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Alveolar fibroblasts in acute lung injury: biological behaviour and clinical relevance

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ABSTRACT: Although fibroblasts are key cells in the lung repair/fibrosis process, their characteristics are poorly studied in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). The aims of our study were to: 1) determine the biological behaviour of alveolar fibroblasts during ALI; and 2) to evaluate the clinical relevance of positive alveolar fibroblast culture from patients with ALI/ARDS.

Cells were cultured from bronchoalveolar lavage (BAL) obtained from 68 critically ill, ventilated patients: ALI n=17; ARDS n=31; and ventilated controls n=20. Patients were followed for 28 days and clinical data was recorded. We studied proliferation, migration and collagen-1 synthesis capacities of fibroblasts.

Cells expressing fibroblast markers were cultured from BAL obtained in six (35%) ALI patients and six (19%) ARDS patients, but never from ventilated controls. Alveolar fibroblasts exhibited a persistent activated phenotype with enhanced migratory and collagen-1 production capacities, with hyporesponsiveness to prostaglandin E_2 compared to normal lung fibroblasts ($p \leqslant 0.04$). Positive fibroblast culture was associated with both an increased collagen-1 concentration and monocyte/macrophage percentage in BAL fluid ($p \leqslant 0.01$), and with a reduced duration of mechanical ventilation ($p < 0.001$).

We conclude that activated alveolar fibroblasts can be cultured either in ALI or ARDS and that their presence might reflect the initiation of the organising phase of ALI.

KEYWORDS: Acute respiratory distress syndrome, bronchoalveolar lavage fluid, collagen 1, fibrocyte, migration, repair

Acute lung injury (ALI) and its extreme form, acute respiratory distress syndrome (ARDS), are important causes of mortality in critically ill patients [1]. ARDS is characterised by an acute pulmonary inflammation that contributes to alveolar-capillary membrane injury with concomitant tissue repair and remodeling which can evolve to lung fibrosis. Histological evidence of lung fibrosis is associated with a poor outcome in patients with ARDS during intensive care unit (ICU) stay [2, 3]. Fibroblasts play a key role in normal and pathological repair [4]. These mesenchymal cells produce large quantities of extracellular matrix components, cytokines and repair growth factors during wound healing [5, 6]. After lung injury, fibroblasts proliferate, differentiate into myofibroblasts expressing α-smooth muscle actin (α-SMA) and migrate in the fibrinous exudate inside the alveolar airspace [7, 8]. In resolutive ALI/ARDS, repair normally proceeds with complete re-establishment of the normal alveolar architecture. In non-resolutive ALI/ARDS, normal healing is disrupted with persistent mesenchymal cells in the interstitial/alveolar spaces, chronic myofibroblast activation and excessive extracellular matrix deposits such as collagen 1 [4]. Despite their fundamental role in lung repair, lung fibroblasts obtained either from lung explants or from bronchoalveolar lavage (BAL) fluid have been the subject of a limited number of studies [9, 10]. Many aspects of fibroblast functional properties and the clinical relevance of their detection in the alveolar lumen in patients with different stages of lung injury, such as ALI or ARDS, remain unknown. We hypothesised that in critically ill, ventilated

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patients, alveolar fibroblasts with an activated phenotype may be predominantly cultured from BAL of the most severe patients with ARDS and could adversely affect their outcome. Therefore, the aims of our study were to: 1) determine the biological behaviour of alveolar fibroblasts during acute lung injury; and 2) evaluate the clinical relevance of positive alveolar fibroblast culture from patients with ALI/ARDS.

METHODS

Study population

The protocol was approved by the ethical committee of the Paris-Hotel-Dieu Hospital (Paris, France). Ventilated patients from three ICUs were prospectively enrolled if a BAL procedure was performed to confirm clinically suspected ventilator-associated pneumonia [11]. The patients were classified into three groups after inclusion (ALI, ARDS and a ventilated-control group without ALI/ARDS) according to the criteria of the American–European Consensus Conference on ALI/ARDS [12]. All the patients were ventilated and weaned from the ventilator according to current guidelines [13]. Patients with pre-existing fibrotic lung disease, corticosteroid medication use, HIV infection, end stage cancer, or who were aged <18 yrs or currently pregnant were excluded. On the day of inclusion, the following clinical data were recorded: age; sex; reason for ICU admission; presence or absence of sepsis and pulmonary infection; arterial oxygen tension (P_{a,O_2})/inspiratory oxygen fraction (F_{i,O_2}) ratio; arterial carbon dioxide tension; Simplified Acute Physiologic Score II on admission to ICU [14]; Sequential Organ Failure Assessment score [15]; and lung injury score [16]. In addition, the length of time between the onset of the mechanical ventilatory support and the BAL, as well as the length of ventilatory support, the time spent in the ICU and mortality 28 days after the BAL were recorded for all patients.

BAL protocol and BAL fluid sample processing

The BAL was performed and processed as previously described [17]. A differential BAL cell count was performed on a cytocentrifuge smear with a Diff-Quik stain kit (Dade International, Miami, FL, USA). BAL fluid and plasma protein concentrations were measured with the analyst Hitachi-911 (Roche, Meylan, France), and the protein ratio was determined to evaluate the alveolar permeability induced by lung injury.

Isolation and culture of fibroblasts

The BAL cell viability was determined before initial plating by Trypan blue vital staining (viability always >92% in the different groups tested). The BAL cells (n=68) were cultured at a density of 2.10^5 cells·cm^{-2} in a 25-cm^2 flask (Corning Costar, Lowell, MA, USA) in 5 mL of RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat decomplemented fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (Invitrogen, Cergy Pontoise, France) for at least 28 days. Cells grown from BAL were used for immunocytochemical characterisation 24 h and 21 days after initial plating (n=5), and then at passage 1 and 3 (n=12). Nine BAL cell lines were randomly chosen and their proliferation, migration and collagen 1 production capacities were compared to those of fibroblasts derived from normal lung explants obtained from five patients (two females and three males, mean age 60 yrs) undergoing lung surgery for removal of a primary lung tumour. Three patients had never smoked and two were ex-smokers. As previously described [18], normal lung from a healthy segment distant from the solidary lesion was obtained. Fibroblasts were cultured with complete medium and 10% FCS supplemented with antibiotics and were used at passage 3.

Measurement of C-terminal propeptide of type I pro-collagen, monocyte chemoattractant protein-1, interleukin-8 and transforming growth factor-β1

C-terminal propeptide of type I pro-collagen (PICP) was measured by enzyme immunoassay (OSTEOmedical, Paris, France). The detection threshold of the assay was 0.2 ng·mL^{-1}. Transforming growth factor (TGF)-β1, monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8 concentrations were measured by ELISA (R&D systems, Minneapolis, MN, USA). The detection thresholds of TGF-β1, IL-8 and MCP-1 assays were 5 pg·mL^{-1}, 3.5 pg·mL^{-1} and 5 pg·mL^{-1}, respectively.

Immunocytochemistry

Alveolar fibroblasts cultured on Lab-Tek slides (Nunc, Naperville, IL, USA) were fixed with acetone. Anti-human antibodies (Ab) directed against collagen 1 (Sigma Aldrich, St Louis, MO, USA), vimentin (Immunotech, Marseille, France), prolyl-4-hydroxylase (α-4H), desmin, pancytokeratin, CD31, α-SMA, CD45 (Dako SA, Trappes, France), smooth muscle myosin 1 (Abcam, Paris, France), CD14, CD34 (BD Pharmingen, Le Pont-De-Claix, France) or isotype-matched control Ab were used. Positive cells were revealed using the Vectastain ABC-alkaline phosphatase kit mouse immunoglobulin G (Vector Abcys, Paris, France) and the fast red substrate (Dako SA).

Confocal microscopy analysis

Cells cultured on Lab-Tek slides were fixed after 21 days of culture with acetone and saturated with 50 mM NH_4Cl and permeabilised with 0.1% Triton X100. After saturation with normal horse serum, slides were incubated with appropriate dilution of anti-collagen 1 monoclonal Abs (1:290) (Sigma Aldrich) prior to the addition of fluorescent-labelled Abs (biotinilated horse antimouse (1:300) plus Alexa Fluor 568-conjugated streptavidin (1:100); Invitrogen, Carlsbad, CA, USA). After washing, the cells were incubated with anti-CD45-Alexa Fluor 488 Abs (1:25) (Santa Cruz Biotechnology, San Diego, CA, USA). Cell nuclei were localised by DNA staining with thiazole orange protein 3 (0.4 μg·mL^{-1}; Molecular Probes, Eugene, OR, USA). Slides were mounted with Prolong Gold anti-fade reagent (Invitrogen) and examined by confocal laser scanning microscopy (LSM-510-META microscope; Zeiss, Oberkochen, Germany). To test for the specificity of immunostaining, Abs were replaced by an isotype-matched control Ab.

Cell proliferation assay

After 72 h of incubation with 0.1% FCS alone (basal condition) or with prostaglandin (PG) E_2 (10^{-6} M), recombinant human (rh)IL-1β (10 ng·mL^{-1}), rh platelet-derived growth factor (PDGF)-BB (10 ng·mL^{-1}) or rhTGF-β1 (10 ng·mL^{-1}; R&D systems), proliferation was measured as bromodeoxyuridine (BrdU) incorporation according to the manufacturer's protocol (Roche).

Cell migration assay

Cell motility was examined using a modified Boyden chamber apparatus (Transwell Costar, Cambridge, MA, USA) fitted with an 8-μm pore membrane coated with fibronectin (10 $\mu g \cdot mL^{-1}$). Serum deprived fibroblasts (2.10^5 per well) were placed into the upper well and allowed to migrate for 24 h in the presence or absence (basal condition) of PGE_2 (10^{-6} M), Oleoyl-L-α-lysophosphatidic acid sodium salt (LPA, 10^{-6} M; Sigma Aldrich), or rhPDGF-BB (10 $ng \cdot mL^{-1}$) into the lower well. Non-migrated cells were removed and the membrane was fixed and stained with the Diff-Quick staining kit (Dade Behring, Paris, France). The number of cells that migrated to the lower surface of the membrane was counted (400× magnification). Five high-power fields (HPF) were counted per sample and run in duplicate. The chemotactic index was determined as the ratio of cells moving in response to a chemoattractant relative to cells moving in response to media control.

Assessment of collagen I and TGF-β1 protein secretion

Cells (5×10^4 $cells \cdot well^{-1}$) were cultured in serum-free medium (basal condition) or with mediators: PGE_2 (10^{-6} M), rhIL-1β (10 $ng \cdot mL^{-1}$) or rhTGF-β1 (10 $ng \cdot mL^{-1}$) for 48 h. The concentration of PICP or TGF-β1 in fibroblast supernatants was divided by the total protein amount of the cell monolayer (Bio-Rad Laboratories, Hercules, CA, USA).

Assessment of collagen I or TGF-β1 mRNA expression

After 18 h of culture, the relative content of mRNA was analysed after reverse transcription by RT-PCR as previously described [17]. The following primer sequences were used. Collagen 1-α1 (COL1A1): CGAGGTAGTCTTTCAGC AACACAGT and AGCCACCAGCCCCTCACT; collagen 1-α2 (COL1A2): CAGTTCTTGGCTGGGATGTTT and TTGAGACTC AGCCACCCAGAGT; TGF-β1: TGCTGTCACAGGAGCAGTG and AGGGGAAATTGAGGCTTT; Ubiquitin C: TTTTTTGG GAATGCAACAACTTT and CACTTGGTCCTGCGCTTGA.

Statistical analysis

Demographic data were expressed as mean ± SD and biological data as median (range). All proportional values were compared with the Fischer's exact test or the Chi-squared test for multiple comparisons. The continuous variables were compared by the Mann–Whitney test or Wilcoxon paired test when appropriate. Multiple comparisons were tested by the Kruskal–Wallis or Friedman test followed by Dunn's multiple-comparison *post hoc* analysis. Durations of ventilation after the BAL procedure were analysed by Kaplan–Meier survival curves and compared by log-rank analysis. Correlations were assessed with the Spearman rank-order test. Statistical significance was accepted as $p \leqslant 0.05$.

RESULTS

Characteristics of the study population

31 patients with ARDS, 17 with ALI and 20 ventilated patients without ALI/ARDS criteria were enrolled. The patient characteristics are shown in table 1. ALI/ARDS patients were admitted for septic shock (n=23), multiple organ failure (n=18), cerebral stroke or convulsive state (n=4), severe trauma (n=2) or cardiac arrest (n=1). The ventilated patients without ALI/ARDS were admitted for acute heart failure and post-operative complications following cardiopulmonary bypass (n=14), post-operative localised pneumonia (n=3), acute renal failure (n=1), haemorrhagic shock (n=1) or acute intoxication (n=1).

Cells cultured from BAL expressed fibroblast markers

All BAL cell pellets (n=68) were maintained in culture for 28 days without any bacterial or fungal contamination. At the time of initial plating, BAL cells were mainly neutrophils and mononuclear cells (macrophage/monocyte). In positive cell cultures (n=12), isolated spindle-shaped cells were usually detected after the first week of culture (median time 10 (4–23) days) (fig. 1a). After 3 weeks of culture, spindle-shaped cells formed foci (fig. 1b), while round cells (likely macrophages) were quiescent. After 4 weeks of culture, the cell monolayers were harvested to produce a homogenous cell population in the subsequent cultures (fig. 1c).

We characterised the phenotype of adherent BAL cells from five different patients (three ARDS and two ALI) 24 h after initial plating. At that time point, 95% of adherent cells were mononuclear round cells expressing the common leukocyte marker (CD45+) (fig. 1f). The remaining cells were CD45-, including some endothelial cells (CD31+) and epithelial cells (pancytokeratin+). No mature form of fibroblast (CD45-) with large cytoplasm expressing collagen 1 was detectable 24 h after plating. In contrast, we observed a side population (<1% of the total cell population) of very typical elongated spindle-shaped cells with a ratio of cell length/cell width equal to 16. All these cells were CD45+, α-4H + and weakly CD34+ (fig. 1e–g). This pattern of expression is consistent with that of fibrocytes [19] and was confirmed by confocal microscopy showing the co-expression of collagen 1 and CD45 on day 21, before the first passage (fig. 1h–k). At passage 1, most of the spindle-shaped cells were positive for α-4H, collagen 1 and vimentin, but only a few population were still CD45+ (fig. 1l–p). A limited population of cells with large cytoplasm expressing αSMA was also observed at this passage. The cells characterised at passage 3 demonstrated a stable mesenchymal phenotype with positive staining for vimentin, collagen 1 and α-4H (fig. 1q–u). The α-SMA was expressed in eight out of 12 cell lines in which 20% of cells were α-SMA positive. Markers for vascular smooth muscle cells (desmin, smooth muscle myosin 1), epithelial cells (pancytokeratin), endothelial cells (CD31), monocytes (CD14), leukocytes (CD45) and haematopoieteic progenitor cells (CD34) were negative at this passage. Together, this pattern of markers is consistent with a differentiated fibroblast phenotype. According to these characteristics, the cells will be hereby referred to as ''alveolar fibroblasts''.

Proliferative rate and migration of alveolar fibroblasts

After 72 h of culture, no significant difference in the proliferative rate was observed between unstimulated alveolar and control fibroblasts studied at passage 3 (BrdU median absorbance 0.58 *versus* 0.60, $p>0.05$). IL-1β, TGF-β1 or PDGF-BB induced a similar weak mitogenic effect in both types of fibroblasts (125% of basal condition, $p>0.05$). PGE_2 induced a weak and similar inhibitory effect on proliferation in both cell types (75% of basal condition, $p>0.05$). We examined the migration of alveolar fibroblasts in the presence or absence of

TABLE 1 Characteristics of patients

	Ventilated patients#	ALI	ARDS	p-value¶
Subjects n	20	17	31	
Age yrs	66±11	61±18	62±16	0.92
Females/males n	7/13	10/7	11/20	0.23
Simplified Acute Physiologic Score II	45±19	51±22	50±16	0.67
Sepsis %	45	59	58	0.60
Sepsis-related Organ Failure Assessment	7±3	7±3	8±3	0.29
Pulmonary infection %	35	41	45	0.77
Aetiology of ALI/ARDS+ n		5/12	13/18	0.54
Lung Injury Score		1.4±0.5	2.0±0.5	<0.001
$P_{a,O_2}/F_{i,O_2}$ ratio	262±70	252±51	156±73	<0.001
P_{a,CO_2} kPa	4.9±0.9	5.7±1.2	5.6±1.3	0.04
Length of ICU stay days	25±21	26±16	33±24	0.40
Length of mechanical ventilation before BAL days	7±7	10±11	9±8	0.52
Total time on mechanical ventilation days	21±18	20±15	27±25	0.60
28-day mortality %	10	35	39	0.07

Data are presented as mean±SD, unless otherwise stated. ALI: acute lung injury; ARDS: acute respiratory distress syndrome; P_{a,O_2}: arterial oxygen tension; F_{i,O_2}: inspiratory oxygen fraction; P_{a,CO_2}: arterial carbon dioxide tension; ICU: intensive care unit; BAL: bronchoalveolar lavage. #: patients with out ALI or ARDS; ¶: Kruskal–Wallis test or Chi-squared test when appropriate; +: extrapulmonary/pulmonary.

mediators known to modulate mesenchymal cell migration. In the basal condition, the migration of alveolar fibroblasts was three-fold higher than that demonstrated by control fibroblasts (26 *versus* 8 cells·HPF^{1}, p=0.04) (fig. 2a). PDGF-BB (10 ng·mL^{-1}) and LPA (10^{-6} M) stimulated control and alveolar fibroblast migration to the same extent (fig. 2b–e). In contrast, alveolar fibroblasts were hyporesponsive to the inhibitory effect of PGE_2 as compared with control fibroblasts (73 *versus* 23% of basal condition, p=0.01).

Collagen I production was elevated in alveolar fibroblasts and correlated with TGF-β1 production

Collagen 1 production was assessed by measuring PICP in cell culture supernatants. PICP secretion by alveolar fibroblasts was four-fold higher than that of control fibroblasts (7.69 *versus* 1.63 ng·μg^{-1}·well^{-1}, p<0.01) (fig. 3a). In addition, COL1A1 and COL1A2 mRNA expression in alveolar fibroblasts was higher than in control fibroblasts (p<0.01, fig. 3b) and was positively correlated with collagen 1 protein secretion (ρ=0.75, p=0.03). Recombinant TGF-β1, and to a lesser extent rhIL-1β, stimulated collagen 1 production by control and alveolar fibroblasts (p<0.05). After stimulation, collagen 1 production remained higher in alveolar fibroblasts than in control fibroblasts (p⩽0.04) (fig. 3a). Interestingly, as compared with fibroblasts obtained from normal lung explants, alveolar fibroblasts were hyporesponsive to the inhibitory effect of PGE_2 (p<0.05). As TGF-β1 is a key factor in collagen 1 production by fibroblasts, its role was specifically studied. The basal levels of TGF-β1 secretion by alveolar and control fibroblasts were similar (5.3 *versus* 4.8 pg·μg^{-1}·well^{-1}, p=0.24) (fig. 4a). A strong correlation was found between TGF-β1 and collagen 1 production by alveolar fibroblasts at both protein and transcriptional levels (fig. 4b and c). Such a correlation was not found in control fibroblasts (fig. 4d).

Clinical determinants of alveolar fibroblast culture from BAL and influence on outcome

Alveolar fibroblasts were grown from BAL of patients with ALI (n=6; 35%) or ARDS (n=6; 19%) but never from ventilated controls (n=0) (p⩽0.04, control *versus* ALI or ARDS). The proportion of positive fibroblast cultures was higher in ALI than in ARDS without reaching significance (35% *versus* 19%, p=0.30). There was a greater proportion of positive cultures (eight (35%) out of 25 BAL) in the early phase (<7 days) of ALI/ARDS when compared to the late phase (⩾7 days) (four (17%) out of 23 BAL), although this difference was not statistically significant (p=0.32) (fig. 5a).

To clarify the determinants of positive fibroblast culture, the ALI/ARDS patients were divided into two groups according to the cell culture results (positive culture (n=12) *versus* negative culture (n=36)). As summarised in table 2, the clinical parameters were similar in the two groups. There was no difference in $P_{a,O_2}/F_{i,O_2}$ ratio, severity scores, aetiology of ALI, length of ICU stay and 28-days mortality. However, the total duration of mechanical ventilation was notably reduced in patients with positive culture (14 *versus* 28 days, p=0.03). This difference was explained by a reduction of mechanical ventilation duration after BAL procedure (8 *versus* 21 days, p<0.001) (fig. 5b) and was observed in patients with early (8 *versus* 22 days, p=0.0024) and late (9 *versus* 21 days, p=0.0068) ALI/ARDS.

Patients with positive fibroblast culture exhibited specific BAL fluid characteristics (table 3). In this group, a higher collagen 1 concentration associated with a reduction of inflammatory response assessed by a decreased neutrophil percentage and a lower IL-8 concentration in BAL was noted. In parallel, an increased percentage of monocytes/macrophages was found, which was positively correlated with BAL fluid MCP-1 concentration (ρ=0.67, p=0.02).

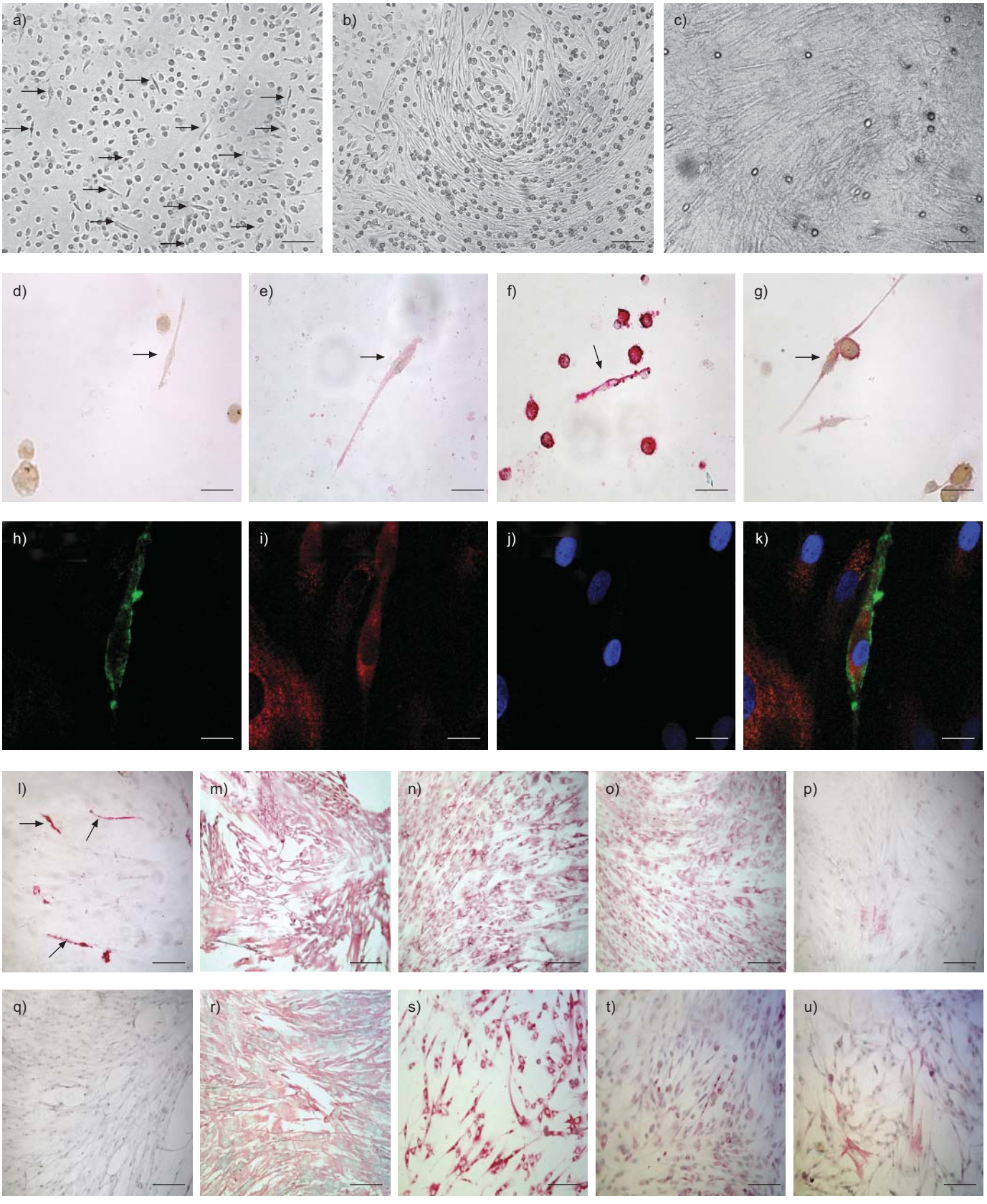
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FIGURE 1. a–c) Morphology of adherent bronchoalveolar lavage (BAL) cells at amplification when a positive culture occurred. a) After 8 days, b) at 21 days and c) at passage 1. The arrows indicate typical adherent spindle-shaped cells. d–g) Immunocytochemical characteristics of adherent BAL cells after initial plating (H24) with d) control antibody, e) CD34, f) CD45, g) prolyl-4-hydroxylase (α-4H). The arrows indicate typical adherent spindle-shaped cells. h–k) Characterisation of fibrocytes (spindle-shaped cells co-expressing CD45 and collagen 1) by confocal microscopy analysis of BAL cell culture at day 21. h) CD45, i) collagen 1, j) thiazole orange protein 3 (TO-PRO-3) and k) merge of the three fluorescence channels. l–p) Representative immunocytochemical stain prepared from alveolar fibroblasts at passage 1. l) CD45, m) vimentin, n) α-4H, o) collagen 1, p) α-smooth muscle actin (α-SMA). The arrows indicate CD45+ spindle-shaped cells. q–u) Representative immunocytochemical stain prepared from alveolar fibroblasts at passage 3. q) CD45, r) vimentin, s) α-4H t) collagen 1 and u) α-SMA. a–c) Scale bars=250 μm. d–g) Scale bars=25 μm. h–k) Scale bars=15 μm. l–u) Scale bars=100 μm.

DISCUSSION

We demonstrate for the first time in patients with ALI/ARDS that: 1) A limited population of fibrocytes is detectable during the initial phase of BAL culture; 2) alveolar fibroblasts can be cultured from BAL in 25% of ALI/ARDS patients but never from ventilated patients without ALI/ARDS; 3) alveolar fibroblasts can be isolated either in ALI and ARDS. Alveolar fibroblasts exhibit a persistent activated phenotype with enhanced migratory and collagen 1 production capacities and are hyporesponsive to PGE_2 in comparison with control fibroblasts. In addition, collagen 1 concentration and monocyte/macrophage percentage is increased in BAL with positive culture whereas neutrophil percentage and IL-8 are decreased. The presence of alveolar fibroblasts is associated with a lower duration of ventilatory support.

In this study we have evaluated the ability to culture fibroblasts from a high number of BAL of ventilated critically ill patients (n=68). For the first time, we show that alveolar fibroblast culture is not limited to the most severe form of ARDS as we observed an even higher proportion of positive fibroblast cultures in BAL from ALI. Owing to the lack of positive culture in the control group, our findings argue that BAL fibroblast culture is independent of mechanical ventilation *per se*. However, a possible selection bias linked to the observational design of our study could occur. As BAL was performed for the clinical suspicion of pneumonia, we may have included the most severe ALI patients with clinical worsening. Otherwise, the equal proportion of fibroblast culture in patients with or without confirmed bacterial pneumonia does not support a significant role of infection in this process.

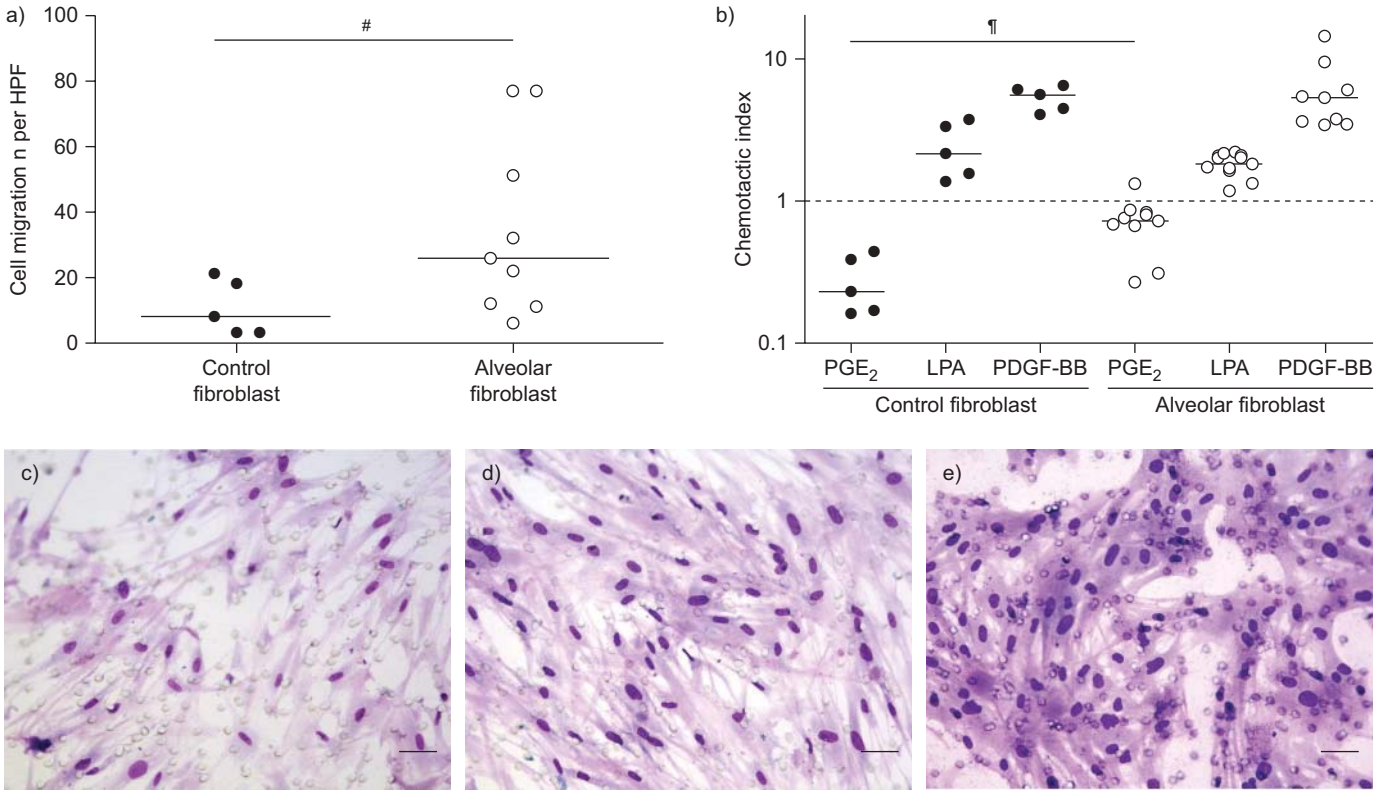

FIGURE 2. a) Alveolar fibroblast migration capacities after 24 h. Basal migratory capacity of alveolar fibroblasts (n=9) was assessed in comparison with control fibroblasts (n=5) by cell count in five random high-power fields (HPF) run in duplicate. The horizontal bars indicate the median of individual values. b) Cell migration in the presence of prostaglandin (PG) E_2 (10^{-6} M), lysophosphatidic acid (LPA; 10^{-6} M) and platelet-derived growth factor (PDGF)-BB (10 ng·mL^{-1}). Results are expressed on a logarithmic scale as chemotactic index (ratio of the basal condition performed in the same assay). c–e) Representative observations of alveolar fibroblasts traversing the chamber membrane. c) Basal condition, d) lysophosphatidic acid (10^{-6} M) and e) PDGF-BB (10 ng·mL^{-1}). Scale bars=30 μm. #: p=0.04; ¶: p=0.01.

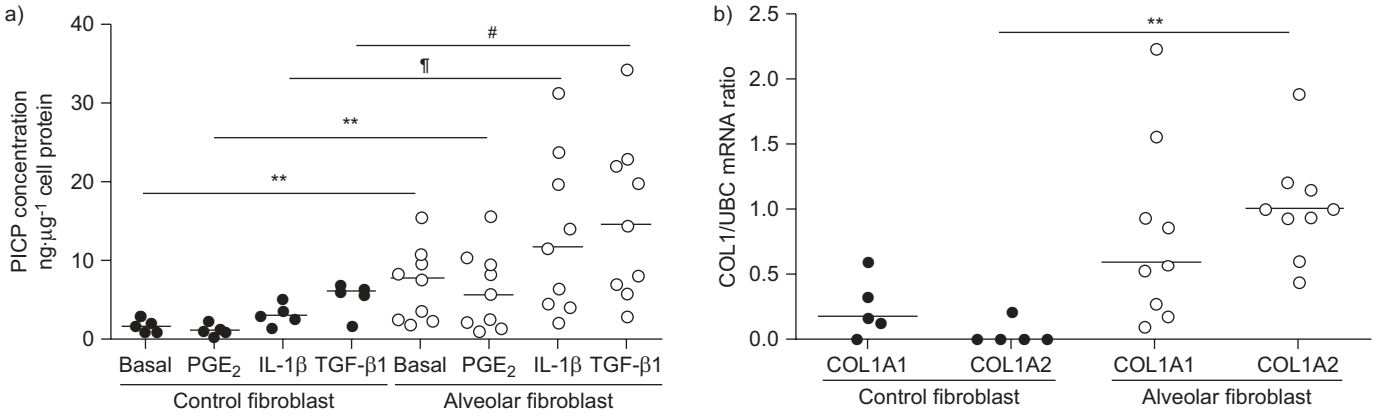

FIGURE 3. Alveolar fibroblast type I collagen production capacities. a) Type I collagen synthesis of alveolar fibroblasts (n=9) in comparison with control fibroblasts (n=5). Fibroblasts were cultured in duplicate for 48 h in serum-free medium alone (basal) or with prostaglandin (PG) E_2 (10^{-6} M), interleukin (IL)-1β (10 ng·mL^{-1}) or transforming growth factor (TGF)-β1 (10 ng·mL^{-1}). b) Unstimulated alveolar and control fibroblast type I collagen α-1 (COL1A1) and α-2 (COL1A2) mRNA relative content analysed at 18H by quantitative real time RT-PCR and expressed as a ratio to the Ubiquitin C (UBC) mRNA. The horizontal bars indicate the median of individual values. #: p=0.04; ¶: p=0.03. **: p<0.01.

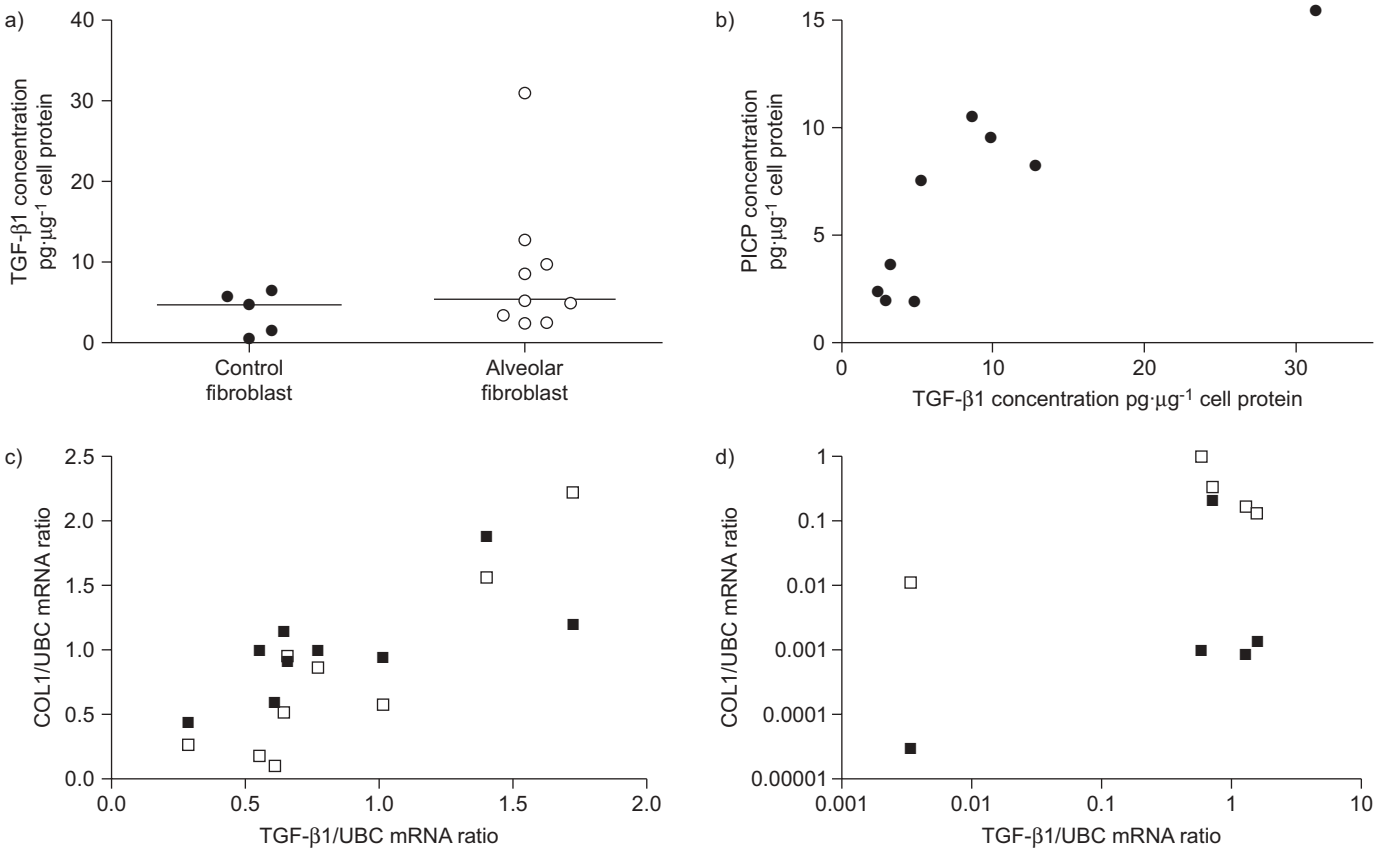

FIGURE 4. Correlation between transforming growth factor (TGF)-β1 and type I collagen production in alveolar fibroblasts. a) TGF-β1 synthesis of alveolar fibroblasts (n=9) in comparison with control fibroblasts (n=5). Fibroblasts were cultured in duplicate for 48 h in serum-free medium alone (basal condition). The horizontal bars indicate the median of individual values. b) Correlation between TGF-β1 and type I collagen concentration in unstimulated alveolar fibroblast (n=9) supernatants. PICP: propeptide of type I pro-collagen. ρ=0.82; p=0.01. c) Correlation between both type I collagen alpha-1 (COL1A1) and alpha-2 (COL1A2) mRNA and TGF-β1 mRNA expression in unstimulated alveolar fibroblasts (n=9). ρ=0.87 and 0.72, respectively; p<0.04. d) Lack of correlation between COL1A1 and COL1A2 mRNA and TGF-β1 mRNA expression in unstimulated control fibroblasts (n=5, p>0.05). Results are represented on a logarithmic scale. UBC: Ubiquitin C. COL1A1; □; COL1A2; ■.

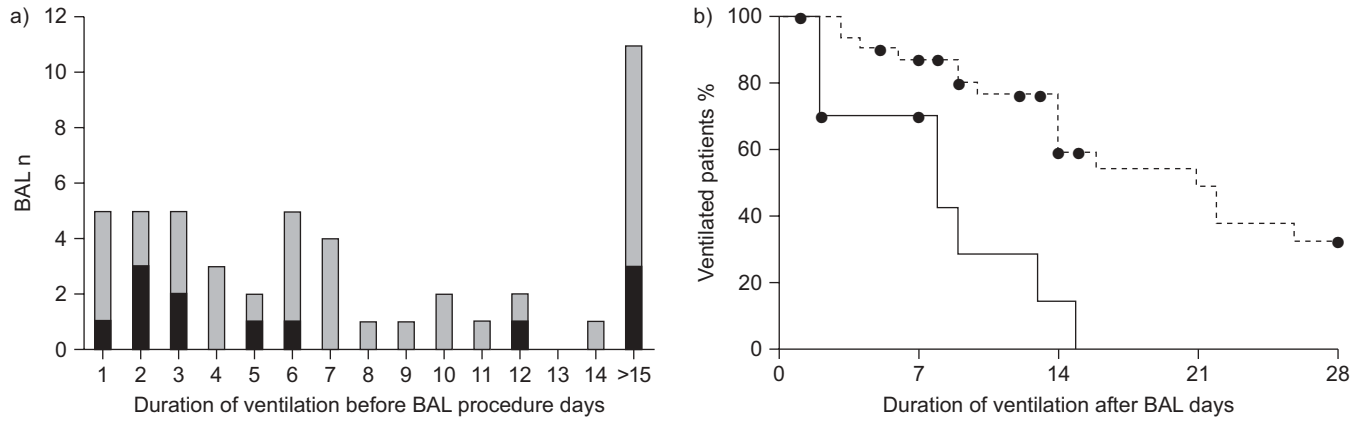

FIGURE 5. a) Kinetics of fibroblast isolation in the acute lung injury and acute respiratory distress syndrome groups. Positive (■) and negative (■) fibroblast cultures were plotted as a function of mechanical ventilation duration before bronchoalveolar lavage (BAL). b) Duration of mechanical ventilation after BAL procedure according to the growth of alveolar fibroblasts. Kaplan–Meier survival curves comparing duration of mechanical ventilation after BAL procedure for patients with positive (n=12: ——) and negative fibroblast culture (n=36: - - - -). ●: censored data (death during ventilation or ventilation continuation > 28 days after BAL procedure). p<0.001, Log-rank test.

Alveolar fibroblasts could potentially originate from a local precursor as interstitial fibroblasts, from pneumocytes after epithelia–mesenchymal transition or from a circulating precursor derived from bone-marrow and recruited to the lung [5, 8, 20]. In our study, 24 h after initial plating we observed a population of spindle-shaped cells expressing fibrocyte markers (CD45+, α-4H+ and weakly CD34+) [19]. These cells accounted for <1% of total adherent cells. To our knowledge, this is the first demonstration that cells with characteristics of fibrocytes (plastic adherent, spindle-shaped cells, CD45+ and α-4H+) can be detected within 24 h among adherent cells from BAL fluid during ALI/ARDS in humans. In agreement with other studies in human BAL [10, 21, 22], we found that most of the mesenchymal cells that proliferated were CD45- at the first passage. These cells could result from differentiation of fibrocytes after loss of CD45 expression [23], and/or from the proliferation of CD45- progenitors cells, such as mesenchymal stem cells [21, 22]. Our data, do not allow us to appreciate the relative contribution of each cellular pathway in this process.

We observed variability in the percentage of cells expressing α-SMA at passage 3. Despite this phenotypic variability, all the isolated fibroblast lines showed an activated phenotype in comparison with fibroblast derived from normal lung explants.

TABLE 2 Clinical characteristics and outcome of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) patients associated to the alveolar fibroblast culture

	Alveolar fibroblast culture		p-value#
	Negative	Positive	
Subjects n	36	12	
Age yrs	65±14	52±20	0.07
Females/males n	11/25	7/5	0.10
Simplified Acute Physiologic Score II	51±18	49±21	0.70
Sepsis %	58	58	1.00
Pulmonary infection %	44	42	0.40
Sepsis-related Organ Failure Assessment	8±3	7±4	0.39
Aetiology of ALI/ARDS¶ n	14/22	5/7	0.90
Lung injury classification ALI/ARDS n	11/25	6/6	0.30
Lung Injury Score	1.9±0.5	2.0±0.9	0.92
$P_{a,O_2}/F_{i,O_2}$ ratio	181±56	217±128	0.44
P_{a,CO_2} kPa	5.6±1.3	5.9±1.1	0.49
Length of ICU stay days	33±23	23±14	0.22
Length of mechanical ventilation before BAL days	10±9	8±8	0.36
Total time on mechanical ventilation days	28±24	14±9	0.03
28-days mortality %	39	33	0.73

Data are presented as mean±SD, unless otherwise stated. P_{a,O_2}: arterial oxygen tension; F_{i,O_2}: inspiratory oxygen fraction; P_{a,CO_2}: arterial carbon dioxide tension; ICU: intensive care unit; BAL: bronchoalveolar lavage. #: Mann–Whitney test or Fischer exact test when appropriate; ¶: extrapulmonary/pulmonary.

TABLE 3 Characteristics of bronchoalveolar lavage fluid (BALF) from acute lung injury/acute respiratory distress syndrome patients associated to the alveolar fibroblast culture

	Alveolar fibroblast culture		p-value#
	Negative	Positive	
Subjects n	36	12	
BALF protein g·L^{-1}	0.44±0.32	0.61±0.37	0.24
Protein ratio BALF/serum	0.009	0.011	0.19
BALF total cell count ×10^6	17±31	20±23	0.51
Neutrophils %	85±17	58±31	<0.01
Macrophages %	13±15	38±31	<0.01
IL-8 pg·mL^{-1}	6344 (177–35988)	2233 (145–9150)	0.03
MCP-1 pg·mL^{-1}	284 (<5–1060)	337 (85–1100)	0.32
TGF-β1 pg·mL^{-1}	46.8 (<5–294)	54.8 (5.4–213)	0.59
Pro-collagen 1 ng·mL^{-1}	4.78 (<0.2–51)	11.38 (2.3–25)	0.01

Data are presented as mean±SD or mean (absolute range), unless otherwise stated. IL-8: interleukin-8; MCP-1: monocyte chemotactic protein-1; TGF-β1: transforming growth factor-β1. #: Mann–Whitney test.

We found a constant up-regulation of collagen production, both at baseline and after TGF-β1 stimulation. A similar observation was made in systemic sclerosis and attributed to an up-regulation of the TGF-β signalling axis [24]. We observed a specific correlation between collagen 1 and TGF-β1 expression in alveolar fibroblasts suggesting the possible implication in ALI/ARDS of such an autocrine TGF-β1 loop in collagen overproduction. However, *in vivo*, the picture is certainly more complex as other TGF-β1 producing cells may affect fibroblasts functions as previously described [25, 26]. The alveolar fibroblast activation was also confirmed by the strong enhancement of their migratory capacity, which is a crucial step in repair and in fibrotic evolution after acute lung injury [27]. We observed that the alveolar fibroblasts had a relative insensitivity to PGE_2 a major anti-fibrotic mediator, both for migration and collagen 1 production. This finding was in agreement with observations made in lung injury model [28] and in chronic pulmonary fibrosis [29] where a lower PGE_2 susceptibility was explained by PGE_2 receptor down regulation.

Our results must be interpreted with caution as the functional differences observed between alveolar fibroblasts cultured from BAL fluid of patients with ALI/ARDS and fibroblasts derived from normal lung explants might represent either an activated state of alveolar fibroblasts, or a difference in phenotype between two distinct populations of fibroblast originating from two distinct areas of the lung. The latter hypothesis is consistent with observations made in asthma or systemic sclerosis, where alveolar fibroblasts and interstitial fibroblasts obtained from the same patient exhibited different phenotypes [30–32]. We were not able to perform such a comparison as obtaining fibroblasts from BAL and biopsy in the same patient with ALI/ARDS was not possible in the clinical setting for ethical concerns. Furthermore, alveolar fibroblasts cannot be grown from BAL in healthy subjects [10, 30, 33]. In some experiments (see online supplementary data), we found that ALI/ARDS alveolar fibroblasts had proliferation and collagen 1 secretion capacities very similar to those of idiopathic pulmonary fibrosis fibroblasts cultured from BAL.

Neutrophils were the main cell population in BAL from ALI/ARDS patients included in our study. However, BAL associated with positive fibroblast culture contained a three-fold higher percentage of monocyte/macrophage and an increased collagen 1 concentration as compared to BAL with negative fibroblast culture. These observations might be interpreted in two nonexclusive ways. First, a cross-talk between fibroblast precursors and monocytes/macrophages may be required for *in vitro* proliferation of fibroblasts. This hypothesis is supported by *in vitro* studies showing that: 1) monocytes/macrophages stimulated by T-helper 2 cytokines can interact with fibroblasts through CCL18 signalling [34]; and 2) monocytes/macrophages produce mediators such as PDGF-BB and TGF-β1, which promote mesenchymal progenitor recruitment, proliferation and fibroblast survival [10]. Secondly, reduced neutrophils and increased macrophages together with increased collagen 1 concentration suggest that BAL was performed at a time where the organising phase of alveolar repair was engaged [35] with a reduction of inflammatory response. This interpretation is supported by the analysis of consecutive BAL from patients showing that alveolar macrophages increased in ARDS survivors [36], and by the capacity of macrophages to control neutrophil recruitment to the lung [37].

In our study, the beneficial role of alveolar fibroblasts is suggested by the reduction of the duration of mechanical ventilation observed in ALI/ARDS survivors with a positive fibroblast culture. This finding could reflect the participation of alveolar fibroblasts in lung repair by production of keratinocyte growth factor and hepatocyte growth factor [6, 17]. Otherwise, the fibroblasts are known to play a specific role in inflammation resolution after an acute injury [38]. In addition, an optimal myofibroblast differentiation also seems necessary for efficient lung repair [39] and is associated with better outcome during ALI/ARDS [25].

In conclusion, our study demonstrated that fibroblast population could be isolated from alveolar lumen during the early and late phase of ALI and ARDS in 25% of the patients. These cells showed a persistent activated phenotype with enhanced collagen 1 production and migratory capacities. Their presence is associated with a reduction of ventilation duration and with a decrease of inflammatory markers and could reflect an adapted repair process contributing to the resolution of acute lung injury. Our results and other recent works refocus the role of fibroblasts as an essential player of lung repair during ALI.

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STATEMENT OF INTEREST

None declared.

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