STAT3 regulates cytokine expression in peripheral blood mononuclear cells from asthma patients

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Abstract: Asthma is a common long term inflammatory disease of the airways. This disease affected millions of people worldwide. Recently, it is demonstrated that signal transducer and activator of transcription 3 (STAT3) plays critical role in asthma occurrence. In the current study, we isolated peripheral blood mononuclear cells (PBMCs) from patients with mild and moderate asthma, and then determined the correlation between STAT3 and cytokine expression. We found that the concentration and mRNA level of cytokines was increased in PBMCs from asthma patients. The concentration and mRNA level of cytokines was altered by the regulation of STAT3 expression and the concentration and mRNA expression level of cytokines was positively correlated with STAT3 activation. Furthermore, phosphorylated STAT3 expression in PBMCs from asthma patients was increased compared with the control. Collectively, this study directly proved that STAT3 was correlated with cytokine expression in PBMCs from asthma patients, providing a potential linkage between STAT3 and pathogenesis of asthma.

Key words: STAT3; Asthma; PBMCs; p-STAT3; Cytokine.

Introduction

Asthma, which is known as a common chronic inflammatory disease of the airways involving cells of the innate and adaptive immune system, affects more than 300 million people worldwide (1). Generally, there are two types of clinically defined asthma including non-allergic (or intrinsic) asthma and allergic (atopic) asthma, among which allergic asthma occurs in almost all children cases and nearly 50% of adult asthma patients (2). The allergic asthma is triggered by proteins of common inhaled or ingested allergens such as house dust mite, animal dander or plant and tree pollen (3). Currently, the treatment to asthma includes the inhaled corticosteroids as well as between healthy control and asthma patients, as difference in the phosphorylation of STAT3 was regulated with the regulation of STAT3. We also found that the phosphorylation of STAT3 was regulated with application of STAT3 inhibitor or activator. Furthermore, the alteration of cytokine concentration or mRNA expression as well as between healthy control and asthma patients, as well as between patients with mild and moderate asthma. Therefore, these results demonstrated that STAT3 might be involved in pathogenesis of asthma, contributing to our understanding for the underlying mechanism regarding the correlation between asthma and STAT3.

Materials and Methods

Peripheral Blood Mononuclear Cells (PBMCs) Isolation and Stimulation

PBMCs were isolated from blood of asthma patients and healthy people by using density gradient centrifugation (Ficoll-Paque, GE Healthcare). Each group contains 15 persons. Briefly, cells were washed in PBS twice, and resuspended in RMPI culture medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. For in vitro stimulations, cells
were cultured in pure RPMI culture medium, with 1µM STAT3 inhibitor Stattic or STAT3 activator Colivelin, which was dissolved in DMSO. Same amount of DMSO was used as blank control.

Enzyme-linked immunosorbent assay (ELISA)
ELISA kits (Elabscience) were used to measure the alteration in concentration of IL-2, IL-6, IL-23, IL-17, IFN-γ, IFN-α or TNF-α in PBMCs treated with different stimulations. After coating, plates were sequentially washed with PBST buffer and blocked with 1% BSA and incubated for 1 h at 37 °C. Then, anti- IL-2, IL-6, IL-23, IL-17, IFN-γ, IFN-α or TNF-α antibody and HRP-conjugated antibody were sequentially added and incubated for 1 h at 37 °C. The detection was achieved by adding chromogenic substrate, 3,3',5,5'-Tetramethylbenzidine (TMB). Absorbance was measured at 450 nm with an EnSpire multimode plate reader (Perkin Elmer, Waltham, Massachusetts).

Real time PCR
Total RNA extraction was performed using TRIzol reagent (Life Technologies) according to the manufacturer’s instruction. Two microgram of total RNA extracted from ovarian tissue was subjected to reverse transcription (RT). Synthesis of cDNA was performed by using one-step RT-PCR kit from Takara. SYBR Green (Toyobo) RT-PCR amplification and real time fluorescence detection were performed using ABI 7300 real-time PCR thermal cycle instrument (ABI, USA), according to the supplied protocol. Relative gene expression was calculated by the ∆∆Ct method. The relative expression levels were normalized to expression of endogenous GAPDH.

Western blotting
2 µg cell lysates were loaded on each lane of 10% polyacrylamide gel, and then blotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking with a PBST containing 5% nonfat dry milk, the membrane was incubated with primary antibodies against p-STAT3 (Cell Signaling Technologies), STAT3 (Chemicon), or GAPDH (Cell Signaling Technologies). Peroxidase-linked IgG (Life Technologies) were used as secondary antibodies. These proteins were visualized by using an ECL western blotting detection kit (Amersham Biosciences).

Statistical analysis
Data were presented as mean ± SEM. One way ANOVA was used to determine significant differences. A p value less than 0.05 is considered as significantly different.

Results
STAT3 regulates concentration of cytokines in PBMCs from asthma patients
Firstly, we determined whether Stat3 could regulate the concentration of cytokines in asthma patients. ELISA data demonstrated that the concentration of IL-2, IL-6, IL-23, IL-17, IFN-γ, IFN-α or TNF-α in PBMCs from patients with mild asthma was increased compared to these in PBMCs from healthy people, P<0.01. In the presence of STAT3 inhibitor Stattic, the concentration of these cytokines was significantly decreased compared to the untreated controls (Fig.1), P<0.001. By contrast, the concentration of these cytokines in PBMC from patients with moderate asthma was drastically increased in the presence of STAT3 activator Colivelin compared to the untreated controls (Fig.1), P<0.01. Similar results were obtained in PBMCs from patients with moderate asthma (Fig.1). However, the concentration of cytokines was increased in patients with moderate asthma compared to those in patients with mild asthma. These data indicated that STAT3 might be associated with the alteration of concentration of cytokines in PBMCs from patients with asthma.

STAT3 controls mRNA level of cytokines in PMMCs from asthma patients
Then, we checked the mRNA level alteration of above described cytokines through quantitative real time PCR. Compared to the healthy controls, mRNA expression level of IL-2, IL-6, IL-23, IL-17, IFN-γ, IFN-α or TNF-α was significantly increased (Fig.2), P<0.01. In the presence of Stat3, the mRNA level of...
these cytokines was drastically decreased compared to those in untreated controls (Fig.2), \( P<0.01 \). By contrast, the application of STAT3 activator Colivelin could significantly increase the mRNA expression level of IL-2, IL-6, IL-23, IL-17, IFN-\( \gamma \), IFN-\( \alpha \) or TNF-\( \alpha \), compared to the untreated controls (Fig.2), \( P<0.001 \). Furthermore, these qPCR data also showed that mRNA expression level of cytokines was increased in PBMCs from patients with moderate asthma than those in patients with mild asthma (Fig.2), \( P<0.01 \). These data was consistent with our findings through ELISA, further validating the conclusion the critical role of STAT3 in regulating the cytokines in PBMCs from patients with asthma.

**STAT3 phosphorylation was altered in PBMCs from asthma patients**

Next, we determined whether the level of phosphorylated STAT3 was altered in patients with asthma. The western blotting data showed that the expression level of p-STAT3 was not significantly changed in PBMCs from patients with mild asthma compared to healthy controls, while the p-STAT3 level in PBMCs from patients with moderate asthma was drastically increased (Fig.3). Furthermore, application of Stattic could significantly decrease the p-STAT3 level in PBMCs from patients with both mild and moderate asthma. By contrast, the presence of Colivelin increased the p-STAT3 level in PBMCs from patients with mild asthma compared to the untreated control. However, this increase was not shown in PBMCs from patients with moderate asthma (Fig.3). Similar results were obtained through flow cytometry. In untreated PBMCs, p-STAT3 level in moderate but not mild patients was apparently increased compared to that in PBMCs from healthy controls (Fig.4A). In PBMCs treated with Colivelin, p-STAT3 level was significantly increased in patients with both mild and moderate asthma compared to the healthy controls (Fig.4B). By contrast, the p-STAT3 level was not drastically altered in Stattic-treated PBMCs from patients with mild or moderate asthma, compared to the PBMCs from healthy patients (Fig.4C). Collectively, these data indicated that p-STAT3 level was altered in PBMCs from patients with asthma.

**Discussion**

Through working on the PBMCs from asthma patients, we elucidated the role of STAT3 in regulating cytokine concentration and expression in PBMCs from asthma patients. Also, the phosphorylation of STAT3 was altered in PBMCs with the regulation of STAT3. Furthermore, both cytokines and p-STAT3 were differentially expressed among healthy controls, patients with mild asthma, and patients with moderate asthma. All these data supported the potential role of STAT3 in pathogenesis of asthma.

STAT3 expression was aberrantly elevated in the airway smooth muscle tissues of patients with asthma (6). STAT3 is activated by cytokines and is critical in airway smooth muscle cell proliferation, inflammation and asthmatic airway remodeling (9-11). Based on these findings, we determined the correlation between STAT3 and cytokine expression in PBMCs from asthma patients. The cytokine concentration and expression is positively correlated with STAT3 activation (Fig.1, 2). This is consistent with the previous finding demonstrating these cytokines was drastically decreased compared to those in untreated controls (Fig.2), \( P<0.01 \). By contrast, the application of STAT3 activator Colivelin could significantly increase the mRNA expression level of IL-2, IL-6, IL-23, IL-17, IFN-\( \gamma \), IFN-\( \alpha \) or TNF-\( \alpha \), compared to the untreated controls (Fig.2), \( P<0.001 \). Furthermore, these qPCR data also showed that mRNA expression level of cytokines was increased in PBMCs from patients with moderate asthma than those in patients with mild asthma (Fig.2), \( P<0.01 \). These data was consistent with our findings through ELISA, further validating the conclusion the critical role of STAT3 in regulating the cytokines in PBMCs from patients with asthma.
ting the activation of STAT3 is resulted from cytokine application (9). The correlation between STAT3 and cytokine has been widely reported in other cell types (12, 13). The current study provides new pieces evidence to support the pro-inflammatory role of STAT3 in the asthma. Based on the results in the current study, we will focus on whether STAT3 regulation could affect the proliferation/apoptosis in PBMCs from asthma patients, considering that STAT3 and NF-κB signals are closely interactive.

In patients with moderate asthma, the expression level of p-STAT3 was significantly higher than healthy control and patients with mild asthma (Fig.3). This is the first report to show the significance of p-STAT3 in PBMCs from asthma patients. Previous study indicated that STAT3 phosphorylation level was upregulated in fibroblasts and endothelial cells with the treatment of cytokines (14), STAT3 phosphorylation was also associated with the Sema3A inhibitory effect on human airway smooth muscle cells (15). Phosphorylation of STAT3 is important in the cell proliferation (16, 17). Thus, we hypothesized that the p-STAT3 level alteration in asthma patients may impair the cell proliferation and lead to apoptosis in airway, and the involved signaling pathways are likely to include p38 MAPK (18), phosphoinositide-3-kinase, Janus kinase 2(19) and AKT (20).

In conclusion, this study elucidated the role of STAT3 in the pathogenesis of asthma. This work may facilitate us understanding the correlation between inflammation and asthma, providing new insights into the asthma therapy.

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