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Increased Plasminogen Activator Inhibitor-1 Concentrations in Bronchoalveolar Lavage Fluids Are Associated with Increased Mortality in a Cohort of Patients with Pseudomonas aeruginosa

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Background: **Increased plasminogen activator inhibitor-1 (PAI-1) concentrations are found in bronchoalveolar lavage (BAL) fluids from patients with ventilator-associated pneumonia or acute respiratory distress syndrome. The authors hypothesized that PAI-1 concentrations were associated with increased mortality in patients with either *Pseudomonas aeruginosa*–induced ventilator-associated pneumonia or tracheobronchial colonization.**

Methods: **In a prospective cohort study, daily aspirates from intubated patients were cultured for *P. aeruginosa*. Positive patients had blind BAL (bBAL) that was processed for biomarker concentrations. Secretion of type III secretion cytotoxins were also analyzed from the *P. aeruginosa* strains.**

Results: **Thirty-three patients were enrolled. Ten of the 33 patients died. bBAL PAI-1 concentrations were significantly increased in nonsurvivors compared with survivors (31.7 *vs.* 3.4 ng/ml, $P = 0.001$ for hospital mortality; 35.9 *vs.* 4.7 ng/ml, $P = 0.02$ for 28-day mortality). Even when acute respiratory distress syndrome patients were excluded, there was a significant difference between the survivors and nonsurvivors for bBAL PAI-1 concentrations ($P = 0.005$). Eighty-three percent of *P. aeruginosa* strains isolated from patients with high concentrations of bBAL PAI-1 also had strains that secreted cytotoxins.**

Conclusions: **PAI-1 concentrations in bBALs correlated with mortality in ventilated patients with positive cultures for *P. aeruginosa*. Elevated bBAL PAI-1 concentrations also correlated with the secretion of type III exotoxins by *P. aeruginosa*.**

BIOMARKERS of lung injury have been actively sought because they could serve as objective diagnostic tools as well as prognostic indicators. Biomarkers that have been investigated in patients with ventilator-associated pneumonia (VAP) include biomarkers reflecting the activity of the coagulation/fibrinolysis cascade. Concentrations of plasminogen activator inhibitor 1 (PAI-1), a protease inhibitor that blocks fibrinolysis, were increased in the airspaces of patients who were diagnosed with VAP.[1,2] In patients with VAP and infiltration of only one lung, PAI-1 concentrations were only increased in the infected lung.[1] In contrast, patients with acute respiratory distress syndrome (ARDS) have increased PAI-1 concentrations in their plasma, suggesting a breakdown of the lung epithelial and endothelial barrier functions in ARDS.[3,4] Furthermore, the patients with the highest plasma concentrations of PAI-1 had increased mortalities.[3,4] PAI-1 concentrations were elevated in the bronchoalveolar lavage (BAL) fluids obtained from patients with aspiration pneumonia that subsequently progressed to ARDS,[5] suggesting that fibrin deposition may be occurring in the airspaces of patients who develop ARDS.

Plasminogen activator inhibitor-1 concentrations have not been evaluated as prognostic indicators in mechanically ventilated patients without ARDS. As part of a prospective investigation of *Pseudomonas aeruginosa* colonization and infection of intubated, mechanically ventilated patients, we collected samples of blind BAL (bBAL) obtained from *P. aeruginosa* culture–positive patients suspected of having VAP. We were able to investigate the association of PAI-1, as well as two other biomarkers that have been evaluated in patients suspected of having VAP, protein C and soluble triggering receptor expressed on myeloid cells (sTREM-1),[6,7] with patient mortality and with quantitative cultures.

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

Materials and Methods

Patient Enrollment Criteria and Study Design

This study was approved by the Institutional Review Board of University of California, San Francisco, California. The study was conducted in a single-center tertiary care hospital. The patients originated from three intensive care units (ICUs), including a combined medical–surgical ICU, a neurointensive care unit, and a vascular–cardiac intensive care unit. The inclusion criteria were any mechanically ventilated adult older than 18 yr who was not pregnant and required a fraction of inspired oxygen (F_{IO_2}) of 0.80 or less. Only patients that remained

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ventilated for 48 h were cultured, and if they had a positive culture for *P. aeruginosa*, they were approached for consent. All patients in the three ICUs had daily endotracheal aspirates obtained, which were then cultured selectively for *P. aeruginosa* on *Pseudomonas* isolation agar in our laboratory. Patients with positive endotracheal aspirate cultures were approached for enrollment; written informed consent was obtained from either the patient or the patient's surrogate before collecting the bBALs. The day of enrollment was the first day of a positive endotracheal aspirate culture for *P. aeruginosa*. Patients with positive *P. aeruginosa* cultures were requested to undergo bBAL (when they were suspected to have VAP based on clinical symptoms such as fever, purulent sputum, and increased leukocyte count), and we were permitted to retain a portion of the fluid from enrolled patients for our studies.

All patients were followed up for a minimum of 28 days, until hospital discharge or death. Quantitative cultures of *P. aeruginosa* were performed daily on the endotracheal aspirates for as long as the patient was intubated or for 28 days, whichever came first. bBALs were conducted with a sterile catheter (Combicath; Plastimed Lab, Le Plessis Bouchard, France; see Materials and Methods, Collection of bBAL and Quantitative Bacteria Culture, second sentence). Other patient data obtained included Simplified Acute Physiology Score II and Acute Physiology and Chronic Health Evaluation Score II, Lung Injury Scores, Multiple Organ Dysfunction Score, Logistic Organ Dysfunction System Score, Sequential Organ Failure Assessment, antibiotic usage, tidal volume, mode of ventilation, and other physiologic data.

Definitions Used

Ventilator-associated Pneumonia. The diagnosis of VAP was established according to published criteria.[8,9] Mechanical ventilation for 48 h or more was required, the occurrence of a new and persistent pulmonary infiltrate on chest radiograph had to be seen, and two of the following criteria had to be present: (1) temperature greater than 38°C or less than 36°C, (2) leukocyte count of greater than 10×10^9/l or less than 4×10^9/l, or (3) purulent tracheal secretions. Finally, quantitative cultures with a threshold of 10^4 colony-forming units/ml in the bBAL were required to confirm the diagnosis.

Colonization. Colonization was defined as isolation of *P. aeruginosa* with or without other bacteria, from at least one endotracheal aspirate culture in the absence of clinical signs or symptoms of infection.

Bacteremia and Fungemia. Bacteremia and fungemia were defined as positive blood cultures for bacteria or fungi.

Septic Shock. Patients were diagnosed with septic shock if they presented with the following two criteria[10]: (1) confirmed positive blood culture; (2) systolic blood pressure less than 90 mmHg, a mean artery pressure less than 60 mmHg, or a reduction of 40 mmHg in the systolic blood pressure from baseline. These two criteria in combination with two or more of the following criteria defined septic shock: (1) heart rate greater than 90 beats/min; (2) body temperature less than 36°C or greater than 38°C; (3) hyperventilation greater than 20 breaths/min or, on blood gas, arterial carbon dioxide tension ($Paco_2$) less than 32 mmHg; (4) leukocyte count less than 4,000 cells/mm^3 or greater than 12,000 cells/mm^3 ($< 4 \times 10^9$ cells/l or $> 12 \times 10^9$ cells/l).

Adequate Antibiotic Treatment. This term was defined as a regimen that included at least one anti-pseudomonal antibiotic that the organism was sensitive to. The antibiotic had to have been administered 3 days before the bBAL and/or 1 week after bBAL.

Collection of bBAL and Quantitative Bacteria Culture

The bBAL was performed by experienced respiratory therapists working in the ICUs.[11] Briefly, a sterile plugged telescoping catheter was inserted into the lung blindly through the endotracheal tube until the tip of the catheter met resistance. The catheter was withdrawn a short distance to allow advancement of the inner catheter displacing the plug and allowing infusion of the saline. Up to 60 ml sterile warm saline was infused into the airway (average instillation 20 ml). Approximately 3–5 ml was then suctioned with a sterile syringe. The BAL fluid sample was centrifuged at 5,000 rpm for 10 min, and the supernatant was aliquoted and immediately stored at −70°C. Approximately 1 ml bBAL fluid was transferred to the clinical microbiology laboratory for quantitative cultures of all bacteria. Calibrated loops (which have been shown to be as accurate as serial dilution[12]) were used in the clinical laboratory to quantify bacteria in the bBAL fluid.

Protein C, PAI-1, and sTREM-1 Measurement

Three biomarkers were compared in the bBAL samples. All were measured in duplicate according to the manufacturer's protocol. Briefly, PAI-1 was measured by an enzyme-linked immunosorbent assay kit (Catalog no. REF822[3]; American Diagnostica Inc., Stamford, CT) without any dilution. Protein C concentrations were measured by an enzyme-linked immunosorbent assay kit without dilution (Helena Laboratories, Beaumont, TX; Ca# 5291[13]). Each patient's bBAL protein C level was calculated as percentile of normal plasma protein C activity. sTREM-1 was measured using a self-coated 96-well plate (R&D Systems, Inc., Minneapolis, MN; Ca# DY1278) without additional dilution. The sensitivity of PAI-1 detection was 1 ng/ml for undiluted samples. Intraassay variation was 4.3%, 6.1%, and 6.8% for PAI-1, protein C, and sTREM-1 assays, respectively.

Multiplex PCR Analyses of Type III Secretion System Genotype

Pseudomonas aeruginosa clinical isolates were cultured in 200 μl Luria broth in 96-well plates overnight at 37°C without shaking. Cultures were diluted 1:10 or 1:100 in sterile water before addition into the polymerase chain reaction (PCR) mixture. Multiplex PCR reactions consisted of 5 μl diluted culture, 3.75 pmol of each primer, (*exoU*, CCGTTGTGGTGCCGTTGAAG, CCAGATGTTCACCGACTCGC; *exoS*, GCGAGGTCAGCAGAGTAT, TTCGGCGTCA CTGTGGATGC; *exoY*, CGGATTCTATGGCAGGGAGG, GCCCTTGATGCACTCGAC CA; *exoT*, AATCGCCGTCCAACTGCA TGCG, TGTTCGCCGAGGTACTGCTC), 12.5 μl *Taq*PCR Mastermix (Qiagen, Valencia, CA), and 5.5 μl sterile H_2O to give a final volume of 25 μl. Reactions were performed in a GeneAmp thermocycler (Applied Biosystems, Foster City, CA) as follows: 94°C for 2 min, followed by 36 cycles of 94°C for 30 s, 58°C for 30 s, 68°C for 1 min, and a final extension step of 68°C for 7 min. PCR products were visualized on a 3% agarose gel, stained with ethidium bromide, and viewed under ultraviolet light.

Immunoblot Analysis of PcrV, ExoS, and ExoU

Representative isolates from each patient were cultured under type III secretion system–inducing conditions in MIN-S medium.[14] Cultures were incubated with shaking overnight at 37°C before dilution to optical density at 600 nm wavelength of 0.1 in fresh MIN-S medium and cultured for a further 5 h. Bacterial cells were harvested by centrifugation and the supernatant removed. Cell-free supernatant from each sample was concentrated using Centricon tubes (molecular weight cutoff 10 kd; Millipore, Bedford, MA). The cell pellet was lysed using sodium dodecyl sulfate lysis buffer (1% sodium dodecyl sulfate and 50 mM Tris-HCl, pH 8.0) and boiling. Protein concentration in both cell lysate and secreted fractions was determined by the Biorad Dc protein quantification kit (Bio-Rad Laboratories, Hercules, CA). Standardized protein concentrations were loaded onto polyacrylamide gels and run under denaturing conditions. Polyacrylamide gels were transferred to polyvinylidene fluoride membrane and immunoblotted with anti-PcrV, anti-ExoS, or anti-ExoU as previously described.[15] ExoS and ExoU secretion was quantified by densitometry and normalization to a known concentration of pure cytotoxin loaded on the same gel. Immunoblot analysis sensitivity was determined to be greater than 20 ng, greater than 13 ng, and less than 20 ng for anti-PcrV, anti-ExoS, and anti-ExoU, respectively.

Statistics

Data were analyzed using STATA 9.0 Software (StataCorp LP, College Station, TX). Student *t* tests were used for the comparisons of approximately normally distributed continuous variables and Wilcoxon–Mann–Whitney tests were used for the comparisons of skewed continuous variables. Categorical variables were compared by chi-square tests or Fisher exact tests, as appropriate. Logistic regression was used to calculate odds ratios for the association between the biomarkers' (PAI-1, protein C, sTREM-1) concentrations in the bBAL and mortality. Spearman analysis was used to correlate between bBAL PAI-1 and ExoS and ExoU secretion. All tests were two-sided. Statistical significance was defined as *P* values of 0.05 or less.

Table 1. Patient Characteristics

Item	Total (n = 33)
Age, yr	70 ± 15
Sex	
Male	13 (39%)
Female	20 (61%)
Race	
White	23 (70%)
Asian	5 (15%)
Hispanic	4 (12%)
African American	1 (3%)
Days BAL performed after enrollment	4 [2–10]
Hospital mortality	10 (30%)
ICU mortality	7 (21%)
Mortality within 28 days	5 (15%)
Organ failure* before BAL	
Cardiac (SBP ≤ 90 mmHg) and/or vasopressin, unresponsive to fluids	17 (51%)
Liver (bilirubin, mg/dl, ≥ 2)	3 (9%)
Hematology (platelets, $10^3/\mu l$, ≤ 80)	1 (3%)
Kidney (creatinine, mg/ml, ≥ 2)	8 (24%)
Respiratory (ARDS)	6 (18%)
Mechanical vent days before enrollment	5 [2–10]
Admission diagnosis	
After surgery	3 (9%)
Lung infection/respiratory failure	11 (33%)
Neuromuscular disease	8 (24)
Other diseases	11 (34%)

Other diseases includes include heart, digestive disease, *etc.*

* Modified from Brussels Organ Failure: Moderate Organ Dysfunction.[27]

ARDS = acute respiratory distress syndrome; BAL = bronchoalveolar lavage; ICU = intensive care unit; SBP = systolic blood pressure.

Results

Patient Characteristics

Thirty-three patients who underwent BAL were enrolled in this study from August 2002 to May 2005. Table 1 summarizes the patient characteristics. Among the 33 patients whose endotracheal aspirate cultures were positive for *P. aeruginosa* at enrollment, 31 were positive for *P. aeruginosa* and/or other bacteria in the bBAL samples, and 2 patients had no bacterial growth in their bBAL fluid. The overall ICU mortality was 21.2%, the 28-day mortality was 15.2%, and the hospital mortality was 30.3%.

Antibiotic Administration

All 33 patients received antibiotics during their stay in ICU, 26 (81.3%) patients received antibiotics that were

Table 2. PAI-1, Protein C, and sTREM-1 Concentrations in VAP and Non-VAP Patients

	Including ARDS Patients			Excluding ARDS Patients		
	VAP Patients (n = 11)	Non-VAP Patients (n = 22)	*P* Value	VAP Patients (n = 9)	Non-VAP Patients (n = 18)	*P* Value
PAI-1	3.4 [1.0–10.9]	8.3 [0–28.2]	0.59	1.6 [0.87–10.49]	6.1 [0–11.7]	0.92
Protein C	0.53 [0.28–2.35]	0.43 [0.1–1.1]	0.37	0.97 [0.21–1.07]	0.43 [0.05–1.07]	0.20
sTREM-1	203 [35–498]	145 [0–364]	0.60	380 [0–545]	118 [0–321]	0.41
SAPS at enrollment	47 ± 13	40 ± 15	0.23	49 ± 13	37 ± 12	0.05
APACHE at enrollment	22 ± 5	21 ± 7	0.49	23 ± 5	19 ± 6	0.11
P/F	195 ± 75	211 ± 72	0.56	209 ± 77	200 ± 76	0.78
LODS	7 [6–8]	4 [2–7]	0.02	7 [5.5–8]	3 [2–6.5]	0.02
MODS	6 [5–8]	4 [3–5]	0.03	5 [4.5–7]	4 [3–5.5]	0.16
SOFA	7 [6–8]	5 [4–6]	0.03	7 [6–8]	5 [3.5–6]	0.03
Ventilation days	16 [13–27]	12 [7–17]	0.07	16 [14–27.5]	11.5 [7–18]	0.10
Ventilation-free days	1 [0–4]	1 [0–4]	0.61	1 [0–4.5]	1 [0–3.75]	0.79
Mortality at day 28	0%	23%	0.14	0%	17%	0.53
Tidal volume, ml/kg	9.3 ± 1.8	8.5 ± 1.9	0.41	9.7 ± 1.9	8.6 ± 2.1	0.34
Mortality at hospital discharge	36%	27%	0.70	33%	67%	0.65

Mean ± SD was expressed for normally distributed variables; median [25–75% interquartile] was expressed for nonnormally distributed variables. *P* values were given by univariate analysis. Dead patient was defined as 0 for vent-free days (see Le Manach *et al.*[26]). Tidal volume is setting tidal volume divided by ideal body weight. Ventilation-free days are days without ventilation within 28 days after enrollment.

APACHE = Acute Physiology and Chronic Health Evaluation Score II; LODS = Logistic Organ Dysfunction System; MODS = Multiple Organ Dysfunction Score; PAI-1 = plasminogen activator inhibitor 1; P/F = oxygen index, artery oxygen partial pressure divided by fraction of inspired oxygen; SAPS = Simplified Acute Physiology Score II; SOFA = Sequential Organ Failure Assessment; sTREM-1 = soluble triggering receptor expressed on myeloid cells; VAP = ventilator-associated pneumonia.

active against the *P. aeruginosa* cultured before the bBAL was performed, and 30 (96.8%) received the antibiotics after the bBAL was performed. Antibiotics were changed in 20 patients within 72 h of the performance of the bBAL, and there was no association between the antibiotics the patients received and the bBAL fluid concentrations of PAI-1, protein C, or sTREM-1 (data not shown).

Biomarker Concentrations in Patients Who Met and Did Not Meet VAP Criteria

Classification of patients according to the criteria for VAP resulted in 11 patients who met the criteria and 22 patients who did not met these criteria (table 2). There were no significant differences between the concentrations of the bBAL PAI-1, protein C, or sTREM-1 between the patients who met the criteria for VAP and those who did not. There was a significant difference between the clinical scores; the Logistic Organ Dysfunction System Score ($P < 0.02$), the Multiple Organ Dysfunction Score ($P < 0.03$), and the Sequential Organ Failure Assessment ($P < 0.03$) scores obtained on the day of bBAL were different between the patients who met the VAP criteria and the patients who did not meet the criteria (table 2).

Biomarker Concentrations and Mortality

Table 3 summarizes the clinical parameters on the day of study enrollment between the survivors and the nonsurvivors. There was no significant difference for age, arterial oxygen tension (Pa_{O_2})/F_{IO_2}, Simplified Acute Physiology Score II, or Acute Physiology and Chronic Health Evaluation Score II on the day of enrollment, or clinical scores on the day of enrollment between the survivors and nonsurvivors (table 3). The survivors had significantly more ventilator-free days than the nonsurvivors (table 3).

bBAL PAI-1

Figures 1A and B illustrate that bBAL PAI-1 concentrations were significantly higher in the patients who died than in the patients who lived. This was true for both hospital mortality ($P = 0.001$) and 28-day mortality ($P = 0.003$). Figure 1C shows that there was a continuous increase of mortality as PAI-1 concentration increased. Analysis of quartile bBAL PAI-1 concentrations documents a stepwise increase of mortality with increasing bBAL PAI-1 concentrations (fig. 1D). Comparisons between the concentrations of bBAL PAI-1 from patients with different quantitative cultures of *P. aeruginosa* and other bacteria showed that there was no correlation between the bBAL PAI-1 concentrations and the BAL bacteria burdens (fig. 1E).

Blind bronchoalveolar lavage PAI-1 concentrations in ARDS patients were significantly higher than the concentrations measured in patients without ARDS ($P = 0.003$; fig. 2A). We therefore performed a secondary analysis excluding patients with ARDS. A significant difference between survivors and nonsurvivors in bBAL fluid PAI-1 concentration remained, even after the exclusion of the ARDS patients ($P = 0.005$ for hospital mortality, fig. 2B; and $P = 0.078$ for 28-day mortality, fig. 2C).

Other possible confounders that might increase PAI-1 concentrations in the circulation and in the lungs of patients include the presence of bacteremia, fungemia,

Table 3. Patient Characteristics for bBAL PAI-1 Measurements*

	Hospital Mortality			28-Day Mortality		
	Survivors (n = 23)	Nonsurvivors (n = 10)	*P* Value	Survivors (n = 28)	Nonsurvivors (n = 5)	*P* Value
Age, yr	69 ± 17	71 ± 11	NS	70 ± 16	70 ± 9	NS
Male, %	9 (39%)	4 (40%)	NS	11 (39%)	2 (40%)	NS
White, %	17 (74%)	6 (60%)	NS	22 (71%)	3 (60%)	NS
SAPS (day 1)	41.1 ± 16	45.4 ± 12.7	NS	38 [31–50]	47 [40–63]	NS
APACHE (day 1)	20.8 ± 6.9	21.8 ± 5.5	NS	19 [15–24]	23 [20–29]	NS
P/F ratio (day 1)	217.6 ± 69.3	171.7 ± 62.9	0.09	210 ± 69	168 ± 69	NS
MODS (BAL)	4 [3–5]	5.5 [3–8]	NS	4 [3–6]	6 [3-13]	NS
LODS (BAL)	5 [2–7]	6 [5–8]	NS	6 [2–7]	7 [5–9]	NS
SOFA (BAL)	6 [4–7]	6 [5–9]	NS	6 [4–7]	6 [5.5–13.5]	NS
Lung injury score (BAL)	1.33 [0.92–1.67]	1.33 [0.92–1.76]	NS	1.33 [1.00–1.67]	1.67 [1.17–2.17]	NS
Vent days before PA (+)	3.5 [2–8.5]	6 [3.5–12]	NS	4 [2–9]	6 [5–12]	NS
Vent-free days	7 [0–16]	0 [0–0.5]	0.01	4 [0–15]	0 [0–1]	0.07
Tidal volume, ml/kg	8.9 ± 2.0	9.1 ± 2.1	NS	8.8 ± 1.9	9.5 ± 2.4	NS
Leukocytes	11.8 [8.6–15.9]	11.6 [10.6–18.5]	NS	11.7 [8.9–16.9]	12.6 [10.2–19]	NS
Temperature, °C	38.1 ± 1.0	38.0 ± 0.4	NS	38.1 ± 0.9	37.8 ± 0.4	NS
Adequate antibiotics—before	19 (86%)	7 (70%)	NS	22 (82%)	4 (80%)	NS
Adequate antibiotics—after	20 (95%)	10 (100%)	NS	25 (96%)	5 (100%)	NS
Vent days	21 [12–28]	25 [17–28]	NS	24 [14–28]	20 [10–24]	NS
ICU days	23 [17–39]	26 [18–49]	NS	17 [19–47]	21 [11–25]	NS
Hospital days	36 [25–61]	36 [21–148]	NS	43 [29–70]	21 [17–26]	< 0.01

* Mean ± SD was expressed for normally distributed variables; median [25–75% interquartile] was expressed for nonnormally distributed variables. *P* values were given by univariate analysis. Day 1 is the first day of enrollment. Tidal volume means setting tidal volume divided by ideal body weight. Vent days are total ventilation days within 28 days after enrollment. Vent-free days are days without ventilation within 28 days after enrollment.

APACHE = Acute Physiology and Chronic Health Evaluation Score II; BAL = at day of bronchoalveolar lavage; bBAL = blind bronchoalveolar lavage; ICU = intensive care unit; LODS = Logistic Organ Dysfunction System; MODS = Multiple Organ Dysfunction Score; NS = not significant; PA = *Pseudomonas aeruginosa*; PAI-1 = plasminogen activator inhibitor 1; P/F = oxygen index, artery oxygen partial pressure divided by fraction of inspired oxygen; SAPS = Simplified Acute Physiology Score II; SOFA = Sequential Organ Failure Assessment.

and septic shock. Only one patient had bacteremia within 1 week of the bBAL. The bacteremic patient's bBAL PAI-1 concentration was not elevated, so this patient was not excluded. None of the patients met the criteria for septic shock before the performance of the bBAL; therefore, septic shock does not seem to be a confounding factor in this group of patients.

Coinfection by other bacteria *e.g.*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Stenotrophomonas*, *Enterococcus* species, and/or *Candida albicans*, could affect patient outcomes[16]; therefore, we analyzed the PAI-1 concentration of the bBALs with respect to colony-forming units of other bacterial species identified in the patient bBALs (less or more than 10^4 colony-forming units/ml). No difference between the bBAL PAI-1 concentrations was found even when these other bacteria were considered. Further, no difference in patient mortality was detected when these other bacteria were considered. This suggests that the quantity of *P. aeruginosa* colony-forming units on the day of mini-BAL and the presence of other bacterial species did not seem to affect bBAL PAI-1 concentration or patient mortality.

The majority of patients were ventilated with a volume control ventilation mode, either assist control or synchronized intermittent mandatory ventilation. Post end-expiratory pressure was set at 5 cm H_2O almost uniformly. Because high tidal volume may induce lung injury,[17] we compared the tidal volumes between the survivors and nonsurvivors. Tidal volume per ideal body weight did not differ between survivors and nonsurvivors, suggesting that tidal volume was not a confounding factor in this study (tables 2 and 3).

Association of Type III Virulence Secretion and bBAL PAI-1 Concentrations

Type III virulence is dependent on the expression and active secretion of both a cytotoxin and the PcrV protein.[15] Thirty strains (a representative strain for each patient) were chosen from the cohort. Of the strains analyzed, 24 possessed the *exoS* gene, and 6 strains possessed *exoU* (determined by multiplex PCR); all strains carried the gene for *pcrV*. The ability to produce and secrete cytotoxins (ExoS and ExoU) and PcrV was determined by immunoblot analysis. Figure 3A illustrates a typical immunoblot analysis of the secreted fraction using anti-ExoS, anti-ExoU, and anti-PcrV immunoglobulin G to detect each respective protein. The majority of strains secreted PcrV. Strains associated with high BAL PAI-1 exhibited PcrV secretion and high concentrations of ExoS in the extracellular fraction. Those associated with low PAI-1 concentrations exhibited low or nondetectable concentrations of ExoS and/or PcrV in the secreted fraction (fig. 3B; the Spearman correlation between ExoS and PAI-1 is 0.44 with $P = 0.03$). Because

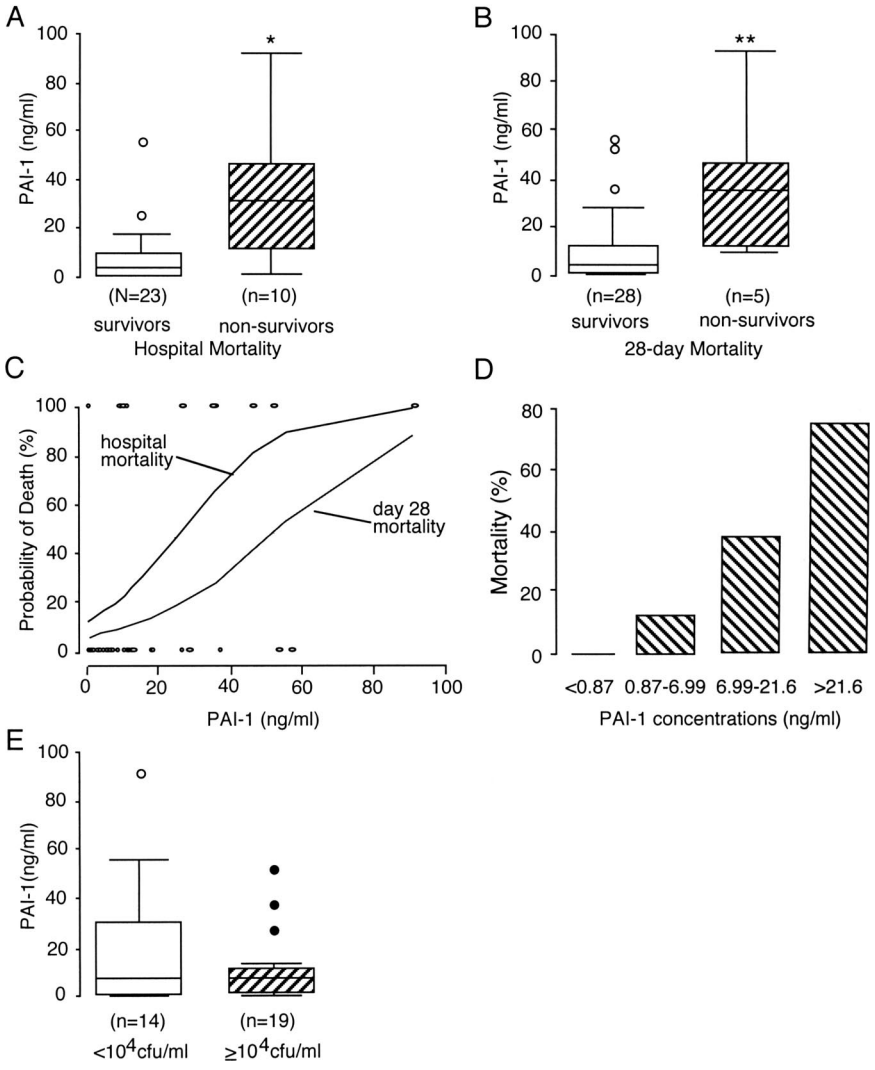

Fig. 1. Blind bronchoalveolar lavage plasminogen activator inhibitor-1 (PAI-1) concentrations in survivors and nonsurvivors and their association with quantity of bacteria. (*A*) PAI-1 concentrations in survivors and in nonsurvivors evaluating hospital mortality. (*B*) PAI-1 concentrations in survivors and in nonsurvivors evaluating 28-day mortality. (*C*) Logistic regression of continuous mortality prediction of blind bronchoalveolar lavage PAI-1 concentrations. (*D*) Patients mortality by PAI-1 quartile classification. (*E*) Average PAI-1 concentrations in patients with lower and higher blind bronchoalveolar lavage quantitative cultures (below and above or equal to 10^4 colony-forming units [cfu]/ml). There were statistically significant differences in PAI-1 concentrations for the groups evaluated for hospital mortality and for 28-day mortality. * $P = 0.001$. ** $P = 0.003$. *Open and closed circles* signify numbers out of range (median [25, 75]) in each group of patients.

ExoU is a significantly more potent cytotoxin compared with ExoS, considerably less ExoU cytotoxin secretion is necessary to cause significant lung damage. Analysis of ExoU-containing strains demonstrated that all strains secreted PcrV. Strains associated with low PAI-1 concentrations exhibited secretion of very low ExoU concentrations. Those with elevated PAI-1 levels (these were among the highest concentrations of PAI-1 detected in the total cohort) demonstrated the highest concentrations of the potent ExoU cytotoxin in the extracellular fraction (fig. 3C; the Spearman correlation between ExoU and PAI-1 is 0.83 with $P = 0.04$).

bBAL Protein C Level

The protein C levels of the bBALs measured in this study were substantially lower than those previously reported in either lung edema or plasma samples.[13] This is likely due to the fact that unlike the previous two samples, bBAL samples are diluted with saline. In contrast to the bBAL PAI-1 concentration differences in the two patient groups (survivors *vs.* nonsurvivors), we did not find a significant difference in protein C concentrations between these two groups (fig. 4A). There was also no correlation between the bBAL protein C concentrations and the quantitative cultures of *P. aeruginosa* in the bBAL fluid (data not shown).

bBAL sTREM-1 Concentrations

We developed an enzyme-linked immunosorbent assay to quantify the BAL sTREM-1 concentration. Previous reports used an immunoblot technique that was not standardized to the amount of protein in the various fluids.[7] We measured sTREM-1 concentrations that were much higher than had been reported using the immunoblot. There was huge variability of bBAL sTREM-1 concentrations, which ranged from near 0 to around 1,653 pg/ml. There were no significant differences between the bBAL sTREM-1 concentrations between the survivors and nonsurvivors (fig. 4B). No association was found between bBAL sTREM-1 concentrations and quantities of bacteria in bBAL fluid (data not shown).

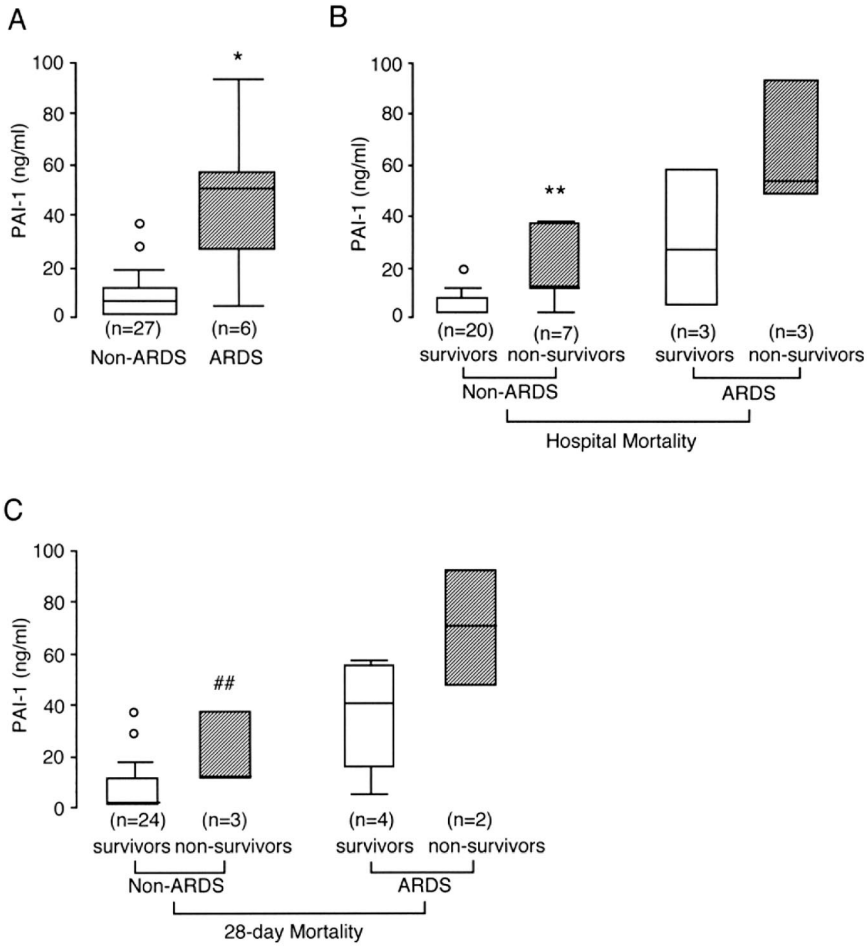

Fig. 2. Blind bronchoalveolar lavage plasminogen activator inhibitor-1 (PAI-1) concentrations in acute respiratory distress syndrome (ARDS) and patients without ARDS. (*A*) Blind bronchoalveolar lavage PAI-1 concentrations in ARDS *versus* non-ARDS patients were significantly different. * $P = 0.003$. (*B*) Blind bronchoalveolar lavage PAI-1 concentrations in survivors (ARDS or non-ARDS) and nonsurvivors (ARDS or non-ARDS) patients evaluating hospital mortality. ** $P = 0.005$. (*C*) Blind bronchoalveolar lavage PAI-1 concentrations in survivors (ARDS or non-ARDS) and nonsurvivors (ARDS or non-ARDS) evaluated for 28-day mortality. There were no significant differences between the survivors and patients who died when evaluated for 28-day mortality, whether they had ARDS or not. ## $P = 0.078$. This may be due to the small number of patients in the groups. *Open and closed circles* signify numbers out of range (median [25, 75]) in each group of patients.

Univariate Analysis of Mortality and Concentrations of the Measured Biomarkers

Table 4 details the univariate analysis of bBAL PAI-1, protein C, and sTREM-1 concentrations and mortality. From the analysis, it is evident that only PAI-1 concentrations predicted hospital and 28-day mortality.

Discussion

Biomarker Concentration and Mortality

The most significant finding of this investigation is that elevated concentrations of PAI-1 in bBAL fluids are associated with increased hospital and 28-day mortality in mechanically ventilated patients who had positive bBAL cultures for *P. aeruginosa* and/or other bacteria. We did not find an association between mortality and elevated concentrations of either sTREM-1 or of protein C in the bBALs. This result documents that elevated PAI-1 concentrations in bBALs are similar to the finding of elevated PAI-1 in edema fluids from ARDS patients, suggesting that elevated PAI-1 concentrations are associated with increased mortality in a variety of patients, with and without severe lung injury.

This result further suggests that the presence of airspace *P. aeruginosa* might specifically affect the coagulation/fibrinolysis system. In fact, Kipnis *et al.*[18] demonstrated that the instillation of *P. aeruginosa* activated alveolar thrombin in isolated lungs. Whether *P. aeruginosa* activates the coagulation system more than other bacteria has not been shown, but the ability of *P. aeruginosa* to injure both the epithelial and endothelial barriers of the lung[19,20] explains why it could activate the coagulation system and increase PAI-1.

Notably, the concentrations of PAI-1 in the bBAL did not correlate with concentrations of total protein or myeloperoxidase concentrations in the bBAL (data not shown). The PAI-1 concentrations also did not correlate with the clinical markers of lung injury, nor did the PAI-1 concentrations correlate with the physiologic Pao_2/Fio_2 ratio used to detect lung injury. Previous reports in patients with ARDS[21] also noted that plasma PAI-1 did not correlate with lung injury scores in that population, but elevated PAI-1 concentrations were also associated with increased mortality in the ARDS patients.[3,4] The cause of death of the patients in this study (similar to that of patients who have ARDS) was primarily due to multisystem organ failure that always included lung injury. Six of the 10 patients who died in our study had more than two organs failing before death. Our results are also similar to those of a recent investigation[5] where the

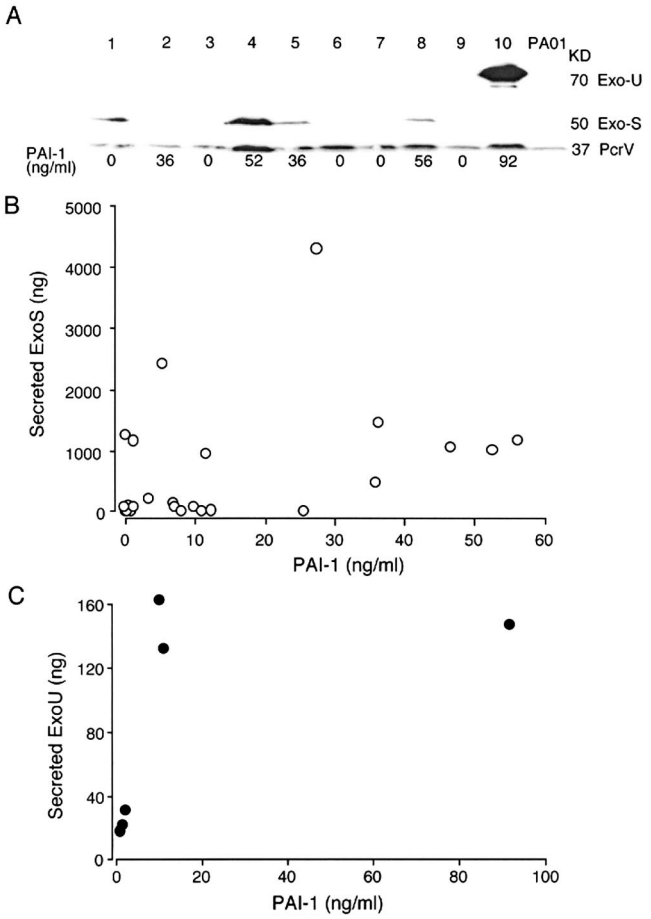

Fig. 3. Immunoblot of secreted ExoU, ExoS, and PcrV from *Pseudomonas aeruginosa* strains. (*A*) *Top line numbers* indicates number of strains, and *bottom line numbers* indicate the blind bronchoalveolar lavage plasminogen activator inhibitor-1 (PAI-1) concentrations. *Top line immunoblot* indicates ExoU secretion, with a KD of approximately 70; *middle line immunoblot* indicates ExoS secretion, with a KD of approximately 50; and *bottom line immunoblot* indicates PcrV secretion, with a KD of approximately 37. PA01 served as the control *P. aeruginosa* strain. (*B*) Correlation between PAI-1 concentration and ExoS secretion. Spearman analysis showed a significant correlation between PAI-1 and ExoS ($P = 0.03$). (*C*) Correlation between PAI-1 concentration and ExoU secretion. Spearman analysis showed a significant correlation between PAI-1 and ExoU ($P = 0.04$).

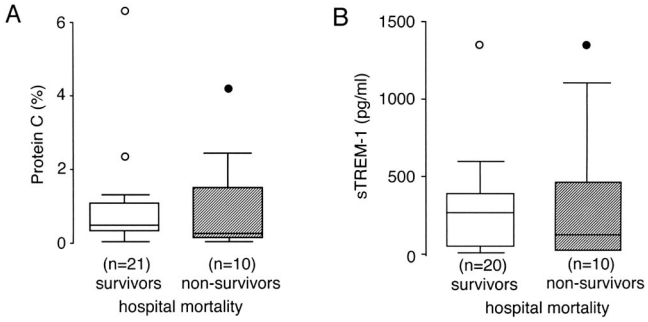

Fig. 4. Protein C and soluble triggering receptor expressed on myeloid cells (sTREM-1) concentrations in blind bronchoalveolar lavage. (*A*) Protein C concentration in survived and nonsurvived patients. (*B*) sTREM-1 concentration in survived and nonsurvived patients. There was no significant difference between survived and nonsurvived patients for both blind bronchoalveolar lavage protein C and sTREM-1. *Open and closed circles* signify numbers out of range (mean [25, 75]) in each group of patients.

median bBAL PAI-1 concentrations were approximately fivefold higher in the ARDS patients than the concentrations found in patients without ARDS.

Elevated PAI-1 concentrations have also been previously found in the airspaces of patients with unilateral lung infections and not found in the airspaces of the opposite, uninfected lungs.[1] The patients with the unilateral lung infections clearly had a contained infection that was not associated with other organ failure. In contrast to that investigation, we obtained bBAL fluid from patients with more diffuse lung infiltrates. Our patients also usually had multisystem organ failure (see previous paragraph). Because the presence of ARDS is associated with elevations in the concentrations of PAI-1, we performed analyses from which ARDS patients were excluded. Elevations of PAI-1 in the bBAL of patients without ARDS were still associated with increased mortality.

Elevations of PAI-1 Concentrations and Quantitative Cultures

Several reports have shown that PAI-1 levels increase in patients who develop VAP but are not elevated in patients who do not develop VAP.[1,22] The 11 patients in the current study who met the criteria for VAP did not have different PAI-1 concentrations compared with the concentrations in the patients who did not meet the criteria for VAP. All patients received antibiotics before and after the bBAL. This may have influenced the quantitative culture results. Therefore, we cannot be sure whether PAI-1 concentrations correlate with bacterial number in the absence of antibiotics. However, this investigation documents that the elevated PAI-1 concentrations in the bBAL fluids, despite antibiotic treatments, correlates with mortality.

bBAL PAI-1 Concentrations and P. aeruginosa *Secretion of Toxins*

Pseudomonas aeruginosa binds to airway epithelium through lipopolysaccharide, flagella, and pili. Upon cell contact, the type III secretion system, which delivers toxins directly into host cells through a needle-like apparatus, is activated.[23] Key component proteins of the needle-like translocator include PopB, PopD, and PcrV. The known *P. aeruginosa* cytotoxins injected include ExoU, ExoS, ExoT, and ExoY. These cytotoxins vary in potency with ExoU, a phospholipase/lysophospholipase, being more than 100 times more virulent than ExoS.[24] ExoU is responsible for decompartmentalization of the inflammatory response in a septic mouse model,[25] and its secretion (in conjunction with PcrV secretion)

Table 4. Univariate Analysis between Mortality and bBAL PAI-1, sTREM-1, and Protein C Concentrations

	Hospital Mortality			28-Day Mortality		
Univariate Analysis	Survivors	Nonsurvivors	*P* Value	Survivors	Nonsurvivors	*P* Value
PAI-1, ng/ml	3.4 [0–10.1]	31.7 [10.6–48.1]	0.001	4.7 [0.2–11.9]	35.9 [10.6–69.2]	0.02
Protein C, % of control	0.5 [0.3–1.2]	0.2 [0.1–1.5]	NS	0.4 [0.1–1.1]	0.4 [0.1–2.9]	NS
sTREM-1, pg/ml	170 [0–380]	115 [0–670]	NS	150 [0–382.5]	357 [112.5–1,225]	NS

bBAL = blind bronchoalveolar lavage; NS = not significant; PAI-1 = plasminogen activator inhibitor 1; sTREM-1 = soluble triggering receptor expressed on myeloid cells.

correlates strongly with patient mortality.[15] Mutual exclusive secretion between ExoS and ExoU suggest that ExoS is also an important toxin in certain strains. In all cases, secretion of PcrV is crucial to efficient intoxication of eukaryotic cells.

Analysis of the toxin secretion by the *P. aeruginosa* strains documented a correlation between cytotoxin and PcrV secretion and the measurement of elevated bBAL PAI-1 concentrations. It seems that a relatively linear correlation exists between the quantity and potency of cytotoxin secreted and the PAI-1 levels recorded. Strains secreting ExoU exhibited some of the highest PAI-1 concentrations in the cohort, whereas the majority of those secreting low concentrations of ExoS exhibited nondetectable PAI-1 concentrations. These results suggest that effective type III cytotoxin secretion results in increased PAI-1 concentrations in lung airspaces. The explanation for the nonsecreting strains maybe that alternative epithelium-damaging virulence factors, such as pyocyanin or elastase, may be responsible for the elevated PAI-1 concentrations in these strains. Furthermore, there may be secretion of nonfunctional cytotoxins or PcrV that account for the small number of strains that exhibit cytotoxin secretion but had low levels of bBAL PAI-1. Further characterization of these anomalous strains is necessary to confirm or disprove these hypotheses.

Limitations of the Investigation

The investigation included a small number of patients in a single institution, limiting the generalizability of the conclusions. The universal administration of antibiotics in these patients may have affected the quantitative cultures, preventing a discovery of an association between the biomarkers and quantitative cultures. Because we did not measure PAI-1, protein C, and sTREM-1 in the patients' plasma samples, we could not compare the local *versus* systemic effects of these biomarkers. However, the robust association seen between elevated PAI-1 bBAL concentrations and mortality in patients with and without ARDS, despite ongoing antibiotic treatment, suggests that elevations of PAI-1 concentrations in the airspaces of patients' lungs reflects a grave prognosis. Also, the association of the secretion of type III cytotoxins by the *P. aeruginosa* strains with the highest elevations of PAI-1 suggests that the virulence of the *P. aeruginosa* may affect the concentrations of PAI-1 in the airspaces of patients. Clearly further investigation is needed to determine whether a causal relation exists between these or other cytotoxins and PAI-1 elevations.

Summary

Elevations of PAI-1 concentrations in bBAL fluids were significantly associated with increased mortality in a cohort of patients with positive endotracheal cultures for *P. aeruginosa*. The association with mortality remained even after excluding patients with ARDS from the analyses. Results demonstrated an association between patients with the highest elevations of PAI-1 concentrations in their bBAL and secretion of type III cytotoxins by the *P. aeruginosa* strains. These observations suggest that PAI-1 concentrations may be affected by the virulence factors produced by *P. aeruginosa*.

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