

Impaired Capacity of Fibroblasts to Support Airway Epithelial Progenitors in Bronchiolitis Obliterans Syndrome

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Abstract

Background: Bronchiolitis obliterans syndrome (BOS) often develops in transplant patients and results in injury to the respiratory and terminal airway epithelium. Owing to its rising incidence, the pathogenesis of BOS is currently an area of intensive research. Studies have shown that injury to the respiratory epithelium results in dysregulation of epithelial repair. Airway epithelial regeneration is supported by stromal cells, including fibroblasts. This study aimed to investigate whether the supportive role of lung fibroblasts is altered in BOS.

Methods: Suspensions of lung cells were prepared by enzyme digestion. Lung progenitor cells (LPCs) were separated by fluorescence-activated cell sorting. Lung fibroblasts from patients with BOS or healthy controls were mixed with sorted mouse LPCs to compare the colony-forming efficiency of LPCs by counting the number of colonies with a diameter of $\geq 50 \mu\text{m}$ in each culture. Statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., USA). The paired Student's *t*-test was used to test for statistical significance.

Results: LPCs were isolated with the surface phenotype of CD31⁺CD34⁺CD45⁺EpCAM⁺Sca-1⁺. The colony-forming efficiency of LPCs was significantly reduced when co-cultured with fibroblasts isolated from patients with BOS. The addition of SB431542 increased the colony-forming efficiency of LPCs to 1.8%; however, it was still significantly less than that in co-culture with healthy control fibroblasts ($P < 0.05$).

Conclusion: The epithelial-supportive capacity of fibroblasts is impaired in the development of BOS and suggest that inefficient repair of airway epithelium could contribute to persistent airway inflammation in BOS.

Key words: Bronchiolitis Obliterans Syndrome; Fibroblasts; Lung Progenitor Cells; Proliferation

INTRODUCTION

Obliterative bronchiolitis (OB) describes a rare clinical condition leading to irreversible airway obstruction. The main manifestations of OB include progressive dyspnea and cough, eventually resulting in damage to the respiratory and terminal bronchioles.^[1] In patients who received lung transplant or human stem cell transplant, bronchiolitis obliterans syndrome (BOS) represents the main form of chronic allograft rejection and is associated with major morbidity and mortality.^[2,3] In recent years, as a result of the rising incidence of end-stage lung diseases, lung transplantation is more common than ever. Clinicians have been treating increasingly more patients with BOS but without a uniformly accepted treatment

protocol. The pathogenesis of BOS also remains poorly understood.

Kelly *et al.* discovered that BOS presents a specific alteration in the distribution and function of bronchiolar club cells, believed to be lung progenitor cells (LPCs) in the airways.^[4] A recent study has also shown that injury to the

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respiratory epithelium results in dysregulation of epithelial repair.^[5] Incomplete epithelial repair normally leads to chronic inflammation, resulting in tissue damage. In addition, it is increasingly evident that the epithelial-mesenchymal interaction is pivotal for tissue regeneration in the adult lung.^[6,7] It is also well known that fibroblasts are one of the major stromal cell types that can regulate LPC function directly.^[8] However, it is unclear how fibroblasts and LPCs interact specifically during the development of BOS.

In the present study, we hypothesize that alterations to fibroblasts lead to the functional change of LPCs. Co-culture assays of mouse/human cells have been broadly used to investigate functions of stem cells *in vitro*.^[9,10] This mixed cell model was adopted in the present study because of a lack of human source LPC. We have analyzed the colony formation of LPCs co-cultured with lung fibroblasts from BOS or from normal lung tissues *in vitro*. These data may provide a new understanding of the pathogenesis of BOS.

METHODS

Mice

ACTB-EGFP mice were purchased from the Jackson Laboratory and maintained in specific pathogen-free conditions. Mice were raised in a 12 h light/dark cycle and had free access to food and water. Adult mice between the ages of 8–12 weeks were sacrificed for experiments according to the protocol approved by the Animal Care and Use Committee of the Tianjin Haihe Hospital.

Lung cell preparations and flow cytometry

Lung cell suspensions were prepared for fluorescence-activated cell sorting (FACS) on the basis of a previously published method.^[11] Briefly, mice were anesthetized, the chests were opened, and the tracheae were cannulated. Lungs were perfused with phosphate-buffered saline/0.2 mmol/L EGTA (Sigma, Germany) and then removed completely. Elastase (Worthington Biochemical Corporation, USA) digestion was performed at 37°C in three instillations for 5 min each. Lungs were removed, minced, and incubated with DNase I (Sigma) for 15 min at 37°C. Cells were then passed through a 70-µm cell strainer, and red blood cells were removed using RBC Lysis Buffer (eBioscience Inc., San Diego, USA). Cells were resuspended in Hanks' balanced salt solution (HBSS+) buffer (HBSS supplemented with 2% fetal bovine serum [FBS], 10 mmol/L HEPES, 0.1 mmol/L ethylenediaminetetraacetic acid [Sigma], 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone) following centrifugation (600 ×g,

5 min) and were incubated with primary antibodies including CD31-biotin, CD34-biotin, CD45-biotin, EpCAM-PE-Cy7, and Sca-1-APC. The properties and source of antibodies used are described in Table 1. Dead cells were distinguished by 7-amino-actinomycin D (0.25 mg/100 ml staining buffer; BD Biosciences, San Diego, USA) staining. Sorting experiments were performed on an FACS Aria III (BD Biosciences) sorter.

Fibroblasts-lung progenitor cell co-culture

Sorted LPCs were mixed with lung fibroblasts from patients with BOS or from healthy controls (kindly provided by Dr. Barry Stripp from Cedars-Sinai Medical Center) in Matrigel (BD Pharmingen, USA)/basic medium (1:1). Basic medium includes Dulbecco's modified Eagle's medium/F12 (Gibco, USA) supplemented with insulin/transferrin/selenium (Invitrogen, USA), 10% FBS (Invitrogen), 0.25 µg/ml amphotericin B, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells in Matrigel were added to 24-well transwell chamber filter inserts (Greiner Bio-One, Germany) and placed in 24-well plates, containing basic medium with or without SB431542 (Ascent Scientific LLC, USA). Fibroblasts were added to the Matrigel at 2×10^6 cells/ml. Cultures were maintained in a humidified 37°C incubator. Colonies were visualized with an inverted fluorescent microscope (OLYMPUS IX73, Japan) on days 4 and 6. Colony-forming efficiency was examined by counting the number of colonies with a diameter of ≥ 50 µm in each culture.

Statistical analysis

Data from three independent experiments were collected and analyzed as the mean \pm standard error of mean. Statistical analysis was performed using the SPSS 17.0 software (SPSS Inc., USA). The significance of the results was assessed using the paired Student's *t*-test between two groups and *P* values below 0.05 were considered statistically significant.

RESULTS

Isolation of lung progenitor cells

We used an FACS-based strategy to separate LPCs according to the green fluorescent protein fluorescence levels. Lung cells from ACTB-EGFP mice were isolated and stained with fluorescent antibodies to surface markers and a viability dye. Dead cells and cell debris were discriminated by 7-amino-actinomycin D staining [Figure 1a]. We used surface staining for CD31, CD34, and CD45 for negative selection of endothelial, stromal, and hematopoietic

Table 1: Antibodies used for flow cytometry

Antigen	Host	Titer	Source
Sca1	Rat IgG2A, clone D7	1:200 (FC-eBioscience)	BioLegend (San Diego, CA, USA)
EpCAM (PE-Cy7)	Rat IgG2A, clone G8.8	1:200 (FC)	BioLegend
CD45 (biotinylated)	Rat IgG2B, clone 30-F11	1:200 (FC-eBioscience)	eBioscience (San Diego, CA, USA)
CD31 (biotinylated)	Rat IgG2A, clone 390	2.5:100 (FC)	eBioscience
CD34 (biotinylated)	Rat IgG2A, clone RAM34	6.5:100 (FC)	eBioscience

cells [Figure 1b]. LPCs were further enriched by surface Sca-1 staining [Figure 1c].

Colony-forming ability of lung progenitor cells

To investigate the colony-forming ability of LPCs in BOS, we mixed LPCs with lung fibroblasts from patients with BOS or healthy controls. In the absence of SB431542, a

transforming growth factor- β (TGF- β) inhibitor, the ability of LPCs co-cultured with healthy control fibroblasts to form colonies was low [Figure 2a and 2b]. However, following the addition of SB431542, the colony-forming ability was significantly promoted. By day 6, the colony-forming efficiency of LPCs reached 6.8% [Figure 2c and 2d]. Consistent with previous data,^[11] TGF- β exhibits an inhibitory

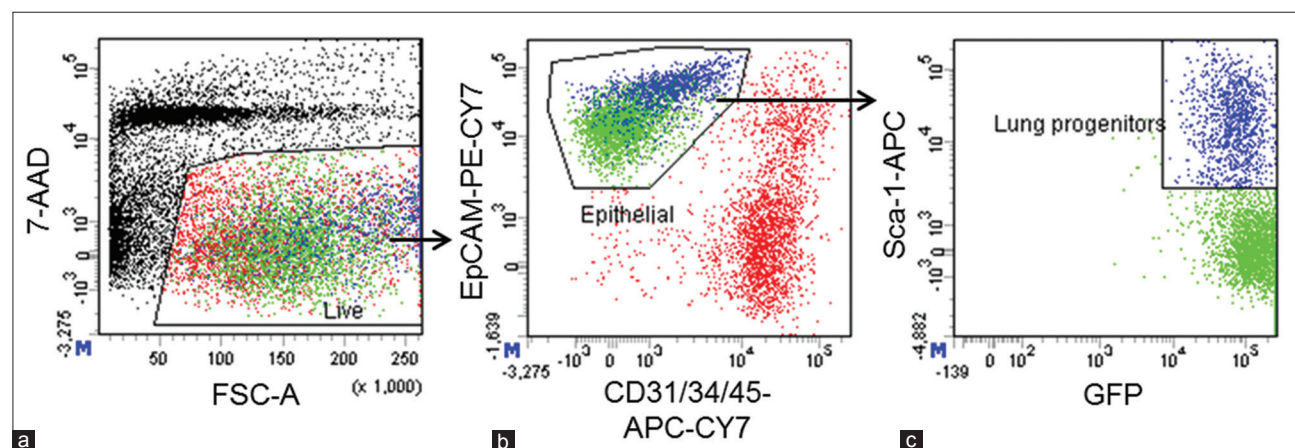


Figure 1: Fractionation of mouse lung progenitor cells. Live cells were sorted after removing cell debris and dead cells positive for 7-amino-actinomycin D staining (a). Epithelial cells were CD31/CD34/CD45-APC-CY7 negative and EpCAM-pE-CY7 positive (b). Among epithelial cells, those double positive for green fluorescent protein and Sca-10-APC are lung progenitor cells (c).

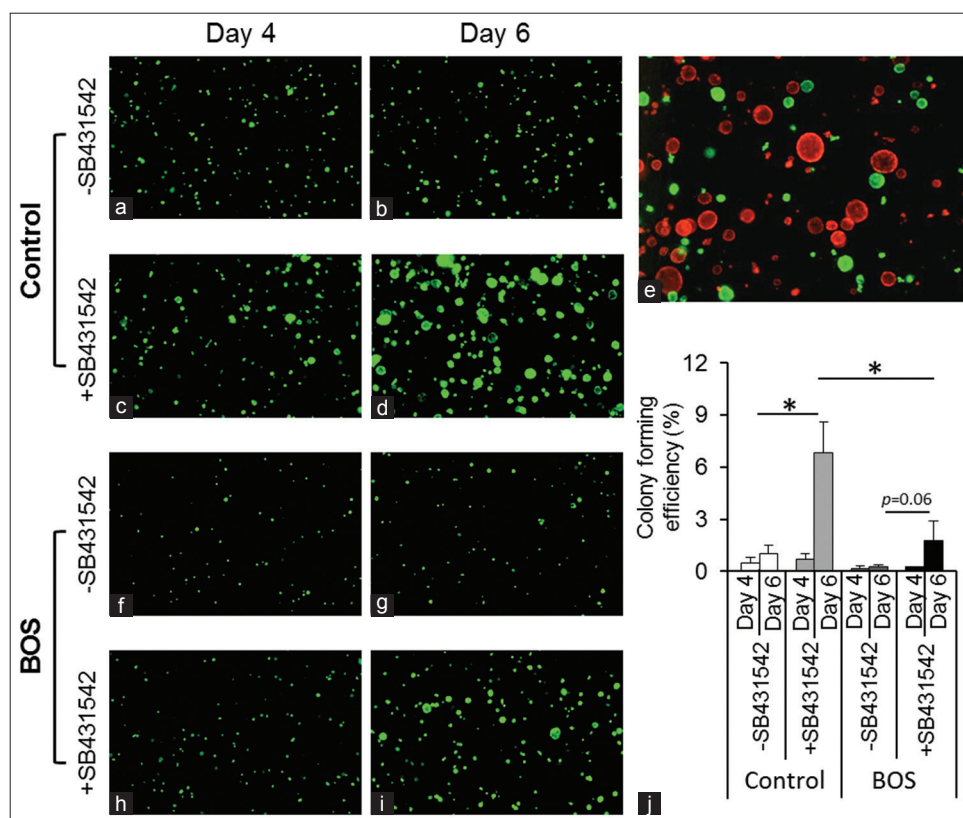


Figure 2: The colony formation of lung progenitor cells ($\times 40$ of lung progenitor colonies). Green fluorescent protein fluorescence images of lung progenitor cells co-cultured with fibroblasts from healthy controls in basic medium (a and b), basic medium supplemented with SB431542 (c and d). Analysis of the formation of lung progenitor colonies (e). Lung progenitor cells were isolated from mice expressing ubiquitous green fluorescent protein (green signal) or Td-Tomato (red signal) and co-cultured with fibroblasts. Images of lung progenitor cells co-cultured with fibroblasts from patients with bronchiolitis obliterans syndrome in basic medium (f and g), basic medium supplemented with SB431542 (h and i). Colony-forming efficiency of lung progenitor cells is summarized in j ($P < 0.05$).

effect on LPC growth. Co-culture of equivalent numbers of LPCs from ACTB-EGFP or ROSA-R/G mice led to the formation of either green or red colonies without any evidence of mixing. These data suggest that colonies are derived from a single PLC [Figure 2e]. When LPC was co-cultured with fibroblasts isolated from patients with BOS, the colony-forming efficiency of LPCs was significantly reduced. The addition of SB431542 was able to promote the colony-forming ability of LPCs, but the colony-forming efficiency of LPCs was only 1.8%, significantly less than that in co-culture with healthy control fibroblasts [Figure 2f and 2j]. These data suggest that fibroblasts from patients with advanced BOS exhibited an impaired capacity to support LPC growth.

DISCUSSION

In lung transplant recipients, BOS is the major cause of death in the 1st year following transplantation.^[12] Immune responses mediated by antibodies and lymphocytes initiate epithelial damage, inflammatory infiltration, and the activation of fibrotic pathways, ultimately resulting in bronchiolar obstruction.^[13,14] Although the precise etiology of BOS is unclear, epithelial injury is thought to be central to its development. Studies have shown that epithelial injury, including basal cells and club cells, resulted in continuous damage and abnormality of epithelial repair. Findings from blood samples of lung transplant recipients have demonstrated that patients with BOS have more circulating fibrocytes and fewer epithelial progenitors compared to those without evidence of the disease.^[15,16] Furthermore, the migration and proliferation of fibroblasts following epithelial repair are thought to underpin the development of BOS.^[17]

In the present study, we use an *in vitro* model to determine how fibroblasts from patients with BOS affect the behavior of LPCs. Our data show that the colony-forming ability of LPCs co-cultured with fibroblasts from patients with BOS significantly decreased compared with LPCs co-cultured with fibroblasts from healthy controls. This indicates that the epithelial-supportive capacity of fibroblasts is impaired during the development of BOS. Our observation that the addition of the supplement SB431542 strengthened the colony-forming ability of LPCs is consistent with previous studies.

BOS has been associated with bronchial epithelial-mesenchymal communication, a process that has been shown to be intensified by TGF- β .^[18,19] For example, in a model of lung epithelial injury, the administration of TGF- β significantly enhanced the features of epithelial-mesenchymal communication.^[20] Furthermore, findings from a rat model of OB showed a significant upregulation of TGF- β and downstream genes of fibrotic airways.^[21] As a vital factor in the BOS fibrotic process, TGF- β signaling has been shown to inhibit the proliferation of epithelial cells in several organs, including the lung.^[22] The activation of lung stromal cells mediated by TGF- β suppresses their epithelial-supportive

capacity via the downregulation of epithelial growth factors such as FGF-10.^[23] As a result, the regeneration and repair of the lung epithelial are interrupted, which may play a part in the development and progression of BOS. Although our study was the first to examine selective alteration of fibroblasts in BOS, a limitation of the study is that the LPCs were from mice rather than humans. However, human LPCs are rather difficult to acquire and culture successfully. The study of LPCs of BOS is indispensable and will have considerable influence. Here, we co-cultured the mouse stem cells and human supportive cell-fibroblasts allowing the study of functional alterations in LPCs *in vitro*. Finally, as well as demonstrating a quantitative change, our results showed functional variation in the development of BOS.

In conclusion, these preliminary data demonstrated that the dysregulation of epithelial repair in BOS partially results from the functional alteration of stromal cells. The epithelial-supportive capacity of fibroblasts in patients with BOS is significantly impaired. We used a new method involving FACS and co-culture to explore the potential role of fibroblasts in the development of BOS. In view of the deficiency of existing treatments for BOS, the regulation of stromal cells may represent a promising area of research in future studies.

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Conflicts of interest

There are no conflicts of interest.

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