

Regulation of hepatocyte growth factor secretion by fibroblasts in patients with acute lung injury

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Quesnel C, Marchand-Adam S, Fabre A, Marchal-Somme J, Philip I, Lasocki S, Leçon V, Crestani B, Dehoux M. Regulation of hepatocyte growth factor secretion by fibroblasts in patients with acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 294: L334–L343, 2008. First published December 7, 2007; doi:10.1152/ajplung.00096.2007.—The mechanisms of pulmonary repair in acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) are poorly known. Hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) are key factors involved in alveolar epithelial repair, present in the bronchoalveolar lavage fluid (BALF) from patients with ALI/ARDS. The role of BALF mediators in their production remains to be determined. We evaluated the overall effect of BALF from 52 patients (27 ventilated patients with ALI/ARDS, 10 ventilated patients without ALI, and 15 nonventilated control patients) on HGF and KGF synthesis by lung fibroblasts. Fibroblasts were cultured in the presence of BALF. HGF and KGF protein secretion was measured using ELISA, and mRNA expression was evaluated using quantitative real-time RT-PCR. Only BALF from ALI/ARDS patients upregulated both HGF and KGF mRNA expression and protein synthesis (+271 and +146% for HGF and KGF, respectively). BALF-induced HGF synthesis from ALI/ARDS patients was higher than that from ventilated patients without ALI ($P < 0.05$). HGF secretion was correlated with BALF IL-1 β levels ($\rho = 0.62$, $P < 0.001$) and BALF IL-1 β /IL-1 receptor antagonist ratio ($\rho = 0.54$, $P < 0.007$) in the ALI/ARDS group. An anti-IL-1 β antibody partially (>50%) inhibited the BALF-induced HGF and PGE₂ secretion, whereas NS-398, a specific cyclooxygenase-2 (COX-2) inhibitor, completely inhibited it. Anti-IL-1 β antibodies as well as NS-398 reversed the COX-2 upregulation induced by BALF. Therefore, IL-1 β is a main BALF mediator involved in HGF secretion, which is mediated through a PGE₂/COX-2-dependent mechanism. BALF mediators may participate in vivo in the production of HGF and KGF by lung fibroblasts during ALI/ARDS.

keratinocyte growth factor; acute respiratory distress syndrome; bronchoalveolar lavage fluid; interleukin-1 β ; cyclooxygenase-2; prostaglandin E₂

ACUTE LUNG INJURY (ALI) and its most extreme form, acute respiratory distress syndrome (ARDS), are characterized by a diffuse alveolar injury associated with widespread endothelial and epithelial disruption (36). The restoration of healthy alveolar epithelium requires the proliferation and migration of type II alveolar epithelial cells (AEC2) and their differentiation into type I alveolar epithelial cells (AEC1) (30, 36). This tightly

regulated mechanism is crucial to the effective repair of the alveolus after injury (30). Lung fibroblast migration and proliferation occur early after lung injury and are necessary for ongoing lung healing or fibrosis (6, 16, 22). These mesenchymal cells have been shown to control proliferation and differentiation of AEC through direct contact (31) as well as the secretion of soluble mediators such as hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) (37). To date, HGF and KGF are the most potent growth factors known to be involved in lung repair. Both are heparin-binding growth factors secreted by fibroblasts and regulate the differentiation and proliferation of alveolar epithelial cells. Whereas KGF secretion is strictly limited to lung fibroblasts, inflammatory cells such as neutrophils (15, 17) and possibly macrophages (38) are also involved in pulmonary HGF secretion. In patients with ALI/ARDS, KGF and HGF concentrations in bronchoalveolar lavage fluid (BALF) and edema fluid have been shown to be increased when compared with controls (32, 35). Despite the key role played by HGF and KGF during lung repair, little is known about the regulation of their production by lung fibroblasts during acute lung injury in humans. In vitro, interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and prostaglandin E₂ (PGE₂) stimulate their production, whereas transforming growth factor (TGF)- β and corticosteroids are known inhibitors (37). Elevated levels of IL-1 β and TNF- α are found in the alveolar space soon after lung injury in patients (10, 27). A kinetics study in an animal model with ALI showed that pulmonary production of both KGF and HGF increased at day 3 after initial injury, peaked on day 7, and remained elevated through day 14 (1). This time course suggests that this pulmonary production might be related to the early proinflammatory mediators, such as IL-1 β and TNF- α (27). Recent studies have also documented an increase of TGF- β together with evidence of fibroblast activation at the same period (5, 33). The early inflammatory response can be counterbalanced by anti-inflammatory mediators such IL-1 receptor antagonist (IL-1Ra) (10, 14, 27). Altogether, these studies suggest that there is a balance in vivo between stimulants and inhibitors of HGF and KGF production. We hypothesize that during the early phase of ALI, this balance will promote the production of growth factors for the alveolar epithelium by lung fibroblasts. Therefore, the aim of this study was 1) to evaluate ex vivo the overall effect of

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BALF from patients with acute lung injury on HGF and KGF production by lung fibroblasts and 2) to determine the main mediator involved in this effect.

MATERIALS AND METHODS

Study population. The protocol was approved by the ethical committee of Paris-Hotel-Dieu Hospital. The study was conducted prospectively in a surgical intensive care unit (ICU) and in the department of chest medicine of a tertiary teaching hospital. All the patients were enrolled in the study after a bronchoalveolar lavage (BAL) procedure without modification of current clinical practice guidelines. The patients included ($n = 52$) were separated into three groups: a group of ventilated patients without ALI, a group of ventilated patients with ALI or ARDS, and a control group of nonventilated patients. Patients with fibrotic lung disease, corticosteroid medication (methylprednisolone >1 mg/kg), human immunodeficiency virus infection, end-stage cancer, age <18 yr, and current pregnancy were excluded.

In mechanically ventilated patients ($n = 37$), the BAL was performed to diagnose a ventilator-associated pneumonia (VAP). The lung injury classification was established by following the criteria as defined by the American-European Consensus Conference on ALI and ARDS (4). The ALI/ARDS group ($n = 27$, 13 ALI and 14 ARDS patients) was defined by clinical criteria of bilateral infiltrates on chest X-ray, a ratio of arterial partial pressure of O_2 to fractional inspired O_2 (P_{aO_2}/F_{iO_2}) <300 mmHg, and no clinical evidence of left atrial hypertension or a pulmonary capillary wedge pressure <18 mmHg when available. The group of ventilated patients without ALI ($n = 10$) was composed of subjects intubated and ventilated for cardiogenic edema ($n = 5$) or polytrauma ($n = 5$). In this group, the BAL procedure was only performed in patients with clinical suspicion of VAP, none of whom had a confirmed VAP diagnosis after clinical evolution and results of microbiological cultures were reviewed.

Nonventilated patients ($n = 15$) underwent a bronchoscopy and BAL for the evaluation of chronic cough ($n = 10$) or hemoptysis ($n = 5$). Bronchoscopy, chest CT, and microbiological examination gave normal results for all control patients.

On the day of inclusion in the study, the following data were recorded in the various groups when appropriate: age, sex, reason for ICU admission and mechanical ventilation, presence or absence of sepsis, the P_{aO_2}/F_{iO_2} ratio, the Simplified Acute Physiologic Score II (SAPS II) (20), Organ System Failure Score (OSF) (18), and Lung Injury Score (LIS) (24). The length of time between the onset of the mechanical ventilation support and the BAL, as well as the length of stay in ICU and mortality 28 days after the BAL, were recorded for all patients.

BAL protocol and BALF sample processing. The BAL was performed as previously described (34). Briefly, six aliquots of 20 ml of sterile saline solution were injected through a bronchoscope wedged in a distal bronchus and gently aspirated. The first aliquot was discarded; the others were filtered on a sterile gauze and pooled. Ten milliliters of the BALF were processed for quantitative bacterial culture. A bacterial growth ≥ 10.4 colony-forming units/ml was consistent with lung infection. The remaining BALF (≥ 10 ml) was kept on ice and immediately centrifuged at 1,500 rpm for 10 min. A protease inhibitor, aprotinin (Bayer, Leverkusen, Germany), was added to the supernatant (5% vol/vol), and aliquots were frozen at -80°C until analysis. A differential cell count was performed on a cytocentrifuge smear with a Diff-Quik stain kit (Dade International, Miami, FL). BALF and serum protein concentrations were measured with a Hitachi 911 analyzer (Roche, Meylan, France), and the protein ratio was determined to evaluate the alveolar permeability induced by lung injury.

Cytokines, antibodies, and chemicals. Recombinant human IL-1 β , monoclonal anti-IL-1 β , and nonspecific mouse IgG were purchased from R&D Systems (Minneapolis, MN). Indomethacin, NS-398, PGE₂, and

dimethyl sulfoxide were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, amphotericin B, penicillin G, streptomycin, fetal calf serum (FCS), and phosphate-buffered saline (PBS) were purchased from Invitrogen (Cergy Pontoise, France). Aprotinin (Trasyolol) was purchased from Bayer Pharma (Sens, France).

Measurement of HGF, KGF, IL-1 β , IL-1Ra, and PGE₂. HGF and KGF were measured in BALF and in the fibroblast supernatants by ELISA according to the manufacturer's recommendations (HGF and KGF Duoset Kit; R&D Systems). The detection threshold of the assay was 15 and 40 pg/ml for KGF and HGF, respectively. IL-1 β and IL-1Ra were measured in BALF by ELISA (IL-1 β and IL-1Ra Quantikine; R&D Systems). The detection threshold of the assay was 1 and 22 pg/ml for IL-1 β and IL-1Ra, respectively. PGE₂ was measured in BALF and in the fibroblast supernatants by enzyme immunoassay (PGE₂ HS Quantikine; R&D Systems). The detection threshold of the assay was 8.5 pg/ml.

Cell culture. The human lung fibroblast MRC-5 cell line (CCL MRCOO/OR) obtained from Eurobio (les Ulis, France) was used to evaluate the effect of BALF. In preliminary experiments, we verified that BALF-induced HGF and KGF production were similar in normal adult lung fibroblasts and MRC-5 cells. Cells were cultured with DMEM and 10% FCS supplemented with antibiotics (2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin). Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C .

HGF and KGF protein secretion. Cells were seeded on 12-well tissue culture plates at a density of 5×10^4 cells/well. Fibroblasts were grown to 80% confluence and then washed twice in PBS and cultured in 375 μl of serum-free DMEM with either 125 μl of saline and 5% aprotinin (control condition) or with 125 μl of BALF for 48 h. The optimal concentration of BALF (25% of final culture volume) was determined in preliminary assays, demonstrating a BALF dose-dependent effect on KGF and HGF fibroblast production. After a 48-h incubation period for all the experiments, the supernatants were preserved with aprotinin (50 $\mu\text{l}/\text{ml}$), aliquoted, and stored at -20°C before HGF protein, KGF protein, or PGE₂ determination. HGF and KGF concentrations in the fibroblast supernatant were corrected by withdrawing the amount of HGF and KGF contained in each BALF tested to specifically determine the fibroblast production. This net production of HGF or KGF by stimulated fibroblasts was divided by the total protein amount of the cell monolayer (Bio-Rad protein assay, Hercules, CA) to take into account the possible variation in the cell numbers between wells. The results were expressed in picograms of KGF or HGF per microgram of protein in the cell monolayer per well. The results shown in Figs. 1A and 2A have been transformed and are expressed as percentages of the control condition to standardize the results of different experiments. In Figs. 3–7, all other results are directly expressed as picograms per microgram per well.

To evaluate the specific involvement of IL-1 β contained in BALF on HGF secretion, eight BALF (4 ALI and 4 ARDS), selected from a wide range of increase in HGF, were preincubated for 1 h with anti-IL-1 β neutralizing antibody (10 $\mu\text{g}/\text{ml}$) or nonspecific IgG (10 $\mu\text{g}/\text{ml}$). The treated BALF were then added to 375 μl of serum-free DMEM as previously described.

To evaluate the role of the cyclooxygenase-2 (COX-2) pathway, indomethacin (2.5 $\mu\text{g}/\text{ml}$; Sigma), a COX-1 and COX-2 inhibitor, and NS-398 (25 μM ; Sigma), a selective COX-2 inhibitor, were added to the culture serum-free medium before the addition of BALF ($n = 4$) or recombinant human (rh)IL-1 β (10 ng/ml). In this experiment, PGE₂ (10^{-6} M) was used as a positive control for HGF upregulation. Both net HGF and PGE₂ levels were measured in cell supernatants and expressed as previously described. In the inhibitory experiments, the mouse monoclonal anti-IL-1 β and control IgG interfered in the PGE₂ enzyme immunoassay. They were therefore extracted before PGE₂ measurement in fibroblast supernatants. The cell culture supernatants were applied to Ab SpinTrap affinity columns (GE Healthcare Europe, Orsay, France) according to the manufacturer's recommendations.

The columns contained protein G-Sepharose and were designed for rapid purification of mouse monoclonal IgG antibodies. To evaluate drug-induced and BALF cytotoxicity, lactate dehydrogenase (LDH) activity was measured in the cell culture supernatants on the Hitachi 911 analyzer (Roche).

HGF, KGF, and COX-2 mRNA expression: RNA preparation and RT-PCR. To evaluate HGF and KGF mRNA expression, fibroblasts were grown to confluence in a 25-cm² culture flask and then washed twice in PBS and cultured for 18 h in 2 ml of serum-free DMEM in the presence of either saline with aprotinin (control condition) or in the presence of BALF as described above. To evaluate COX-2 mRNA expression, the medium was changed to serum-free DMEM for 24 h to reduce serum-induced expression of COX-2, and fibroblasts were then stimulated with BALF alone or in combination with anti-IL-1 β antibody and/or NS-398 for 6 h.

The relative content of HGF, KGF, and COX-2 mRNA was analyzed after reverse transcription by real-time RT-PCR and expressed as a ratio to ubiquitin C mRNA (UBC) as a housekeeping gene. Fibroblast mRNA extraction was performed using the Nucleospin RNA II kit (Macherey Nagel, Hoerd, France). Reverse transcription was performed with random hexamer primers, oligo(dT), and reverse transcriptase SuperScript II (Invitrogen, Carlsbad, CA) as previously described (21). Each amplification reaction was performed on an ABI Prism 7700 (Applied Biosystems, Foster City, CA) in duplicate with SYBR green mix (Sigma) and specific primers (see Table 1). The data were analyzed using Sequence Detection software (Applied Biosystems). A negative control with water only was used for each primer. The results are expressed as HGF/UBC mRNA, KGF/UBC mRNA, or COX-2/UBC mRNA ratios.

Statistical analysis. Demographic data are expressed as means \pm SD and biological data as median and range values. The continuous variables in the different patient groups were compared using the Kruskal-Wallis test followed by Dunn's multiple-comparison post hoc analysis. All proportional values were compared using Fischer's exact test. To compare different conditions in inhibitory assays, we used the Friedman test followed by Dunn's multiple-comparison post hoc analysis. Correlations were assessed using the Spearman rank-order test (Spearman's rho). Statistical significance was accepted as $P \leq 0.05$.

RESULTS

Patient characteristics. Patient characteristics are depicted in Table 2. There were more men than women in both ventilated groups compared with the nonventilated group (28/9 vs. 6/9, respectively; $P < 0.05$). The mean age, the severity score (SAPSII), and the number of organ failures (OSF) were similar in ventilated patients groups ($P > 0.05$). The delay in perform-

ing the BAL was similar in ventilated patients with ALI/ARDS and in patients without ALI (3 vs. 4 days, respectively; $P > 0.05$). In the ALI/ARDS group, the ARDS patients ($n = 14$) were more severely ill than the ALI patients ($n = 13$), as demonstrated by their respective SAPSII (60 vs. 47), LIS (2.6 vs. 1.5; $P < 0.001$), and mortality (78 vs. 38%; $P < 0.001$).

BALF characteristics. BALF cell analysis showed a neutrophilic alveolitis in the ALI/ARDS group. As expected, the protein level in BALF and the protein ratio between alveoli and blood was higher in the ALI/ARDS group than in the other groups and depended on the degree of lung injury.

KGF was detectable in BALF from 11 of 27 patients in the ALI/ARDS group and in none of the other groups ($P < 0.01$; Table 3). HGF was detectable in BALF from all patients in the ALI/ARDS group, in 8 of 10 patients in the ventilated group without ALI, and in 1 of 15 patients in the nonventilated group ($P < 0.001$).

KGF protein secretion and mRNA expression. BALF from 13 of 15 nonventilated patients and from 4 of 10 ventilated patients without ALI either inhibited or had no effect on KGF fibroblast secretion. The BALF from ventilated patients without ALI induced no significant increase in KGF secretion compared with BALF from nonventilated patients ($P > 0.05$; Fig. 1). By contrast, 20 of 27 BALF from ALI/ARDS patients had a modest stimulating effect on KGF secretion (median effect [range effect]: 146% [60–308%]). This moderate stimulating effect was significantly different from that induced by BALF from nonventilated patients ($P < 0.05$). No difference was found between ventilated patients without ALI or with ALI/ARDS (125% [49–192%] vs. 146% [60–308%], respectively, $P > 0.05$) and between ALI and ARDS patients. No correlation was found between BALF KGF levels and BALF-induced KGF secretion.

The BALF from ALI/ARDS patients induced a 1.4-fold increase of KGF mRNA expression in fibroblasts, whereas BALF from nonventilated patients and ventilated patients without ALI had little or no effect on KGF mRNA expression. No correlation was found between KGF protein synthesis and mRNA expression.

HGF protein secretion and mRNA expression. BALF from 12 of 15 nonventilated patients and from 2 of 10 ventilated patients without ALI either inhibited or had no effect on HGF fibroblast secretion. By contrast, 26 of 27 BALF from ALI/ARDS patients stimulated HGF production (271% [87–749%]). The stimulating effect was higher in this group than in ventilated patients without ALI (271 vs. 137%, $P < 0.05$; Fig. 2). However, no difference was observed between ALI and ARDS patients. BALF HGF levels were positively correlated with BALF-induced HGF secretion ($\rho = 0.59$, $P < 0.0005$) in the ventilated patients.

BALF from nonventilated and ventilated patients without ALI has little or no effect on HGF mRNA expression by fibroblasts, whereas the BALF from ALI/ARDS patients led to a fourfold increase in HGF mRNA expression ($P < 0.05$). There was a positive correlation between HGF protein synthesis and mRNA expression for all the patients ($\rho = 0.414$, $P = 0.017$).

IL-1 β and IL-1Ra concentrations in BALF. In vitro, IL-1 β is a powerful inducer of HGF and KGF secretion by fibroblasts. IL-1 β is produced in large quantities in the alveoli during the acute phase of lung injury (14, 27). Before evaluating the role

Table 1. Sequence of primers pairs used for quantitative PCR

Gene	Sequence, 5'-3'	Size, bp
KGF		
Reverse	TTTTGTCTTTCT	201
Forward	GAACAAGGAAGGAAAACCTCTATGCAA	
HGF		
Reverse	CAGAGGGACAAAGGAAAAGAA	167
Forward	GCAAGTGAATGGAAGTCCTTTA	
COX-2		
Reverse	GCCGAGGCTTTTCTACCAGAA	152
Forward	TTAATGAGTACCAGCAACGCTTTAT	
UBC		
Reverse	TTTTTTGGGAATGCAACAACCTTT	105
Forward	CACCTTGGTCCTGCGCTTGA	

KGF, keratinocyte growth factor; HGF, hepatocyte growth factor; COX-2, cyclooxygenase-2; UBC, ubiquitin C.

Table 2. Clinical characteristics of patients with and without ALI/ARDS

	Not Ventilated	Ventilated w/o ALI	Ventilated		P
			ALI	ARDS	
n	15	10	13	14	
Age, yr*	50±14	64±18	60±16	64±15	NS
Sex ratio, F/M	9/6	1/9	6/7	2/12	<0.05
SAPS II*		44±13	47±16	60±19	NS
OSF*		2±1	2±1	3±1	NS
Sepsis, %			31	79	NS
Pulmonary infection, %			38	71	NS
Length of stay in ICU before BAL, days*		3±3	4±5	4±3	NS
LIS*			1.5±0.4	2.6±0.3	<0.001
PaO ₂ /FiO ₂ ratio, mmHg*		232±129	248±54	133±35	<0.01
28-day mortality, %		20	38	78	<0.001

*Values are means ± SD. ALI, acute lung injury; ARDS, acute respiratory distress syndrome; F/M, female/male; SAPS II, Simplified Acute Physiologic Score II; OSF, organ system failure score; BAL, bronchoalveolar lavage; ICU, intensive care unit; LIS, lung injury score; PaO₂/FiO₂ ratio, ratio of arterial partial pressure of O₂ to fractional inspired O₂; NS, not significant.

of this mediator, we first measured IL-1β in the BALF from patients. Since IL-1Ra functions as a competitive inhibitor of IL-1β, BALF IL-1Ra and the IL-1β/IL-1Ra ratio were also determined. As shown in Fig. 3A, IL-1β was detectable in only two patients from the nonventilated group and three patients from the ventilated group without ALI, whereas IL-1β was detectable in all except one patient from the ALI/ARDS-group. IL-1β concentrations were higher in BALF from ALI/ARDS patients (123 pg/ml [1–1,157 pg/ml]) than in BALF from other groups ($P < 0.001$). A positive correlation was observed between IL-1β and HGF concentration in the BALF from ALI/ARDS patients ($\rho = 0.57$, $P < 0.01$; Fig. 4A). Furthermore, IL-1β in BALF from ALI/ARDS patients was positively correlated with the HGF secretion induced by BALF in fibroblasts ($\rho = 0.62$, $P < 0.001$; Fig. 4B). IL-1Ra was detected in BALF from all patients (Table 3). IL-1Ra concentrations were higher in BALF from ventilated patients with ALI/ARDS than in those from ventilated patients without ALI and nonventilated patients ($P < 0.0001$ for both). No correlation was found between IL-1Ra and HGF either in BALF or in cell culture supernatant. To better analyze the contribution of IL-1Ra, we evaluated the relationship between IL-1β/IL-1Ra ratio and HGF secretion. The higher the IL-1β/IL-1Ra ratio, the more pronounced the overall effect of the BALF on HGF synthesis by fibroblasts. In the ALI/ARDS group, the IL-1β/IL-1Ra ratio was correlated to both HGF concentration in

BALF ($\rho = 0.37$, $P = 0.03$) and BALF-induced HGF secretion by fibroblasts ($\rho = 0.54$, $P = 0.007$). No correlation was found between BALF IL-1β or IL-1Ra and KGF levels (measured in BALF or in fibroblast supernatant).

Participation of IL-1β in the stimulation of HGF production. Because the BALF-induced KGF production was very modest, we focused our study on the mediators involved in HGF production. We then evaluated the role of IL-1β in the stimulating effect of BALF on HGF secretion. Eight BALF (4 ALI and 4 ARDS) were chosen among samples with sufficient volumes to perform the experiment. These BALF varied in their stimulating effect on HGF secretion (from 140 to 271%) and in their BALF IL-1β concentrations (from 3 to 570 pg/ml). The higher the BALF IL-1β level or the IL-1β/IL-1Ra ratio, the more pronounced the inhibition of HGF production in the presence of the anti-IL-1β antibody. As shown in Fig. 5, the stimulating effect of BALF on HGF secretion was strongly inhibited in the presence of anti-IL-1β antibodies (the median decrease of BALF-induced HGF was 89%, ranging from 22 to 100%, $P < 0.05$), whereas the control IgG had no effect.

BALF induced HGF and PGE₂ secretion through a COX-2-dependent mechanism. IL-1β is known to upregulate COX-2 expression and PGE₂ synthesis in fibroblasts (9). Because PGE₂ is a potent inducer of HGF synthesis in lung fibroblasts (23), we wondered whether IL-1β from BALF directly stimu-

Table 3. BALF characteristics of patients with and without ALI/ARDS

	Not Ventilated	Ventilated w/o ALI	Ventilated		P
			ALI	ARDS	
n	15	10	13	14	
BALF protein, g/l	0.129	0.16	0.48	0.85	<0.05
Protein ratio BALF/serum		0.003	0.01	0.02	<0.05
BALF total cell count, 10.6/ml*	4±3	4±3	17±21	16±19	NS
Neutrophil, %*	3±5	13±22	83±14	78±30	<0.001
Macrophage, %*	96±5	85±22	13±14	20±30	<0.001
HGF, pg/ml†	≤40 [<40 –127]	86 [<40 –144]	1035 [116–3,108]	819 [65–3,634]	<0.0001
KGF, pg/ml†	ND	ND	15 [<15 –64]	18 [<15 –81]	<0.01
IL-1β, pg/ml†	1.5 [1–8.5]	21.7 [1–189]	250 [3–994]	218 [1–1,157]	<0.0001
IL-1Ra, pg/ml†	842 [246–2,674]	384 [86–806]	8,250 [1,626–27,735]	11,822 [274–51,329]	<0.0001
PGE ₂ , pg/ml†	86 [17–513]	87 [9–360]	227 [12–562]	199 [11–697]	NS

*Values are means ± SD. †Values are means [extreme range values]. BALF, BAL fluid; IL-1Ra, IL-1 receptor antagonist; PGE₂, prostaglandin E₂; ND, not detectable.

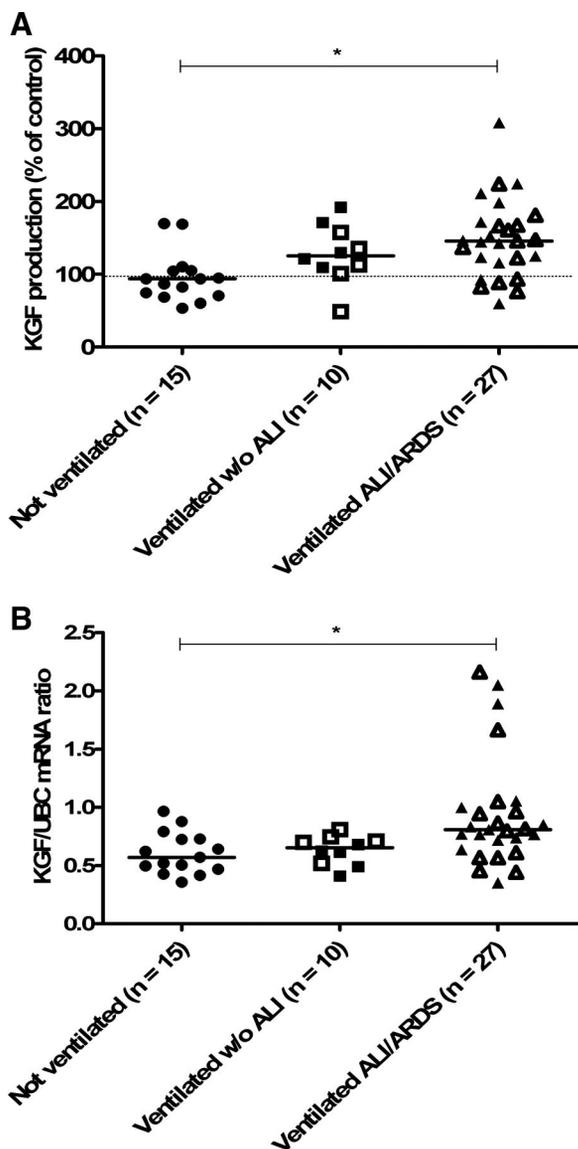


Fig. 1. Effect of bronchoalveolar lavage fluid (BAL) on keratinocyte growth factor (KGF) synthesis by lung fibroblasts. **A:** human lung fibroblasts (MRC-5) were cultured in triplicate for 48h in serum-free medium and stimulated with 25% (vol/vol) saline with 5% aprotinin (control condition) or with BALF from nonventilated patients (\bullet , $n = 15$), ventilated patients without acute lung injury (ALI) [\square , cardiogenic edema ($n = 5$); \blacksquare , polytrauma ($n = 5$)], and ventilated patients with ALI (Δ , $n = 13$) or ARDS (\blacktriangle , $n = 14$). KGF concentration in fibroblast supernatants was measured by ELISA and divided by μg of total protein measured in the cell monolayer per well. Results are expressed as a percentage of KGF production in control condition. Horizontal bars indicate the median of individual values. $*P < 0.05$. **B:** KGF mRNA expression. Lung fibroblasts were cultured in serum-free medium for 18 h in the presence of either saline with aprotinin (control condition) or in the presence of BALF as described in A. The relative content of KGF mRNA was analyzed by quantitative real-time RT-PCR and expressed as a ratio to the ubiquitin C mRNA (UBC). Horizontal bars indicate the median of individual values. $*P < 0.05$.

lated HGF production or whether IL-1 β could act through the stimulation of COX-2 and PGE₂ secretion.

To evaluate the role of IL-1 β and COX-2 in BALF-induced HGF secretion, we measured both HGF and PGE₂ in fibroblast supernatants stimulated by rhIL-1 β or BALF in the presence or absence of COX-2 inhibitors or of anti-IL-1 β antibodies ($n =$

4, Fig. 6). In addition, COX-2 mRNA expression was evaluated (Fig. 7).

Before measuring PGE₂ in fibroblast supernatants, we measured BALF PGE₂ concentrations (Table 3). PGE₂ was detectable in all BALF. PGE₂ concentration tended to be higher in BALF from patients with ALI/ARDS (119 pg/ml [11–697 pg/ml]) than in those from ventilated patients without ALI (20 pg/ml [9–360 pg/ml]) and nonventilated patients (26 pg/ml [17–513 pg/ml]) ($P > 0.05$).

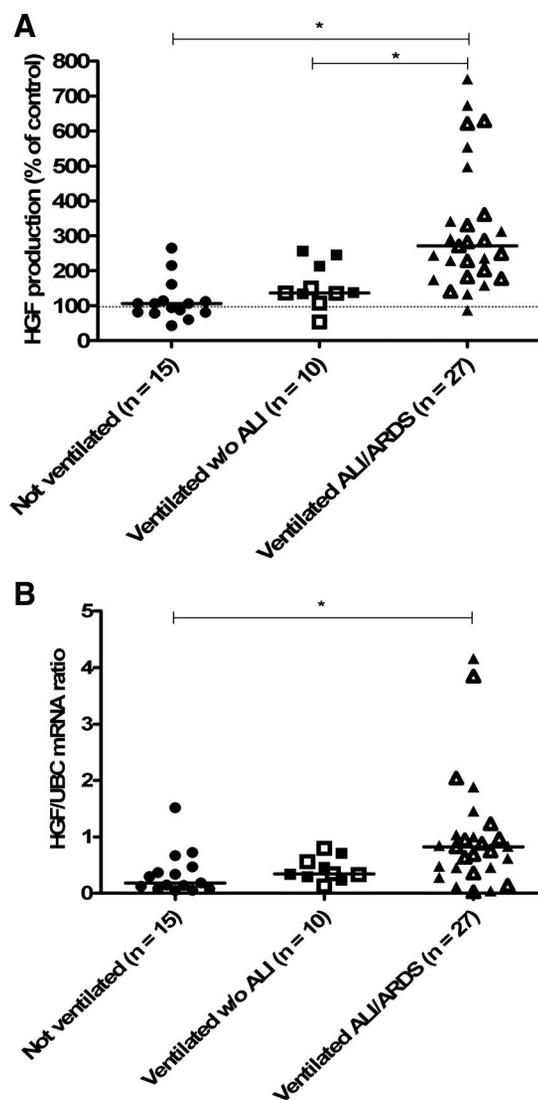


Fig. 2. Effect of BALF on hepatocyte growth factor (HGF) synthesis by lung fibroblasts. **A:** human lung fibroblasts (MRC-5) were cultured in triplicate for 48 h in serum-free medium and stimulated with 25% (vol/vol) saline with 5% aprotinin (control condition) or BALF from nonventilated patients (\bullet , $n = 15$), ventilated patients without ALI [\square , cardiogenic edema ($n = 5$); \blacksquare , polytrauma ($n = 5$)], and ventilated patients with ALI (Δ , $n = 13$) or ARDS (\blacktriangle , $n = 14$). HGF concentration in fibroblast supernatants was measured by ELISA and divided by μg of total protein measured in the cell monolayer per well. Results are expressed as a percentage of HGF production in control condition. Horizontal bars indicate the median of individual values. $*P < 0.05$. **B:** HGF mRNA expression. Lung fibroblasts were cultured in serum-free medium for 18 h in the presence of either saline with aprotinin (control condition) or in the presence of BALF as described in A. The relative content of HGF mRNA was analyzed by quantitative real-time RT-PCR and expressed as a ratio to the UBC mRNA. Horizontal bars indicate the median of individual values. $*P < 0.05$.

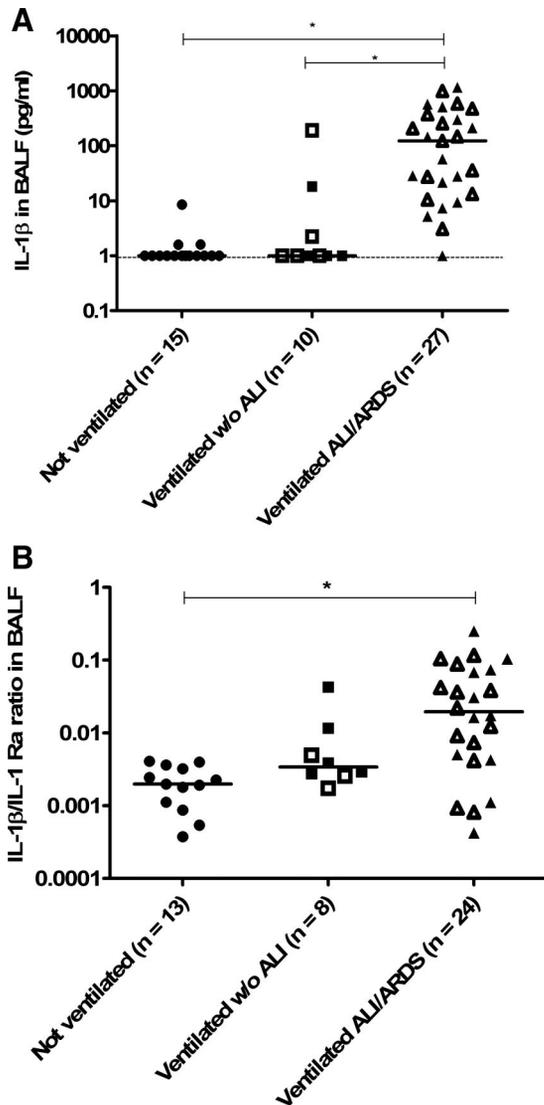


Fig. 3. IL-1 β concentrations and IL-1 β /IL-1 receptor antagonist (IL-1Ra) ratio in BALF. A: IL-1 β was measured by ELISA in the BALF from nonventilated patients (\bullet , $n = 15$), ventilated patients without ALI [\square , cardiogenic edema ($n = 5$); \blacksquare , polytrauma ($n = 5$)], and ventilated patients with ALI (\triangle , $n = 13$) or ARDS (\blacktriangle , $n = 14$). IL-1 β levels, expressed as pg/ml, are represented on a logarithmic scale. Horizontal bars indicate the median of individual values; dotted line represent the sensitivity threshold of the assay. * $P < 0.05$. B: BALF IL-1Ra was measured by ELISA and expressed as pg/ml. IL-1 β /IL-1Ra ratios are represented on a logarithmic scale. Horizontal bars indicate the median of individual values. * $P < 0.05$.

In basal condition, both PGE₂ and HGF are secreted by fibroblast and COX-2 mRNA is expressed even in serum-free culture (Figs. 6 and 7). HGF basal secretion was inhibited by COX-2 inhibitors. These results showed that COX-2 pathway is involved in the autocrine HGF production by fibroblast. We confirmed that rhIL-1 β induced an increase of HGF and PGE₂ that was completely inhibited by COX-2 inhibitors, suggesting that IL-1 β -induced HGF secretion was COX-2 dependent. BALF from ALI/ARDS patients ($n = 4$) increased both PGE₂ and HGF levels in fibroblast supernatants. Furthermore, BALF from ALI/ARDS patients induced a 24-fold increase of COX-2 mRNA expression compared with the control condition (Fig. 7). In the set of experiments shown in Fig. 6, BALF-induced

HGF and PGE₂ release by fibroblasts was inhibited by 66 and 57%, respectively, in the presence of anti-IL-1 β antibodies and by >90% in the presence of COX-2 inhibitors. BALF-induced COX-2 mRNA expression was inhibited by 54% in the presence of anti-IL-1 β antibody and by 100% in the presence of both anti-IL-1 β antibody and NS-398. LDH was measured in all cell supernatants. Although LDH concentrations varied from 13 to 52 UI/l (median value) in the various conditions, no significant difference was found and no cytotoxic effect can be shown.

DISCUSSION

In this study, we evaluated ex vivo the overall effect of BALF from patients with ALI/ARDS on HGF and KGF production by human pulmonary fibroblasts. The main results of this study are 1) BALF from ventilated patients upregulates the secretion of HGF at the transcriptional level; 2) BALF from patients with ALI/ARDS has a higher capacity to induce HGF than BALF from ventilated patients without lung injury; 3) in ALI/ARDS, BALF-induced HGF secretion by fibroblasts is mediated through a COX-2/PGE₂ pathway; 4) IL-1 β contained in BALF is a main mediator involved in this effect; and 5) other BALF mediators are likely involved in BALF-induced PGE₂ autocrine production.

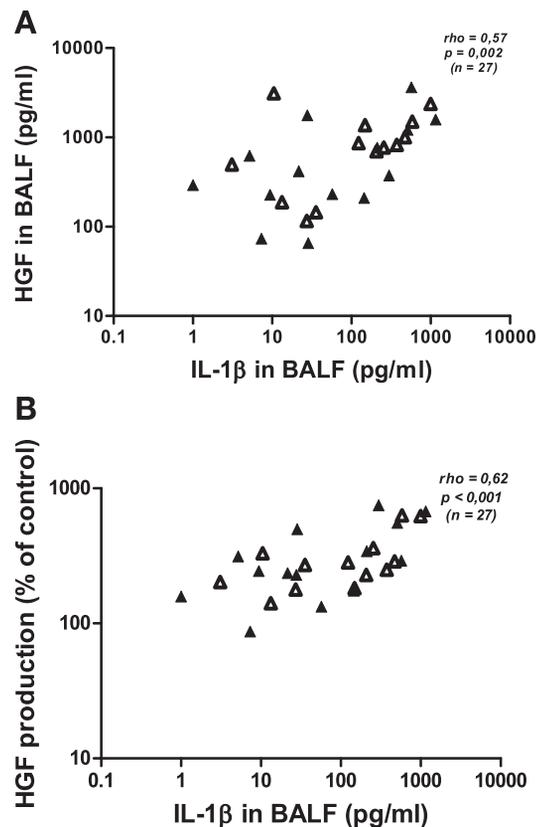


Fig. 4. Correlation between BALF IL-1 β and HGF concentrations in the ALI/ARDS group. A: correlation between BALF IL-1 β and BALF HGF levels [\triangle , ventilated patients with ALI ($n = 13$); \blacktriangle , ARDS patients ($n = 14$)]. BALF IL-1 β and BALF HGF levels, expressed as pg/ml, are represented on a logarithmic scale ($n = 27$, $\rho = 0.57$, $P < 0.01$). B: correlation between BALF IL-1 β and BALF-induced HGF secretion ($n = 27$, $\rho = 0.62$, $P < 0.001$). IL-1 β in BALF (pg/ml) and HGF production by stimulated fibroblasts (% control) are represented on a logarithmic scale.

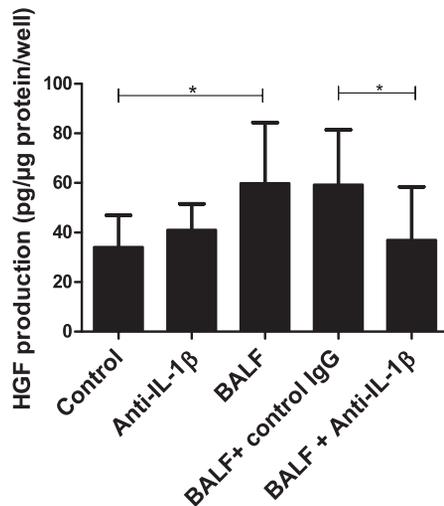


Fig. 5. Effect of anti-IL-1 β antibodies on BALF-induced HGF secretion by fibroblasts. Fibroblasts were incubated in triplicate in serum-free culture medium and stimulated with 25% BALF (vol/vol) for 48 h. Results (means \pm SE) are representative of 8 experiments with BALF from patients with ALI ($n = 4$) or with ARDS ($n = 4$) in the presence of either anti-IL-1 β antibodies (10 μ g/ml) or IgG controls (10 μ g/ml). Results are expressed as HGF concentration in cell culture supernatants divided by μ g of total protein measured in the cell monolayer per well. * $P < 0.05$.

HGF and KGF play a key role in alveolar epithelial repair (2, 37), thereby allowing the restoration of normal lung architecture after injury. Although several mediators have been shown to modulate positively or negatively HGF and KGF production in vitro (37), the regulation of HGF and KGF production during

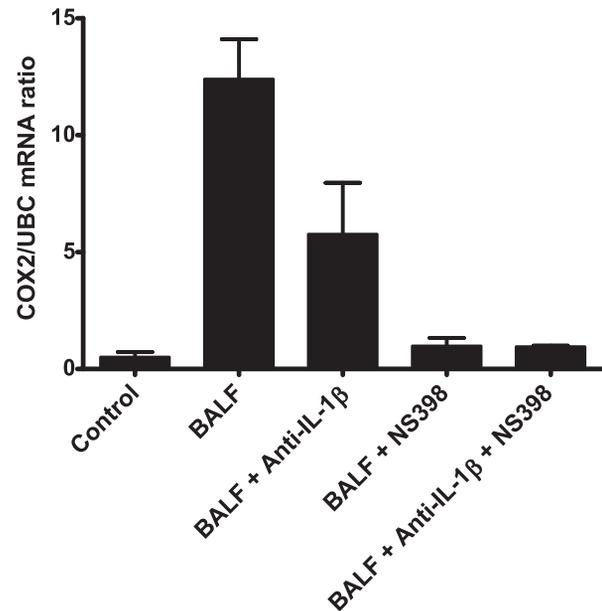


Fig. 7. Effect of BALF and IL-1 β on COX-2 mRNA expression in lung fibroblasts. Lung fibroblasts were cultured for 6 h in serum-free medium in the presence of either saline with aprotinin (control condition) or in the presence of BALF (25% vol/vol) with or without anti-IL-1 β antibody (10 μ g/ml), NS-398 (25 μ M), or both. Results (means \pm SE) are representative of 2 experiments performed in triplicate using BALF from 2 ARDS patients. The relative content of COX-2 mRNA was analyzed using real-time quantitative RT-PCR and expressed as a ratio to UBC mRNA.

acute lung injury in humans remains poorly known. In this study, we demonstrated for the first time that the BALF obtained from patients with ALI/ARDS stimulated HGF and KGF synthesis by human lung fibroblasts in vitro. BALF induced an upregulation of HGF and KGF synthesis at the transcriptional level. However, this effect was more consistent with HGF than with KGF.

To avoid bias of interpretation, we performed our experiments with the MRC-5 fibroblast cell line because of the large number of BALF we had to test. We performed preliminary experiments using primary cultures of fibroblasts from human lung explants (21) and have confirmed the results obtained with the MRC-5 cells. Since pulmonary fluid from patients with ALI has been shown to stimulate the proliferation of human fibroblasts in culture (25) and therefore may have influenced our results, we verified this potential effect. We found no significant mitogenic properties of BALF in our culture condition. These results may be explained by the use of BALF instead of concentrated fluid such as edema fluid. As described by Olman et al. (25), the dilution of the edema fluid was associated with a decrease in its proliferating effect. Moreover, Olman et al. stimulated fibroblasts for 4–6 days; in our study, fibroblasts were stimulated for only 48 h. Our experimental conditions likely led to an underestimation of the BALF-induced HGF/KGF production that may be observed in vivo, taking into account the dilution of the epithelial fluid induced by the BAL procedure.

We observed that BALF from nonventilated patients had no effect on HGF/KGF production. The moderate secretion of HGF/KGF induced by some BALF from ventilated patients without ALI could result from the release of mediators following mechanical stretch and inflammatory response induced by ventilation as previously reported (28). The most important

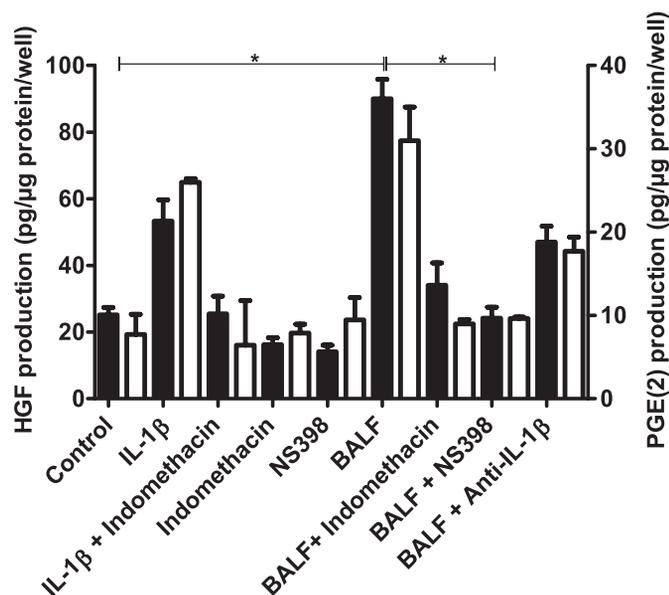


Fig. 6. Effect of cyclooxygenase-2 (COX-2) inhibition on BALF-induced HGF and PGE₂ secretion by fibroblasts. Fibroblasts were cultured in serum-free culture medium stimulated with BALF from patients with ARDS ($n = 4$, 25% vol/vol) for 48 h in the presence of the following modulators, used alone or in combination: PGE₂ (10⁻⁶ M), IL-1 β (10 ng/ml), indomethacin (2.5 μ g/ml), NS-398 (25 μ M), or anti-IL-1 β (10 μ g/ml). Results (means \pm SE) are representative of 4 experiments performed in triplicate. Results are expressed for each condition as HGF concentration (solid bars, corresponding to left axis) and PGE₂ concentration (open bars, corresponding to right axis) in cell culture supernatants divided by μ g of total protein measured in the cell monolayer per well. * $P < 0.05$.

upregulation of HGF/KGF secretion was observed with BALF from ALI/ARDS patients. Despite the difference in severity of alveolar injury and outcome, no difference was found between ALI and ARDS patients. Indeed, the stimulating effect of the BALF was correlated with neither the level of lung injury (estimated by LIS) nor the severity scores (OSF, SAPS II). This could be due to a lack of statistical power, to the pitfall of ALI and ARDS classification, which differentiates two steps of a continuum ranging from ALI to ARDS, and/or to an imbalance between lung injury severity and alveolar repair process.

Besides fibroblasts, several cells are known to produce HGF in the alveolus such as macrophages and neutrophils (15, 17). In this study, we confirm the strong correlation between BALF HGF concentrations and total neutrophil count in BALF in the whole population ($\rho = 0.88$, $P < 0.001$), which likely indicates the participation of neutrophils in HGF production in the lung (32). In addition, BALF from ALI patients contains many proteases (29) capable of releasing KGF and HGF that are tethered to the extracellular matrix proteoglycans. We found a correlation between BALF HGF concentrations and the capacity of the corresponding BALF to stimulate the production of this factor by fibroblasts, suggesting that these cells might participate *in vivo* in HGF production during ARDS. Even if KGF synthesis is known to be limited to fibroblasts, we did not find such a correlation with KGF. This might be due to the moderate effect of BALF on KGF secretion and to the low levels of KGF produced during ALI/ARDS (32, 35).

The BAL was performed only because of suspicion of VAP. Therefore, the time to BAL following mechanical ventilation was not chosen but occurred naturally. Since the alveolar inflammatory response in ALI/ARDS is a highly dynamic process, a kinetics study would have been of interest but could not be prospectively evaluated for ethical concerns. Within the time frame studied, the alveolar inflammatory response might favor the elaboration of either a highly proinflammatory environment or an overall anti-inflammatory environment. This might explain the variation observed in the BAL-induced HGF/KGF secretion and even the inhibitory effect found with some BALF from the control or ventilated groups. Since all the BALF except one from ALI/ARDS triggered HGF secretion, we have assumed that proinflammatory mediators participate in this effect. We therefore evaluated the role of IL-1 β and of IL-1 β /IL-1Ra ratio on HGF secretion. We demonstrated the main participation of BALF IL-1 β in the induction of HGF by lung fibroblasts by using a specific neutralizing antibody. The role of IL-1 β in the upregulation of HGF synthesis was further strengthened by the strong correlations 1) between BALF IL-1 β levels and the BALF-induced HGF secretion and 2) between IL-1 β and HGF levels in the BALF. The IL-1 β /IL-1Ra ratio was also correlated to both BALF HGF levels and BALF-induced HGF secretion. This suggests that IL-1Ra counteracts the inducible effect of IL-1 β and that HGF secretion by fibroblasts is dependent, at least in part, on the IL-1 β /IL-1Ra balance in the alveolar environment during ALI/ARDS.

In vitro, PGE₂ is known as the most potent inducer of HGF secretion in fibroblasts (21, 23). The BALF PGE₂ levels (equivalent to 10⁻⁹–10⁻⁸ M) that we found are known to slightly, if at all, induce *in vitro* HGF production by fibroblasts (21). Our results do not suggest a simple, direct participation of

BALF PGE₂ on HGF production but do not exclude it *in vivo*, since BAL procedure led to an ~100-fold dilution of alveolar fluid. Moreover, it might be hypothesized that BALF mediators could either potentiate the effect of low PGE₂ level or induce it through COX-2 upregulation. In human lung fibroblasts, IL-1 β induces COX-2 expression and PGE₂ synthesis (9). We confirmed that rhIL-1 β (10 ng/ml) induced an increase of PGE₂ and HGF in agreement with previous studies (7, 23). The secretion of both PGE₂ and HGF was completely inhibited by COX-2 inhibitors, suggesting that IL-1 β -induced HGF secretion was COX-2 dependent. To our knowledge, this has been shown in only one study using gastric fibroblasts (3). BALF from ALI/ARDS patients increased both PGE₂ and HGF levels in fibroblast supernatants and induced an upregulation of COX-2 mRNA expression (Figs. 6 and 7). BALF-induced HGF and PGE₂ released by fibroblasts as well as BALF-induced COX-2 mRNA expression were partially inhibited in the presence of anti-IL-1 β antibodies and completely inhibited in the presence of COX-2 inhibitors. Together, our results show that in ALI/ARDS, 1) BALF-induced HGF production by fibroblast is mediated through a COX-2/PGE₂ pathway; and 2) BALF IL-1 β is a main mediator involved in COX-2 upregulation leading to HGF production. Since anti IL-1 β antibodies did not completely inhibit COX-2 upregulation and PGE₂ secretion induced by BALF, other mediators are likely involved and might participate through different mechanisms. TNF- α , present at high levels in BALF from ALI/ARDS patients, has been shown to induce the transcription of COX-2 mRNA (9). Diaz et al. (2) have shown that *in vitro*, the combination of TNF- α and IL-1 β is synergistic, with lung fibroblasts producing a 110-fold increase in PGE₂ (9). Moreover, if TGF- β decreased HGF secretion when used alone, they demonstrated that TGF- β potentiates PGE₂ production induced by IL-1 β or TNF- α by stabilization of COX-2 mRNA (9). Therefore, even if IL-1 β is one of the mediators involved in BALF-induced HGF secretion, other mediators acting on COX-2 pathway likely modulate its effect.

Few studies have highlighted the dual role of IL-1 β in lung repair process. The pulmonary expression of IL-1 β in a rat model induces an acute lung injury and an evolution toward fibrosis by raising the expression of TGF- β (19). Conversely, IL-1 β supports the migration of alveolar type II cells (12, 13) and decreases their apoptosis *in vitro* (8). In this study, we showed that IL-1 β from alveolar fluid of patients with ALI is a main inducer of growth factors produced by lung fibroblasts involved in pulmonary repair. Furthermore, we demonstrated that BALF IL-1 β acts through COX-2 stimulation to induce HGF secretion. COX-induced mediators are thought to contribute to the pathobiology of ALI. In large-scale clinical studies, global inhibition of COX is not an effective prevention for ARDS (11). As recently reviewed, a possible explanation is that COX-2 products have a protective role in ALI (26). Indeed, both genetic disruption and pharmacological blocking of COX enzymes induce an exaggerated fibrotic response in the murine model of bleomycin induced lung injury. In line with this beneficial effect, we demonstrated that BALF from ALI patients induce the epithelial repair factor through an IL-1 β /COX-2 axis. However, to definitively determine whether these effects are beneficial or harmful, this needs to be addressed in future work.

In summary, our study shows that BALF from patients with ALI/ARDS trigger the HGF and KGF synthesis by lung fibroblasts. BALF-induced HGF synthesis is mediated by the autocrine production of PGE₂ through a COX-2-dependent mechanism. IL-1 β is one of the main BALF mediators involved in COX-2 upregulation. Our results suggest that BALF mediators may participate in vivo in the production of epithelial repair factor by lung fibroblasts during ALI/ARDS.

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