Published in final edited form as:

J Burn Care Res. 2012 ; 33(1): 57–64. doi:10.1097/BCR.0b013e31823dc559.

Ubiquitin and stromal cell-derived factor-1 α in bronchoalveolar lavage fluid after burn and inhalation injury

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Abstract

Objective—To determine whether the CXC chemokine receptor (CXCR) 4 ligands ubiquitin and stromal cell-derived factor (SDF)-1 α are detectable in bronchoalveolar lavage fluid (BALF) after burn and inhalation injury and whether their concentrations in BALF are associated with injury severity, physiological variables or clinical outcomes.

Methods—BALF was obtained on hospital admission from 51 patients (48 $\pm$ 18 years) with burn (TBSA: 23 $\pm$ 24%) and inhalation injury (controls: 10 healthy volunteers, 42 $\pm$ 8 years). BALF was analyzed for total protein and for ubiquitin and SDF-1 α by ELISA. Ubiquitin/SDF-1 α levels were normalized to total BALF protein content. The extent of inhalation injury was determined during bronchoscopy using a standardized scoring system. Percent TBSA, Baux scores, revised Baux scores and clinical variables were documented.

Results—Ubiquitin and SDF-1 α were detectable in 40% of normal BALF specimens. After injury, ubiquitin was detectable in 90% ($p < 0.01$ vs. control) and SDF-1 α in 10% of the specimens ($p < 0.05$ vs. control), respectively. While SDF-1 α levels were reduced in patients ($p < 0.01$), ubiquitin levels were increased ($p < 0.01$). Ubiquitin concentrations correlated inversely with grade of inhalation injury, revised Baux scores and resuscitation fluid requirements (Spearman correlation coefficients (r): -0.3, -0.33 and -0.45, respectively). Ubiquitin levels correlated positively with arterial oxygenation at the time of bronchoscopy (r : 0.35).

Conclusions—BALF levels of CXCR4 agonists are differentially regulated after burn and inhalation injury. Increases in BALF ubiquitin after inhalation injury may maintain CXCR4 mediated lung protection and repair processes. The finding that BALF ubiquitin decreased with higher grades of inhalation injury may provide a biological correlate for an insufficient local inflammatory response after severe inhalation injury.

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Keywords

Extracellular ubiquitin; chemokine (CXC motif) ligand 12; CXC chemokine receptor 4; fusin; CD184; bronchoscopy

INTRODUCTION

Ubiquitin, a small (8.6kDa) and highly conserved protein inside all eukaryotic cells, fulfills essential intracellular functions as a post-translational protein modifier (1-3). Ubiquitin is also a natural constituent of extracellular fluids, such as plasma, cerebrospinal or bronchoalveolar lavage fluid, and various diseases have been associated with increased concentrations of extracellular ubiquitin, locally and in the systemic circulation (4). Recently, we showed that extracellular ubiquitin functions as an endogenous immune modulator with anti-inflammatory properties and as a natural agonist of the CXC chemokine receptor (CXCR) 4 (5-7). In addition, multiple studies demonstrated that treatment with exogenous ubiquitin and with the cognate ligand of CXCR4, stromal cell-derived factor (SDF)-1 α (CXCL12), have therapeutic potential to reduce inflammation and organ injury in animal models of trauma, hemorrhage and ischemia-reperfusion injury (8-15).

After burns in patients, systemic ubiquitin and SDF-1 α levels are significantly elevated and correlate with the burn size (16, 17). Moreover, burn patients who develop multiple organ failure or die were found to have a relative deficiency of plasma ubiquitin during the first week after injury (16), suggesting a protective role of the ubiquitin/SDF-1 α /CXCR4 axis during the early inflammatory response. In contrast to these observations in systemic circulation, information about extracellular ubiquitin and SDF- α at local sites of injury is sparse. It has been shown that ubiquitin concentrations are also significantly elevated in cerebrospinal fluids after traumatic brain injuries in animals and patients (9, 18), and in bronchoalveolar lavage fluid (BALF) after blunt chest trauma in an animal model (19). Increased SDF-1 α concentrations have been described in burn blister fluids in patients (20). However, it is not known whether ubiquitin and SDF-1 α are also released into the lung epithelial lining fluid in burn patients with inhalation injury, and thus, could play a pathophysiological role during the local inflammatory response to inhalation injury in the lung. Therefore, it was the aim of this study to determine whether ubiquitin and SDF-1 α are detectable in BALF after burn and inhalation injury in patients and whether their concentrations in BALF are associated with the severity of the injury, physiological variables or outcomes. Based on the previously described increases in ubiquitin and SDF-1 α concentrations in the systemic circulation after burns in patients (16, 17) and of ubiquitin in BALF in a blunt chest trauma model (19), we hypothesized that inhalation injury is also associated with increased amounts of ubiquitin and SDF-1 α in BALF.

MATERIALS AND METHODS

Patients and volunteers

This study was approved by the Institutional Review Boards for human subjects and informed consent was obtained from all participants. Burn patients admitted to the burn intensive care unit (ICU) of the Loyola University Medical Center requiring bronchoscopy for diagnosis of inhalation injury were recruited between August 2007 and August 2010. Patients were excluded from the study for the following reasons: age less than 18 years, known malignancy, immunosuppressive medications, or known autoimmune or chronic inflammatory diseases.

Fifty-one patients (age: 48 ± 18 years (mean $\pm$ SD)) who met eligibility criteria and gave informed consent were prospectively enrolled for collection of BALF and review of electronic medical records for entry in a clinical database. The clinical characteristics of the patient population are shown in Table 1. Ten healthy volunteers (age: 42 ± 8 years (mean $\pm$ SD), $p > 0.05$ vs. patients, 60% male) were recruited at the Department of Medicine, University of Colorado School of Medicine, for bronchoscopy and collection of normal BALF under conscious sedation with topical endotracheal anesthesia. Volunteers were free of pulmonary, cardiac, infectious, and allergic disease and had no history of chemotherapy, radiation therapy, and were non-smokers.

All patients requiring surgical intervention received standard surgical care and postoperative intensive care according to the standard treatment protocols. All patients were endotracheally intubated and mechanically ventilated with positive end expiratory pressure of 5 cm of H₂O. Bronchoalveolar lavage was performed in patients within 14 hours of injury according to standardized ICU protocols (21). A flexible bronchoscope was directed into a lower subsegmental bronchus and 20 mL of 0.9% saline were instilled, aspirated and discarded. The bronchoscope was then repositioned into another subsegmental bronchus within the same lobe and an additional 20 mL of saline was instilled and aspirated into a sterile Leuken's fluid trap. Up to four 20 mL samples were collected from the remaining lobes as tolerated by the patient. The samples were placed on ice and filtered using a cell 100-micron membrane (BD Falcon). Filtered BALF was centrifuged for 5 min at $250 \times g$ and the cell-free fluid was stored at -80°C until further analyses. Total protein content in BALF was measured according to Lowry et al. (22).

Injury severity was assessed by the percentage of partial or full thickness burned total body surface area (%TBSA), as documented in the patient records. The extent of inhalation injury was determined using a standardized scoring system based on the morphologic appearance of the endobronchial lumen during bronchoscopy (grade 0: absence of carbonaceous deposits, erythema, edema, bronchorrhea, or obstruction; grade 1: minor or patchy areas of erythema and carbonaceous deposits in proximal or distal bronchi; grade 2: moderate degree of erythema, carbonaceous deposits, bronchorrhea, or bronchial obstruction; grade 3: severe inflammation with friability, copious carbonaceous deposits, bronchorrhea, or obstruction; and grade 4: evidence of mucosal sloughing, necrosis, and endoluminal obliteration), as described in detail previously (23, 24).

The following clinical variables and outcomes were documented and entered into a database: age, gender, %TBSA, grade of inhalation injury, Baux score (sum of age in years and %TBSA) (25), revised Baux score (sum of age in years and %TBSA and $(17 \times (\text{inhalation injury}; 1 = \text{yes}, 0 = \text{no}))$) (26), ratio of partial pressure of oxygen and inspired fraction of oxygen (P/F ratio) at the time of BAL collection, volume of crystalloid required for fluid resuscitation over the first 24 - 72h following injury, initial base deficit, BAL leukocyte count, ICU length of stay, duration of mechanical ventilation, incidence of ventilator associated pneumonia (VAP), sepsis and mortality. VAP was defined using the American Burn Association Consensus Conference criteria (27). Sepsis was diagnosed according to the ACCP/SCCM consensus conference criteria (28).

Ubiquitin assay

Ubiquitin BALF concentrations were measured with a competitive enzyme linked immunosorbent assay (ELISA), as described in detail previously (16, 29). In this assay, biotinylated ubiquitin and unlabeled ubiquitin in the test sample compete for a limited number of binding sites on the anti-ubiquitin capture antibody. In brief, high binding microtiter plates (Greiner Bio One) were coated with anti-ubiquitin (Sigma-Aldrich) and incubated for 18 h at 4°C . The plates were washed three times with 0.05% tween 20 in

phosphate buffered saline (PBS) and incubated with blocking buffer (0.5 % bovine serum albumin (Sigma-Aldrich), 0.05 % tween 20, in PBS) for 1.5 h. After washing three times, 50 μ l of the standards or samples were mixed with 50 μ l of biotinylated Ubiquitin (Boston Biochem) and placed in the plates. Each sample was tested in eight dilutions. Dilutions for the standard curve and the test samples were prepared in blocking buffer. After incubation for 1.5 h the plates were washed again and a peroxidase-labeled anti-biotin antibody (Sigma-Aldrich) was added. After incubation for 1.5 h the plates were washed again and 100 μ L tetramethylbenzidine (TMB) ELISA solution (Sigma-Aldrich) was added. After incubation for 20 - 40 min, the reaction was stopped by addition of 100 μ L HCl and optical densities were measured using a micro-ELISA autoreader (Synergy 2, Bio-Tek Instruments Inc., test filter: 450 nm; reference filter: 540 nm). The Ubiquitin concentration in the test sample was calculated with the Gen 5 for Windows program, Version 3.02 (Bio-Tek Instruments Inc.) from a four parameter logistic fit employing ubiquitin as standard (0 - 1700 ng/mL, Sigma-Aldrich). The correlation coefficients for each standard curve were 0.98 - 1 and the lower detection limit of the ELISA was 11 ng/mL.

SDF-1 α assay

SDF-1 α concentrations were quantified utilizing a human SDF-1 α DuoSet ELISA Development kit (R&D Systems). Microtiter plates were coated with 100 μ L per well of capture antibody diluted to 1.0 μ g/mL and incubated for 18 h at 4°C. The plates were washed three times with 0.05% tween 20 in PBS and incubated with 300 μ L per well of blocking buffer (1% bovine serum albumin (Sigma-Aldrich), 0.05 % tween 20 in PBS) for 1h. After washing, 100 μ L of the standards or samples were added to each well and incubated for 2h at room temperature. Plates were washed and incubated for 2h with 100 μ L of anti-SDF-1 α detection antibody (400 ng/ml in 1% bovine serum albumin, 2% heat inactivated (30 min, 56°C) normal goat serum (Jackson ImmunoResearch) in PBS) at room temperature. Microtiter plates were washed followed by addition of 100 μ L streptavidin HRP diluted 1:200 for 20 min at room temperature. Plates were washed again and 100 μ L of TMB was added to each well. After 20 min, 50 μ L of 2N HCl was added and optical densities were measured using a micro-ELISA autoreader (Synergy 2) at 450 nm and analyzed using the Gen 5 program. The correlation coefficients for each standard curve were 0.99 - 1 and the lower detection limit of the ELISA was 9 pg/mL.

Statistical Analyses

Data are described as mean $\pm$ SD or median with interquartile ranges (25th/75th percentile), as appropriate. Normal distribution was assessed with the Kolmogorov-Smirnov test. Because not all data sets passed the normality test ($\alpha = 0.05$), the Mann-Whitney U test and Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparisons test to control for multiple testing were used for comparisons between groups. Dichotomous categorical variables were compared using Fischer's exact test. Nonparametric monotonic correlations were assessed among variables using Spearman's rank correlation coefficient (r_s). Variables which significantly correlated with ubiquitin concentrations were then analyzed with partial correlation coefficients that describe the relationship between two variables while controlling for the effects of one or more additional variables. A two-tailed $p < 0.05$ was considered significant. Statistical analyses were calculated using GraphPad Prism 5 (GraphPad Software Inc.) and SPSS 16.0 (SPSS Inc.) software.

RESULTS

Total protein content was significantly elevated in BALF from patients (0.84 (0.56/1.31) mg/mL), when compared with BALF obtained from healthy volunteers (0.13 (0.04/0.3) mg/mL; $p < 0.0001$, Fig. 1A). Ubiquitin and SDF-1 α were detectable in 4 of 10 BALF

specimens from volunteers. In BALF from patients, ubiquitin was measurable in 45 of 51 BALF specimens ($p = 0.0023$ vs. volunteers), whereas SDF-1 α was detectable in only 5 of 51 BALF specimens ($p = 0.032$ vs. volunteers).

Ubiquitin concentrations per mL of BALF (patients: 51 (9/122) ng/mL; volunteers 0 (0/3.3) ng/mL; $p < 0.0001$, Fig. 1B) and per mg of BALF protein (patients: 54 (18/132) ng/mg; volunteers 0 (0/13) ng/mg; $p < 0.0001$, Fig. 1C) were significantly elevated in patient specimens. In contrast, SDF-1 α BALF concentrations were significantly reduced in patients (volunteers: 0 (0/14) pg/mL, 0 (0/234) pg/mg; patients: 0 (0/0) pg/mL ($p = 0.027$ vs. volunteers), 0 (0/0) pg/mg ($p = 0.0074$ vs. volunteers); Fig. 1D/E).

Because of the small proportion of BALF specimens with detectable SDF-1 α concentrations from patients, further analyses of associations with clinical variables were limited to BALF ubiquitin concentrations.

The correlations between ubiquitin concentrations per mg of total BALF protein and clinical parameters are shown in Table 1. Although there were no statistically significant associations between age, gender, %TBSA and Baux scores, BALF ubiquitin levels correlated negatively with the grade of inhalation injury and with revised Baux scores, in which patients with inhalation injury of grade 0 were scored as having no inhalation injury. In addition, ubiquitin BALF concentrations correlated negatively with intravenous fluid requirements within the first 48 h after burns and positively with P/F ratios at the time of BALF collection.

When patients were grouped according to the grade of inhalation injury, BALF protein content increased from 0.6 (0.5/1.0) mg/mL with grade 0 to 0.8 (0.6/1.3) mg/mL and 1.2 (1.1/1.7) mg/mL with grades 1-2 and 3-4, respectively ($p = 0.0043$, Fig. 2A). In contrast, ubiquitin BALF concentrations per mg of protein decreased with increasing severity of the inhalation injury (grade 0: 172 (68/266) ng/mg, grade 1-2: 42 (9/112) ng/mg, grade 3-4: 28 (0/130) ng/mg; $p = 0.029$, Fig. 2B).

BALF protein content did not show differences between patients with revised Baux scores < 80 and ≥ 80 (Fig. 3A) and between survivors and non-survivors (Fig. 3C). Ubiquitin BALF concentrations were significantly lower in BALF from patients with revised Baux scores ≥ 80 (60 (29/155) ng/mg vs. 31 (0.13/98) ng/mg, $p = 0.022$; Fig. 3B). Although ubiquitin levels were higher in survivors (61 (19/136) ng/mg) than in non-survivors (27 (0.6/44) ng/mg), this difference did not reach statistical significance ($p = 0.06$, Fig. 3D).

The correlations between ubiquitin concentrations per mg of BALF protein, initial fluid requirements and P/F ratios at the time of bronchoscopy are shown in Fig. 4. BALF ubiquitin concentrations increased with increases in P/F ratios (Fig. 4A). Inversely, BALF ubiquitin levels decreased as fluid requirements within the first 24h after burn injury increased (Fig. 4B).

Ubiquitin BALF concentrations did not correlate independently in partial correlation analyses with either age, grade of inhalation injury, fluid requirements or P/F ratios ($p > 0.05$ for all partial correlation analyses).

DISCUSSION

In the present study, we provide the initial assessment of ubiquitin and SDF-1 α in BALF from patients after burn and inhalation injuries. There are several new findings in this study. First, ubiquitin levels are increased in BALF following burn and inhalation injury, whereas SDF-1 α levels are decreased. Second, BALF ubiquitin levels are inversely correlated with

clinical grading scores that are used to assess the severity of the inhalation injury or the overall severity of the combined burn and inhalation injury. Third, ubiquitin concentrations in BALF correlate positively with oxygenation and negatively with required volumes of resuscitation fluids.

The low concentrations of ubiquitin and SDF-1 α that we detected in BALF from volunteers are in agreement with previously reported levels in normal BALF (19, 30-32). The findings of the present study are also consistent with the observations that ubiquitin was increased in BALF after blunt chest trauma in animals and in BALF from patients with other lung pathologies (19, 32, 33). This suggests that release of ubiquitin into the lung epithelial lining fluid is a typical local response in the diseased or injured lung. Our finding that SDF-1 α levels in BALF decreased after burn and inhalation injury, whereas increased BALF SDF-1 α levels have been described in patients with asthma (31), suggests that the local inflammatory response in the lung is disease specific. Elevated SDF-1 α levels have been described in the systemic circulation after burns in patients (17). This argues against the possibility that reduced SDF-1 α BALF levels in the present study are due to plasma protein leakage into the lung epithelial lining fluid after inhalation injury and in favor of the assumption that inhalation injury inhibits pulmonary SDF-1 α expression. Although we cannot exclude that protein leakage into the alveolar space partially contributes to increased ubiquitin levels in BALF after burn and inhalation injury, the observation that protein and ubiquitin content of BALF fluid showed inverse relationships with grade of inhalation injury points towards release of ubiquitin from local cells and tissues as its predominant origin in BALF. The missing association of BALF ubiquitin levels with %TBSA and Baux scores and ubiquitin's correlations with grade of inhalation injury and revised Baux scores further support this assumption.

Besides important roles in the pathogenesis of human immunodeficiency virus infection and cancer metastases, CXCR4 plays pleiotropic roles in the immune system and during tissue repair processes (34-39). In the lung, CXCR4/SDF-1 α -mediated recruitment of circulating progenitor epithelial cells has been shown to be necessary for the re-establishment of a normal pseudostratified epithelium after airway injury (40). Interestingly, our findings demonstrate that the expression of both natural ligands of CXCR4 in BALF after burn and inhalation injury is shifted into opposite directions. This suggests that increased levels of extracellular ubiquitin may compensate for reduced SDF-1 α expression and maintain CXCR4 signaling during the early inflammatory response to inhalation injury in the lung.

Previously, we showed that systemic ubiquitin levels correlate inversely with sequential organ failure assessment (SOFA) scores in burn patients (16). Our observations that ubiquitin concentrations in BALF from patients with inhalation injuries are negatively associated with resuscitation fluid requirements and positively associated with P/F ratios further support the notion that extracellular ubiquitin may play a protective role in the systemic circulation and at local sites of injury (4). The findings that BALF ubiquitin levels decrease with higher grades inhalation injury and are lower in patients with higher revised Baux scores suggest that a high severity of injury is associated with the inability to mount sufficient host defense and repair processes. This assumption is further supported by previous findings, which demonstrated lung protective effects of exogenous ubiquitin in trauma/hemorrhage and lung ischemia-reperfusion injury models (9, 10).

Obvious limitations of the present study are the cross-sectional design and its descriptive nature. Although the present and any other clinical observational study is inherently descriptive and unable to address mechanistic aspects, clinical association studies provide the opportunity to develop, test and validate novel pathophysiological concepts. Our observations in the present study support the concept that extracellular ubiquitin is involved

in the regulation of the local inflammatory response to inhalation injury in the lung. Furthermore, the invasiveness of the BAL procedure mandates initial explorative studies with BALF specimens obtained during routine diagnostic procedures before large-scale longitudinal studies are justified.

In conclusion, in the present study, we provide the first assessment of ubiquitin and SDF-1 α in BALF after inhalation injury. We demonstrate that the expression of the natural CXCR4 agonists is differentially regulated. The distribution of ubiquitin and SDF-1 α concentrations in BALF after inhalation injury and the associations of BALF ubiquitin levels with clinical variables demonstrate that both CXCR4 ligands will probably not be able to serve as clinically useful prognostic or diagnostic biomarkers. Nevertheless, our observations support the novel concept that increases in BALF ubiquitin after inhalation injury are required to maintain CXCR4 mediated lung protection and repair processes. The finding that BALF ubiquitin decreased with higher grades of inhalation injury may provide a biological correlate for an insufficient local inflammatory response after severe inhalation injury, which is accompanied by a higher degree of lung dysfunction or failure. In combination with the previously described association between systemic ubiquitin levels and organ failure in burn patients and the lung protective effects of ubiquitin treatment in animal studies (9, 10, 16), the results of the present study provide further clinical evidence to support the therapeutic use of ubiquitin and justify preclinical studies on therapeutic properties of CXCR4 ligands after trauma and burns.

Acknowledgments

Supported by NIHT32GM008750, NIHT32AA13672 and Dr. Ralph and Marian Falk Medical Research Trust

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Fig. 1. SDF-1 α : stromal cell-derived factor-1 α . Boxes extend from the 25th to 75th percentile; the horizontal line shows the median. Error bars show the minimum and maximum. (control: n=10; burn: n=51). The asterisk indicates statistical significance between groups ($p < 0.05$). (A) Bronchoalveolar lavage fluid (BALF) protein content. (B) BALF ubiquitin/mL. (C) BALF ubiquitin/mg protein. (D) BALF SDF-1 α /mL. (E) BALF SDF-1 α / mg protein.

Fig. 2.

Boxes extend from the 25th to 75th percentile; the horizontal line shows the median. Error bars show the minimum and maximum. Boxes not sharing the same letter are statistically significant ($p < 0.05$). **(A)** BALF protein content and **(B)** BALF ubiquitin/mg protein stratified by grade of inhalation injury (grade 0: $n=9$, grades 1-2: $n=23$, grades 3-4: $n=19$).

Fig. 3.

Boxes extend from the 25th to 75th percentile; the horizontal line shows the median. Error bars show the minimum and maximum. (A) BALF protein content and (B) BALF ubiquitin/mg protein from burned patients stratified according by Revised Baux Scores (<80: n=27, RBS >80: n=24). (C) BALF protein content and (D) BALF ubiquitin/mg protein in survivors (n=40) and non-survivors (n=11). The asterisk indicates statistical significance between groups (p<0.05).

Fig. 4.

(**A**) BALF ubiquitin/mg protein plotted against P:F ratio (partial pressure of oxygen / inspired fraction of oxygen) at the time of bronchoscopy. The line was calculated by linear regression analysis ($r^2=0.1004$, $p=0.0282$). (**B**) BALF ubiquitin/mg protein plotted against the volume of fluid resuscitation required in the initial 24h following injury. The line was calculated by linear regression analysis ($r^2=0.0937$, $p=0.0344$).

Table 1

Correlations with ubiquitin [*] in BALF:	n = 51	r _{spearman}	p value
Age (yrs)	50 (34 / 61)	-0.0125	0.93
Male (%)	65	-0.0531	0.72
%TBSA	15 (2 / 35)	-0.2452	0.09
Inhalation Injury Grade	2 (1 / 3)	-0.3065	0.03
Baux Score	50 (19 / 89)	-0.0884	0.55
Revised Baux Score	79 (62 / 106)	-0.3303	0.02
P / F Ratio	327 (259 / 425)	0.3501	0.01
Resuscitation Volume / kg Initial 24 h	125 (21 / 189)	-0.4584	0.001
Resuscitation Volume / kg Initial 48 h	181 (113 / 299)	-0.3278	0.02
Resuscitation Volume / kg Initial 72 h	222 (162 / 353)	-0.2812	0.06
Base Deficit (Initial)	1 (-2 / 4)	-0.1605	0.29
BALF Leukocyte Count (per μ L)	220 (47 / 352)	-0.3552	0.06
ICU Length of Stay (days)	13 (7 / 31)	0.0044	0.98
Mechanical Ventilation (days)	3 (8 / 24)	-0.2427	0.12
Pneumonia (%)	45	-0.2508	0.09
Sepsis (%)	12	-0.0227	0.88
Mortality (%)	20	-0.2453	0.09

* Correlations were calculated using the BALF ubiquitin concentrations normalized to total BALF protein content. Data are median with 25th/75th percentile in parenthesis or percentages. BALF: bronchoalveolar lavage fluid; r_{spearman}:Spearman correlation coefficient; TBSA: total body surface area; P/F ratio: partial pressure of oxygen / fraction of inspired oxygen at the time of bronchoscopy. ICU: intensive care unit. Significant correlations are highlighted.