Effect of Dexamethasone on STAT6-Dependent Ym1/2 Expression

in Vivo and in Vitro

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Asthma is one of the most common chronic diseases affecting children and adults and the incidence of asthma in developed countries is increasing.2) Allergic asthma is characterized by airway hyperresponsiveness (AHR) associated with an elevated serum IgE level and bronchial inflammation. Numerous eosinophils and Th2 lymphocytes infiltrate into the airways of asthmatics and cytokines such as IL-4, via Th2 cytokine production, Th2 cell differentiation, and in cells, indicating that the inhibition of Ym1/2 expression was not a direct effect of Dex.

Ym1 and Ym2 (Ym1/2) are chitinase-like proteins and we reported previously that IL-4 induced Ym1/2 in mouse bone marrow-derived mast cells. In the present study, ovalbumin-induced asthmatic mice were used to investigate the effect of glucocorticoids on Ym1/2 expression. Ym1/2 were highly induced in bronchoalveolar lavage fluid (BALF) and the lung. Ym1/2 expression was completely inhibited by dexamethasone (Dex) in BALF and weakly inhibited in the lung. Primary cultured macrophages were used to investigate the inhibition of Ym1/2 expression at the cellular level. Although Dex pretreatment inhibited the Ym1/2 expression level in an animal model, it did not reduce IL-4 induction of Ym1/2 expression in vitro. Next, we tested whether Dex blocks IL-4 induced STAT6 signaling and found that it had no inhibitory effect on the phosphorylation level of STAT6 in macrophages. The luciferase reporter assay also revealed that Dex did not inhibit IL-4 induction of Ym1/2 promoter activity. These results indicate that the inhibitory effect of Dex on Ym1/2 protein expression in the murine model of asthma does not involve the STAT6 signaling pathway.

Key words Ym1; Ym2; bronchoalveolar lavage fluid; dexamethasone; asthma

MATERIALS AND METHODS

Cell Culture and Reagents The mouse lung fibroblast cell line MLg was obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in complete medium (RPMI 1640 supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% FBS). Ovalbumin (OVA) and dexamethasone (Dex) were purchased from Sigma (St. Louis, MO, U.S.A.). RPMI 1640 and antibiotics were obtained from Invitrogen (Carlsbad, CA, U.S.A.). FBS was purchased from HyClone (Logan, UT, U.S.A.). Goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Ab) was obtained from Bio-Rad (Hercules, CA, U.S.A.). Recombinant mouse (rm) IL-4 was obtained from their expression in the baculovirus system, as described previously.4) The concentration of IL-4 was determined by the Bank for Cytokine Research, Chonbuk National University (Jeonju, Korea). RmIL-13 was purchased from R&D Systems (Minneapolis, MN, U.S.A.). Anti-Ym1/2 polyclonal Ab (1:500 dilution) was kindly provided by Dr. Shioko Kimura of NCI, National Institute of Health, MD.11) Anti-STAT6 and anti-phosphorylated STAT6 were purchased from Cell Signaling (Beverly, MA, U.S.A.). Alum (Imject® Alum) was obtained from Pierce Biotechnology (Rockford, IL, U.S.A.)

Allergen Sensitization/Challenge Protocol Six week old female BALB/c mice (Hyochang Science, Daegu, Korea) were used. Antigen sensitization, challenge, and treatment were performed as previously described.15)

Reverse Transcription (RT)-PCR and Northern Blot Analysis Total RNA from mouse lung and MLg cells were extracted with TRIZOL® reagent (Invitrogen) according to the manufacturer’s instructions. First strand cDNA was generated from 1 µg of total RNA using an RNA PCR kit (Takara, Kyoto, Japan) and amplified. Primers used were as follows: Ym1 primers, 5’-AGA ATG AGA TCA CTT
ACA CAC A-3’ and 5’-AAT GTC TTT CTC CAC AGA CTT-3’; Ym2 primers, 5’-GAG GAA GAA TCC ACT TTG AAC C-3’ and 5’-GTC CAG CAC TAA CAG TAG GGT C-3’. PCR amplifications were performed for 35—40 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 55—60°C for 30 s, and extension at 72°C for 30 s. For Northern blot analysis, 30 μg of total RNA from mouse lung was separated by gel electrophoresis and transferred to a nylon membrane (Schleicher & Schuell, Keene, NH, U.S.A.). Probe was generated by RT-PCR using the primers 5’-CTG ATC TAT GCC TTT GCT GG-3’ and 5’-CAG AGA TTC TTC CTC AAA AGC T-3’. The labeling and hybridization were performed as previously described.15

**SDS-PAGE and Western Blot Analysis** Centrifuged cells were resuspended and lung tissues were homogenized in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) and centrifuged. Supernatants were used as whole cell lysates. Protein concentration was quantified with BCA protein assay reagent (Pierce Biotechnology) using bovine serum albumin as the standard. Western blotting was performed as previously described.7

**Preparation and Activation of Macrophages** Thioglycollate-elicited mouse peritoneal macrophages were obtained as previously described.16 The harvested peritoneal macrophages were plated into 6-well culture plates at 2×10⁶ cells per well. Alveolar macrophages from bronchoalveolar lavage fluid (BALF) of normal mice were collected as described above and plated into 24-well plates at 3.5×10⁵ cells per well. Following 2—3 h adherence at 37°C, the non-adherent cells were removed. The macrophages were activated by treatment with IL-4 (20 ng/ml) in complete medium for 18 h.

**Plasmid Construct, Transient Transfection and Luciferase Activity Assays** The promoter regions of Ym1 and Ym2 were amplified by PCR using pairs of primers (forward primers, 5’-CTG TCA CTG AGT TTG TAG CTT CC-3’ for Ym1 and 5’-CTG AGT GTG TGC TTC AAT TTA TCA TC-3’ for Ym2; reverse primer, 5’-TTG TGT CTG CAG GAT TGC TTC-3’) and ligated with KpnI and XhoI sites of pGL2 basic vector. MLg cells were seeded into 6-well plates (2×10⁵) and allowed to grow to 50—70% confluence. Cells were transiently transfected with the Ym1 or Ym2 construct using LipofectAMINE reagent (Invitrogen) according to the manufacturer’s protocol. After transfection, cells were incubated in complete media for 24 h at 37°C, and stimulated with cytokines. In some experiments, cells were preincubated with various concentrations of Dex for 1 h at 37°C prior to cytokine stimulation. Cell lysates were assayed for luciferase activity using a luminometer (Promega) according to the manufacturer’s protocol.

**Quantification of Cytokine** Spleen cells from control (OVA sensitization and no-challenge), asthmatic and Dex treated mice were isolated and suspended in complete culture medium. Cells (4×10⁵/ml/well) were cultured in 24-well plates in the presence of OVA (50 μg/ml). Supernatants were collected after 72 h of culture. The levels of IL-4 and IL-13 in spleen culture supernatants were determined by ELISA according to the manufacturer’s protocols (Biosource, Camarillo, CA, U.S.A.).

**Statistical Analysis** All values are expressed as the mean±S.D. One-way ANOVA was used to determine the statistical significance.

**RESULTS AND DISCUSSION**

**Dex Decreased Induced Expression of Ym1/2 in Lung and BALF in OVA-Challenged Mice** After verifying the murine asthma model, the total RNA from the mouse lung tissues was subjected to Northern blot (Fig. 1A) and RT-PCR (Fig. 1B) analysis in order to confirm the effect of Dex on Ym1/2 mRNA expression. As shown in Fig. 1A, Ym1/2 mRNA levels were significantly increased in the lung of OVA-induced mice compared to controls and Dex reduced the transcript levels of Ym1/2. The levels of Ym1 and Ym2 were also analyzed by RT-PCR using the specific primers because they have a high sequencing homology and can not be differentiated by Northern blot analysis. The Ym1 mRNA was constitutively expressed in the lungs of control mice and increased by OVA challenge whereas the Ym2 gene was absent and markedly induced in the lungs of asthmatic mice (Fig. 1B). Dex treatment of the OVA-challenged mice decreased Ym1 and Ym2 mRNA expression in the lung tissues. The protein expression of Ym1/2 in the BALF and lung from OVA-induced asthmatic mice was examined. As shown in Fig. 1C, induction of Ym1/2 protein from BALF of asthmatic mice was completely inhibited by Dex treatment. In contrast, Dex treatment only slightly decreased Ym1/2 expression in lung tissue (Fig. 1D). Based on published papers, we assume that the Ym1/2 mRNA and proteins are mainly expressed by lung macrophages.

**Ym1/2 Expression Was Not Reduced by Dex Treatment**

![Fig. 1. Expression of Ym1/2 in Lung and BALF](image-url)

(A) 30 μg of total RNA isolated from lungs of mice (n=5 mice/group, +/-; sensitized and challenged with PBS —/+; sensitized and challenged with OVA +/+; sensitized, challenged with OVA and treated with Dex) was used for electrophoresis and Ym1/2 mRNA was detected by Northern blot analysis. Sample loading in each lane was verified by visualizing the ribosomal RNA. Representative data from two independent experiments are shown. (B) 1 μg of total RNA from lungs of mice was reverse-transcribed into complementary DNA and PCR was performed. The specific PCR primers were designed to distinguish the Ym1 and Ym2 gene in the RT-PCR experiment. β-Actin was used as a control for equality of all RNA preparations used for these experiments. The results are representative of 3 independent experiments. (C) BALF was collected 18 h after the last OVA challenge and analyzed for protein expression. Each panel represents BALF from a different mouse. (D) The lungs were removed and homogenized in cold lysis buffer. The supernatant was quantified and protein was separated by SDS-PAGE and probed with Ym1/2 Ab (1:500 dilution). The membrane was stripped for reprobing with an anti-actin Ab to verify the equal loading of the protein in each lane. Densitometric bands of was determined with a Kodak Gel Logic 100 Imaging System. A positive control (second lane) divided by β-actin was set at 1.0 and densitometric values were compared to it.
in Macrophages. We evaluated the effect of Dex on Ym1/2 expression using primary cultured macrophages in vitro. When peritoneal macrophages were pretreated with Dex for 1 h and stimulated with IL-4 for 18 h, RT-PCR analysis revealed that the Ym1 transcript level was not changed while Ym2 mRNA was slightly affected by Dex pretreatment (Fig. 2A). The protein expression level of Ym1/2 in peritoneal (Fig. 2B) and alveolar macrophages (Fig. 2C) was not decreased by Dex treatment. These data demonstrated that oral administration of Dex significantly inhibited the increased Ym1/2 expression in the BALF and lung of OVA-challenged mice. However, the increased expression of Ym1/2 upon IL-4 stimulation in vitro using cells was not affected by Dex pretreatment. The increased Ym1/2 protein by IL-4 stimulation (Fig. 2B) may be mostly due to Ym1 upregulation in peritoneal macrophages because the level of Ym2 mRNA was much weaker than with Ym1 transcript (Fig. 2A). In alveolar macrophages, two different bands were detected with Ym1/2 polyclonal Ab. We speculated that Ym1 is mainly expressed in peritoneal macrophages by IL-4 stimulation. In alveolar macrophages, however, Ym1 protein is constitutively expressed and increased by IL-4 whereas Ym2 protein is only induced in the presence of IL-4.

Dex Did Not Repress IL-4-Induced Phospho-STAT6 and Ym1/2 Promoter Activities. STAT6 acts as a signal transducer immediately downstream of IL-4 receptor activation, leading to Ym1/2 expression. Therefore, we analyzed the effects of Dex on the levels of STAT6 and phosphorylated STAT6. Western blot analysis using whole cell lysates showed that there was no change in the levels of cellular STAT6 in the peritoneal macrophages (Fig. 3A). To characterize the molecular mechanisms underlying the transcriptional response of Ym1/2 to IL-4, we investigated whether Dex would repress the activity of Ym1/2 promoter. MLg cells were transfected with Ym1 or Ym2 construct, stimulated with IL-4 after pretreatment with Dex, and then luciferase activity was measured. As shown in Fig. 3C, IL-4 activated the promoter and Dex did not inhibit this induction of the Ym1 promoter. Similar results were obtained when experiments with Ym2 reporter gene assays were performed. These data suggest that decreased Ym1/2 expression in the lung of the asthmatic mouse by Dex pretreatment is not due to the inhibition of STAT6 phosphorylation or transcriptional repression. The ability of glucocorticoids to regulate IL-4-dependent STAT6 activation is controversial. In contrast to our result that Dex had no effect on STAT6 phosphorylation in MLg cells, other studies have demonstrated that glucocorticoids suppress IL-4-induced STAT6 activation.17,18 How-

![Fig. 2. Effect of Dex on IL-4-Induced Ym1/2 Expression](image)

(A, B) Thioglycollate-elicited peritoneal macrophages or (C) alveolar macrophages were pretreated with the indicated concentrations of Dex for 1 h and cultured without or with IL-4 for 18 h. (A) Total RNA was extracted for the detection of Ym1 and Ym2 by RT-PCR analysis. (B, C) Western blot analysis was performed to detect Ym1/2 expression. Representative data from 3 repetitive experiments are shown.

![Fig. 3. Effect of Dex on STAT6 Tyrosine Phosphorylation and Transcription from Ym1 and Ym2 Promoter](image)

(A) Peritoneal macrophages were prepared and stimulated with IL-4 for 30 min after pretreatment with the indicated concentrations of Dex. Whole cell extracts were used for Western blot analysis. (B) Structures of Ym1 and Ym2 promoter luciferase constructs. (C) MLg cells were transiently transfected with the Ym1 or Ym2 promoter reporter construct illustrated in A. The cells were pretreated with Dex or DMSO (□) for 1 h and then stimulated for 24 h with IL-4 or left untreated as a control. The luciferase activity in cell lysates was determined in samples either treated with IL-4 alone (■) or with Dex and IL-4 (□). Responsiveness was calculated as the ratio of luciferase activity of IL-4-stimulated over unstimulated cells. Data are shown as the mean±S.D. *p<0.05, NS (p>0.05).

![Fig. 4. Cytokine Measurement](image)

Cell suspensions prepared from spleens of negative, OVA-challenged, or Dex-treated mice were cultured with OVA (50 μg/ml) and cultured splenocyte supernatant was collected after 72 h to determine the IL-4 and IL-13 levels. Data are shown as the mean±S.D. of each group of mice (n=5 mice/group). Asterisk denotes statistically significant difference from culture with Dex and IL-4 treatment. **p<0.01.
ever, two published papers support our results. Franchimont et al., found that STAT6 phosphorylation of T lymphocytes was not inhibited by Dex treatment. A recent study also showed that glucocorticoids such as budesonide did not interfere in the phosphorylation of STAT6 induced by IL-4 in the human airway epithelial cell line, BEAS-2B.

**Th2 Cytokine Level from Splenocyte Supernatant Was Markedly Decreased by Dex Treatment** To determine the effect of Dex on T cell responses, we measured cytokine levels in supernatant of spleens (IL-4 and IL-13). Figure 4 shows that cytokine levels were markedly increased in cultures from OVA-challenged mice compared to controls, indicating a Th2 response. However, IL-4 production and IL-13 production were abolished in supernatants of spleen from Dex-treated mice. From this result, we speculate that Dex treatment in the mouse model of asthma may cause T cell alteration to decrease the production of cytokines such as IL-4 and IL-13, which leads to the decreased Ym1/2 expression. Our findings suggest that STAT6 is not the central target of Dex in the allergic response despite the potent inhibitory effect of the glucocorticoid on STAT6-induced genes in the present animal model.

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**REFERENCES AND NOTES**

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