Leptin Is Oversecreted by Respiratory Syncytial Virus-Infected Bronchial Epithelial Cells and Regulates Th2 and Th17 Cell Differentiation

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Key Words
Human bronchial epithelial cells · Leptin · Respiratory syncytial virus · Th2 · Th17

Abstract

Background: Infection of human bronchial epithelial cells (hBECs) with respiratory syncytial virus (RSV) has been shown to induce a Th lymphocyte subset drift, e.g. enhanced differentiation of Th2 and Th17 subsets, which is a classic characteristic of asthma. However, the molecules responsible for the drift in Th subsets remain unknown. This study aims to determine the expression of leptin in RSV-infected hBECs, and its role in Th2 and Th17 cell differentiation and extracellular regulated kinase (ERK) 1/2 phosphorylation. Methods: Cultured hBECs were infected with RSV. mRNA expression of the LEP gene in cells was measured by real-time PCR while LEP protein secretion in culture medium was measured by ELISA. Th differentiation was investigated in cultured human peripheral blood mononuclear cells following stimulation with recombinant human leptin. Th2 and Th17 subsets were examined by flow cytometry. Phosphorylation of the ERK1/2 protein in lymphocytes was detected by Western blot and immunofluorescence. Results: LEP mRNA expression was significantly upregulated in RSV-infected hBECs while the leptin protein level in the supernatants of RSV-infected hBECs was significantly increased. Stimulation of lymphocytes with leptin increased the differentiation of the Th17 subset and ERK1/2 phosphorylation, but suppressed Th2 subset differentiation. Conclusion: Leptin was oversecreted by RSV-infected hBECs, which promoted Th17 subset differentiation but suppressed Th2 subset differentiation possibly via regulating ERK1/2 phosphorylation.

Introduction

Respiratory syncytial virus (RSV) infection has been widely recognized to be involved in the development of asthma [1, 2]. A Th lymphocyte subset drift, such as the enhanced differentiation of Th2 and Th17 subsets, is a classic characteristic of asthma [3–5]. However, how RSV induces the drift in Th subsets remains unclear. Bronchial epithelial cells (BECs) are the primary target of RSV after infection [6]. Our previous study demonstrated that co-culture of lymphocytes with RSV-infected human BECs (hBECs) significantly increased cell apoptosis of lymphocytes and the secretion of IL-4, IFN-γ and IL-17. The supernatants from RSV-infected hBECs significantly increased the differentiation of Th2 and Th17 subsets, and decreased the differentiation of T_{reg} subsets [7]. However, the molecules in the supernatants of RSV-infected hBECs that exert a stimulatory effect during cell secretion have not been identified.

Leptin is a 16-kDa protein composed of 167 amino acids and is produced by the Ob gene on chromosome 7...
and mainly secreted by fatty tissue [8, 9]. Recently, leptin expression has been found in a variety of organs, including lung tissue. BECs, alveolar epithelial cells and pulmonary macrophages can produce a small amount of leptin [10, 11]. Leptin is a protein hormone and exerts potential functions in immune regulation and the pathogenesis of autoimmune diseases. It has been demonstrated that leptin receptors are found in many tissues and cells, including peripheral blood T lymphocytes [12, 13]. Leptin is involved in immune responses via the activation of monocytes, macrophages, neutrophils and T lymphocytes [14]. In a preliminary microarray assay, a significant increase in LEP gene mRNA levels was observed in RSV-infected hBECs compared to uninfected hBECs. In this study, we investigated the role of leptin in the differentiation of Th subsets and the associated signaling.

Materials and Methods

Cell Culture

The hBEC line was obtained from the Department of Physiology, Central South University (Changsha, China) and cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mM glutamine and 4,500 mg/l of D-glucose. A human cervical cancer cell line (HeLa cells) was obtained from the Cell Center of the Central South University and cultured in DMEM containing 2% FBS. Cells were cultured at 37 °C in 5% CO2.

RSV Preparation

RSV (long strain/A2 type) was obtained from the Guangzhou Medical College (Guangzhou, China) and propagated in HeLa cells overlaid with DMEM containing 2% FBS until the cytopathic effects reached 80%. Cells were were refrigerated, thawed and centrifuged. The supernatant was harvested, and the dose of RSV was determined by an acute cytopathic effect in 50% of cells.

RSV Infection

hBECs in confluent monolayer were infected with RSV at a multiplicity of infection of 0.0001 pfu/cell, as previously described [7]. The viral suspension was added to the cells for 3 h. hBECs were then continuously cultured in fresh medium and passaged. The passaged cells were designated as 'hBECs with persistent RSV infection'. hBECs without RSV infection were used as controls.

Identification of RSV-Infected hBECs

RSV infection in hBECs was identified by examining cell morphology under a phase contrast microscope and immunofluorescence staining of RSV F protein using FITC-labeled anti-F protein monoclonal antibody (Santa Cruz, Santa Cruz, Calif., USA).

Real-Time PCR

Total RNA was isolated from RSV-infected hBECs and uninfected hBECs using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA). RNA was reverse transcribed using the EnergeticScript® cDNA synthesis kit and analyzed using an FTC 2000 PCR detection system (Funglyn, Toronto, Ont., Canada). qRT-PCR was performed in a total of 50 μl of reaction volume containing 1 μl of cDNA, 15 pmol of forward primer of LEP: 5′-GCGGATCCCTGGCCTTTGTTG-3′ and reverse primer: 5′-GGAGTTTCTCTCTTTGATGT-3′, 25 μl of 2× PCR buffer, 0.3 μl of SYBR green I and 22.5 μl of DEPC water. The β-actin was amplified using forward primer: 5′-TGACCTGACATCGGAAAG-3′ and reverse primer: 5′-GGAGCACGGTGAAGTCC-3′ as an internal control. LEP expression was normalized to β-actin and quantified using the comparative Ct method, as previously described [15].

ELISA

The leptin protein level in the cell culture medium of hBECs was measured using human leptin ELISA kits (R & D Systems, Minneapolis, Minn. USA).

Isolation of Peripheral Lymphocytes and Leptin Treatment

Fifty milliliters of heparinized whole blood was collected from each healthy adult volunteer. Peripheral blood mononuclear cells were isolated using density gradient centrifugation, as previously described [16]. Subsequently, they were incubated in RPMI 1640 containing 10% FBS at 37 °C in 5% CO2 for 2 h to remove adherent monocytes [16].

Fluorescent Staining and Flow-Cytometric Analysis

Lymphocytes were incubated with 2 μl of monensin (×1,000; BioLegend, San Diego, Calif., USA) for 6 h to inhibit the secretion of newly produced cytokines. Monensin is a protein transport inhibitor commonly used to enhance intracellular cytokine staining signals by blocking transport processes during cell activation. Being especially useful for the intracellular staining of cytokines, monensin leads to the accumulation of most cytokines at the Golgi complex/endo-plasmic reticulum [17]. Cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and incubated in permeabilizing solution (eBioscience, San Diego, Calif., USA). After blocking with 3% BSA for 15 min, cells were stained with PE anti-human IL-4 and PE anti-human IL-17A antibodies (eBioscience) on ice for 45 min. Cells were then subjected to flow-cytometric analysis. TH and TH17 cells have no specific cell surface antigens. TH2 and TH17 cells were usually identified through the specific cytokines secreted by them. The TH2 subset secretes IL-4 while the TH17 subset secretes IL-17. Therefore, the cells can be stained by PE-anti-IL-4 and PE-anti-IL-17 [18].

Western Blot

Total protein was isolated from lymphocytes. 20 μg of total protein were loaded onto each lane. Western blot was performed as previously described [19]. Anti-extracellular regulated kinase (ERK) 1/2, anti-phospho-ERK1/2, anti-β-actin and horse radish peroxidase-conjugated anti-IgG were purchased from Cell Signaling Technology (Danvers, Mass., USA).

Immunofluorescence

The collected lymphocytes were fixed, permeabilized and then attached to the slides. Immunofluorescence was performed as previously described [20]. Briefly, slides were incubated with primary antibodies against ERK1/2 or phospho-ERK1/2 for 1 h and then incubated with FITC-conjugated or PE-conjugated anti-IgG.
antibodies (Sigma, St. Louis, Mo., USA) in the dark after washing with PBS. The cells were mounted on slides and observed under a fluorescence microscope (Olympus America, Melville, N.Y., USA).

Statistical Analysis
Data are presented as means ± SEM and analyzed using SPSS 13.0 software. Statistical analysis was performed by paired t test followed by post hoc testing for the data from normal hBECs and RSV-infected hBECs. Univariate ANOVA followed by Dunnett’s t test was applied to the data from lymphocytes treated with leptin. A value of p < 0.05 was considered statistically significant.

Ethics Statement
The study was approved by the Ethics Committee of the Xiangya Hospital. Peripheral blood was drawn from healthy adult volunteers. Written informed consent was obtained from all participants. Healthy adult volunteers with any current acute or persistent chronic disease, on any medication and/or with a history of severe immune system diseases were excluded from the study.

Results
Study of RSV-Infected hBECs
Consistent with our previous study [7], RSV infection in hBECs was established. Variation in cell shape, cell shrinkage and enhanced refraction were observed. Enlarged fusion cells were regarded as evidence of RSV infection (Fig. 1a). Fluorescence staining showed the distribution of differently sized viral particles within the cytoplasm (Fig. 1b).

Fig. 1. Morphological study of RSV-infected hBECs using phase contrast (×100; a) and fluorescence microscopy (×40; b). In RSV-infected hBECs, variation in cell shape, cell shrinkage and enhanced refraction were present. The enlarged fusion cells and intracytoplasmic eosinophilic inclusion were considered proof of RSV infection (a). Green color indicates positive staining for RSV (see online version for colors).
Expression of Leptin mRNA and Protein

Real-time PCR showed that leptin mRNA expression was significantly increased in RSV-infected hBECs compared to uninfected controls (fig. 2a). ELISA showed that the level of leptin in the culture medium of RSV-infected hBECs was significantly increased compared to controls (fig. 2b).

Effects of Leptin on Th2 and Th17 Subsets

Preliminary experiments demonstrated that 100 μg/ml of phytohemagglutinin (PHA-P; Sigma) had no significant effect on the differentiation of Th subsets. Most peripheral blood lymphocytes were in the G0 phase after PHA treatment for 24 h although PHA can stimulate the mitosis of lymphocytes; 100 μg/ml of PHA were then used to stimulate lymphocyte mitosis, as previous reported [21–23]. Recombinant human leptin (ProSpec-Tany TechnoGene Ltd.) was added to 2 ml of lymphocyte suspension (2 × 10^6 cells/ml) at 0, 0.2, 0.5 and 1 ng/ml. After culturing for 24 h, Th2 and Th17 differentiation was analyzed by flow cytometry using anti-IL-4 and anti-IL-17 antibodies [18]. Monensin was used to inhibit the secretion of newly produced cytokines in Golgi bodies [17]. Results showed that Th2 differentiation was suppressed and Th17 differentiation was increased after leptin treatment (fig. 3). No significant difference in Th2 and Th17 differentiation was observed among the three groups treated with three different concentrations of leptin, suggesting that the effects of the different concentrations (0.2–1.0 ng/ml leptin) on lymphocyte differentiation did not differ.

Leptin Increased Phosphorylation of ERK1/2 in Lymphocytes

The total protein and phosphorylation levels of ERK1/2 in lymphocytes were determined by Western blot and immunofluorescence (fig. 4). There was no significant difference in the total ERK1/2 level among the four groups, but the phosphorylated ERK1/2 level in three leptin-treated groups was higher than in the control group, respectively. No difference in phosphorylation of ERK1/2 was observed among the groups treated with different concentrations of leptin, suggesting that 0.2–1.0 ng/ml leptin increased phosphorylation of ERK1/2 but not in a concentration-dependent manner.

Discussion

Although our previous study showed that secretions of RSV-infected hBECs can induce an abnormal drift in Th subsets in vitro [7], the possible molecules involved in this drift have not been identified. A preliminary microarray screening showed significant elevation in LEP mRNA in RSV-infected hBECs [our unpubl. data]. In this study, we validated the stimulatory effect of RSV on LEP mRNA and leptin protein expression in hBECs, and observed that recombinant human leptin (0.2–1.0 ng/ml) increased Th17 differentiation, but decreased Th2 differentiation through increasing ERK1/2 phosphorylation in lymphocytes.

Increased differentiation of Th2 and Th17 subsets has been widely observed in asthma [3–5]. In obese children with asthma, IFN-γ levels were significantly elevated and IL-4 levels were decreased with a significant positive association with serum leptin levels [15]. Our previous study and the present study demonstrated that the level of leptin was significantly increased in BECs after RSV infection, a crucial factor of asthma [our unpubl. data]. The direct roles of leptin in lymphocyte proliferation and differentiation have been revealed in cell culture and ani-
Fig. 3. Distribution of Th2 and Th17 subsets in lymphocytes. 

a Cytometric assays of IL-4 and IL-17 lymphocytes after leptin treatment. After being treated with 0.2, 0.5 and 1.0 ng/ml of leptin, the number of Th2 cells was reduced and Th17 cell numbers were increased compared with control. 

b Percent of Th2 and Th17 subsets in lymphocytes. n = 5, * p < 0.05 vs. control. There were no significant differences among the three different leptin treatment groups.

Fig. 4. ERK1/2 and phosphorylated (p-)ERK1/2 protein levels. Representative Western blots (a) and immunofluorescence (b) of ERK1/2 protein and p-ERK1/2 protein after treatment with leptin.

Leptin-Regulated Th2 and Th17 Differentiation

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eral models [24–27]. For example, a combination of leptin intervention with exogenous stimulus has been revealed to promote T-lymphocyte proliferation and secretion of IL-2 and IFN-γ [24]. Leptin stimulated IFN-γ production in naïve cells and memory T cells, but inhibited IL-4 synthesis in memory T cells [25]. Yu et al. [26] reported a positive association between leptin and Th17 responses. In addition, leptin was identified to enhance Th17 cell responses and exacerbate joint inflammation in mice with collagen-induced arthritis [27]. In this study, recombining human leptin suppressed Th2 differentiation and improved Th17 differentiation. The Th2 cytokine IL-4 strongly inhibits Th17 development both in vitro and in vivo [28]. For example, JAK inhibitor P6 could ameliorate allergic skin inflammation of NC/Nga mice via suppression of Th2 and enhancement of Th17 [29]. Therefore, the inhibition of Th2 differentiation and the improvement in Th17 differentiation may be a result of both the direct effect of leptin on lymphocytes and the mutual antagonism between Th2 and Th17 subsets. However, how leptin stimulates lymphocyte proliferation and differentiation has not been elucidated.

ERK1/2 is a key molecule transferring signals from the cell surface to the nucleus. Phosphorylated ERK1/2 mediates the activation of transcription factors such as NF-AT, AP-1 and NF-κB [30]. Recent studies have shown that activation of the ERK1/2 signaling pathway can promote the activation and proliferation of T lymphocytes, and inhibit their apoptosis. ERK participates in the transcriptional regulation of IL-4, which is also indispensable for human Th2-cell differentiation [31]. Blockade of ERK activation inhibited Th17-cell development, and interfering with the ERK pathway could present a therapeutic treatment for inflammatory bowel diseases and other Th17-related autoimmune diseases [32]. In this study, leptin increased ERK1/2 phosphorylation in peripheral lymphocytes. We, therefore, hypothesize that leptin may regulate Th subset differentiation by activating ERK1/2 signaling. Goplen et al. [33] found that Th2-type cytokines IL-4 and IL-5 were significantly reduced in ERK1−/− asthmatic mice and presumed that ERK1 played a nonredundant role in Th2 differentiation and the development of experimental asthma.

In conclusion, oversecretion of leptin by hBECs after RSV infection may mediate the abnormal drift in Th subsets. Leptin may increase the differentiation of Th17 cells and Th2 subsets via ERK1/2 activation. Leptin could lead to increased asthma susceptibility. Due to the close relationship between RSV and asthma, the results helped to elucidate the pathogenesis of asthma.

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Disclosure Statement
All authors declared no conflict of interest.

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