Activation of signal transducer and activator of transcription factor 1 by interleukins-13 and -4 in cultured human bronchial smooth muscle cells

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Abstract

The family of signal transducer and activator of transcription (STAT) factors play a critical role in the signaling of many cytokines. In addition to the involvement of STAT6 in allergic bronchial asthma, both STAT1 and STAT3 have also been implicated. However, there is little information whether or not the T helper 2 cytokines, which cause several key features of allergic asthma, really induce the activation of STAT1 and/or STAT3 in bronchial smooth muscle (BSM) cells. In the present study, the effects of interleukin-13 (IL-13) and IL-4 on activation of these STAT molecules were examined in cultured human bronchial smooth muscle cells (hBSMCs). After a starvation period, the hBSMCs were treated with 100 ng/ml of IL-13 or IL-4. Total protein samples were prepared at intervals of 1, 3, 6, 12 and 24 hours after the cytokine treatment, and Western blot analyses for total and tyrosine-phosphorylated STATs molecules were conducted. As a result, it was found that both IL-13 and IL-4 caused a significant increase in the levels of phosphorylated STAT1. Examination of the time-course revealed a peak of STAT1 phosphorylation at 1 hr after cytokine application. In contrast, neither IL-13 nor IL-4 induced phosphorylation of STAT3. Neither of these cytokines changed the protein expression of the STATs themselves. These findings suggest that STAT1, but not STAT3, might also be one of the crucial signal transducers in the development of BSM hyper-responsiveness, which is one of the causes of AHR in asthmatics.

Key words: signal transducers and activators of transcription (STATs), human bronchial smooth muscle cells, bronchial smooth muscle hyper-responsiveness, interleukin-13 (IL-13), interleukin-4 (IL-4)

Introduction

The dramatic increase in the number of asthma cases over the last decade is of great concern for public health in the world (Eder et al., 2006). It has been shown that CD4+ T helper 2 lymphocytes (Th2 cells) are closely associated with the severity of the disease, suggesting that...
these cells play an integral role in the pathophysiology of allergic bronchial asthma (Bochner et al., 1994; Cohn et al., 2004; Robinson et al., 1992; Walker et al., 1991). Th2 cells secrete various cytokines, termed Th2 cytokines, which cause several key features of allergic bronchial asthma, including airway hyperresponsiveness (AHR) (Bochner et al., 1994; Cohn et al., 2004; Robinson et al., 1992; Walker et al., 1991). Increasing evidence indicates that interleukin-13 (IL-13), one of the members of the Th2 cytokine family, is a crucial mediator in the development of AHR (Grunig et al., 1998; Wills-Karp et al., 1998; Wills-Karp, 2004). In addition, another Th2 cytokine IL-4 is also believed to play a role in asthma (Bryborn et al., 2004; Dabbagh et al., 1999; Steinke and Borish, 2001). Interestingly, IL-4 shares many functional properties with IL-13, presumably because they share a common receptor composed of the IL4Ra chain as one of the two hetero chains (Callard et al., 1996).

Most of the activities of IL-13 and IL-4 can be ascribed to the activation of signal transducer and activator of transcription factor 6 (STAT6) (Chatila, 2004; Hebenstreit et al., 2006; Kuperman and Schleimer, 2008). Indeed, a critical role for STAT6 signal transduction in the development of AHR has been suggested in STAT6-deficient mice (Akimoto et al., 1998; Kuperman et al., 1998). Similarly, a cell-penetrating dominant-negative STAT6 peptide could inhibit AHR in a mouse model of allergic bronchial asthma (McCusker et al., 2007). In cultured bronchial smooth muscle cells, IL-13 can activate STAT6 directly, resulting in an upregulation of RhoA (Chiba et al., 2009a; 2009b), a crucial protein responsible for AHR (Gosens et al., 2006; Kume, 2008; Schaafsma et al., 2008). On the other hand, recent studies also indicated the implication of other members of the STAT family of molecules, such as STAT1 (Quarcoo et al., 2004) and STAT3 (Simeone-Penney et al., 2007), in the development of AHR. However, there is little information whether or not these Th2 cytokines really cause the activation of STAT1 and/or STAT3 in bronchial smooth muscle cells. In the present study, the effects of IL-13 and IL-4 on activation of these STAT molecules were examined in cultured human bronchial smooth muscle cells (hBSMCs).

**Methods**

**Cell culture and sample collection**

Normal human bronchial smooth muscle cells (hBSMCs; Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) were maintained in SmBM medium (Cambrex) supplemented with 5% fetal bovine serum, 0.5 ng/ml human epidermal growth factor (hEGF), 5 μg/ml insulin, 2 ng/ml human fibroblast growth factor-basic (hFGF-b), 50 μg/ml gentamicin and 50 ng/ml amphotericin B. Cells were maintained at 37°C in a humidified atmosphere (5% CO₂), fed every 48–72 hours, and passaged when cells reached 90–95% confluence. Then the hBSMCs (passages 5–7) were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 3,500 cells/cm² and, when 80–85% confluence was observed, cells were cultured without serum for 24 hr before addition of recombinant human IL-13 or IL-4 (each 100 ng/ml; PeproTech EC, Ltd., London, UK). At the indicated time after the cytokine treatment, cells were washed with PBS, immediately collected and disrupted with 1× SDS sample buffer (250 μl/well), and used for Western blot analyses.
Western blot analyses

The immunoblot analyses were carried out as previously described (Chiba et al., 2009a; 2009b). In brief, protein samples of the cells were subjected to 7.5% SDS-PAGE and the proteins were then electrophoretically transferred to a PVDF membrane. After blocking with 1% BlockAce™ (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), the PVDF membrane was incubated with the primary antibody. The primary antibodies used in the present study were monoclonal mouse anti-STAT1 (1:1,000 dilution; BD Biosciences, San Jose, CA, USA), anti-phospho-STAT1 (1:1,000 dilution; BD Biosciences), anti-STAT3 (1:2,500 dilution; BD Biosciences), anti-phospho-STAT3 (1:500 dilution; BD Biosciences), polyclonal rabbit anti-STAT6 (1:1,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-phospho-STAT6 (1:1,000 dilution; Santa Cruz Biotechnology, Inc.) antibodies. Then the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG or anti-mouse IgG (1:2,500 dilution; Amersham Biosciences, Co., Piscataway, NJ, USA), detected by an enhanced chemiluminescent system (Amersham Biosciences, Co.), and analyzed by a densitometry system. Detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was also performed on the same membrane by using monoclonal mouse anti-GAPDH (1:10,000 dilution; Chemicon International, Inc., Temecula, CA, USA) to confirm the same amount of proteins loaded.

Statistical analyses

All the data were expressed as the mean with S.E. Statistical significance of difference was determined by two-way analysis of variance (ANOVA) using the post hoc Bonferroni/Dunn test (StatView for Macintosh ver. 5.0, SAS Institute, Inc., Cary, NC, USA). A value of P<0.05 was considered to be significant.

Results

STAT1 phosphorylation induced by IL-13 in cultured hBSMCs

We have previously demonstrated that treatment with IL-13 (100 ng/ml) caused a rapid activation of STAT6 in the hBSMCs (Chiba et al., 2009a; 2009b). Using identical experimental conditions in the present study, the activation of STAT1 and STAT3 in the IL-13-stimulated hBSMCs was determined by measuring the phosphorylated products of these proteins. As shown in Fig. 1A, a distinct increase in the level of phosphorylated STAT1 was observed by IL-13 stimulation. Examination of the time course revealed a peak of STAT1 phosphorylation at 1 hr after the application of IL-13 (Fig. 1, A and B). The IL-13 stimulation did not alter the expression of the STAT1 protein itself (Fig. 1C). On the other hand, however, no significant increase in the level of phosphorylated STAT3 was observed by the IL-13 stimulation in the hBSMCs (Fig. 2).

STAT1 phosphorylation induced by IL-4 in cultured hBSMCs

Next, the effect of IL-4 on the activation of these STAT molecules was subjected to immunoblot analysis. As was the case for IL-13, a distinct phosphorylation of STAT1 was also
induced by the IL-4 stimulation (Fig. 3, A and B). A peak of the STAT1 phosphorylation was observed at 1 hour after the application of IL-4 (Fig. 3B). The IL-4 stimulation did not affect the expression of the STAT1 protein itself (Fig. 3C). As well as IL-13, no significant increase in the level of phosphorylated STAT3 was observed by IL-4 stimulation of the hBSMCs (Fig. 4).

Discussion

The current study clearly demonstrates that STAT1 is rapidly activated by IL-13 and IL-4 in cultured hBSMCs. Both IL-13 and IL-4 are upregulated in airways during allergic bronchial asthma and have been suggested to play a critical role in the development of AHR (Bryborn et
STAT1 activation by ILs-13/-4 in hBSMCs  

It is thus possible that activation of STAT1 in BSMCs themselves might be involved in the development of BSM hyperresponsiveness, which is one of the causes of AHR in asthmatics.

Airway smooth muscle is an important effector tissue regulating bronchomotor tone. It has been suggested that one of the factors that contribute to AHR is an abnormality in the properties of airway smooth muscle (Martin 

Fig. 2. The time-course of change in the level of phosphorylated signal transducer and activator of transcription factor 3 (STAT3) induced by interleukin-13 (IL-13) in cultured human bronchial smooth muscle cells. Cells were incubated with IL-13 (100 ng/ml) for the indicated time, and total protein samples of the cells were subjected to immunoblot analyses. (A) Typical immunoblots of phosphorylated STAT3 (pSTAT3; upper), total STAT3 (middle) and GAPDH (lower). The levels of pSTAT3/STAT3 (B) and STAT3/GAPDH (C) were calculated. Each column represents the mean ± S.E. from 3 independent experiments.
Schleimer, 2008), is a key in the development of AHR in allergic bronchial asthma. In allergic asthmatics, the level of phosphorylated STAT6 was increased in peripheral CD4+ CD161+ T cells (Gernez et al., 2007). An increase in phosphorylated STAT6 in the airways was also observed after allergen inhalation in atopic asthma (Phipps et al., 2004). In mouse models of allergic bronchial asthma, development of AHR was inhibited by STAT6 gene knockout (Akimoto et al., 1998; Kuperman et al., 1998) and by treatment with a STAT6 inhibitory peptide (McCusker et al., 2007), with a short interfering RNA against STAT6 (Darcan-Nicolaisen et al., 2009), or by a selective STAT6 inhibitor (Chiba et al., 2009b).

In addition to the phosphorylation of STAT6 (Chiba et al., 2009a; 2009b), a significant
The time-course of change in the level of phosphorylated STAT3 induced by interleukin-4 (IL-4) in cultured human bronchial smooth muscle cells. Cells were incubated with IL-4 (100 ng/ml) for the indicated time, and total protein samples of the cells were subjected to immunoblot analyses. (A) Typical immunoblots of phosphorylated STAT3 (pSTAT3; upper), total STAT3 (middle) and GAPDH (lower). The levels of pSTAT3/STAT3 (B) and STAT3/GAPDH (C) were calculated. Each column represents the mean ± S.E. from 3 independent experiments.

Fig. 4. The time-course of change in the level of phosphorylated signal transducer and activator of transcription factor 3 (STAT3) induced by interleukin-4 (IL-4) in cultured human bronchial smooth muscle cells. A recent suggestion has implicated STAT1 in the pathogenesis of allergic airway diseases as in allergic asthmatics, as an increase in the level of phosphorylated STAT1 was observed in airway structural cells (Sampath et al., 1999). In a mouse model of allergic bronchial asthma, the inhibition of STAT1 by local application of decoy oligonucleotide into airways could attenuate antigen-induced AHR (Quarcoo et al., 2004). More recently, Hattori et al. (2007) found that STAT1-deficient mice failed to develop the hyperresponsiveness of nasal airways that is usually induced by topical antigen challenge. In contrast, however, a recent study raised the possibility that activated STAT1 may have the
ability to inhibit the expression of RhoA (Wang and Koromilas, 2009), an important protein responsible for BSM hyperresponsiveness (Chiba et al., 1999; 2005; 2008; 2009a; 2009b; Gosens et al., 2006; Kume, 2008; Schaafsma et al., 2008). Further studies are required to make clear the role of STAT1 in the development of BSM hyperresponsiveness.

Recently, it has also been suggested that STAT3 is implicated in the development of AHR in a mouse model of allergic bronchial asthma (Simeone-Penney et al., 2007). In the present study, however, neither IL-13 nor IL-4 caused phosphorylation of STAT3 in cultured hBSMCs (Figs. 2 and 4). Our findings indicate that STAT3 is not activated in hBSMCs, at least by these Th2 cytokines. It cannot be denied however that activation of STAT3 in airway cells other than BSMCs plays a role in the pathogenesis of allergic bronchial asthma.

In conclusion, the current study clearly shows that in cultured hBSMCs, STAT1 is phosphorylated directly by Th2 cytokines, IL-13 and IL-4. In addition to the importance of STAT6, the activation of STAT1 might thus also be involved in the development of BSM hyperresponsiveness, which is one of the causes of AHR in allergic bronchial asthma.

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