

Proteome analysis of differential protein expression in allergen-induced asthmatic mice lung after dexamethasone treatment

Gu Seob Roh^{*1}, YoungHyun Shin^{*1}, Seong-Won Seo¹, Bo Ra Yoon¹, SinKoo Yeo¹, Sung Jin Park², Jin Won Cho² and KyuBum Kwack¹

¹National Genome Research Institute, National Institute of Health, Seoul, Korea

²Department of Biology, Protein Network Research Center, Yonsei University, Seoul, Korea

Asthma has become substantially more prevalent in recent decades and is one of the foremost contributors to morbidity and mortality in industrialized countries. Corticosteroids are among the most effective medications for the treatment of asthma, but some patients do not respond well to corticosteroid treatment. In this study, we characterized the responses to an allergen and identified potential molecular targets of dexamethasone (Dex) treatment in acute asthma. Female BALB/c mice sensitized to ovalbumin (OVA) were challenged with aerosolized OVA for 1 week. During the challenge period, mice were treated daily with Dex by intraperitoneal injection. Phosphate-buffered saline treated and non-challenged mice served as control. Histological evaluation of OVA-induced mice revealed airway inflammation and goblet cell hyperplasia. In addition, interleukin 4 levels and interferon- γ levels were increased and decreased, respectively. These changes were moderated by Dex treatment. Protein expression profiles were compared in each experimental group by two-dimensional gel electrophoresis and identified using matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry. Some proteins were increased, while others were decreased by Dex treatment. These results indicated that the regulation of protein expression might play a role in the immunological and pathological development of asthma and could be targeted for therapeutic intervention. These results may assist in the development of quantitative diagnostic markers to monitor disease progression or responses to therapy using proteomic approaches.

Keywords: Asthma / Dexamethasone / Matrix-assisted laser desorption/ionization-time of flight / Mice / Two-dimensional gel electrophoresis

Received: March 31, 2004; revised: May 31, 2004; accepted: June 16, 2004

1 Introduction

Asthma is a chronic inflammatory disease characterized by airway inflammation, goblet cell hyperplasia with mucus hypersecretion, and hyper-responsiveness to various nonspecific stimuli [1]. Corticosteroids are currently

the most potent anti-inflammatory agents used to treat chronic inflammatory diseases such as asthma [2, 3]. At the cellular level, corticosteroids significantly decrease the accumulation and activity of eosinophils, lymphocytes, macrophages, and other cell types [4]. Recruitment of eosinophils to the airways occurs in asthma, and the degree of eosinophilia is correlated with the severity of asthma [5]. Corticosteroids potently inhibit airway eosinophilia [6], and synthesis of inflammatory mediators [7]. Corticosteroids prevent airway hyper-reactivity and inhibit mucus secretion by suppressing the inflammatory process in antigen-challenged animals and in humans with asthma [8, 9].

Correspondence: Dr. KyuBum Kwack, National Genome Research Institute, National Institute of Health, 5 Nokbun-dong, Eunpyung-gu, Seoul 122-701, Korea

E-mail: kkwack@nih.go.kr

Fax: +82-2-354-1063

Abbreviations: ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; Dex, dexamethasone; ECM, extracellular matrix; EH, EspIS-homology; H&E, hematoxylin and eosin; IFN, interferon; IL, interleukin; OVA, ovalbumin; PAS, periodic acid-Schiff

* These authors contributed equally.

Although most patients with asthma respond to corticosteroid treatment, small subsets of patients demonstrate persistent tissue inflammation despite treatment with high doses. Also, these treatments impart only symptomatic relief in the early stage of asthma. Patients with chronic asthma who take these drugs for a long and indefinite period of time may take alternative drugs, as well as develop serious side effects [10, 11]. This corticosteroid resistance or failure results from interaction between susceptibility genes and the host's environment, in addition to immunologic factors. A systemic approach must rule out underlying conditions that lead to resistance or failure and alternative strategies must be employed to inhibit tissue inflammation. The exact therapeutic mechanism remains a matter of controversy. Therefore, we employed a proteomics approach to better understand the molecular basis of this pharmacology.

In this study, BALB/c mice sensitized and challenged to ovalbumin (OVA) served as a murine model of acute asthma. To investigate the effects of the corticosteroid dexamethasone (Dex) on airway inflammation and goblet cell hyperplasia, we measured the levels of total IgE in serum and cytokines in bronchoalveolar lavage fluid (BALF), respectively. We also evaluated the histologic effects of Dex on airway inflammation and goblet cell hyperplasia with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stains. Therefore, we compared protein expression profiles among experimental groups to detect these changes in protein expression. Lung protein samples from PBS- and Dex-treated mice were analyzed by 2-DE. The proteins were digested by trypsin, and then identified by MALDI-TOF/TOF MS.

2 Materials and methods

2.1 Animals

Female BALB/c mice (5 weeks old) purchased from Japan SLC (Hamamatsu, Japan) were maintained in the animal facility at the National Genome Research Institute of Korea National Institute of Health. All mice were provided water and standard chow *ad libitum* for 1 week prior to any experimental procedures to allow them to acclimatize to their new surroundings.

2.2 Antigen sensitization, challenge, and treatment

Mice were sensitized on days 0 and 6 by intraperitoneal injection of 20 µg chicken egg OVA (grade V, Sigma, St. Louis, MO, USA) emulsified in 1 mg of aluminum hydroxide (Pierce, Rockford, IL, USA) in a total volume of 200 µL. Two weeks after the last sensitization, mice were chal-

lenged with an aerosol of 1% w/v OVA in saline using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) for 30 min/day, 3 days/week for 1 week. Dex (Sigma)-treated mice were administered daily intraperitoneal injections of 5.0 mg/kg. Sham-treated mice were intraperitoneally administered PBS. Sensitized and non-challenged mice served as controls. Experimental groups were sacrificed 48 h after the last exposure.

2.3 Sample preparation

Mice were anesthetized by intraperitoneal administration of pentobarbital sodium (100 mg/kg) 48 h after the last challenge and then bled by retroorbital puncture using heparinized capillary tubes. Blood samples were centrifuged and plasma was stored at -70°C until used. After removal of blood, mice were used either for BALF ($n = 4$) or for tissue collection ($n = 4$). The BALF was performed for measurement of cytokines. The tracheas of mice were cannulated and lavaged with two 0.8 mL aliquots of PBS. The BALF was centrifuged and supernatants were collected and stored at -70°C until used. For protein extraction, right lungs were isolated and frozen in liquid nitrogen and stored at -70°C . Lung tissues were cut into slices and these slices were transferred to sterile 1.5 mL microcentrifuge tubes containing 400 µL of sample buffer (7 M urea, 2 M thiourea, 4% w/v Triton X-100, 25 mM DTT, 0.5% w/v IPG buffer, protease inhibitor cocktail). These mixtures were homogenized using disposable pestles attached to a Pellet Pestle Motor (Fisher Scientific, Pittsburgh, PA, USA) [12]. Homogenized tissues were incubated for 1 h at 4°C , then sonicated 10 times at the interval setting 0.3. Next, samples were centrifuged for 20 min at 14 000 rpm at 4°C . Supernatants of each group were pooled and transferred to clean vials. Protein concentrations were quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) and stored at -70°C . For histologic examination, left lungs were slowly perfused with 4% w/v paraformaldehyde and immersed in fixative solution overnight. Lungs were embedded in paraffin, and 5 µm sections were stained with H&E and PAS. The slides were evaluated by microscopy. Section analysis for PAS staining was performed in a blind fashion using a quantitative scoring system (0 to 4), where 0 = no epithelial staining, 1 = slight epithelial staining, 2 = moderate epithelial staining, 3 = heavy epithelial staining, and 4 = massive epithelial staining.

2.4 Measurement of total IgE, IL-4, and IFN- γ

Total serum IgE and BALF cytokines interleukin 4 (IL-4) and interferon-gamma (IFN- γ) from each experimental group were measured by standardized sandwich ELISA according to the manufacturer's directions.

2.5 Protein separation and visualization

Protein (400 µg) was applied to a 24 cm Immobiline Dry-Strip, pH 4–7 (Amersham Biosciences, Roosendaal, Netherlands). Strips were rehydrated for 12 h at 30 V, followed by focusing for; 1 h at 100 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 5000 V and subsequently at 8000 V to 140 kVh on an IPGPhor (Amersham Biosciences) and stored at –70°C. Focused IPG strips were equilibrated twice for 10 min with gentle shaking in equilibration buffer containing 50 mM Tris-HCl buffer, pH 8.8, 6 M urea, 20% v/v glycerol, 0.1% w/v SDS and 1% w/v DTT. In the second equilibration buffer, DTT was replaced with 2.5% w/v iodoacetamide in order to remove excess DTT which causes point streaking in silver-stained patterns [12]. The equilibrated IPG strips were gently rinsed with distilled water, blotted to remove excess equilibration buffer and then applied onto a 12.5%T polyacrylamide gradient gel (26 × 20 cm). The second dimension separation was performed sequentially with a constant voltage of 70 V for 1 h, 150 V for 2 h, and 400 V for 6 h using the Ettan DALT II system (Amersham Biosciences) [13]. After SDS-PAGE, the separated gels were visualized by silver staining according to the kit procedures (PlusOne™ silver staining kit, Amersham Biosciences).

2.6 Image analysis and protein identification

Gels were scanned on ImageScanner (Amersham Biosciences). Images of scanned gels were analyzed using Progenesis Workstation (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). To remove silver ions, in-gel digestion and peptide extraction were carried out as described by Gharahaghi *et al.* and Joo *et al.* [14, 15]. The proteins of interest were excised from the 2-D gels and destained for 10 min in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. They were washed three times for 5 min with distilled water and then dehydrated repeatedly in 100% ACN until they turned opaque white. The gel fragments were dried in a speed vacuum concentrator (Heto-Holten, Camberley, UK) for approximately 1 h. Destained gel fragments were incubated with 5–10 ng/mL of trypsin (Promega) and 50 mM ammonium bicarbonate overnight at 37°C, and then peptides were collected by sequential extraction with 20 µL of 5% v/v TFA/50% v/v ACN. Finally, the peptide extracts were dried by a speed vacuum concentrator for approximately 4 h. After removal of ACN in a vacuum centrifuge, peptides were concentrated and desalted using ZipTip_{µ-C18} (Millipore, Bedford, MA, USA), and eluted directly on the MALDI-target with 2 µL of a saturated solution of CHCA in 50% v/v ACN/0.5%

v/v TFA. Peptides were analyzed using a MALDI-TOF MS (Voyager-DE STR, Applied Biosystems) in reflection mode with delayed extraction. Mass calibration was performed using trypsin autodigestion products (internal calibration, 842.5099 Da and 2211.1046 Da) and calibration mixture 2 (Sequazyme™ Peptide Mass Standards Kit, PE Biosystems, Foster City, CA, USA). When peptides from autolytic degradation of trypsin were detected, an internal calibration of the spectra was performed. For MALDI peptide mapping, Mascot (www.matrixscience.com) search engines were employed. The Swiss-Prot (us.expasy.org) and NCBItr (www.ncbi.nlm.nih.gov) databases were searched. A MALDI-TOF/TOF tandem mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems) in reflectron mode with delayed extraction was also used to perform MS/MS of selected peptide ions from the MALDI spectra acquired for peptide mass fingerprint analysis. For protein identification, the mass spectra were saved in Mascot generic file format, and submitted to the Mascot database search engine.

2.7 Statistical analysis

Values are expressed as the mean ± SEM. Differences between the groups, control, PBS-, and Dex-treated, were determined by analysis of variance (ANOVA), followed by Bonferroni's method for all pairwise multiple comparisons. A *p* value < 0.05 was considered statistically significant.

3 Results

3.1 Effect of Dex on total IgE in serum and cytokines in BALF

Levels of total IgE and cytokines were determined by ELISA in each experimental group 48 h after the last challenge. Total serum IgE levels in PBS-treated mice were significantly higher than levels in control mice. Increased total IgE levels in the PBS-treated mice were reduced by Dex treatment (Fig. 1A). This result demonstrates that the corticosteroid Dex affects serum IgE concentrations in acute asthma resulting from acute allergen exposure. To detect a possible effect of Dex on T-cell responses, we evaluated IL-4 and IFN-γ levels in BALF. In accordance with total IgE, increased IL-4 levels in the PBS-treated mice were reduced by Dex treatment (Fig. 1B). However, levels of IFN-γ did not differ significantly between experimental groups. This result demonstrates that Dex suppresses the Th2 response.

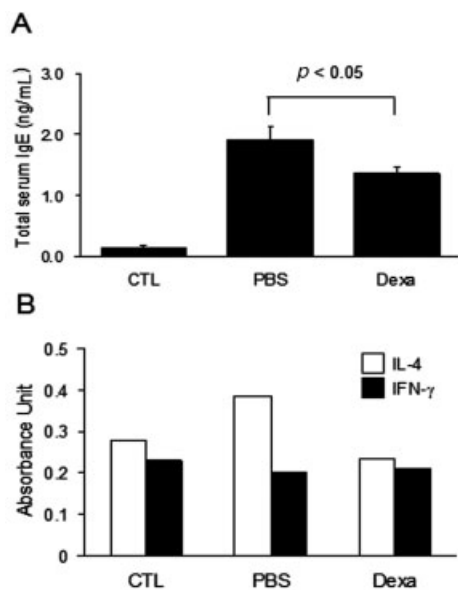


Figure 1. Effect of Dex on total IgE in serum and cytokines in BALF. (A) Total plasma IgE was measured 48 h after the last challenge using ELISA. Data are represented as mean $\pm$ SEM from seven to eight individual mice in each group. (B) IL-4 and IFN- γ were measured using ELISA. Data are represented as absorbance unit from four mixed BALF in each group. The p values were determined by ANOVA. CTL, control mice; PBS, PBS-treated mice; Dex, Dex-treated mice.

3.2 Effect of Dex on lung histology

Dex treatment did not induce any observed side effects in the experimental groups studied. We evaluated the histologic effects of Dex on airway inflammation and goblet cell hyperplasia with H&E and PAS stains (Fig. 2). To evaluate airway inflammation, we stained lung sections with H&E. We found marked peribronchial and perivascular inflammatory infiltrates in the airways of PBS-treated mice compared with those of control mice (Figs. 2A and C). These inflammatory infiltrates were significantly reduced in the Dex-treated mice (Figs. 2E). To evaluate for mucus hypersecretion, we stained lung sections with PAS. Mucus-secreting goblet cells, which were virtually absent in the airways of control mice, were clearly increased in PBS-treated mice (Figs. 2B and D). Dex treatment decreased the number of goblet cells in the airways (Figs. 2F). PAS staining scores were calculated for each experimental group as described in Section 2.3. PBS- and Dex-treated mice had staining scores of 2.95 ± 0.17 and 2.32 ± 0.06 (mean $\pm$ SEM, $n = 4$), respectively. In accord with inflammatory changes, the percentage of goblet cells in the airways was reduced by Dex treatment. These results demonstrate that Dex prevents airway inflammation and goblet cell hyperplasia in the airways.

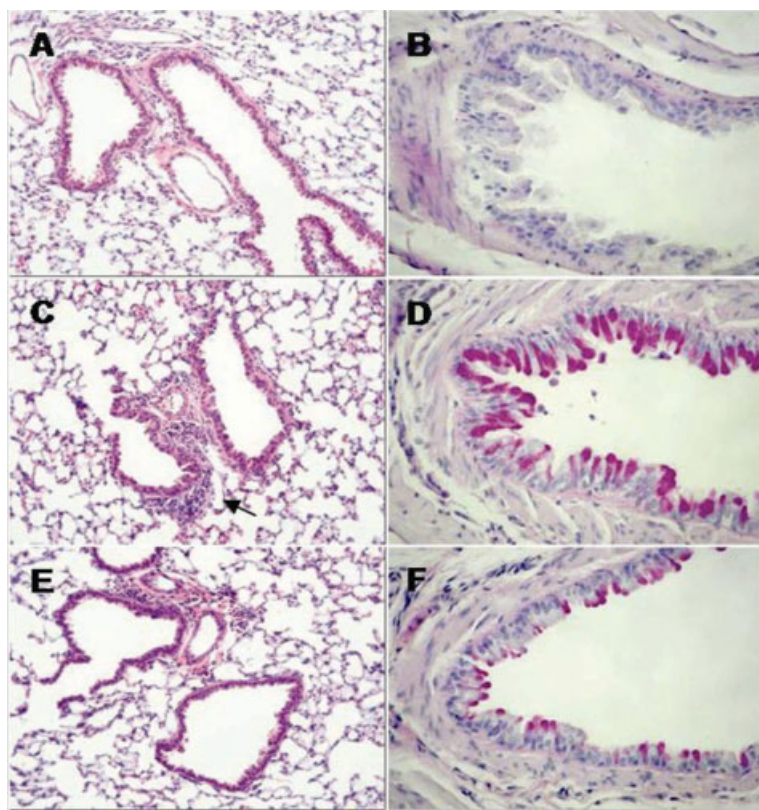


Figure 2. Effect of Dex on histological changes. Representative photomicrographs of lung sections from control mice (A, B), PBS- (C, D), and Dex-treated mice (E, F). Lung sections were stained with H&E (A, C, E), objective $\times 200$ and PAS (B, D, F), objective $\times 400$. Arrow indicates areas of peribronchial and perivascular inflammation.

3.3 2-DE analysis of control mice, PBS- and Dex-treated mice

Lungs were isolated from each mouse 48 h after the last challenge. Proteins from individual mice were combined in each experimental group. Quadruplicate gels were used for each experimental group. Proteins were resolved on 2-D gels and visualized by silver staining (Fig. 3). 2-D gel patterns were analyzed using a Progenesis Workstation. Dramatic differences were not observed within groups. The average spot numbers in the control mice, PBS- and Dex-treated mice were 892, 898, and 1028, respectively. There were no typical or characteristic spots among the extra 130 spots. We believe that many proteins expressed during Dex treatment, even if they are only present in very small amounts, may relate to the effect of Dex. These proteins will be studied in the future.

3.4 Differential protein expression

Each group was run on quadruplicate gels and the average spot intensities were compared between groups. The expressed spots in at least three out of four gels were

used to calculate the average spot number and average differences in protein expressions were compared between groups. Increased, decreased, and a few unchanged spots are labeled in Fig. 4.

3.5 Identification of differential proteins by MALDI-TOF/TOF MS

The 36 selected or labeled spots were identified by MALDI-TOF/TOF MS and are enumerated in Table 1. Spots 1, 3, 4, 6, 11, 19, and 25 are related to cytoskeletal proteins. Spots 2 and 17 are unknown proteins. Proteins related to selenium, an antioxidant important in asthma, are represented by spots 5, 20, 28, and 33. Proteins important in apoptosis and asthma were identified as spots 9, 10, 14, 21, 23, 29, and 35. The functions of other spots have not been identified until now. We analyzed the changed or unchanged spots. The spot intensities which were analyzed statistically are depicted with mean $\pm$ SEM (Fig. 5). In particular, tubulin α chain (spot 1), dihydropyrimidase-related protein 2 (spots 9 and 10), Esp15 homology (EH)-domain containing protein 4 (spot 11), vesicle

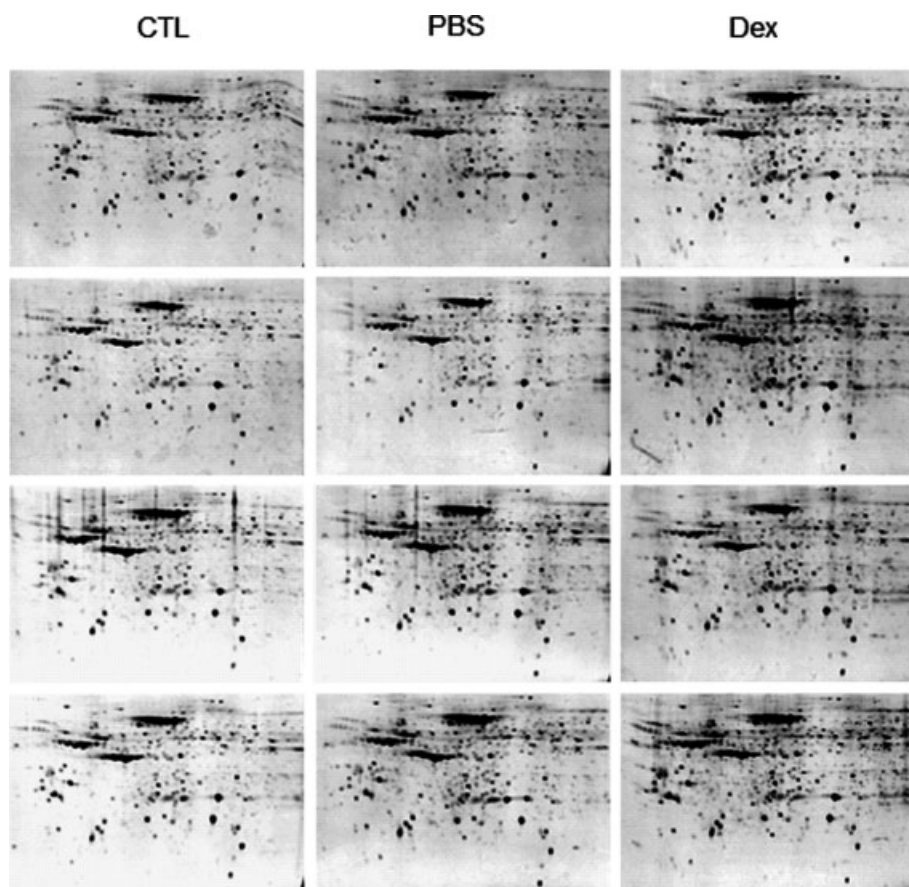


Figure 3. Representative 2-DE profiles of lung samples from control mice, PBS, and Dex-treated mice. Proteins on 2-DE (pH 4–7) gels were visualized by silver staining. CTL, control mice; PBS, PBS-treated mice; Dex, Dex-treated mice.

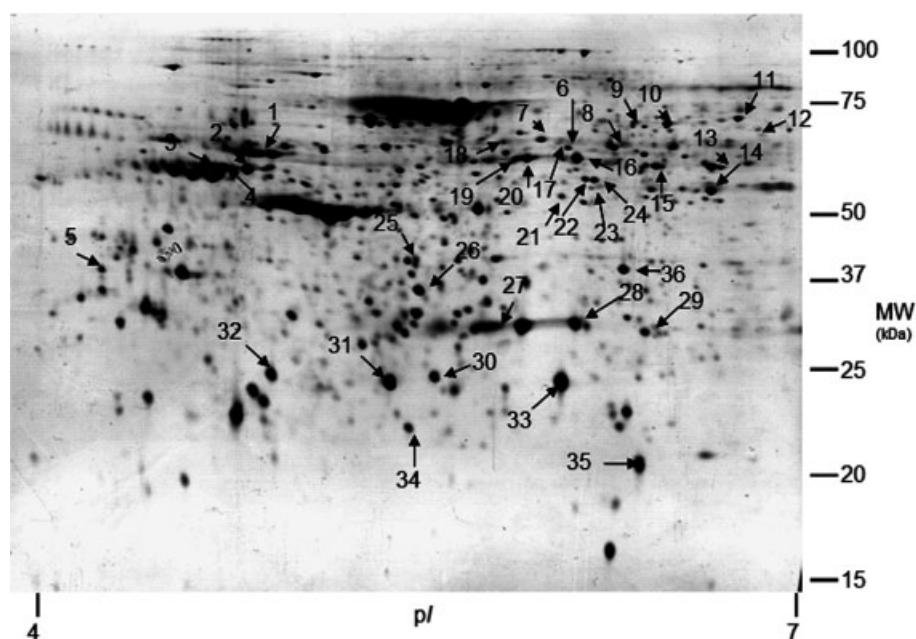


Figure 4. Effect of Dex on differential protein expression in 2-DE. Differential protein expressions from each average gel were compared with each experimental group. The spots identified by MALDI-TOF/TOF MS were enumerated.

amine transport protein (spot 24), and plasminogen (spot 25) were present at higher intensity in PBS-treated mice compared to control mice. Dex normalized the asthma-induced increased spot intensity. In contrast, the asthma-induced decreased intensity of selenium-binding protein 1 (spot 20) was increased in Dex-treated mice. These results demonstrate that the therapeutic mechanisms of Dex involve modification of protein expression in allergen-induced asthmatic mice.

4 Discussion

Corticosteroids are reportedly the most potent anti-inflammatory agents used to treat asthma [2, 16]. We demonstrated that Dex inhibits airway inflammation and goblet cell hyperplasia in a murine model of acute asthma. To investigate the therapeutic mechanism of Dex, we used a proteomics approach to identify differential protein expressions. Most of the identified spots are proteins involved in the epithelial cytoskeleton, oxidative stress, apoptosis, and other metabolic functions. Some of these proteins responded to Dex treatment.

4.1 Cytoskeleton and extracellular matrix (ECM)

Injury to the airway epithelium is a common pathologic finding in asthmatic patients [17]. The ciliated epithelial cells and goblet cells of large airways provide the primary defense against allergens [18], and impairment of muco-

ciliary activity increases pulmonary exposure and contributes to the pathophysiology. Accordingly, cytoskeletal alterations modify cell shape and facilitate cell migration and invasion [19]. These changes could allow greater access of allergen-modified molecules and cells to the underlying tissue and favor stimulation of immune cells. In this study, we identified three tubulin isoforms. Among these, tubulin α chain was increased in PBS-treated mice, which was reduced by Dex treatment, but isoforms of tubulin β chain were not significantly changed. Tubulin, a protein subunit of ciliary microtubules, integrates ciliary motion with cellular function via the cytoskeleton [20]. Lange *et al.* demonstrated toluene diisocyanate-adducted tubulin might contribute to the etiology of asthma by impairing ciliary motion and affecting cytoskeletal signaling [21]. Also, keratin 7 in the columnar and glandular epithelium of the lung, cervix, and breast was identified by MS [22]. T complex polypeptide 1 is important in folding to complete actin and tubulin [23]. Thus, the altered cytoskeletal protein expression induced by Dex may be an important role in detachment of epithelium and in goblet cell hyperplasia.

Airway remodeling in asthma is characterized by sub-epithelial fibrosis due to extensive deposition of the extracellular matrix (ECM). EH-domain containing protein and plasminogen are related to the ECM and tissue fibrosis. Expression of these proteins was higher in PBS-treated mice than in control mice. Dex normalized the levels of these up-regulated proteins. Thus, Dex may prevent or inhibit the progression to airway remodeling. To

Table 1. Identification of differential protein expression

Spot no.	Protein name	Swiss-Prot acc. no.	NCBI	Identified method ^{a)}	MS results ^{b)}	Theoret. mass (kDa/pI) ^{c)}	Observed mass (kDa/pI) ^{d)}	Density ratio Dex/PBS ^{e)}
1	Tubulin alpha chain isotype M-alpha-6		177428	MS/MS	3	49/4.9	58/4.9	0.5**
2	Unnamed	Q8C165		PMF	30%	56/5.24	56/4.85	0.7**
3	Tubulin beta-3	P29512		MS/MS	4	50/4.7	56/4.7	0.4*
4	Tubulin, beta 5 homolog	Q9CVR0		MS/MS	3	32/5.56	55/4.75	0.7
5	Thioether S-methyltransferase	P40936		MS/MS	4	29/6.0	40/4.3	1.7*
6	t complex polypeptide 1		JC1443	MS/MS	6	60/6.2	60/6.15	1.5
7	Glucose regulated protein	Q8C2F4		MS/MS	6	56/5.78	62/6.0	1.4
8	Fibrinogen	Q8K0E8		MS/MS	5	54/6.68	60/6.4	0.5*
9	Dihydropyrimidinase-related protein 2	O08553		MS/MS	3	62/5.95	72/6.45	0.5*
10	Dihydropyrimidinase-related protein 2	O08553		MS/MS	4	62/5.95	72/6.6	0.8
11	EH-domain containing protein 4	Q9EQP2		MS/MS	4	61/6.33	75/6.85	0.6***
12	Olfactory receptor MOR263-4	Q8VFQ0		PMF	40%	35/8.28	70/6.95	1.1
13	Aldehyde dehydrogenase		148966	MS/MS	3	56/7.5	56/6.8	1.2
14	Alpha enolase	P17182		MS/MS	3	47/6.36	54/6.75	0.8*
15	Aldehyde dehydrogenase		148966	MS/MS	3	56/7.5	56/6.53	1.0
16	Hypothetical protein	Q91X87		MS/MS	4	52/5.87	58/6.2	1.0
17	Unnamed		BAC4057	MS/MS	4	58/–	61/6.1	1.2
18	Glucose regulated protein	Q8C2F4		MS/MS	3	56/5.78	61/5.8	2.1*
19	Keratin 7	Q9DCV7		PMF	41%	50/5.67	58/5.85	0.9
20	Selenium-binding protein 1	P17563		PMF	43%	52/5.97	58/5.9	1.3
21	Ornithine-oxo-acid-transaminase	Q988J6		MS/MS	2	47/5.98	52/6.1	1.5
22	Rab GDP dissociation inhibitor beta	P50397		PMF	33%	50/6.02	55/6.2	1.1
23	Fe-S protein 2	Q91WDS		MS/MS	5	52/6.52	54/6.23	1.1
24	Vesicle amine transporter	Q8R3G0		MS/MS	1	43/5.95	55/6.24	0.8
25	Plasminogen	P20918		PMF	30%	90/6.21	40/5.45	0.4***
26	Annexin A3	Q8C1X9		MS/MS	4	36/5.5	37/5.5	0.9
27	AKO13010		BAB28594	MS/MS	2	30/–	30/5.8	0.2*
28	Thioether S-methyltransferase	P40936		MS/MS	3	29/6.0	30/6.15	1.2
29	Heat shock protein 25		JN0679	MS/MS	3	29/6.0	30/6.5	1.0
30	Apolipoprotein A-1	Q00623		MS/MS	5	30/5.64	25/5.56	1.1
31	Apolipoprotein A-1	Q00623		MS/MS	5	30/5.64	24/5.4	0.8
32	RHOGDI-1	Q8BP10		PMF	48%	22/5.3	26/5.0	0.9
33	Peroxiredoxin 6	O08709		MS/MS	4	24/5.72	24/6.1	0.9
34	ATP synthase D chain	Q9DCX2		MS/MS	1	18/5.53	22/5.5	1.3
35	Superoxide dismutase	P08228		MS/MS	2	15/6.03	18/6.5	1.0
36	Malate dehydrogenase	P14152		MS/MS	3	36/6.16	39/6.4	0.9

a) PMF, peptide mass fingerprinting; MS/MS, MALDI-TOF/TOF MS

b) PMF, % protein coverage; MS/MS, number of assigned peptides

c) Calculated from the corresponding Swiss-Prot entry. Signal sequences documented by Swiss-Prot were removed and theoretical M_r/pI values were recalculated

d) Estimated from the position of the protein spot on the 2-DE gel (Progenesis workstation)

e) ***; $p < 0.0005$, **; $p < 0.005$, *; $p < 0.05$, density ration of Dex/PBS by ANOVA

date, more than 40 EH-domain containing proteins have been reported [24]. These proteins promote the assembly of type VI collagen tetramers or stabilize type VI collagen filaments. Plasminogen is converted to plasmin by plasminogen activator. Many studies previously reported that matrix metalloprotease (MMP) contributes to the pathogenesis of asthma [25]. Converted plasmin activates MMPs directly to degrade ECM components or

degrade ECM by inhibiting MMP inhibitors [26]. Thus, the high level of plasminogen may result from accumulation of ECM components. Cho *et al.* demonstrated that sensitized mast cells release considerable amounts of plasminogen activator inhibitor-1, which blocks fibrinolysis and then promotes fibrin and collagen deposition [27]. In our study we demonstrated that total IgE levels derived from mast cells were increased in PBS-treated mice.



Figure 5. Histograms show the relative intensity of various protein spots on the gels and that compared with each experimental group. The data are represented as mean $\pm$ SEM from four individual 2-DE gels in each experimental group. The p values were determined by ANOVA. CTL, control mice; PBS, PBS-treated mice; Dex, Dex-treated mice.

Thus, these results indicate that the interaction between total serum IgE and plasminogen may be influenced by Dex treatment.

4.2 Oxidative stress and apoptosis

Oxidative stress, or the imbalance between reactive oxygen species and antioxidants, occurs in many allergic and immunologic disorders [28]. Accordingly, asthmatic patients are more susceptible to oxidant-induced apoptosis [29]. Asthma is characterized by reduced antioxidant defenses.

The expression of superoxide dismutase (SOD), enzymatic antioxidants was decreased in PBS-treated mice, but was not statistically increased by Dex treatment. De Raeve *et al.* proved that intracellular Cu, Zn-SOD activity is decreased in the asthmatic airway epithelium, but could be normalized by corticosteroid treatment for 3 weeks [30]. However, our result shows that Dex treatment does not affect the expression of SOD. This suggests that the state of asthmatic disease and duration of corticosteroid treatment may contribute to the therapeutic effects.

We also identified selenium, a nonenzymatic antioxidant, related proteins: selenium-binding protein 1 and thioether S-methyltransferase, which contributes to sulfur and selenium metabolism [31]. Pretreatment with selenite prevented airway inflammation in OVA-induced asthma mice [32]. In this study, the altered expression pattern of selenium-binding protein 1 appeared to have a therapeutic effect. However, two spots of thioether S-methyltransferase differed. Spot 5 was identified close to pI 4, and spot 28 was close to pI 6 with respective molecular weights of about 40 kDa and 30 kDa, respectively. This means that protein modification may affect pI and molecular weight of this protein in this asthma model. Although two spots have changed in response to therapeutic effect of Dex, the results were not statistically significant.

Alzheimer's disease is a neurodegenerative disorder in which oxidative stress has been implicated as important in the pathological progression [33]. Dihydropyrimidinase-related protein 2 and α -enolase were reported targets in the brain of protein oxidation in Alzheimer's disease [34]. We suggest that these two proteins contribute to the process of free radical-mediated protein modification in asthma.

Although the effect of HSP on mucous secretion has not been fully elucidated, the microbial HSP is known to exhibit anti-inflammatory functions and suppress goblet cell hyperplasia and mucin hypersecretion [35]. Kim *et al.* reported that HSP is up-regulated in the IL-1 β treated lung carcinoma cell line [36]. Histologic changes of goblet cell

hyperplasia were significant, but levels of HSP 25 measured by 2-DE did not differ in the control and PBS-treated mice. In contrast, we showed previously that the basal epithelium cells express a high level of HSP 27 without apoptotic morphology in asthmatic human [37]. Thus, the role of HSP in the mechanism of asthma remains unclear.

Biosynthesis of nitric oxide activates iron regulatory factor in macrophages [38]. Drapier *et al.* demonstrated that the [Fe-S] cluster of iron regulatory factor mediates iron-dependent regulation of nitric oxide synthesis after IFN- γ and/or lipopolysaccharide stimulation [39]. In this study, Fe-S protein 2 was up-regulated in PBS-treated mice compared to control mice, but did not differ between PBS- and Dex-treated mice. Thus, Fe-S protein 2 might not respond to Dex-treated asthmatic mice.

4.3 Metabolism

α -Enolase is one of the subunits of the enzyme enolase, which interconverts 2-phosphoglycerate and phosphoenolpyruvate in glycolysis [40]. Recent results demonstrated that α -enolase-mediated plasminogen activation is the major extracellular proteolytic executor of necrotic cell death [41]. Some cellular metabolism-related proteins were identified: aldehyde dehydrogenase, glucose regulated protein, malate dehydrogenase, ATP synthase D chain, and apolipoprotein A-1.

4.4 Various functions

Rab GDP dissociation inhibitor β and vesicle amine transport protein-1 are involved in the regulation of vesicle-mediated cellular transport [42, 43]. These proteins also contribute to neural specific synaptic vesicles. Although not expressed in the lungs, their functional and physiological roles may contribute to airway hyperresponsiveness in asthma.

Ornithine-oxo-acid transaminase is a pyridoxal-P (PLP)-dependent mitochondrial matrix protein that catalyzes the breakdown of ornithine to δ -pyrroline-5-carboxylic acid, which is then converted into proline [44]. Defects in this protein lead to gyrate atrophy of the choroids and retina [45].

Annexin A3 belongs to the annexin superfamily of calcium- and phospholipid-binding proteins [46]. This particular protein represents $\sim 1\%$ of the total protein comprising human neutrophils [47]. In particular, among the annexin family, annexin1 is an important mediator of glucocorticoid action in the hypothalamo-pituitary axis [48]. Thus, we suggest that the calcium-binding related protein, annexin A3, may play an important role in apoptosis in asthma.

5 Concluding remarks

In this study we demonstrated differential protein expression in OVA-induced asthmatic mice in response to Dex treatment. Most identified proteins were related to the cytoskeleton, oxidative stress, apoptosis, and metabolism. We proposed therapeutic mechanisms of Dex in airway inflammation and goblet hyperplasia in this model. However, we did not investigate the immunologically relevant cytokines, chemokines, and immune cell-specific proteins. 2-DE did not detect the differential expression of low molecular weight and abundant key proteins. High-throughput performance techniques are needed to identify other proteins. On the other hand, we identified some altered protein profiles that were altered in response to Dex treatment important in the cytoskeleton and oxidative stress. Additional studies are needed to characterize the precise mechanism of action of corticosteroids in asthma therapy.

This work was supported by a grant from the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea (00-PJ9-PG1-CO02-0003).

6 References

- [1] Kay, A. B., *J. Allergy Clin. Immunol.* 1991, 87, 893–910.
- [2] Djukanovic, R., Wilson, J. W., Britten, K. M., Wilson, S. J. *et al.*, *Am. Rev. Respir. Dis.* 1992, 145, 669–674.
- [3] Pitzalis, C., Pipitone, N., Bajocchi, G., Hall, M. *et al.*, *J. Immunol.* 1997, 158, 5007–5016.
- [4] Schleimer, R. P., *Glucocorticosteroids: their mechanisms of action and use in allergic diseases*. Mosby Ltd., St. Louis 1998, pp. 638–660.
- [5] Bousquet, J., Chanez, P., Lacoste, J. Y., Barneon, G. *et al.*, *N. Engl. J. Med.* 1990, 323, 1033–1039.
- [6] Schleimer, R. P., Bochner, B. S., *J. Allergy Clin. Immunol.* 1994, 94, 1202–1213.
- [7] Kibe, A., Inoue, H., Fukuyama, S., Machida, K. *et al.*, *Am. J. Respir. Crit. Care Med.* 2003, 67, 50–56.
- [8] Eum, S. Y., Maghni, K., Hamid, Q., Eidelman, D. H. *et al.*, *J. Allergy Clin. Immunol.* 2003, 111, 1049–1061.
- [9] Blyth, D. I., Pedrick, M. S., Savage, T. J., Bright, H. *et al.*, *Am. J. Respir. Cell Mol. Biol.* 1998, 19, 38–54.
- [10] Tug, T., Kamanli, A., Tug, E., *J. Investig. Allergol. Clin. Immunol.* 2001, 11, 300–302.
- [11] Dubus, J. C., Marguet, C., Deschildre, A., Mely, L. *et al.*, *Allergy* 2001, 56, 944–948.
- [12] Jang, H. S., Oh, C. K., Jo, J. H., Kim, Y. S., Kwon, K. S., *J. Korean Med. Sci.* 2001, 16, 623–629.
- [13] Görg, A., Postel, W., Weser, J., Gunther, S. *et al.*, *Electrophoresis* 1987, 8, 122–124.
- [14] Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai, B. S., Mische, S. M., *Electrophoresis* 1999, 20, 601–605.
- [15] Joo, W. A., Kang, M. J., Son, W. K., Lee, H. J. *et al.*, *Proteomics* 2003, 3, 2402–2411.
- [16] Busse, W. W., Lenfant, C., Lemanske, R. F. Jr., *J. Allergy Clin. Immunol.* 2002, 110, 703–705.
- [17] Laitinen, L. A., Heino, M., Laitinen, A., Kava, T., Haahtela, T., *Am. Rev. Respir. Dis.* 1985, 131, 599–606.
- [18] Pavia, D., *Lung mucociliary clearance*, Butterworth, London 1984, pp. 127–155.
- [19] Kheradmand, F., Werner, E., Tremble, P., Symons, M., Werb, Z., *Science* 1998, 280, 898–902.
- [20] Wheatley, D. N., Wang, A. M., Strugnell, G. E., *Cell Biol. Int.* 1996, 20, 73–81.
- [21] Lange, R. W., Lantz, R. C., Stolz, D. B., Watkins, S. C. *et al.*, *Toxicol. Sci.* 1999, 50, 64–71.
- [22] Ramaekers, F., Huysmans, A., Schaart, G., Moesker, O., Vooijs, P., *Exp. Cell. Res.* 1987, 170, 235–249.
- [23] Llorca, O., Martin-Benito, J., Grantham, J., Ritco-Vonsovici, M. *et al.*, *EMBO J.* 2001, 20, 4065–4075.
- [24] Santolini, E., Salcini, A. E., Kay, B. K., Yamabhai, M., Di Fiore, P. P., *Exp. Cell Res.* 1999, 253, 186–209.
- [25] Kelly, E. A., Jarjour, N. N., *Curr. Opin. Pulm. Med.* 2003, 9, 28–33.
- [26] Matrisian, L. M., *Trends Genet.* 1990, 6, 121–125.
- [27] Cho, S. H., Tam, S. W., Demissie-Sanders, S., Filler, S. A., Oh, C. K., *J. Immunol.* 2000, 165, 3154–3161.
- [28] Bowler, R. P., Crapo, J. D., *J. Allergy Clin. Immunol.* 2002, 110, 349–356.
- [29] Bucchieri, F., Puddicombe, S. M., Lordan, J. L., Richter, A. *et al.*, *Am. J. Respir. Cell Mol. Biol.* 2002, 27, 179–185.
- [30] De Raeve, H. R., Thunnissen, F. B., Kaneko, F. T., Guo, F. H. *et al.*, *Am. J. Physiol.* 1997, 272, 148–154.
- [31] Mozier, N. M., McConnell, K. P., Hoffman, J. L., *J. Biol. Chem.* 1988, 263, 4527–4531.
- [32] Jeong, D. W., Yoo, M. H., Kim, T. S., Kim, J. H., Kim, I. Y., *J. Biol. Chem.* 2002, 277, 17871–17876.
- [33] Markesbery, W. R., *Free Radic. Biol. Med.* 1997, 23, 134–147.
- [34] Castegna, A., Aksenov, M., Thongboonkerd, V., Klein, J. B. *et al.*, *J. Neurochem.* 2002, 82, 1524–1532.
- [35] Schoolink, G. K., *Adv. Microb. Physiol.* 2002, 46, 1–45.
- [36] Kim, C. H., Kim, do K., Choi, S. J., Choi, K. H. *et al.*, *Proteomics* 2003, 3, 2454–2471.
- [37] Merendino, A. M., Paul, C., Vignola, A. M., Costa, M. A. *et al.*, *Cell Stress Chaperones* 2002, 7, 269–280.
- [38] Weiss, G., Goossen, B., Doppler, W., Fuchs, D. *et al.*, *EMBO J.* 1993, 12, 3651–3657.
- [39] Drapier, J. C., Hirling, H., Wietzerbin, J., Kaldy, P., Kuhn, L. C., *EMBO J.* 1993, 12, 3643–3649.
- [40] Canback, B., Andersson, S. G., Kurland, C. G., *Proc. Natl. Acad. Sci. USA* 2002, 99, 6097–6102.
- [41] Bizik, J., Kankuri, E., Ristimäki, A., Taieb, A. *et al.*, *Cell Death Differ.* 2004, 11, 183–195.
- [42] Sedlacek, Z., Shimeld, S. M., Munstermann, E., Poustka, A., *Mol. Biol. Evol.* 1999, 16, 1231–1237.
- [43] Linial, M., Miller, K., Scheller, R. H., *Neuron* 1989, 2, 1265–1273.
- [44] Ames, B. N., Elson-Schwab, I., Silver, E. A., *Am. J. Clin. Nutr.* 2002, 75, 616–658.
- [45] Wang, T., Steel, G., Milam, A. H., Valle, D., *Proc. Natl. Acad. Sci. USA* 2000, 97, 1224–1229.
- [46] Sopkova, J., Raguene-Nicol, C., Vincent, M., Chevalier, A. *et al.*, *Protein Sci.* 2002, 11, 1613–1625.
- [47] Comera, C., Rothhut, B., Cavadore, J. C., Vilgrain, I. *et al.*, *J. Cell Biochem.* 1989, 40, 361–370.
- [48] Cover, P. O., Baanah-Jones, F., John, C. D., Buckingham, J. C., *Endocrine* 2002, 18, 33–39.