



Enhancement of ovalbumin-induced pulmonary eosinophilia by intranasal administration of α_1 -proteinase inhibitor type 2 antisense oligonucleotides

Ha-Na Sung, Chang-Hwan Jeon, Byoung-Chul Gill, Hye-Rin Kim, Sun-Woo Cheong, Joo-Hung Park*

Department of Biology, Changwon National University, #9, Sarim-dong, Changwon, Kyungnam 641-773, South Korea

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ABSTRACT

To identify asthma-susceptibility genes, we did proteome analyses of the lung from control and ovalbumin-sensitized BALB/c mice. Among the 6 up-regulated proteins is α_1 -protease inhibitor (α_1 -PI) type 2, which is a member of the serine protease inhibitor superfamily of protease inhibitors that participate in a variety of physiological functions, including extracellular matrix remodeling and inflammation. The up-regulated expression of α_1 -PI type 2 was confirmed by real-time PCR. Then we examined mRNA expression of five members of the α_1 -PI family genes (α_1 -PI types 1–5) in several organs of BALB/c mice and found that in addition to the liver, all the organs tested also expressed different isoforms of α_1 -PI in a tissue-specific manner, albeit to a lesser extent compared with the liver. When a similar study was performed with C57BL/6 mice, which have been shown to be more susceptible to ovalbumin-induced asthma than BALB/c mice, a pair of remarkable differences between the mouse strains were revealed: (1) the magnitude of α_1 -PI type 2 mRNA in all the organs was much higher in BALB/c than in C57BL/6 mice and (2) α_1 -PI type 2 is the only isoform expressed in the lung of BALB/c, but not of C57BL/6 mice. Using the antisense oligonucleotide technology to specifically down-regulate expression of α_1 -PI type 2, we demonstrated that pulmonary infiltration of eosinophils was significantly increased by intranasal administration of α_1 -PI type 2 antisense oligonucleotides in OVA-sensitized mice, suggesting that α_1 -PI type 2 may suppress the progress of asthma, probably by acting on neutrophil elastase, which can produce many of the pathological features of asthma.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways characterized by reversible airway obstruction, airway hyperreactivity (AHR), and remodeling of the airways. The incidence of asthma is on the rise despite ongoing intensive asthma research. One of the characteristics of asthma is the infiltration of the airways with various cell types including eosinophils, mast cells, and T lymphocytes [1,2]. Several mediators secreted from these cells amplify the inflammatory cascade, increasing AHR and stimulating mucus secretion, which contribute to airway obstruction and airway remodeling [1,3,4]. Several candidate asthma-susceptibility genes, including ADAM33, DPP10, and PHF11, have been identified by genetic association studies and genome screens and the number of them is on the rise [5]. Inbred mouse strains show differential susceptibility to ovalbumin-induced allergic asthma. C57BL/6 mice was shown to be more susceptible to ovalbumin-induced pulmonary eosinophilia

than BALB/c mice [6–9]. Therefore, inbred murine models of allergic asthma have been used to provide a lot of information on quantitative trait loci and pertaining genes controlling allergen-induced airway hyperresponsiveness [10–12].

α_1 -PI, also called α_1 -antitrypsin, is a member of the serine protease inhibitor (serpin) superfamily of protease inhibitors that participate in a variety of physiological functions, including blood coagulation, extracellular matrix remodeling, complementation fixation, and inflammation [13,14]. α_1 -PI is produced primarily in the liver and is secreted into the bloodstream, where it is able to inhibit a variety of serine proteinases including neutrophil elastase [13–15]. Indeed, a genetic deficiency of α_1 -PI in humans leads to pulmonary obstructive diseases such as pulmonary emphysema [16]. In mice, there is strain-dependent variation in the serum levels of α_1 -PI: the serum level of α_1 -PI of C57BL/6 strain is only 70% of that of BALB/c mouse [17], which was shown to be less susceptible to ovalbumin-induced pulmonary eosinophilia than C57BL/6 mice [6–9]. When exposed chronically to cigarette smoke, C57BL/6 strain, which has significantly low levels of elastase inhibitory activity (~40% of that of ICR strain), but not ICR strain, developed emphysema [18,19]. C57BL/6 mice with low levels of α_1 -PI have been shown to be more susceptible to experimentally induced-pulmonary emphysema than control C57BL/6 mice [20].

Abbreviations: α_1 -PI, α_1 -protease inhibitor; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; i.p., intraperitoneally; 2-DE, 2-dimensional gel electrophoresis.

* Corresponding author. Tel.: +82 55 213 3456; fax: +82 55 213 3459.

E-mail address: parkjh@changwon.ac.kr (J.-H. Park).

Considering the involvement of neutrophils in the pathogenesis of asthma, not much is known about the relationship between allergic asthma and the deficiency of α_1 -PI. In humans, it was reported that asthma is more common in patients with α_1 -PI deficiency than in those without it [21,22]. However, the role of α_1 -PI in the pathogenesis of asthma remains to be elucidated. In mice, there have been no studies regarding the regulatory role of α_1 -PI in the pathogenesis of asthma.

Comparative proteome analysis using 2-dimensional gel electrophoresis (2-DE) in association with mass spectrometry (MS) could be used to identify proteins associated with asthma [23,24]. In the present study, using proteomic analysis in conjunction with real-time reverse transcription polymerase chain reaction (real-time RT-PCR) and Western blotting in ovalbumin (OVA)-induced asthma in mice, we have demonstrated that α_1 -PI type 2 in the lung was up-regulated in OVA-sensitized and challenged BALB/c mice and down-regulation of α_1 -PI type 2 in the lung by intratracheal administration of α_1 -PI type 2 antisense oligonucleotides enhanced eosinophil infiltration into the lung, suggesting that in addition to hepatic production of α_1 -PI, local production of α_1 -PI type 2 in the lung also contributes to regulation of eosinophil infiltration into the lung in an OVA-induced asthma.

2. Materials and methods

2.1. Mice

BALB/c and C57BL/6 mice, 6–12 weeks of age, were purchased from the Korean Institute for Chemistry (Taejeon, Korea). The animals were housed 5 mice per cage in a laminar air flow room maintained at $22 \pm 2^\circ\text{C}$ with relative humidity of $55 \pm 5\%$. Mice were cared and treated in accordance with the guidelines established by the Public Health Service Policy on the Human Care and Use of Laboratory Animals.

2.2. Antigen sensitization and challenge

Sensitization and challenge with OVA was performed according to the method of Kung et al. [25] with minor modifications. On day 0, mice were sensitized with an intraperitoneal (i.p.) injection of 15 μg of OVA and 2 mg of alum dissolved in 0.2 ml phosphate buffered saline (PBS). The animals received a booster injection of this alum–OVA mixture 5 days later. Twelve days after the first sensitization, the mice were exposed for 1 h to aerosolized OVA (0.5% dissolved in 0.9% saline) which was produced by an ultrasonic nebulizer at a flow rate of 6 l/min. At the 13th day, mice were challenged again with OVA as performed on day 12. Control mice were exposed to aerosolized OVA as OVA-sensitized mice were. Four mice were used for each group. Mice were sacrificed at day 14.

2.3. Bronchoalveolar lavage fluid (BALF) preparation

The tracheas of mice were cannulated and lavaged with two 0.8 ml aliquots of cold PBS. Number of cells in the BALF was counted and used for cytospin and staining.

2.4. Cytospin preparations and Giemsa staining

Cells (4×10^4 cells) were centrifuged in 100 μl PBS with 3% BSA onto a microscope slide using a CellSpin (Hanil Science, Korea). Cells were fixed in methanol, and stained with 'Hemacolor for microscopy' and identified under the microscope according to the criteria described in the protocol (Merck, USA).

2.5. Cell culture and DNA transfections

CT-26, a murine colon carcinoma, was maintained in DMEM with 10% FBS supplemented with antibiotics and glutamine. Twenty-four hours before transfection at 70–80% confluency, cells were suspended with fresh medium (2×10^5 cells/ml) and transferred to 24-well plates (0.5 ml/well). Transfection of oligonucleotides was performed with Lipofectamine 2000 (Invitrogen, USA), as described by the manufacturer. Before transfection, the culture medium was changed with fresh medium without antibiotics. Oligonucleotides formulated into liposomes were applied to each well (0.5 nM/l). Twenty-four hours after transfection, cells were harvested.

2.6. 2-Dimensional gel electrophoresis and image analysis

Lungs in lysis buffer (8 M urea, 4% CHAPS, 0.5% IPG buffer pH 4–7) were disrupted by a Dounce tissue grinder (Wheaton, USA), sonicated, and centrifuged. The protein separation was performed in duplicate for each sample from two different experiments. 0.5–1 mg of protein was loaded on a IPG strip (18 cm, pH 4–7 nonlinear) which was rehydrated overnight. Isoelectric focusing (IEF) was performed using the following parameters with the Multiphor II system (Amersham Biosciences, USA): 500 V for 1 h, 1000 V for 1 h, 5000 V for 1 h, 8000 V (gradient) for 1 h, and finally 8000 v for a total of 45 kWh. After IEF, the IPG strips were immediately equilibrated in 10 ml equilibration solutions (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) with gentle shaking for 15 min. Vertical SDS-PAGE was run with laboratory-made homogeneous 10% acrylamide gel in an Ettan DALTII apparatus (Amersham Biosciences, USA). The gels were stained with Coomassie blue and image analysis was performed using the Image Master 2-D Platinum software (Amersham Biosciences, USA) according to the protocols provided by the manufacturer.

2.7. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry/mass spectrometry (MALDI-TOF MS/MS)

Spots of interest were excised manually from preparative gels, destained in 50 mM NH_4HCO_3 /acetonitrile (50:50) and dried by vacuum centrifugation. For rehydration, the gel pieces were incubated in 100 mM ammonium bicarbonate and 10 mM DTT for 1 h at 56°C . Alkylation was performed in 100 mM ammonium bicarbonate and 55 mM iodoacetamide for 40 min at room temperature in the dark condition. After dehydration in acetonitrile, the gel pieces were dried and were digested with sequencing grade trypsin (enzyme:substrate (w/w) ratio $> 1:20$) (Promega, USA) at 37°C , overnight, in 50 mM ammonium bicarbonate. After a repeated process of hydration–dehydration and sonication, the resulting tryptic peptides were dissolved in 0.5% trifluoroacetic acid (TFA) solution, and then desalted using ZipTipC18 (Millipore, USA) tip. Peptides were eluted directly onto MALDI target by α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 mg/ml CHCA in 0.5% TFA/50% acetonitrile (1:1, (v/v))). All mass spectra were acquired at a reflection mode by a 4700 Proteomics Analyzer (Applied Biosystems, USA). When the protein spots were not identified by peptide mass fingerprinting (PMF), fragmentation patterns of tryptic peptide molecular ions ($[M + H]^+$) were analyzed by MS/MS methods for obtaining their partial sequences using MALDI-TOF/TOF technique.

2.8. Database searching and identification of proteins

The proteins were identified by searching NCBI nonredundant database using MASCOT Peptide Mass Fingerprint software (Matrixscience, UK) and MS-Fit (Protein Prospector; USA). All mass spectra were searched in the database of rodent species or all entries. The search parameters were considered to allow the mod-

Table 1
Primers used in RT-PCR.

Name	Nucleotide sequence	Annealing temperature (°C)	Cycles ^a	Size ^b (bp)
ma1picomfw ^c	tccagctgggtgctgctgat	58	30	377
ma1pi1rv	catagacataggaaccatttgtaa	58	30	377
ma1pi2rv	tagacataggaacggcttcaaa	58	30	377
ma1pi3rv	atagaataaggaacggctagtaa	58	30	377
ma1pi4rv	catagaataagtagcgacttgtaa	58	30	377
ma1pi5rv	atagacaaaaaacgccttgtaa	58	30	377
mhgprtfw mhgprtrv	gctggtgaaaaggacctctc cacaggactagaacacctgc	58	25	248

^a Indicates the number cycles of amplification.

^b Indicates the expected size of PCR products.

^c Common primer for all isotypes.

ifications of N-terminal Gln to pyroGlu, oxidation of methionine, acetylation of protein N-terminus, carbamidomethylation of cysteine, and acrylamide modified cysteine. The criteria for positive identification of proteins were set as follows: (i) minimum for matching peptide masses, (ii) 50–100 ppm mass accuracy, and (iii) molecular weight and pI obtained from image analysis. For MS/MS search, fragmentation of selected peptide molecular ion peak was used to identify the protein in the same manner by searching NCBI nonredundant database using MASCOT MS/MS ion search program.

2.9. Western blotting

Cells or tissues were homogenized in lysis buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM EDTA (pH 8.0), 50 μ M sodium vanadate, 20 mM p-nitrophenylphosphate, 50 mM sodium fluoride, leupeptin (0.5 μ g/ml), aprotinin (10 μ g/ml), and soybean trypsin inhibitor (10 μ g/ml). Proteins size-fractionated on SDS/PAGE were transferred to PVDF membranes, and the blots were blocked with 3% bovine serum albumin in TBST buffer (20 mM Tris–HCl pH 7.5/137 mM NaCl/0.1% Tween 20). The blots were sequentially treated with primary and secondary antibodies in TBST, with intermittent washing with TBST. Immunodetection was performed with the ECL kit (Amersham Pharmacia Biotech, USA). Antibodies used in the present study are: chicken anti-human α_1 antitrypsin polyclonal IgY (Proscience, USA), HRP-conjugated anti-chicken IgY (Jackson ImmunoResearch, USA), rabbit polyclonal anti- β actin IgG and HRP-conjugated anti-rabbit IgG (Cell Signalling Technology, USA). All antibodies used were 1:1000 diluted.

2.10. RNA preparation, reverse transcription-polymerase chain reaction (RT-PCR), and real-time PCR

Total cellular RNA was extracted from cells using the RNeasy method (Tel-Test, Inc., USA). For PCR analysis, RNA was used after

contaminating DNA was completely removed by DNase I treatment. RT-PCR analysis was performed using pairs of oligonucleotide primers. The PCR products were confirmed to correspond to their original sequence by DNA sequencing. PCR products were comparatively quantitated by GS-700 densitometer (Bio-Rad, USA). Gene specific primers, number of cycles of amplification, annealing temperature, and expected size of PCR product are listed in Table 1. Real-time PCR was performed to confirm the RT-PCR results. Power SYBR green PCR Master Mix and Real-time PCR system (7300, Applied Biosystems, USA) were used.

2.11. Phosphorothioate oligonucleotides

Phosphorothioate oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems Model 8909, USA). The antisense oligonucleotides consisted of 18 mer analogues to the 5' end of the murine α_1 -PI type 2 sequence which spans the translation initiation site. The sequences are as follows (mismatches are underlined): α_1 -PI type 2 antisense DNA, 5'-GGA GGG AGT CAT TTT CAG-3'; α_1 -PI type 2 antisense mismatched DNA, 5'-GGA AGG AGC GGC CTT CAG-3'.

2.12. Antisense oligonucleotides treatment in OVA-sensitized mice

Antisense or control oligonucleotides were given by intranasal administration (86 μ g in 20 μ l PBS/treatment) at days 12 and 13 (30 min before aerosolization with OVA) to OVA-sensitized mice which were anesthetized by i.p. injection of Zoretil (125 mg/kg body weight, Virbac Laboratories, France).

2.13. Histology

Organs were fixed in Bouin's fixative solution. The specimens were dehydrated, embedded in paraffin, and sectioned at a thick-

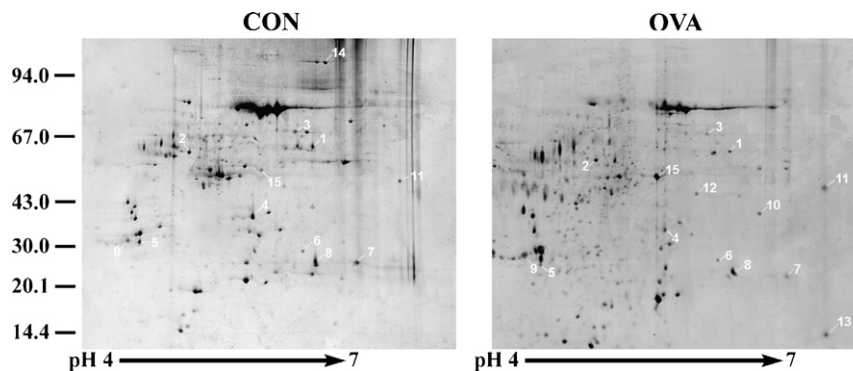


Fig. 1. Representative 2-DE profiles of lung samples of control and OVA-treated mice. Control and OVA-sensitized (4 mice each) female mice were treated as described in Section 2 and four different lung samples were taken for each group. Total lung proteins (1 mg) of control or OVA-treated mice were subjected to 2-DE system (first dimension, IPG strip pH 4–7 NL, 18 cm; second dimension, 10% SDS-PAGE). Proteins were visualized by Coomassie blue staining. Differentially expressed protein spots are numbered and further analyzed by MALDI-TOF MS/MS. Quadruplicate gels were used for each experimental group and one representative gel is shown.

Table 2The list of proteins identified by MALDI-TOF MS/MS^a.

Spot no.	Protein name	Mr (Da)	pI	NCBI accession no.	Number of matched peptides	Sequence coverage (%) ^b
Down-regulated proteins in allergic asthma						
1	Selenium binding protein 1	52,480	5.87	NP033176	4	8
2	ATP synthase beta subunit	56,293	5.19	BAE35088	5	14
3	Albumin 1	68,648	5.75	AAH49971	3	6
4	Annexin III	36,348	5.33	CAA04887	5	17
5	Tropomyosin 1, alpha, isoform h	28,679	4.75	EDL26147	3	12
6	Proteasome activator PA28 alpha subunit	28,685	5.73	AAC53295	1	6
7	1-cys peroxiredoxin protein	24,840	5.98	AAD03716	3	18
8	Nonselenium glutathione peroxidase	24,855	5.71	CAA73383	4	25
14	Vinculin	116,732	5.72	AAB96843	2	3
Up-regulated proteins in allergic asthma						
9	Tropomyosin 5	29,020	4.75	CAA37782	4	19
10	A-X actin	39,161	5.78	CAA27396	4	10
11	zswim 6 protein	35,407	9.34	AAH21311	7	30
12	Polymerase I and transcript release factor	43,927	5.43	AAL60240	1	4
13	Immunoglobulin heavy chain variable domain	13,982	9.52	BAB78785	2	15
15	Alpha-1 protease inhibitor 2	44,747	5.33	AAC28865	4	18

^a Proteins of the lung from control and OVA-treated mice were subjected to 2-DE and identified by MALDI-TOF MS/MS. The proteins were identified by searching NCBI nonredundant database using MASCOT peptide Mass Fingerprint software (Matrixscience, UK) and MS-Fit (protein Prospector, USA).

^b Sequence coverage is defined as the percentage of the whole length of the protein sequence which is covered by matched peptides identified by MALDI-TOF MS/MS.

ness of 6 μ m. The sections were stained with hematoxylin and eosin.

2.14. Statistical analysis

Data were analyzed with the paired Student's *t* test. The level of significance was *p* < 0.05.

3. Results

3.1. Proteomic analysis of lungs after OVA challenge

As a first step to search for asthma-susceptibility genes, we performed proteome analyses of lungs from control and OVA-sensitized female mice. Proteome of the lung was analyzed using 2-DE. Quadruplicate gels were used for each experimental group. Approximately 300 protein spots were detected from a conventional 2-D gel stained with Coomassie blue in the pH range of 4–7 (Fig. 1). The spots detected in all four gels were used to compare average differences in protein expression between the two groups. 15 protein spots, which reproducibly showed marked differences in protein expression between control and OVA-sensitized mice, were identified with MALDI-TOF MS/MS and are listed in Table 2.

Six and 9 proteins were up- and down-regulated, respectively, by OVA treatment.

Among the 6 up-regulated proteins is α_1 -PI type 2, which is a member of the serine protease inhibitor superfamily of protease inhibitors that participate in a variety of physiological functions including extracellular matrix remodeling and inflammation. The up-regulated expression of α_1 -PI type 2 protein was confirmed by Western blotting (Fig. 2a) and real-time RT-PCR (Fig. 2b).

3.2. α_1 -PI protein expression in several organs of mice

So far, most studies about α_1 -PI expression in mice were performed at mRNA levels. Thus, we examined protein levels of α_1 -PI in various organs in BALB/c and C57BL/6 mouse strains, which show differential susceptibility to ovalbumin-induced asthma. In BALB/c mice, as previously reported, α_1 -PI was predominantly expressed in the liver (Fig. 3). In addition to the liver, α_1 -PI was also detected in the heart, kidney, lung, and spleen at lower levels compared to the liver, but barely detectable in the brain. Results analogous to that of BALB/c mice were observed with C57BL/6 mice, except that the α_1 -PI signal in the liver was a little weaker in C57BL/6 mice than in BALB/c mice (data not shown), which

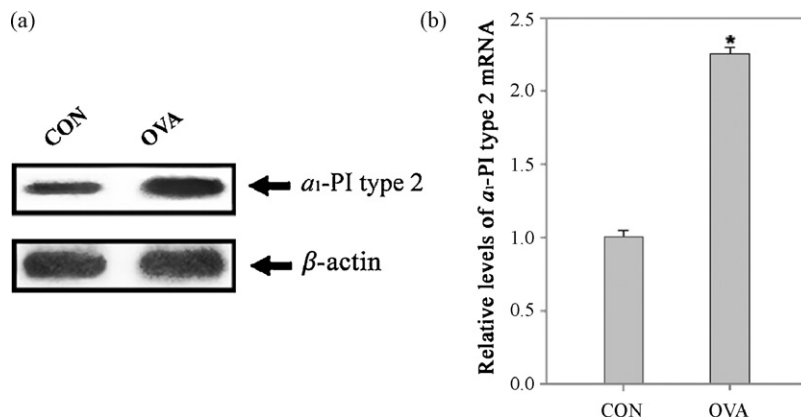


Fig. 2. Increased expression of α_1 -PI in the lung by OVA treatment. Lungs were taken from control and OVA-sensitized (4 mice each) female mice for protein and mRNA. (a) 25–50 μ g of protein extracts were separated by SDS-PAGE and then transferred to a PVDF membrane filter. The filters were primarily incubated with polyclonal anti-human trypsin inhibitor or polyclonal anti- β actin antibody and secondarily with HRP-conjugated anti-chicken IgY or HRP-conjugated anti-rabbit IgG, respectively. Immunodetection was performed by the ECL kit. (b) RNA was examined for α_1 -PI type 2 expression by real-time RT-PCR data are presented as mean $\pm$ SD of relative levels of mRNA (4 mice per group). **p* < 0.05 when compared to control.

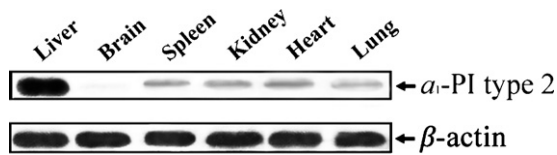


Fig. 3. Immunoblot analyses of α_1 -PI in several organs from mice. 25–50 μ g of protein extracts from BALB/c mice were separated by SDS-PAGE and then transferred to a PVDF membrane filter. The filters were primarily incubated with polyclonal anti-human trypsin inhibitor and secondarily with HRP-conjugated anti-chicken IgY. Immunodetection was performed by the ECL kit.

is consistent with previous findings [17]. It appears that most of α_1 -PI protein detected in organs other than the liver could be from the liver, rather than being synthesized in the organs themselves, because RT-PCR analyses showed little expression of α_1 -PI (Fig. 4).

3.3. RT-PCR analyses of α_1 -PI isotypes in several organs of mice

To investigate which isoform of α_1 -PI contributes to the production of α_1 -PI in organs other than the liver and whether α_1 -PI protein is encoded by mRNAs transcribed in those organs, we did RT-PCR analysis using isotype-specific primers (Table 1). Consistent with the Western blotting results, the mRNA levels of all the isoforms of α_1 -PI in the liver were highest among the organs tested

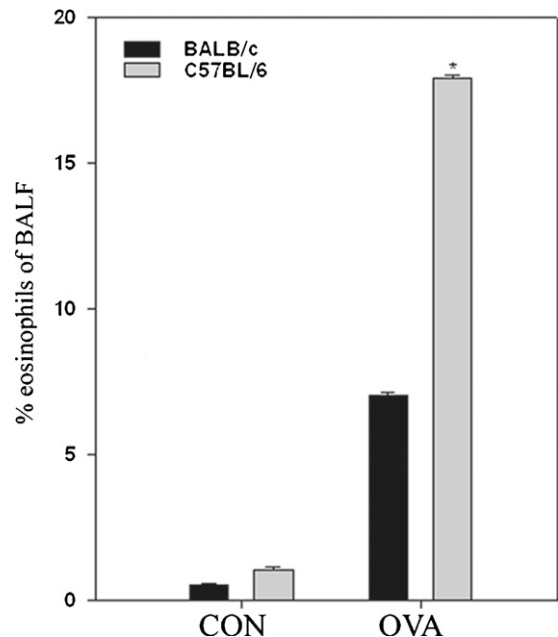


Fig. 5. Strain differences in eosinophil infiltration into the lung. Four mice were sensitized with alum-OVA mixture on days 0 and 5, and challenged on days 12 and 13. On day 14, mice were sacrificed for collection of BALF. Cells were fixed in methanol, and stained with 'Hemacolor for microscopy' and identified under the microscope according to the criteria described in the protocol (Merck, USA). Data are presented as mean $\pm$ SD (4 mice per group) and are representative of three separate experiments. * $p < 0.05$ when compared to BALB/c mice sensitized with OVA.

(Fig. 4). The amount of mRNA used for the liver was 100-fold less than that of other tissues. α_1 -PI 1–4 isotypes were expressed in the liver from both C57BL/6 and BALB/c mice, but α_1 -PI type 5 was expressed only in C57BL/6 mice, as previously reported [26]. In tissues other than the liver, the isotypes were differentially expressed: (1) all the five isoforms were expressed in the kidney and spleen in C57BL/6 and BALB/c except for α_1 -PI type 5, which was detected only in C57BL/c mice; (2) no α_1 -PI isoforms were expressed in the heart and lung in both strains of mice except α_1 -PI type 2, which was expressed in BALB/c but not in C57BL/6; (3) the levels of α_1 -PI type 2 mRNA in all the organs tested were much higher in BALB/c than in C57BL/6; (4) α_1 -PI type 2 is expressed in the lung of BALB/c, but not of C57BL/c mice; (5) the levels of α_1 -PI type 2 in organs other than the liver were much higher than that of other isoforms, suggesting that levels of α_1 -PI protein in the organs other than the liver could be partially determined by α_1 -PI type 2 mRNA synthesized in those organs; and (6) α_1 -PI type 4 isoform was not expressed in the organs other than the liver in BALB/c and α_1 -PI 5 was not expressed in all the organs tested in BALB/c.

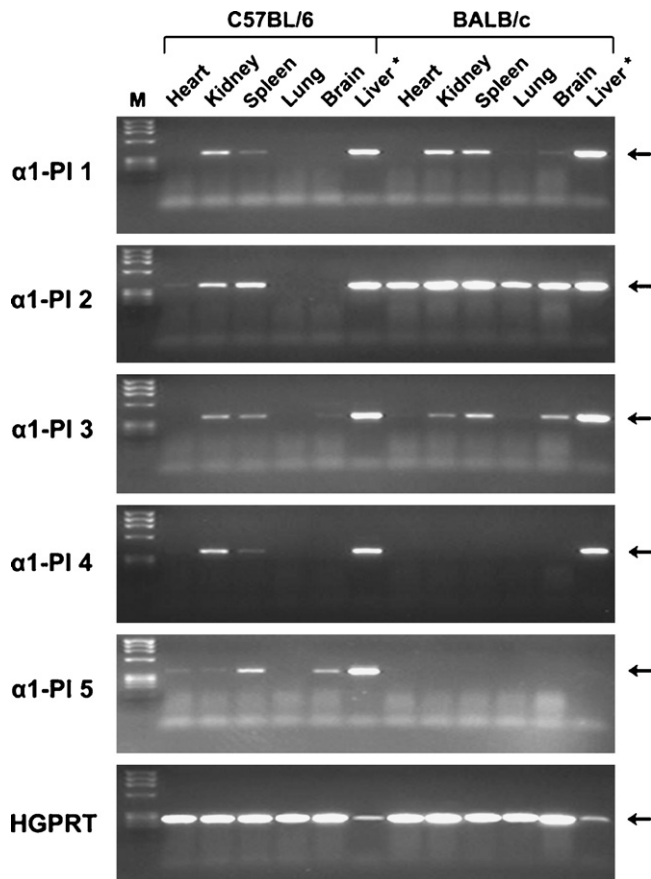


Fig. 4. mRNA analyses of α_1 -PI isoforms in several organs from BALB/c and C57BL/6 mice. RNA was prepared from organs and examined for gene expression by RT-PCR. Four mice for each group were chosen for RT-PCR analysis and a representative gel for each group is shown. The amount of mRNA used for the liver was 100-fold less than that of other organs (1–2 μ g total RNA for each reaction). Hypoxanthine guanine phosphoribosyl transferase (HGPRT) was used as an internal control. Molecular weight markers are shown to the left in each gel photograph. The arrows indicate the expected PCR product for each gene.

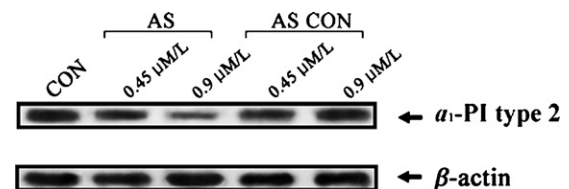


Fig. 6. Reduction of α_1 -PI expression by antisense α_1 -PI type 2 oligonucleotides. CT-26 cells were transfected as described in Section 2. Oligonucleotides formulated into liposomes were applied to each well (0.5 nM/l). Twenty-four hours after transfection, cells were harvested. 25–50 μ g of protein extracts from BALB/c mice were separated by SDS-PAGE and then transferred to a PVDF membrane filter. The filters were primarily incubated with polyclonal anti-human trypsin inhibitor and secondarily with HRP-conjugated anti-chicken IgY. Immunodetection was performed by the ECL kit.

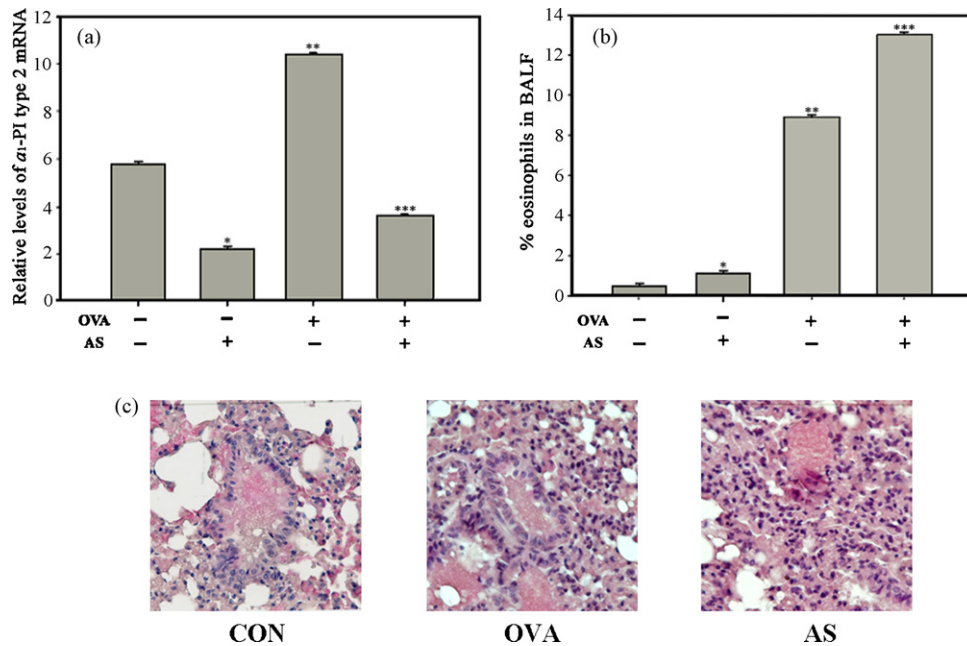


Fig. 7. Increase of lung infiltration of eosinophils by intranasal administration of antisense α_1 -PI oligonucleotides. Antisense α_1 -PI (AS) were given by intranasal administration (86 μ g in 20 μ l PBS/treatment) at days 12 and 13 (30 min before aerosolization with OVA) to OVA-sensitized or control mice (4 mice each group). Mice were sacrificed 24 h later. (a) RNA was examined for α_1 -PI type 2 expression by real-time RT-PCR. Data are presented as mean $\pm$ SD of relative levels of mRNA (4 mice per group). * p < 0.05 when compared to control. (b) Cells in BALF were fixed in methanol, and stained with 'Hemacolor for microscopy' and identified under the microscope according to the criteria described in the protocol (Merck, USA). Data are presented as mean $\pm$ SD (4 mice per group). *, ** p < 0.05 when compared to BALB/c control mice. *** p < 0.05 when compared to BALB/c mice sensitized with OVA. (c) Histology of the lungs. Con: control; OVA: OVA-sensitized; AS: OVA-sensitized and antisense α_1 -PI type 2 oligonucleotides administrated. 400 $\times$.

3.4. Intranasal administration of α_1 -PI type 2 antisense oligonucleotides increases lung infiltration of eosinophils

As described in the previous sections, the increase of the expression of α_1 -PI type 2 in OVA-induced asthma and the strain-dependent differential susceptibility to the OVA-induced asthma in conjunction with differential expression of α_1 -PI type 2 mRNA in the lung raise a possibility that α_1 -PI type 2, which is produced in the lung from BALB/c but not from C57BL/c mice, could regulate pathogenesis of asthma. In an attempt to investigate the role of α_1 -PI type 2 in the pathogenesis of asthma, we used the antisense oligonucleotide technology to specifically down-regulate expression of α_1 -PI type 2. Mice were sensitized with alum-precipitated ovalbumin (OVA) and challenged with aerosolized OVA. Twenty-four hours after the last challenge with OVA, mice were sacrificed. Bronchoalveolar lavage fluid (BALF) cells were centrifuged onto a microscope slide, stained with Hemacolor, and observed under a microscope. Percentages of eosinophils present in the total leukocytes, which is one of the characteristics of asthma, are shown in Fig. 5. Percentages of eosinophils in OVA-sensitized C57BL/6 mice significantly increased compared to OVA-sensitized BALB/c mice, which is consistent with previous findings [9].

We designed antisense phosphorothioate oligonucleotides targeting the translation start site of murine α_1 -PI type 2. First, we examined the ability of such oligonucleotides to down-regulate expression of α_1 -PI type 2 in CT-26 cells, a murine epithelial cell line which constitutively expresses α_1 -PI type 2. Twenty-four hours after transfection with α_1 -PI type 2 antisense oligonucleotides, expression of α_1 -PI type 2 was significantly reduced in CT-26 cells treated with α_1 -PI type 2 antisense oligonucleotides compared with control cells at the protein level (Fig. 6). Treatment with mismatched control oligonucleotides did not affect α_1 -PI type 2 expression.

Then we tested the ability of antisense oligonucleotides to decrease pulmonary expression of α_1 -PI type 2 in OVA-sensitized

mice after intranasal administration of antisense oligonucleotides. As shown in Fig. 7a, type 2 mRNA expression in the lungs was remarkably reduced by antisense oligonucleotides, but not by mismatched control oligonucleotides. The level of α_1 -PI type 2 mRNA was also reduced when α_1 -PI type 2 antisense oligonucleotides were intranasally administrated into control mice (Fig. 7a). However, no such difference was detected on protein levels of α_1 -PI (data not shown), probably because α_1 -PI is mainly produced in the liver and transported to other organs including the lung.

To assess the effects of α_1 -PI type 2 antisense oligonucleotides on pulmonary infiltration of eosinophils, antisense oligonucleotides were intranasally administrated on 2 consecutive days (days 12 and 13) 30 min before OVA challenge and BALF was collected 24 h after the last of two sequential OVA challenges and assayed for eosinophils by morphometric criteria. The influx of eosinophils into the lung was significantly increased in all 4 mice treated with α_1 -PI type 2 antisense oligonucleotides compared with 4 mice treated with OVA alone (Fig. 7b). However, no remarkable effect was observed with mismatched control oligonucleotides (data not shown). The influx of eosinophils into the lung was slightly increased in control mice when α_1 -PI type 2 antisense oligonucleotides were intranasally administrated into those animals (Fig. 7b). Histological examination of the lungs revealed in the perivascular area a remarkable OVA-induced infiltration of inflammatory cells, which was further enhanced by α_1 -PI type 2 antisense oligonucleotides (Fig. 7c).

4. Discussion

In this study we have demonstrated that in an OVA-induced asthma in mice, serine protease inhibitor α_1 -PI type 2 is up-regulated in the lungs of mice by OVA sensitization. Then we found that in addition to the liver, other organs such as the kidney and spleen also expressed different isoforms of α_1 -PI in a tissue-specific manner, to a lesser extent compared with the liver. Considering

the strain difference in susceptibility to OVA-induced asthma in mice, it was of great interest that α_1 -PI type 2 was expressed in the lung of BALB/c mice, but not of C57BL/6 mice, which has been shown to be more susceptible than BALB/c mice. Then we were able to show that pulmonary infiltration of eosinophils was significantly increased by intranasal administration of α_1 -PI type 2 antisense oligonucleotides in OVA-sensitized mice, suggesting that α_1 -PI type 2 may regulate the pathogenesis of asthma, probably by acting on neutrophil elastase, which can produce many of the pathological features of asthma, including epithelial damage and connective tissue destruction.

Plasma concentrations of α_1 -PI protein increase as a result of inflammation, such as post-bone surgery and surgical trauma [27]. However, not much has been reported on the regulation of α_1 -PI in asthmatic patients or experimental animals. Asthmatic patients produced significantly higher levels of sputum α_1 -PI and elastase than control subjects [28], suggesting that the imbalance between elastase and its main inhibitor α_1 -PI may play a critical role in the pathogenesis of asthma and that local synthesis and release of α_1 -PI is of importance at sites of inflammation, particularly at locations not directly in contact with α_1 -PI in the blood. In this study, expression of α_1 -PI, specifically type 2 isoform, was remarkably increased at both protein and mRNA levels. At present, it remains to be known which cell population in the lung produces α_1 -PI type 2. It could be alveolar epithelial cells, macrophages or eosinophils [27,29].

RT-PCR analyses of different organs confirmed the predominant expression of α_1 -PI in the liver. In addition, it was demonstrated that α_1 -PI is also expressed in the kidney and spleen to a much lesser extent. Of interest is the strain-specific expression of α_1 -PI type 2, which is highly expressed in all the organs tested in BALB/c strain, but in only the spleen and kidney from C57BL/6 strain to a lesser extent, raising a possibility that differential expression of α_1 -PI type 2 in the lung, could play a role in strain differences in susceptibility to OVA-induced asthma. It has been shown that tissue-specific expression of α_1 -PI is governed by the methylation status of α_1 -PI genes, particularly at the promoter regions; α_1 -PI genomic sequences are undermethylated in liver DNA compared to kidney or brain DNA from C57BL/6 mice [30]. Promoter sequences and nuclear factors including hepatocyte nuclear factor 3 (HNF-3) and HNF-4, which control α_1 -PI expression, have been characterized [31,32]. How the strain-specific expression of α_1 -PI type 2 is regulated will be of interest. Identification and characterization of the promoters regulating the expression of α_1 -PI isoforms could elucidate the molecular mechanisms underlying the dominant expression of α_1 -PI type 2 over other isoforms in the heart, kidney, spleen, brain, and lung.

Leukocytes and lung epithelial cells contribute to the pathophysiology of asthma by producing numerous mediators including serine proteases and metalloproteinases [33]. Thus, effects of endogenous or synthetic inhibitors for the proteinases on the pathogenesis of respiratory diseases have been studied in animals and human patients. Secretory leukocyte protease inhibitor (SLPI), a product of Clara cells, and several synthetic serine and trypsin inhibitors have been shown to reduce allergen-induced pulmonary responses in animals [34–36]. By analogy, tissue inhibitor of metalloproteinase-1 and a synthetic matrix metalloproteinase inhibitor prevented allergen-induced airway inflammation in a murine model of asthma [37]. Roles of α_1 -PI in the pathogenesis of respiratory diseases have been studied. In a randomized clinical trials, α_1 -PI augmentation therapy reduced lung infections and symptoms of emphysema in patients with α_1 -PI deficiency [38,39]. α_1 -PI attenuated emphysema in a mouse model of emphysema by inhibiting structural alveolar cell apoptosis [40], suggesting that α_1 -PI could attenuate symptoms of lung diseases by acting on molecules other than neutrophil elastase. However, roles of α_1 -

PI in the pathogenesis of asthma remains to be elucidated. In this study, we have demonstrated that local blockade of α_1 -PI expression in the lung by antisense oligonucleotides augmented lung infiltration of eosinophils in a murine model of asthma, suggesting that α_1 -PI locally produced in the lung may also contribute to attenuation of asthmatic symptoms as α_1 -PI produced in the liver and transported to other organs might do so. It was previously reported that inhaled human plasma-derived or recombinant α_1 -PI blocked allergen-induced AHR in sheep [41,42], suggesting that α_1 -PI may regulate allergen-induced AHR and probably asthma. However, roles of α_1 -PI in allergen-induced inflammation such as lung eosinophilia, another feature of asthma, was not addressed in that study. The results in the present study could provide another piece of evidence for the role of α_1 -PI in controlling the pathogenesis of asthma.

While humans have only one functional α_1 -antitrypsin (AT) gene, laboratory mice (*Mus musculus domesticus*) have a family of three to five genes of α_1 -PI genes clustered in the *Serpina1* locus at position 51 of chromosome 12 [43,44]. The isoforms are highly homologous at both nucleotide and amino acid sequences, but are variable in the P1 residue of the reactive center loop, which may determine the target specificity [45,46]. Indeed, at the P1 position, murine α_1 -PI type 1 and 2 isoforms have methionine as does human α_1 -AT, whereas other isoforms have either tyrosine or leucine. Recombinant α_1 -PI proteins showed variable activities on different substrates [47]. Although all five isoforms of α_1 -PI (types 1–5) were active on elastase and chymotrypsin, α_1 -PI type 2 was most active on both substrates, particularly on elastase. In addition, α_1 -PI type 2 is expressed in all the laboratory mouse strains as well as some of the wild-derived *Mus* species [44], suggesting that α_1 -PI type 2 may be primarily responsible for α_1 -PI in the serum and other organs. In this study, we have demonstrated that α_1 -PI type 2 protein level is increased in the lung by antigen stimulation, only type 2 among five isoforms of α_1 -PI is expressed in all the organs examined including the lung, and type 2 is the only α_1 -PI expressed in the lung, suggesting that α_1 -PI type 2 could play a regulatory role in the pathogenesis of asthma.

In conclusion, we demonstrated that expression of α_1 -PI type 2 in the lung was increased in OVA-induced asthmatic mice. Then, in addition to the liver, α_1 -PI protein was also expressed in other organs including the lung, where α_1 -PI type 2 is expressed from BALB/c mice, but not from C57BL/6 mice, which is more susceptible to OVA-induced asthma than BALB/c. Intranasal administration of α_1 -PI type 2 antisense oligonucleotides increased lung eosinophilia in OVA-induced allergic asthma, indicating that α_1 -PI type 2 produced in the lung plays a regulatory role in the pathogenesis of asthma.

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