3) complex ventricular arrhythmias, or 4) endotracheal tube diameter <7.0 mm.

In ALI patients, BMS of pulmonary ELF was performed on *days 0* (onset of ALI) and *1*, unless the patient was extubated or had died. *Day 0* was defined as the day when the first BMS was done within 24 h after the diagnosis of ALI. In addition, blood was sampled on *days 0, 1, 3, 5, 7, and 10*. ELF and plasma were not sampled in two clinically unstable ALI patients on *days 0* and *1*. Control patients and patients with cardiogenic pulmonary edema underwent a single blood sampling and BMS.

**BMS procedure.** All patients with lung edema were sedated and preoxygenated ( $\text{FiO}_2 = 1.0$ ). A flexible bronchoscope (BF-6C240; Olympus, Tokyo, Japan) was inserted into the lung through intratracheal tube for examination of the airway, and excess sputum was suctioned if present. Another identical bronchoscope was then inserted, and its tip was advanced into a segmental bronchus of the right middle lobe (S4 or S5). The BMS probe, recently developed in conjunction with Olympus (Tokyo, Japan), consists of a 1.7-mm-diameter polyethylene outer sheath and an inner fiber rod probe 1.2 mm in diameter and 30 mm in length attached to a stainless steel guide wire 100 cm in length. The BMS procedure has been described in detail previously (16). Briefly, the probe was inserted into the channel and gently advanced. While the outer sheath was held at the target in the subsegmental bronchus, the inner probe was advanced slowly into the peripheral airway until it was in contact with the mucosal surface and was maintained in that position for 5–7 s, thus allowing the fiber rod to absorb ~20  $\mu\text{l}$  of ELF. The inner probe was then withdrawn into the outer sheath, and both were removed together. The wet inner probe was cut, placed in a tube, and stored in a freezer at  $-80^\circ\text{C}$  until analysis. The procedure was performed in triplicate from the same subsegmental bronchus. In control patients, after standard local anesthesia with lidocaine, a flexible fiber-optic bronchoscope was inserted into the right bronchus for the BMS procedure.

The stored frozen probes were weighed before the ELF saline suspension was prepared. We prepared the solution of diluted ELF for

biochemical measurements by adding the three frozen probes, which had been inserted into the same lung subsegment, into a 15-ml polyethylene tube containing 3 ml of saline vortexed for 1 min. The solution was centrifuged for 15 min at 3,000 rpm, and the supernatant was collected. The probe was dried and weighed to calculate the ELF volume recovered from the BMS probes. The dilution factor was calculated as follows:  $\text{ELF volume (ml)} / [3 \text{ ml} + \text{ELF volume (ml)}]$ .

In vitro experiments confirmed that the absorption of 2–20  $\mu\text{l}$  of human serum by the fiber rod probe allowed a >93% recovery of biochemical constituents. The recovery was 96.1% for albumin, 93.7% for lactate dehydrogenase (LDH), and 95.3% for KL-6.

**Measurements of KL-6 and albumin.** KL-6 measurements were performed by a sandwich enzyme-linked immunosorbent assay using anti-KL-6 mouse monoclonal antibodies as solid-phase and enzyme-labeled antibodies. These antibodies were prepared by intraperitoneal injection of anti-KL-6 mouse monoclonal antibody-producing cells. The mouse ascites fluid was purified with a protein A column (18). We prepared the antibody-producing cells by immunizing a mouse with pulmonary adenocarcinoma cells (VMRC-LCR cells) and fusing its splenocytes and myeloma NS1 cells by Kohler's and Milstein's method (17).

KL-6 measurements were performed in duplicate. For plasma samples, we carried out predilution by adding 10  $\mu\text{l}$  of plasma specimen to 2 ml of a diluent (0.05 M Tris-HCl buffer, pH 7.5, containing 1% BSA and 0.1% wt/vol sodium azide). Then, 100  $\mu\text{l}$  of a reaction solution (0.05 M Tris-HCl buffer, pH 7.5, containing 10% normal rabbit serum, 0.1% normal mouse serum, 10 mM EDTA, 0.15 M NaCl, and 0.1% wt/vol sodium azide) were added to each well of a 96-well plate coated with anti-KL-6 monoclonal antibodies. Twenty microliters of the sample and 20  $\mu\text{l}$  of the standard antigen were then added. This preparation was incubated at  $25^\circ\text{C}$  for 2 h. A 0.05 M Tris-HCl buffer, pH 7.5, containing a detergent, was used to wash the inside of each well to remove unreacted substances. Next, 100  $\mu\text{l}$  of an enzyme-labeled antibody solution were added and allowed to react at  $25^\circ\text{C}$  for 1 h. After being washed, 100  $\mu\text{l}$  of an enzyme-substrate solution were added and allowed to react at  $20\text{--}30^\circ\text{C}$  for 30 min. We prepared this enzyme-substrate solution by dissolving 6 mg of 2,2'-azino-di-3-ethyl-benz-thiazoline-6-sulphonic acid in 12 ml of a citrate buffer solution (0.28 M, pH 4.2) and then adding 30  $\mu\text{l}$  of 3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). We stopped the reaction by adding 100  $\mu\text{l}$  of 0.013% sodium azide, measured the absorbance (main  $\lambda$ , 405 nm; secondary  $\lambda$ , 492 nm), and determined the concentration of KL-6 from a standard calibration curve.

The albumin was measured with colorimetric assay kits (Beckman, Fullerton, CA). The concentrations of KL-6 and albumin in ELF obtained by the BMS probe were expressed by unit volume of ELF after correction for the dilution factor.

**Lung KL-6 immunostaining.** Autopsy tissues from 12 patients admitted between 1996 and 2001 to the adult medical ICU of the University of Utah Hospital or Latter-Day Saints Hospital, in Salt Lake City, were analyzed. The samples were collected within 12–14 h of death by standard anatomical pathology approaches optimized to preserve tissue architecture and antigen display. The tissues were collected from six patients who died with ALI or ARDS and from six patients who died of nonpulmonary causes. Patients who died of nonpulmonary disorders served as controls. The autopsy protocol was approved by the Institutional Review Board committees at both participating hospitals. The requirement for written informed consent was waived by both committees.

Two to three tissue cubes ( $>2 \times 2 \times 2$  cm) were obtained from each of the three lobes of the right lung of each patient. The cubes were sliced ( $3 \text{ mm} \times 2 \text{ cm} \times 2 \text{ cm}$ ), and the slices were immersed in 10% buffered neutral formalin (VWR, Media, PA) overnight at  $4^\circ\text{C}$ . The slices were placed in 70% ethanol and processed immediately. Paraffin-embedded tissue sections (5  $\mu\text{m}$ ) were collected on PLUS slides (VWR), and the paraffin was removed from the sections before immunohistochemistry (15). We treated the tissue sections with meth-

anol-H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. We inhibited nonspecific binding of the primary antibody by treating the tissue sections with blocking buffer. The primary anti-KL-6 monoclonal antibody was placed on the tissue sections (1:1,000 and 1:2,000 dilutions). After overnight incubation (4°C), the tissue sections were treated with a secondary antibody (horse anti-mouse). Antigen was detected by a standard peroxidase method (ABC Elite kit; Vector Laboratories, Burlingame, CA). Immunohistochemical staining controls included substitution of the primary antibody with an irrelevant, species-matched, immunoglobulin isotype-matched secondary antibody (anti-insulin), omission of the primary antibody (replaced with blocking buffer), and omission of the secondary antibody (replaced with blocking buffer). We counterstained the tissue sections with Gill's no. 3 hematoxylin and photographed them with a Zeiss Axio-Phot microscope system equipped with a Jenoptik high-resolution color digital camera (ProgRes model 3012). Figure composition was performed with Adobe Photoshop, without alteration of image color or detail.

**Colocalization of KL-6 protein with a marker of alveolar type II epithelial cells.** KL-6 immunolocalization to the apical region of alveolar type II epithelial cells was performed by double immunofluorescence microscopy with both the KL-6 antibody described above and surfactant precursor protein B (SPPB) antibody (catalog no. NCL-SPPB; Novocastra Laboratories, Newcastle, UK). Double immunofluorescence was performed on tissue sections from the same blocks of lung tissue that were used for localization of KL-6 protein alone. All of the tissue sections were incubated with 1:50 dilution of SPPB overnight at 4°C, followed by incubation with 1:250 dilution of anti-mouse-biotin conjugate (catalog no. BA-2000, Vector Laboratories) at room temperature for 30 min. The sections were then incubated with 1:250 dilution of streptavidin-horseradish peroxidase conjugate (catalog no. NEL701, TSA Fluorescein kit; Perkin-Elmer, Boston, MA) at room temperature for 30 min. We performed signal amplification by incubating the tissue sections in a 1:250 dilution of tyramide FITC (TSA Fluorescein kit, Perkin-Elmer) at room temperature for 10 min. After several rinse steps, the same tissue sections were incubated with 1,000 dilution of the anti-KL-6 antibody at room temperature for 2 h, followed by incubation with anti-mouse IgG rhodamine conjugate (605-140; Roche Molecular Biochemicals, Indianapolis, IN) at room temperature for 60 min (1). Immunofluorescence staining controls included substitution of each primary with normal horse serum, substitution of each primary antibody with an irrelevant, species-matched, isotype-matched antibody (anti-insulin), and substitution of the secondary antibody with phosphate-buffered saline (PBS). We used a Zeiss AxioPhot photomicroscope equipped for epifluorescence microscopy. Photographs were recorded on color slide film (Kodak ASA 400). The color slides were scanned (1,200 dpi) to prepare digital images for figure composition, which was then prepared for illustration using Adobe Photoshop, without alteration of image color or detail.

**Isolation and culture of human alveolar type II cells.** Alveolar epithelial type II cells were isolated by a modification of methods previously described (4, 9–11). Briefly, type II cells were isolated from human lungs that were not used by the Northern California Transplant Donor Network. Recent studies indicate that these lungs are in good condition physiologically and pathologically (31). Cells were isolated after the lungs had been preserved for 4–8 h at 4°C. The pulmonary artery was perfused with PBS solution at 37°C, and the distal air spaces were lavaged 10 times with warmed Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free PBS solution containing 0.5 mM EGTA and EDTA. Then, 12.9 U/ml elastase in Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free HBSS were instilled into the distal air spaces through segmental intubation. The lungs were minced finely in the presence of fetal bovine serum (FBS) and DNase (500 µg/ml). The cell-rich fraction was filtered by sequential filtration through one-layer gauze, two-layer gauze, 150-mm, and 30-mm nylon meshes. The resultant pellet was resuspended in DMEM containing 10% FBS, and then the cell suspension was incubated in tissue culture-treated

plastic petri dishes in a humidified incubator (5% CO<sub>2</sub>, 37°C) for 90 min. The cell-rich solution was layered onto a discontinuous Percoll density gradient 1.04–1.09 g/ml solution and centrifuged at 1,500 rpm for 20 min. The recovered upper band contains a mixture of alveolar type II cells and alveolar macrophages. The cell-rich solution containing alveolar type II pneumocytes and macrophages was centrifuged at 800 rpm for 10 min. The resultant pellet was resuspended in DMEM containing 10% FCS. We then incubated the cells containing DMEM in magnet beads coated with anti-CD14 antibodies at 4°C for 40 min during constant mixing and then passed them over a magnetic column to separate alveolar macrophages (CD14 positive). The cell viability was assessed by trypan blue exclusion. The purity of isolated human alveolar type II cells was checked by Papanicolaou staining or by anti-human type II cell antibody (a gift from Dr. Leland Dobbs, University of California, San Francisco) and was >90%. Freshly isolated alveolar type II cells were resuspended in cell preservation fluid and maintained at –80°C.

**KL-6 secretion from human alveolar type II cells and distal lung epithelial cells.** Human alveolar type II cells were thawed and seeded in 24-well collagen I-coated plate. After 48 h, the cells are nearly confluent. At that time, we added to each well Cytomix (Boehringer Mannheim, Indianapolis, IN) containing IFN-γ, IL-1β, and TNF-α, each in final concentrations of 10 or 50 ng/ml (25), and the cells were incubated for 24 h. The supernatant was then recovered to measure the concentration of KL-6. The levels of LDH in the supernatant were also measured by colorimetric assay kit (Jisseikenn, Tokyo, Japan) to assess the degree of cell injury.

Distal lung epithelial cells (Clonetics, San Diego, CA) were subcultured in small airway epithelial cell growth medium (Clonetics). They were then incubated at 37°C in a humidified, 95% air-5% CO<sub>2</sub> atmosphere. They were split upon reaching 60–70% confluence and used before the 4th passage. Cells were grown on 24-well tissue culture plates (Costar, Cambridge, MA). The supernatants were removed when the distal lung epithelial cells were nearly confluent. At that time, we added Cytomix (10 ng/ml or 50 ng/ml) in each well, and the cells were incubated for 24 h. The supernatant was then recovered to measure the concentration of KL-6.

**Statistical analyses.** Statistical significance was defined as  $P < 0.05$ . Differences in variables between control and ALI patients and between survivors and nonsurvivors at each time point were compared by the nonparametric Mann-Whitney  $U$ -test, since the data were not normally distributed. Analyses were performed with receiver operating characteristics (ROC) curves for individual plasma KL-6 and KL-6 in ELF in predicting the prognosis of ALI. The in vitro results of KL-6 production from pulmonary cells were examined by one-way analysis of variance with multiple comparisons and Fisher's least significant difference test.

## RESULTS

**Patient population.** There were 35 patients with ALI, 21 control patients with normal lung function, and 5 control patients with cardiogenic pulmonary edema included for the sampling study. Sampling of ELF was not performed in two clinically unstable patients with ALI at study onset (*day 0*) and *day 1*. In patients with ALI, the primary disorders were sepsis ( $n = 17$ ), pneumonia ( $n = 9$ ), gastric aspiration ( $n = 5$ ), and miscellaneous causes, including massive transfusion and acute pancreatitis ( $n = 4$ ). Criteria for ARDS were met in 27 of the 35 patients. The ALI population was 77% male, with a mean age of 68 yr. The initial mean values ( $\pm$  SE) for PaO<sub>2</sub>/FiO<sub>2</sub> ratio and lung injury score were  $160 \pm 11$  and  $2.4 \pm 0.1$ , respectively. The in-hospital mortality rate was 32%.

The average recovery of ELF was  $17.3 \pm 1.8$  µl in ALI patients,  $16.2 \pm 1.4$  µl in patients without lung disease, and



20.1 ± 2.8 µl in patients with cardiogenic pulmonary edema (mean ± SE, not significantly different among the groups).

**KL-6 and albumin levels in ELF and plasma.** Hemorrhage, pneumothorax, significant changes in SaO<sub>2</sub>, or other complications were not observed during or after BMS. The albumin concentration in ELF at ALI onset was significantly higher than that in control patients ( $P < 0.001$ ; Fig. 1, top). In five reference patients with cardiogenic pulmonary edema, the median concentration of albumin in ELF was 8 mg/ml.

There was no significant difference in plasma KL-6 levels between ALI on day 0 and control patients (median: 171 vs. 147 U/ml). However, the median KL-6 level in ELF at onset of ALI (median: 2,354 U/ml) was 10-fold higher than in controls (median: 233 U/ml,  $P < 0.0001$ ; Fig. 1, bottom). The KL-6 level in ELF on day 1 of ALI slightly decreased from day 0 but was still higher than control levels (median = 1098.1,  $P < 0.001$ ). The median plasma and ELF KL-6 levels in the five patients with cardiogenic pulmonary edema were 181 and 322 U/ml, respectively.

**KL-6 and survival in ALI.** In ALI patients, the KL-6 concentration in ELF was significantly higher in nonsurvivors than in survivors on day 0 and day 1 ( $P < 0.002$ ,  $P < 0.05$ , respectively; Fig. 2). Plasma KL-6 concentrations were elevated on day 0 in ALI patients who died compared with survivors ( $P < 0.0001$ , Fig. 3). Also, the plasma concentration of KL-6 was significantly higher in nonsurvivors than in survivors throughout the entire clinical course (Fig. 3).

**ROC curves.** ROC curves demonstrate that the concentrations of KL-6 in plasma and in ELF at ALI onset were both sensitive and specific predictors of fatal outcomes (Fig. 4). The

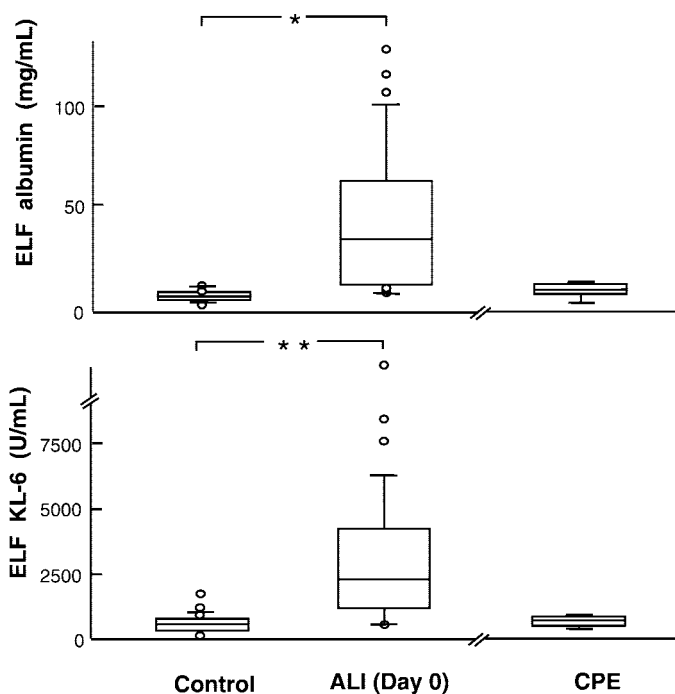


Fig. 1. Albumin (top) and KL-6 concentrations (bottom) in epithelial lining fluid (ELF) in control patients ( $n = 21$ ), patients at the onset of acute lung injury (ALI,  $n = 33$ ), and 5 patients with cardiogenic pulmonary edema (CPE). The Box-whisker plots show the 25th and 75th percentiles, the median (horizontal line within the box), and the 10th and 90th percentiles (whiskers). \* $P < 0.0001$ , \*\* $P < 0.001$  (ALI vs. control patients).

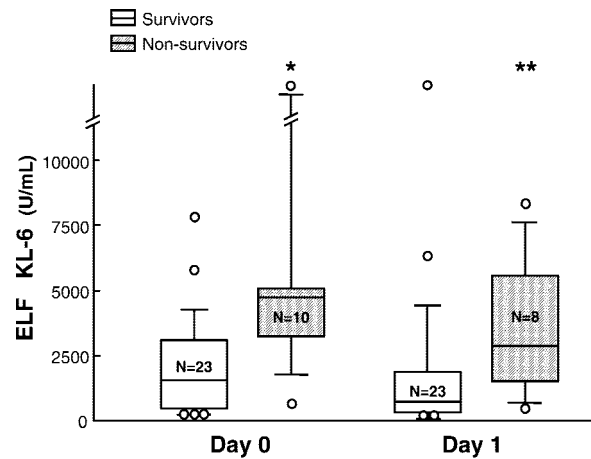


Fig. 2. KL-6 concentrations in ELF obtained from survivors vs. nonsurvivors with ALI on days 0 and 1. The Box-whisker plots show the 25th and 75th percentiles, the median (horizontal line within the box), and the 10th and 90th percentiles (whiskers). \* $P < 0.002$ , \*\* $P < 0.05$  (nonsurvivors vs. survivors).

optimal cut-off value of plasma KL-6 was 253 U/ml, with a sensitivity, specificity, and likelihood ratio of 87%, 100%, and 23, respectively. In 10 of 12 patients (83%) who died, the plasma KL-6 concentration was >253 U/ml, in contrast to no deaths in the 20 patients with values <253 U/ml ( $P < 0.001$ ). The optimal cut-off value of KL-6 in ELF was 2,734 U/ml, with a sensitivity, specificity, and likelihood ratio of 83%, 90%, and 30, respectively. In 9 of 13 patients (69%) who died, the ELF concentration was >2,734 U/ml, whereas only 1 of 19 patients (5%) with an ELF concentration <2,734 U/ml died ( $P = 0.001$ ).

**Lung histopathology.** The lungs of the six patients who died with ALI/ARDS had characteristic pathological changes, including accumulation of neutrophils in the distal air spaces, proliferation of alveolar type II cells, and alveolar edema. The KL-6 protein was predominantly expressed on the surface of epithelial cells, probably type II cells. There was also some KL-6 expression on what appeared to be denuded epithelial cells in the alveolar space in samples from ALI patients (see example in Fig. 5). KL-6-positive cells were considerably more abundant in the lung tissue sections from the six patients who

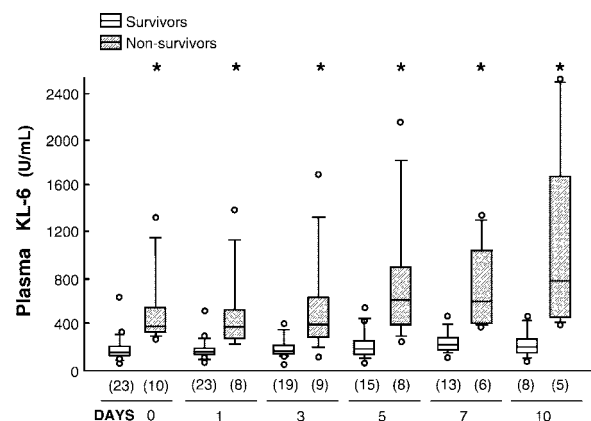


Fig. 3. Serial changes in plasma KL-6 concentrations in survivors vs. nonsurvivors with ALI. Data are presented as in Figs. 1 and 2. Values in parentheses indicate numbers of observations. \* $P < 0.008$  vs. survivors.

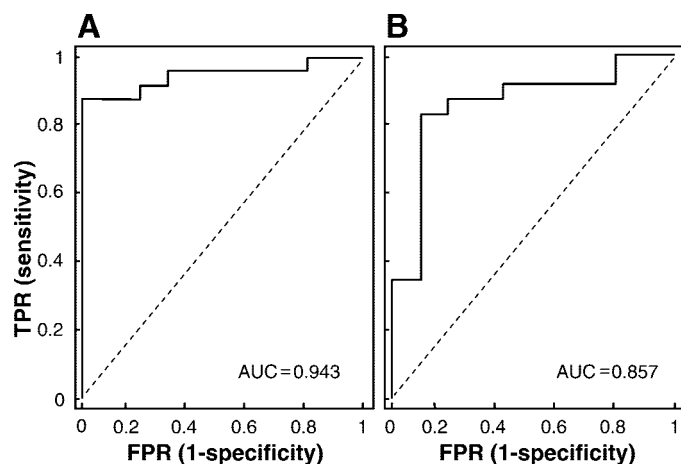


Fig. 4. Receiver operating characteristic curves relating KL-6 concentrations at onset of ALI in plasma (A) and in ELF (B) with survival. The ordinate represents the number of true positive responses (TPR, sensitivity), and the abscissa represents the number of false positive responses (FPR, 1-specificity). Area under the curve (AUC) represents the fraction of nonsurviving ALI patients who would have a positive test (high KL-6 in plasma or in ELF). The optimal value of plasma KL-6 was 253 U/ml, with a sensitivity, specificity, and likelihood ratio of 87%, 100%, and 23, respectively. The optimal value of KL-6 in ELF was 2,734 U/ml, with a sensitivity, specificity, and likelihood ratio of 83%, 90%, and 30, respectively.

died with ALI/ARDS than in the sections from the six patients who died of nonpulmonary causes (see example in Fig. 5).

Double immunofluorescence demonstrated that cells that expressed KL-6 protein (red) were domed cells that lined the normal alveolar epithelium (Fig. 6). Furthermore, the KL-6-expressing cells also expressed pro-SP-B protein (indicated by yellow, Fig. 6). Topographically, KL-6 protein was immunolocalized to the apical region of the alveolar type II epithelial cells, whereas pro-SP-B protein expression was uniform throughout the cytoplasm. Many of the double immunolabeled cells had yellow cytoplasmic staining (red + green), indicating overlap of the two proteins.

**KL-6 secretion from normal human alveolar type II cells and distal lung epithelial cells.** Without stimulation (controls), KL-6 production from normal human alveolar type II cells was  $113 \pm 4$  U/ml (mean  $\pm$  SE). Cytomix increased the KL-6 production from alveolar type II cells significantly and in a dose-dependent manner ( $193 \pm 8$  for 10 ng/ml of Cytomix and  $213 \pm 4$  for 50 ng/ml of Cytomix,  $P < 0.05$ ) compared with

the controls ( $P < 0.0001$ ,  $P < 0.0001$ , respectively; Fig. 7). There was no apparent proliferation of alveolar type II cells during the incubation with Cytomix.

Distal lung epithelial cells also secreted KL-6 without stimulation in a concentration of  $0.7 \pm 0.1$  U/ml after 24 h, although the magnitude was only  $\sim 1\%$  of the secretion measured in alveolar type II cells. Coincubation with Cytomix in concentrations of 10 and 50 ng/ml increased the production of KL-6 ( $3.2 \pm 0.1$  and  $3.8 \pm 0.4$ , respectively). However, the magnitude was  $<2\%$  of that KL-6 secretion measured in alveolar type II cells. The concentrations of LDH in the supernatant of each experimental setting revealed no significant differences among these three groups [ $2.5 \pm 1.5(0.6)$  ng/mg in control,  $2.5 \pm 0.7(0.3)$  in 10 ng/mg Cytomix, and  $1.7 \pm 1.2(0.5)$  IU/l in 50 ng/ml Cytomix group, respectively].

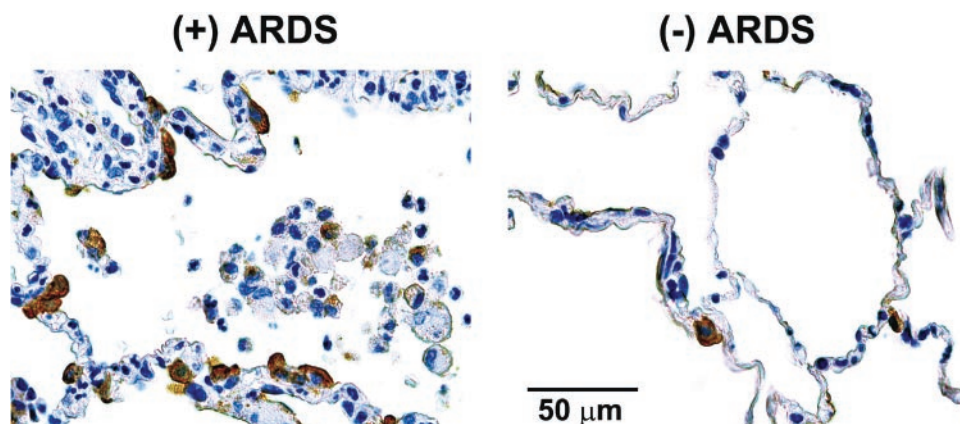
## DISCUSSION

In this study, a new BMS method was used to sample undiluted fluids from the distal airways to test the usefulness of KL-6 as a novel lung epithelial marker in clinical ALI. The BMS procedure was safe, as it did not cause hypoxemia during or after the procedure in any of the patients. The levels of KL-6 were elevated in the ELF and plasma of patients with ALI and were highest in both plasma and ELF in nonsurvivors.

The BMS procedure detected significant changes in alveolar epithelial permeability. The albumin concentration in the ELF of patients with ALI was significantly higher than in controls (Fig. 1), confirming prior results using BAL in patients with ARDS (26). Although ELF sampling with the BMS probe was done in distal airways 2–3 mm in diameter, the presence of high KL-6 levels at that level demonstrates that the BMS procedure can identify pathophysiological changes associated with damage to the alveolar epithelial barrier.

The high-molecular-weight pulmonary epithelial mucin KL-6 is an integral membrane glycoprotein that appears to be predominantly expressed on injured and/or activated alveolar type II cells (19). Recently, we reported that the localization of KL-6 protein appeared primarily on the apical surface of alveolar type II cells in postmortem ARDS patients (1). Accordingly, in the present study, we studied the production of KL-6 with isolated human alveolar type II cells in vitro. These studies indicate that KL-6 release significantly increased by stimulation by a mixture of proinflammatory cytokines, Cytomix. In this setting, LDH levels in the supernatant were normal

Fig. 5. Representative immunohistochemical staining for KL-6 in a postmortem lung specimen obtained from a patient who died with ALI [(+) ARDS] and from a patient who died of nonpulmonary causes [(-) ARDS]. KL-6 protein was predominantly expressed on the surface of epithelial cells (brown stain), probably type II cells. KL-6 was also expressed on apparently denuded epithelial cells in the alveolar space. ARDS, acute respiratory distress syndrome.



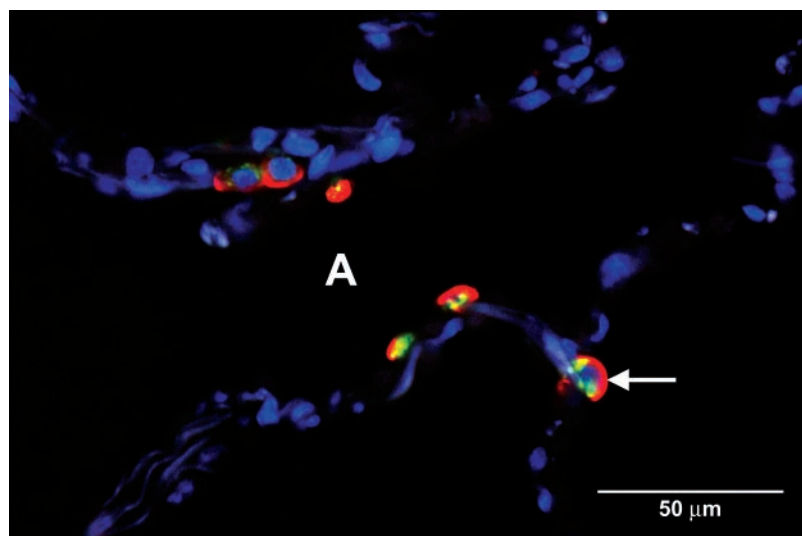


Fig. 6. Double immunofluorescence colocalization of KL-6 (red) and pro-surfactant protein (SP)-B (green) in a lung tissue section from a subject who died of nonpulmonary causes. Domed epithelial cells that line the alveoli (A) express both proteins. KL-6 immunolocalization is along the apical region of the domed cells, whereas pro-SP-B immunolocalization is throughout the cytoplasm. Both proteins colocalized to the same region of the cells, indicated by yellow immunofluorescence (arrow).

even after Cytomix was added, suggesting that the Cytomix had not induced cell death. We did not directly measure cell proliferation in this assay, but it is well known that it is difficult to induce proliferation of primary adult type II cells in culture. Thus it is unlikely that cell proliferation was responsible for the elevation of KL-6 in the *in vitro* experiments, although we cannot rule out the possibility that proliferation of some alveolar type II cells occurred in the injured lungs, which conceivably contribute to elevated KL-6 levels in clinical ALI. Therefore, these results suggest that inflammatory cytokines that accumulate in the lung during ALI might induce the production of KL-6. The *in vitro* study of distal human lung epithelial cells indicated that these cells are also capable of producing KL-6 when stimulated by Cytomix. However, the production of KL-6 was nearly 70 times greater in alveolar type II cells. This result suggests that the distal lung epithelial cells are not the main source of KL-6. The postmortem immunohistochemical differences support this interpretation. We found prominent expression of KL-6 in alveolar type II cells confirmed by colocalization of SP-B in the ALI lungs. Alveolar barrier integrity to albumin was disrupted at the onset of ALI. However, despite the nearly 10-fold higher concentration of KL-6

in ELF of patients with ALI than in controls, the plasma levels at the onset of ALI were similar to controls, perhaps because of the relatively large molecular size of KL-6. The molecular mass of KL-6 antigen, which includes large amounts of saccharides, is estimated to be at least 1,000–2,000 kDa, whereas that of albumin is 67 kDa. This molecular size difference may explain the slower diffusion of KL-6 through the paracellular pathway of lung endothelium and alveolar epithelium compared with albumin. The extent of alveolar septal barrier injury responsible for the leakage of albumin into the alveolar lumen may not have been sufficient to cause significant alveolar KL-6 leakage into the bloodstream. In some patients, increased plasma KL-6 concentrations were present at the onset of ALI. This early increase in plasma KL-6 concentration especially in nonsurvivors suggests that the alveolar barrier might be more disrupted in those patients.

The significant increase in KL-6 concentrations in ELF and plasma at the onset of ALI in nonsurvivors further supports the hypothesis that alveolar epithelial cell injury may be a crucial determinant of prognosis of ALI and that measurements of KL-6 might be useful to predict its outcome. From the ROC curve analyses, KL-6 levels in both ELF and plasma were sensitive and specific markers of fatal outcomes. Because these cut-off values were chosen by a post hoc analysis and the number of patients was rather small, the hypothesis should be validated in a large prospective study.

In conclusion, the BMS procedure is a safe and practical method to identify pathophysiological changes in the alveolar space in patients with ALI. KL-6 concentrations in ELF of patients with ALI were significantly increased at the onset of the disorder, and KL-6 in ELF at ALI onset was significantly higher in nonsurvivors than in survivors. These observations suggest the participation of proliferating, stimulated, and/or injured pulmonary epithelial cell in the pathogenesis of ALI, which may help define the prognosis of the disorder. Furthermore, plasma KL-6 in nonsurvivors remained significantly higher than in survivors throughout the 10-day observation period. This finding suggests that both the production of pulmonary KL-6 and disruption of the alveolar barrier could be associated with a poor prognosis.

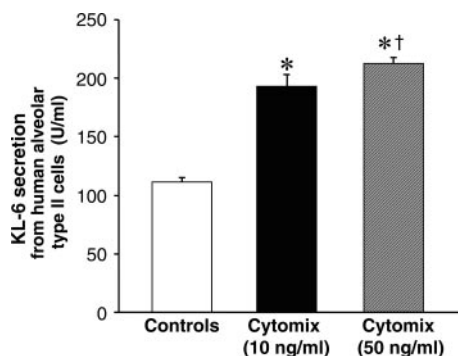


Fig. 7. KL-6 secretion from normal human alveolar type II cells after Cytomix stimulation. The studies were done  $n = 6$  in the control,  $n = 6$  in the low-dose Cytomix (10 ng/ml), and  $n = 6$  in the high-dose (50 ng/ml) groups. \* $P < 0.0001$  vs. controls. † $P < 0.05$  vs. 10 ng/ml Cytomix treatment group.



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