Activation of Signal Transducer and Activator of Transcription 5 (STAT5) in Splenocyte Proliferation of Asthma Mice Induced by Ovalbumin

Guoping Li[1, 2], Zhigang Liu[1, 3], Peixing Ran[2], Jing Qiu[1] and Nanshan Zhong[2]

To investigate the role of signal transducer and transcriptional activator 5 (STAT5) activated in ovalbumin (OVA)-induced splenocyte proliferation of asthma mice, an asthma mouse model was set up by intraperitoneal injection and aspiration of OVA with nebulizer. The proliferation of splenocytes isolated from the asthma mice was detected by [^{3}H] thymidine incorporation. The phosphorytation of STAT5 was examined by Western blotting and STAT5-DNA binding was measured by electrophoretic mobility shift assay (EMSA). OVA could pronouncedly induce the splenocyte proliferation of asthma mice in a dose-dependent manner compared with control groups. Phosphorylation of STAT5 and STAT5-DNA binding were observed in splenocytes from asthma mice induced by OVA at 1 h and 3 h. These results indicated that STAT5 signal pathway played an important role in lymphocyte proliferation of asthma mice induced by OVA. *Cellular & Molecular Immunology*. 2004;1(6):471-474.

Key Words: STAT5, OVA, splenocyte proliferation

Introduction

Asthma is increasing in prevalence around the world. An important feature of asthma is the development of airway inflammation, which is associated with the presence of several cell types such as eosinophils, neutrophils, metachromatic cells and lymphocytes. In addition, airway inflammation is related to the presence of airway hyperresponsiveness (AHR) (1). T lymphocytes are now recognized as a critical participant in airway inflammation leading to asthma. Histopathologic sections from patients with allergic asthma demonstrated peribronchial infiltration of T lymphocytes, and bronchoalveolar lavage (BAL) samples from patients with asthma contained increased numbers of activated T lymphocytes after antigen exposure. These cells were required for the development of eosinophil-rich airway inflammation, mucus production and AHR, and T lymphocyte appeared to be critically important for the events resulting in AHR (2). Waserman et al. demonstrated that the capacity of sensitized peripheral blood lymphocytes to respond to allergens may determine the magnitude of late airway responses (3). T-cell proliferative response to allergen is that it reflects an increased frequency of allergen-specific T cells. The products of allergen-activated T cells, including IL-5, may be implicated in eosinophil recruitment to the airways after allergen exposure (5). Despite these observations, there are some fundamental questions regarding the mechanism of allergen-specific T cells proliferation in the pathogenesis of asthma that remain unanswered.

The recent advances made in dissecting the roles of this signaling pathway in the pathogenesis of asthma (5). STAT proteins play a key role in the regulation of T cell growth and differentiation. STAT proteins of knockout mice either are embryonic lethal or exhibit various defects in proliferation and signal transduction. STAT 5 polypeptides typically are cytoplasmic and quiescent under homeostatic conditions. Their activation results from phosphorylation of the highly conserved C-terminal tyrosine, and permits dimer pair formation and nuclear translocation. The phosphotyrosyl "on-switch" is a generic STAT feature and is triggered when cells with cognate receptors are exposed to a variety of stimuli including cytokines, immune complexes, microbiologic agents or non-peptidyl compounds (6).

Here, our aim in this study, therefore, was to compare allergen-induced cell proliferation and STAT5 tyrosine phosphorylation and DNA-binding of STAT5 in splenocytes isolated from asthmatic mice with normal control subjects. We found that OVA induced splenocytes proliferation and accumulating tyrosine phosphorylation of STAT5 in asthma mice model. Thus, our data support that specific antigen stimulation of TCR induces tyrosine phosphorylation of STAT5 and cell proliferation, and suggest that STAT5 plays a key role in the pathogenesis of asthma.

[1]College of Life Sciences, Shenzhen University, Shenzhen, China.

[2]Guangzhou Institute of Respiratory Disease, Guangzhou Medical College, Guangzhou, China.

[3]Corresponding to: Dr. Zhigang Liu, College of Life Sciences, Shenzhen University, Shenzhen 518060, China. Tel: +86-755-265-58941, Fax: +86-755-265-58940, E-mail: lzg@szu.edu.cn.

Received for publication Oct 17, 2004. Accepted for publication Dec 21, 2004.

Materials and Methods

Experimental procedure of OVA sensitized and challenged mice

Specific pathogen-free male mice, 4-6 weeks old, were purchased from Guangzhou Medical College (Guangzhou, China). Eight BALB/c mice were sensitized by intraperitoneal injection of 20 μg of OVA emulsified in 4 mg alum on day 0. They were challenged with 20 ml nebulized OVA solution (1.0% w/v in saline), using an air-pressure nebulizer for 1 h twice on day 14. Eight mice in the control group (non-challenged group) were sensitized by intraperitoneal (*i.p.*) injection with 0.9% saline on day 0. On day 14, control mice were challenged with 20 ml 0.9% saline. The mice were killed by cervical dislocation 24 h after the last aeroallergen challenge. Lungs were taken from both nonsensitized and sensitized groups for histological analysis. Spleen was aseptically removed from mice. Single cell suspensions were obtained by mincing the spleen and gently pressing the fragments through a 100 μm stainless steel mesh. The resultant suspension was washed twice in Hanks' balanced salt solution. The splenocytes were resuspended in RPMI 1640 (GIBCO) containing 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin. The viability cells (> 98%) were assessed by typan blue dye exclusion. Cell concentration was adjusted to 2.5×10^6 cell/ml using RPMI 1640 medium.

Measurement of splenocyte proliferation

Using sterile 96-well culture plates, 2.5×10^6 cells/well were seeded in 200 μl medium. Cells were cultured for 1 day in the presence of a range of concentrations of OVA. And then 0.5 microcurie [^{3}H] thymidine in 40 μl volumes of sterile working media was added to each of the microcultures. The incubation continued for 4 h. The contents were harvested onto glass filter mats using a cell harvester. The discs representing each well were lunched from the filter mats into 5 ml volumes of scintillation fluid to measure incorporation of [^{3}H] thymidine into the cultured cells using standard scintillation counting procedures.

Detection of STAT5 phosphorytation in splenocytes by Western blotting

The cell cultures were exposed to equivalent amounts of OVA for 30 min, 1 h and 3 h. The complete cell extract was prepared by nuclear extract Kit (Activemitif, USA). The cells were harvested, washed with ice-cold PBS supplemented with phosphatase inhibitors and lysed with ice-cold complete lysis buffer (10 mM DTT, lysis buffer, protease inhibitor cocktail). The lysate was clarified by centrifugation (14,000 g, 4°C, 20 min). The protein (50 μg) from supernatant was fractionated by 10% SDS-PAGE and electrotransferred into a nitrocellulose membrane for Western blotting. The membranes were blocked with 1% BSA in washing buffer for 1 h, incubated with anti-phospho-STAT5 (Y694/699) (1:1,000, upstate, USA) for 2 h, washed with washing buffer for 3 times, incubated with HRP-labeled secondary antibody (1:2,500) for 2 h, washed with washing buffer for 3 times. Finally the membranes were detected with ECL.

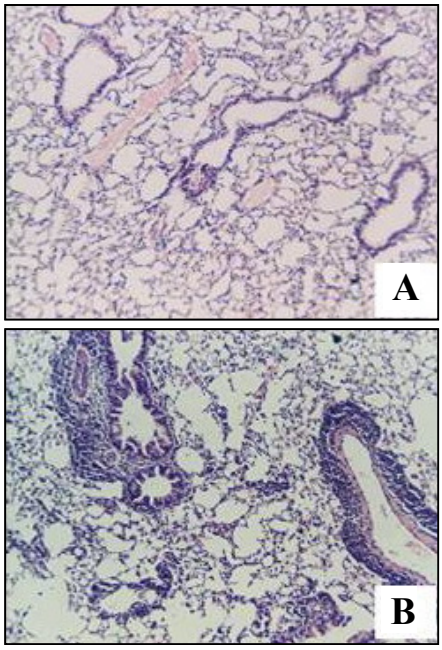

Figure 1. Severe widespread inflammatory infilltration after the OVA-challenge by the histological analysis of lung sections. (A) Control group; (B) Asthma group. Lung tissue was stained with Giemsa's solution.

EMSA for STAT5-DNA binding

The cell cultures were exposed to equivalent amounts of OVA (20 μg/ml) for 30 min, 1 h and 3 h. EMSA was used to determine the binding activity of STAT5. Splenocytes were cultured in the presence of OVA for different time periods. The nuclear extracts were prepared by nuclear extract Kit (Activemitif, USA). Immediately following stimulation, the cells were washed with ice-cold PBS supplemented with phosphatase inhibitors. Nuclear extracts were prepared from nuclear pellets after hypotonic lysis in complete lysis buffer. STAT5 gel shift oligonucleotides were labeled with terminal transferase and DIG-11-dUTP (Roche). The DNA probes were double-stranded oligonucleotides genes, 5'-AGA TTT CTA GGA ATT CAA ATC-3' (Proligo, USA). The EMSA was performed as described in GIG Gel shift Kit instruction manual (Roche). Twenty μg of nuclear extract and 1 μg of poly[d(A-T)] were added to the binding buffer and incubated for 15 min at 15-25°C. For competition assays, nuclear extracts were incubated with a 50-fold excess of unlabeled oligonucleotide over labeled one before the DIG-labeled probe addition. The reaction mixtures were run through a 6% nondenaturing polyacrylamide gel at 4°C in 0.5 × TBE buffer (90 mM Tris-borate, 2 mM EDTA). Transfer was performed by electroblotting for 60 min at 500 mA. The nylon membranes were rinsed briefly in washing buffer, and incubated for 30 min in anti-Digoxigenin-AP (1:10,000) for 30 min after blocked and equilibrated 5 min in detection buffer. The membranes were placed on hybridization bag and CSPD working solution was applied. Finally the results were performed by exposing to X-ray film for 3 h at 15-25°C.

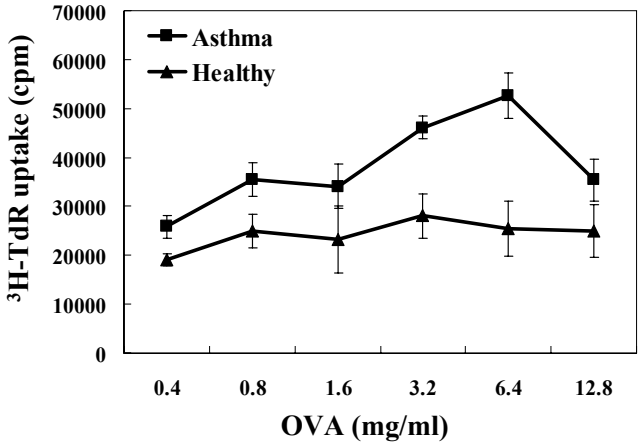

Figure 2. Proliferation of mixed spleen cells during suspension culture with increasing concentrations of OVA as measured by ^{3}H-TdR incorporation. Cells were derived from healthy mice or asthma mice. Results were presented as mean ± SD from five separate experiments (n = 5).

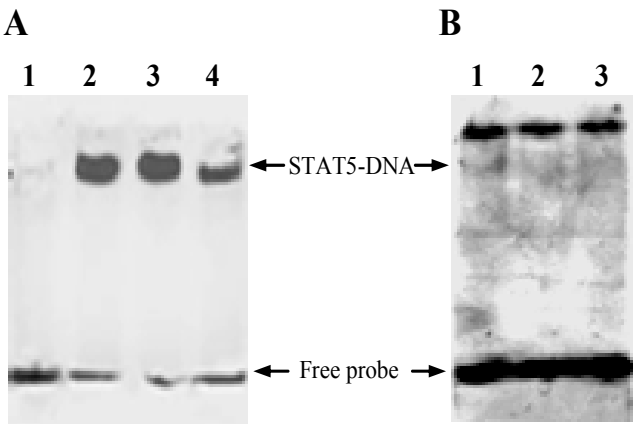

Figure 4. STAT5 activity in cultured spleen cells from healthy mice and asthma mice. (A) STAT5 binding activity was assessed by electrophoretic mobility shift assay (EMSA) in nuclear protein extracts prepared from cultured cells stimulated by OVA 3.2 mg/ml at 30 min (lane 1), 1 h (lane 2) and 3 h (lane 3). As a control, STAT5 binding activity was assessed in the same extracts by IL-2 at 1 h (lane 4). (B) Specificity of STAT5 complexes was not found by competition experiments performed with a 50-fold excess of unlabelled probe (lanes 1-3). Solid arrows indicate specific complexes.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using the Student's *t* test and values of $p < 0.05$ were considered statistically significant.

Results

Lung histology

Compared with control groups, lung sections from aerosolized OVA-challenged mice showed severe wide-spread inflammatory infilltration after the challenge (Figure 1). These infiltration localized primarily around airway walls (bronchi and bronchioli) and blood vessels. Lung tissue in OVA-challenged mice was also characterized by gross alteration in the structural integrity of the airway walls, epithelial cell shedding and extensive mucosal oedema.

Assessment of cell proliferation during coculture of splenocytes with OVA

We detected the proliferation capability of splenocytes from asthma mice. Compared with healthly mice, ^{3}H-TdR uptake in the splenocytes of asthma mice was significantly increased in response to specific OVA stimulation ($p < 0.01$). The effect was in a dose-dependent manner below 6.4 mg/ml OVA dosage. However, the stimulatory role of OVA in splenocyte proliferation of asthma mice was abolished at the dose of 12.8 mg/ml OVA (Figure 2).

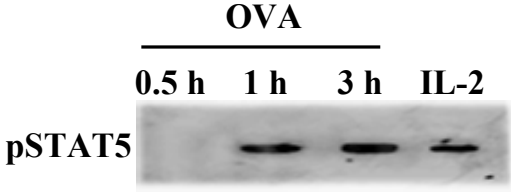

Figure 3. Detection of STAT5 phosphorylation in splenocytes by Western blotting. Cell cultures were exposed to OVA (3.2 mg/ml) for 30 min, 1 h and 3 h, or to IL-2 (20 U/ml) for 1 h. The complete lysis extracts from cultured cells was prepared and 50 μg proteins were evaluated for the presence of phosphorylated STAT5 using anti-phospho-STAT5 (Y694/699) antibody.

Identification of tyrosine phosphorylation of STAT5

To investigate the role of STAT5 activation in OVA-specific lymphocyte proliferation, Western blotting was used to detect the tyrosine phosphorylation of STAT5. As shown in Figure 3, the tyrosine phosphorylation of STAT5 was found in the cell extracts of OVA-stimulated splenocytes for 1 h, and increased in 3 h stimulation group. The phosphorylation of STAT5 was also observed in lysis extracts of IL-2-stimulated splenocytes (Figure 3).

Detection of STAT5 binding activity

In order to clarify the molecular mechanisms involved in cell proliferation stimulated by allergen in asthma, the binding of nuclear proteins isolated from both cultured cells with OVA and cultured cells with IL-2 was tested by gel mobility shift assays. STAT5-DNA binding band could be detected with nuclear extracts from OVA-stimulated splenocytes. As control, one STAT5-DNA binding band also could be found using nuclear extracts of the splenocytes which were stimulated by IL-2 at 1 h (Figure 4). Gel shift competition analysis was performed using two oligonucleotides containing STAT5-binding site. As shown in Figure 4B, both bands could be competed by a 50-fold excess of unlabeled oligonucleotides over labeled ones. The data indicated that STAT5 activation trigged by OVA in asthma, had an ability to bind DNA response element.

Discussion

Several kinds of cells play important roles in chronic inflammation of asthma. Recent studies have shown that allergen could induce inflammatory cells proliferation. These cells traffic through the circulation in the lungs in response to allergen inhalation and inflammatory cells infiltration in lung (7). In this study, we also found OVA could induce severe widespread inflammatory infiltrates. These cells mainly consisted of eosinophils, lymphocytes and monocytes. We also found that OVA-induced lymphocytes proliferation was associated with STAT5 activation.

Our data showed that OVA could pronouncedly induce the splenocytic proliferation of asthma mice in dose-dependent manner. T-cell proliferative response to allergen is that it reflects an increased frequency of allergen-specific T cells. The products of allergen-activated T cells, including IL-5, may be implicated in eosinophil recruitment to the airways after allergen exposure (4). Despite these observations, there are some fundamental questions regarding the mechanism of allergen-specific T cells proliferation in the pathogenesis of asthma that remain unanswered.

STAT5 is activated in OVA-induced splenocytic proliferation of asthma mice. Recent studies found that STAT5 was activated by many cytokines such IL-2, IL-3, IL-12, GM-CSF and IFN-γ, which induced STAT5 phosphorylation and cellular proliferation, respectively (3). It has been reported that STAT5 is an important component of the TCR complex required for optimal sensitivity of the TCR to antigen stimulation for T cell proliferation. STAT5 is specifically recruited to the TCR complex in response to T cell activation *in vivo*. Once bound by upon activation, STAT5 is released from the receptor, dimerizes, translocates to the nucleus, and binds to DNA (8). We hypothesized that STAT5 may play an important role in OVA-induced lymphocyte proliferation in asthma mice. However, it is not clear how expression of STAT5 in cell proliferation is associated with allergen and is responsible for cell proliferation in asthma.

In our study, our data showed OVA could induce STAT5 phosphorylation of splenocytes in asthma mice. This data demonstrated that allergen could induce STAT5 phosphorylation on tyrosine residues, and the tyrosine phosphorylation of STAT5 may play important roles in the regulation of cell proliferation by allergen in asthma. EMSA showed that STAT5-DNA binding was detected in asthma mice group from the nuclear extracts of splenocytes induced by OVA at 1 h and 3 h. These results indicated that STAT5 was activated in splenocytes of asthma mice, dimerized, translocated to the nucleus, and bound to DNA. Recent studies showed STAT5 could induce expression of the growth-related genes *c-myc*, *bcl-2*, and *bcl-x* through a TAD-dependent mechanism. Induction of the *c-myc* and *bcl-x* genes by STAT5 did not require *de novo* protein synthesis, suggesting that STAT5 interacts directly with the promoters of these genes (9, 10).

In this study using Western blotting, we found STAT5 was activated in OVA-induced splenocytes in asthma mice, but not in healthy mice, which was further confirmed by EMSA. Previous studies had shown that several kinds of cytokines such as IL-2, IL-3, IL-12, GM-CSF and IFN-γ could induce STAT5 phosphorylatation. We can not deduce the relationship of STAT5 activation and these related cytokines. In further studies we would detect the serum concentration of these cytokines and evaluate their roles in STAT5 activation. STAT5-deficient mice will be used to analyze the exact role of STAT5 activation in the pathogenesis of OVA-induced asthma.

Acknowledgements

This work was supported by grants from the National 863 High Technology Research and Development Program of China (No.2002AA214011) and the National Natural Science Foundation of China (No. 39860071).

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