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Inhibited proliferation of human lung fibroblasts by LPS is through IL-6 and IL-8 release

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ABSTRACT

Through the consideration of decreased proliferation of lung fibroblasts from subjects with chronic obstructive pulmonary disease (COPD) and the proinflammatory role of lipopolysaccharide (LPS) in the COPD development, we hypothesized that LPS might inhibit proliferation in lung fibroblasts and the possible mechanism was investigated.

Primary human lung fibroblasts were cultured from peripheral lung tissue and then treated with or without LPS. Proliferation was measured by AlamarBlue® assay. Levels of TNF-α, IL-6, IL-8, IL-12p70, IL-1β and IL-10 in the supernatants were measured by ELISA. The mRNA of histone deacetylases 2 (HDAC2) was analyzed using real-time PCR.

LPS appeared to have a dose-dependent inhibitory effect on fibroblasts proliferation. The concentrations of IL-6 and IL-8 in the treatment culture media were significantly increased, accompanied by a reduced mRNA expression of HDAC2. IL-6 or IL-8 itself led to the reduction of fibroblasts proliferation. Treatment with 1 ng/ml TNF-α in fibroblasts also caused a significant decrease in proliferation and an increase in the production of IL-8 and IL-6.

Our data suggest that LPS can inhibit the proliferation of *in vitro* human lung fibroblasts at least through a production of IL-6 and IL-8. The cytokine response is related to the decreased HDAC2 transcription.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality worldwide and results in an economic and social burden that is both substantial and increasing [1]. It is characterized by chronic inflammation and irreversible airflow obstruction which involves structural changes of the lung, including emphysema [2]. Emphysema is a major morphological change of COPD, which is associated with an abnormal pulmonary inflammatory response to stimuli [1].

The development of emphysema is currently thought to be due to the imbalance of tissue injury and repair [3,4]. The chronic abnormal inflammatory process induced by tobacco smoking contributes to increased extracellular matrix degradation and promotes the structural changes in lung parenchyma [1]. On the other hand, studies suggested that impaired tissue repair has been regarded as another pivotal mechanism for emphysema [3,4]. There is increasing evidence supporting that the tissue repair capacity of lung fibroblasts, which are responsible for the extracellular matrix regeneration and maintenance, is decreased in COPD. Lower proliferation of fibroblasts was seen in patients with COPD compared to that in patients with normal lung function despite a comparable smoking history [5–7]. In addition, the response of lung fibroblasts to transforming growth factor (TGF)-β1 was decreased [8]. These suggest that the abnormal lung inflammation might also contribute to the dysfunction of fibroblasts.

Lipopolysaccharide (LPS), a proinflammatory component of the outer cell membrane of Gram-negative bacteria and a contaminant of cigarette smoke, air pollution, and organic dusts [9], is a potent stimulus for the development and progression of various pulmonary diseases including COPD [10–12]. Chronic exposure to significant levels of LPS induced a profound pulmonary inflammatory response resulting in a decline lung function and is associated with the development and/or progression of irreversible airflow obstruction and emphysema [13]. Chronic LPS exposure induced by cigarette smoke has also been shown to cause pathological abnormalities in lung in animal models [11,12].

Chronic inflammation is presumably of major importance in the pathogenesis of emphysema in COPD, and LPS is a ubiquitous inflammatory stimulus and regarded to play a role in the development and progression of COPD. However to our knowledge,

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whether LPS alters the function of the lung fibroblasts has not been well investigated. Since the lung fibroblasts express Toll-like receptor 4 (TLR4) which initiates the cellular response to LPS [14], we hypothesized that LPS might impact the function of lung fibroblasts. Using primary lung fibroblast cultures from human explants, the function of the cells as determined by proliferation was measured and cytokine production was studied in response to LPS.

2. Materials and methods

This study was approved by the Ethics Committee of Zhongshan Hospital of Fudan University. All patient-derived tissues were obtained with written informed consent.

2.1. Subjects

Primary human fibroblasts were isolated from human lung tissue obtained at surgery for biopsy or bronchial carcinoma. Subjects were excluded from the study population if the patient had a clearly documented history of lung diseases such as asthma, bronchiectasis and interstitial lung disease.

2.2. Reagents

All cell culture reagents including culture media, fetal bovine serum (FBS) and AlamarBlue® (AB) were purchased from InVitrogen. LPS (*Escherichia coli* serotype 0111:B4), Phorbol-12-myristate-13-acetate (PMA), PD98059 (p42/44 MAP kinase inhibitor), LY-294002 (PI3 kinase inhibitor) and SB203580 (p38 MAP kinase inhibitor) were purchased from Sigma. Recombinant human TNF-α, IL-6, and IL-8 were purchased from R&D system. All OptEIA™ ELISA kits for measuring cytokines (TNF-α, interleukin-6 (IL-6), IL-8, IL-12p70, IL-1β and IL-10) were purchased from BD.

2.3. Isolation and cultures of human lung fibroblasts

Human lung fibroblasts were isolated from the surgical specimens of peripheral lesion-free and pleura-free lung tissue. The tissues were immediately transferred into complete medium [Dulbecco's modified Eagle medium (DMEM), 10% fetal calf serum (FCS), penicillin (100 ng/ml), and streptomycin (100 ng/ml)]. The tissue was minced with a scalpel (1–2 mm^2) and transferred into T25 cm^2 culture dishes for primary culture at 37 °C in 5% CO_2. Cells were then trypsinised until sub-confluent and passaged. The fibroblasts were only used from passage 2–3 in this study.

Apart from observing the morphology of the fibroblasts, the purity of the cultured primary lung fibroblasts were confirmed by staining with vimentin (fibroblast marker), and cytokeratin (epithelial marker), vonWillebrand factor (endothelial markers) and desmin (smooth muscle marker).

2.4. Collection of conditioned medium that from U937 derived macrophages cultured with LPS

Monocytotic U937 cells (ATCC: CRL 1593) were cultured using DMEM complete medium. U937 derived macrophages were induced by the treatment of U937 cells with PMA (10 μg/ml) for 24 h. After the medium was removed and washed, macrophages were then treated with 1 μg/ml LPS for 24 h. The culture medium was removed and washed, and the cells were then cultured with fresh complete medium for a further 24 h. Supernatants were then collected as the LPS-pre-treated macrophage conditioned.

2.5. Determination of the proliferation of lung fibroblasts

Purified human lung fibroblasts were seeded in 96 well plate and grown to approximately 70% confluence in a complete medium in a humidified atmosphere containing 5% CO2. The doubling time of fibroblasts was 48 h. After an additional culture for 24 h under serum-free conditions (5% CO_2 at 37 °C), the human lung fibroblasts were treated with LPS and other cytokines for 24 h. The proliferation of lung fibroblasts was measured using Alamar blue (AB) assay as described previously [15]. Briefly, AB was added into the culture medium at a final concentration of 10%. After additional 6-h incubation, the %AB reduction was measured. In addition, the fibroblasts that had been treated with LPS were extracted for mRNA measurement. The conditioned media that fibroblasts cultured with LPS were also collected for cytokines analysis.

2.6. ELISA

The levels of cytokines including human TNF-α, IL-6, IL-8, IL-12p70, IL-1β and IL-10 in fibroblasts cell conditioned media were measured using OptEIA™ ELISA kits following the manufacturer's instructions. The cytokines, together with their respective lower limit of detection, in square brackets, were as follows: IL-1β [5.2 pg/ml], IL-6 [15.7 pg/ml], IL-8 [10 pg/ml], IL-10 [1.5 pg/ml], IL-12p70 [11.6 pg/ml] and TNF-α [17.5 pg/ml].

2.7. Real time RT-PCR

Total RNA extraction was performed using Trizol Reagent (Invitrogen Life Technologies) and then 1 μg of total RNA was transcribed using SuperScript® ViLOTM cDNA synthesis kit (Invitrogen Life Technologies) according to the manufacturer's instructions.

Real-time PCR was performed using Express SyBR® GreenERTM SuperMix with Premixed ROX (Invitrogen Life Technologies) following the manufacturer's instructions. The following primers were used: histone acetylation, histone deacetylases2 (HDAC2) forward, 5′-TCC AAG GAC AAC AGT GGT GA-3′; HDAC2 reverse, 5′-GCC CAG AAA AAC AAA AAC GA-3′; GAPDH forward, 5′-TGA GCA CCA GAT TGT CTC CT-3′; and GAPDH reverse, 5′-GCA TCA AAG GTG GAA GAC TG-3′. PCR assays were performed in duplicate on the 7900HT real-time PCR machine (Applied Biosystems) and the cycler conditions were as follows: incubation for 2 min at 50 °C followed by another incubation step at 95 °C for 10 min, afterwards 15 s at 95 °C and 1 min at 60 °C for 40 cycles. Reaction specificity was evaluated by melting curve analysis which was performed by heating the plate from 55 to 95 °C and measuring SYBR Green I dissociation from the amplicons. The calculation of threshold cycles (C_t values) and further analysis of these data were performed by the Sequence Detector software. The relative expression of HDAC2 mRNA in each sample were quantified and normalized to the GAPDH mRNA levels by the 2^{-ddC_t} method.

2.8. Statistical analysis

All experiments were conducted in duplicate and repeated at least three times. Data are presented as mean ± standard deviation. All statistical analysis was performed using GraphPadPrism 5.0 for Windows. D'Agostino and Pearson omnibus normality test was performed at first. The statistical significance of the results was assessed by paired *t*-tests or one way ANOVA. Differences were considered significant at the value of $p < 0.05$.