Intra-airway administration of small interfering RNA targeting plasminogen activator inhibitor-1 attenuates allergic asthma in mice

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Intranasal administration of small interfering RNA targeting plasminogen activator inhibitor-1 attenuates allergic asthma in mice. Am J Physiol Lung Cell Mol Physiol 301: L908–L916, 2011. First published September 30, 2011; doi:10.1152/ajplung.00115.2011.—Recent studies suggest that plasminogen activator inhibitor-1 (PAI-1), a major inhibitor of the fibrinolytic system, may promote the development of asthma. To further investigate the significance of PAI-1 in the pathogenesis of asthma and determine the possibility that PAI-1 could be a therapeutic target for asthma, this study was conducted. First, PAI-1 levels in induced sputum (IS) from asthmatic subjects and healthy controls were measured. In asthmatic subjects, IS PAI-1 levels were elevated, compared with that of healthy controls, and were significantly higher in patients with long-duration asthma compared with short-duration asthma. PAI-1 levels were also found to correlate with IS transforming growth factor-β levels. Then, acute and chronic asthma models induced by ovalbumin were established in PAI-1-deficient mice and wild-type mice that received intranasal administration of small interfering RNA against PAI-1. In PAI-1-deficient mice and wild-type mice that received intranasal administration of PAI-1-siRNA, the expression of PAI-1 in airway epithelial cells (AECs) was found to be upregulated compared with nonasthmatic AECs, and PAI-1 levels in induced sputum samples from asthmatic subjects were increased compared with those from healthy controls. In addition, in a murine model of chronic asthma, PAI-1-deficient mice showed a lesser degree of airway remodeling than wild-type (WT) mice. These findings indicate that PAI-1 may be a therapeutic target molecule for bronchial asthma and suggest that inhibiting PAI-1 activity and/or expression in the airways could become a therapeutic strategy to suppress the development and progression of bronchial asthma.

To further investigate if PAI-1 could be a therapeutic target for bronchial asthma, the present study used the following three strategies. First, with the use of induced sputum samples from asthmatic subjects, the relationships among PAI-1, transforming growth factor (TGF)- β, and duration of asthma were analyzed. Second, the degrees of eosinophilic airway inflammation, AHR, and airway remodeling were evaluated in acute and chronic asthma models generated in WT and PAI-1-deficient mice. Finally, we evaluated the effects of intranasal administration of small interfering RNA against PAI-1 on the development of eosinophilic airway inflammation, AHR, and airway remodeling in murine models of acute and chronic asthma.

MATERIALS AND METHODS

Induced sputum. The patients with previously diagnosed bronchial asthma (n = 31, disease duration <10 yr: 8; 10–20 yr: 11; >20 yr: 12) in stable clinical condition followed up at Hiroshima University Hospital were recruited. The collection of induced sputa was approved by the ethics committee of Hiroshima University, and all subjects provided written, informed consent. There were no statistically significant differences in the age among the three groups of asthmatic subjects (data not shown). Seventeen patients (<10 yr: 4; 10–20 yr: 7; >20 yr: 6) with bronchial asthma were receiving regular inhaled corticosteroids. The nonsmoking healthy volunteers (n = 8) without respiratory symptoms were also recruited as the healthy subjects. Induced sputum was performed as previously described (5, 22). Briefly, to collect sputum samples, the subjects inhaled hypertonic 5.0% NaCl solution for 15 min delivered via an ultrasonic nebulizer with an output of 3.0 ml/min.

Mice. Breeding pairs of homozygous PAI-1−/− mice strains on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). The three to four generations from the breeding pairs of PAI-1−/− mice were used for the experiments to reduce the possibility of changes in genetic background. Age- and sex-matched WT C57BL/6 (PAI-1+/+) mice were purchased from the Charles River Laboratories (Kanagawa, Japan), which, in Asia, is the exclusive, authorized commercial breeder and distributor of mice main-
tain the Jackson Laboratory. Animals were maintained according to guidelines for the ethical use of animals in research at Hiroshima University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Hiroshima University and carried out in accordance with the Hiroshima University Animal Experimental Regulations.

Protocols for ovalbumin-induced allergic airway inflammation. All mice were sensitized on days 0 and 14 by intraperitoneal injection of 10 µg ovalbumin (OVA) (Sigma Aldrich, St. Louis, MO) and 20 mg Al(OH)3 in 0.2 ml of PBS (pH 7.4). After sensitization, control mice (OVA/PBS group for acute model and OVA/5-wk PBS group for chronic model) were exposed to aerosolized PBS. Asthma model mice (OVA/OVA group for acute model and OVA/5-wk OVA group for chronic model) were exposed to 1% OVA aerosols for 20 min once a day following the protocols summarized in Fig. 2.

siRNA designs. siRNAs were designed using the TaKaRa online algorithm (TaKaRa Bio, Tokyo, Japan). The sequences of siRNA directed against mouse PAI-1 (PAI-1-siRNA) were as follows: sense, 5'-CCCAAGGCAAGCACAACCATT-3', and antisense, 5'-UGUUGUGCCUCUUGUGGTT-3'. The sequences of nonspecific scrambled (NS) siRNA were as follows: sense, 5'-ACCACGACGUACAACCAACTT-3', and antisense, 5'-UGUUUGUGUAGCGUGUGGTT-3'. The specificities of these sequences were confirmed by performing a BLAST search against the National Center for Biotechnology Information database.

siRNA administration. PAI-1-siRNA and NS-siRNA were dissolved in RNase-free water and then administered intranasally (2 µM in 50 µl) after anesthesia. The first sensitization with OVA, siRNA was administered on days 15, 18, 21, 24, 27, and 30 for the acute asthma model, and on days 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, and 57 for the chronic asthma model following the protocols summarized in Fig. 5. In preliminary experiments to determine an appropriate dose of PAI-1-siRNA, we intranasally administered different amounts (2, 10, and 20 µM in 50 µl) of PAI-1-siRNA and NS-siRNA and 50 µl of water into mice with acute and chronic asthma. In these experiments, we found that 2 µM of PAI-1-siRNA was an effective dose, and 20 µM of NS-siRNA did not cause any significant influences on lung hydroxyproline levels, and inflammatory cells. PAI-1 levels, and TGF-β levels in bronchoalveolar lavage (BAL) fluids (data not shown).

Measurement of AHR. AHR was assessed 24 h after the final aerosol challenge by calculating respiratory system resistances using various concentrations of methacholine (MCh), as described previously (21). Mice were connected to a ventilator (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) at a rate of 160 breaths/min with a stroke volume of 175 µl. A plethysmograph (Buxco Electronics, Troy, NY) was sealed, and a pressure transducer measured alterations in tracheal pressure. Once stabilized, aerosols containing varying concentrations of MCh (0, 0.5, 1, 2, 4, 8, 16 mg/ml) were delivered to the inspiratory line for 3 min, and pressure and flow data were continuously recorded. A computer program (BioSystemXa, Buxco) was used to calculate respiratory system resistances for each MCh concentration.

Immunohistochemistry for PAI-1. Lung sections from mice prepared from paraffin-embedded tissue were immunohistochemically stained for PAI-1, as previously described (26).

BAL. BALF was obtained as previously described (21, 24). Briefly, mice were killed with a lethal dose of pentobarbital, the tracheas were cannulated with a 20-gauge needle, and the lungs were lavaged three times with 0.5 ml of PBS. Cells in BALF were counted with a standard hemocytometer. Differential cell counts were obtained by Diff-Quik (Kokusai Shiyaku, Kobe, Japan) using cytospin preparations (Shandon, Pittsburgh, PA).

Measurements of PAI-1 and TGF-β, hepatocyte growth factor in BALF and induced sputum. PAI-1, TGF-β, and hepatocyte growth factor (HGF) levels in samples were measured using ELISA kits for PAI-1 (Innovative Research, Novi, MI), for TGF-β (R&D Systems, Minneapolis, MN), and for HGF (Institute of Immunology, Tokyo, Japan), according to the manufacturers’ instructions.

Fluorescence imaging. Rhodamine-labeled PAI-1-siRNA was synthesized by TaKaRa Bio (Tokyo, Japan) and instilled intranasally into mice. Twenty-four hours after the siRNA instillation, frozen lung sections were scanned at an excitation wavelength of 540 nm using a BZ-α fluorescence imaging system (KEYENCE, Osaka, Japan) with an inverted fluorescence phase-contrast microscope BZ-9000 (KEYENCE).

Hydroxyproline assay. To quantify lung collagen contents, hydroxyproline contents in whole mouse lungs were measured in each group 24 h after final exposure of OVA or PBS, as described previously (9).

Histology. After BAL and lung perfusion, the lungs of mice were fixed by inflation with a buffered 10% formalin solution. The lung tissue specimens were embedded in paraffin and cut into 5-µm sections. The sections were stained with hematoxylin and eosin to evaluate airspace eosinophilia, or Elastica-Masson’s trichrome to evaluate peribronchial fibrosis, and examined by light microscopy. Two investigators independently and blindly selected a representative histology by viewing at least 10 high-power fields per lung from all mice of each group. Thereafter, the representative histology for each group was chosen from these two candidates following the discussion and agreement between the two investigators.

Statistical analysis. Results are given as means ± SE. Comparisons between groups were made by a Mann-Whitney U-test. Correlations between biomarkers were determined using Pearson correlation coefficient analysis. A P value of <0.05 was considered to be statistically significant. All analyses were performed using SPSS, version 12.0 (SPSS, Chicago, IL).

RESULTS

PAI-1 levels in induced sputum from asthmatic subjects are elevated and positively correlated with asthma duration and TGF-β levels. We first compared the PAI-1 levels in induced sputum samples from bronchial asthma patients and from healthy subjects. Based on asthma duration, asthmatic patients were divided into three groups: <10 yr, 10–20 yr, >20 yr. As shown in Fig. 1A, the PAI-1 levels in induced sputum from the asthmatic subjects were significantly higher than those in the healthy subjects. In addition, the PAI-1 levels in induced sputum from patients with long asthma duration were significantly higher than those with short asthma duration (Fig. 1B). We also found a correlation between the PAI-1 and TGF-β levels in induced sputum samples from the asthmatic subjects (Fig. 1C).

PAI-1 levels in BALFs are elevated in murine models of acute and chronic asthma. The experimental protocols to establish murine models of acute and chronic asthma are summarized in Fig. 2. BAL was performed on day 31 for the acute asthma model and on day 59 for the chronic asthma model. As shown in Fig. 3A, the PAI-1 levels in the BALFs of the acute and chronic asthma models were significantly increased compared with those of control mice. Interestingly, the chronic asthma model showed significantly higher PAI-1 levels in BALFs than the acute asthma model.

PAI-1 deficiency reduces eosinophilic inflammation and AHR in an acute asthma model. Acute asthma was established in PAI-1-deficient mice, and the degrees of AHR and airway inflammation were analyzed. As shown in Fig. 3B, the numbers of inflammatory cells and eosinophils in BALF were significantly decreased in PAI-1-deficient mice compared with WT mice for the acute asthma model. In accordance with the
analysis of cells in BALFs, histological examinations showed a lesser degree of eosinophil accumulation in the airways of PAI-1-deficient mice compared with WT mice for the acute asthma model (Fig. 3C). Corresponding with the degree of eosinophil accumulation in the airways, the degree of AHR after MCh challenge was significantly reduced in PAI-1-deficient mice compared with WT mice for the acute asthma model (Fig. 3D).

PAI-1 deficiency suppresses airway remodeling in a chronic asthma model. To evaluate the effect of PAI-1 on airway remodeling in a murine asthma model, lung hydroxyproline contents were analyzed for the chronic asthma models generated in PAI-1-deficient and WT mice. The lung hydroxyproline contents were significantly higher for the chronic asthma model compared with control mice (Fig. 3E). When the lung hydroxyproline contents were compared between PAI-1-deficient and WT mice, significantly lower values were observed in PAI-1-deficient mice for the chronic asthma model (Fig. 3E).

In addition, to evaluate the degree of peribronchial fibrosis, lung sections were stained with Elastica-Masson’s trichrome. As shown in Fig. 3F, the accumulation of collagen in peribron-
chial areas was markedly reduced in the PAI-1-deficient mice compared with WT mice for the chronic asthma model.

Localization of PAI-1-siRNA following single intranasal administration into mice with OVA-induced asthma. Based on observations that PAI-1 is predominantly expressed in the airway epithelium, both in asthmatic patients and murine asthma models, the distribution of PAI-1-siRNA in the lungs of mice with OVA-induced bronchial asthma was determined following a single intranasal administration. Rhodamine-labeled PAI-1-siRNA or unlabeled PAI-1-siRNA was intranasally instilled into the mice of the acute asthma model on day 30 after the first OVA sensitization. After 24 h, the localization of fluorescence in the lungs was evaluated by fluorescence microscopy. The highest fluorescence intensity was detected in the bronchial epithelium, and slightly less intense fluorescence emanated from the alveolar epithelium (Fig. 4).

Repeated intra-airway administration of PAI-1-siRNA reduces PAI-1 level and elevates HGF level in BALF and suppresses eosinophilic airway inflammation and AHR in an acute asthma model. For the acute asthma model, siRNAs were administered on days 15, 18, 21, 24, 27, and 30 after the first OVA sensitization (Fig. 5). On day 31, BAL was performed, AHR was measured, and lungs were removed. As shown in Fig. 6, A and B, repeated intranasal administration of PAI-1-siRNA for the acute asthma model significantly reduced the PAI-1 levels and the numbers of eosinophils in BALFs compared with NS-siRNA. A histological examination also showed that eosinophilic accumulation in the airways was prominently inhibited in the mice that were repeatedly administered PAI-1-siRNA compared with those that were administered NS-siRNA (Fig. 6C). As shown in Fig. 6D, repeated intranasal instillation of PAI-1-siRNA significantly limited the increase in AHR compared with NS-siRNA. In addition, we measured the levels of HGF, which has been reported to regulate airway inflammation and AHR (12, 20), in BALFs from mice for the acute asthma model. We found that repeated intranasal instillations of PAI-1-siRNA for the acute asthma model significantly elevated HGF levels in BALFs compared with NS-siRNA (Fig. 6E). We also confirmed that the HGF levels in BALFs from PAI-1-deficient mice were significantly higher than those in WT mice for the acute asthma model (Fig. 6E).

Repeated intra-airway administration of PAI-1-siRNA reduces PAI-1 expression in the lung and suppresses airway remodeling in a chronic asthma model. For the chronic asthma model, siRNAs were administered on days 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, and 57 after the first OVA sensitization.
On day 59, BAL was performed, and lungs were removed. As shown in Fig. 7A, repeated intranasal administration of PAI-1-siRNA significantly reduced the PAI-1 levels in BALFs compared with NS-siRNA. Correspondingly, an immunohistochemical examination showed that the expression of PAI-1 was diminished in the bronchial epithelium, alveolar epithelium, peribronchial areas, and alveolar macrophages in the mice that were administered PAI-1-siRNA compared with mice that received NS-siRNA (Fig. 7B). In addition, to evaluate the effect of PAI-1-siRNA administration on airway remodeling, lung hydroxyproline contents were measured, and histological examinations were made to evaluate the degree of peribronchial fibrosis.

As shown in Fig. 7C, repeated intranasal administrations of PAI-1-siRNA was effective for limiting the accumulation of collagen in the lungs compared with NS-siRNA. A histological examination of sections stained with Elastica-Masson’s trichrome also showed that the fibrotic areas beneath the basement membranes of the bronchi improved in the mice that were repeatedly administered PAI-1-siRNA (Fig. 7D). We also measured the levels of TGF-β, a highly potent profibrotic cytokine, in BALFs and found that repeated intranasal instillations of PAI-1-siRNA significantly reduced its levels compared with NS-siRNA (Fig. 7E). We also confirmed that the TGF-β levels in BALFs from PAI-1-deficient mice were significantly lower than those in WT mice for the chronic asthma model (Fig. 7E).

**DISCUSSION**

There is a growing body of evidence that PAI-1 is involved in the development and progression of allergic airway inflammation, AHR, and airway remodeling. PAI-1 mRNA expression was upregulated in the AECs of asthmatic subjects (27). The concentration of PAI-1 in induced sputum samples of allergic asthmatic subjects was significantly higher than that of healthy nonatopic subjects and was correlated with %forced expiratory volume in 1 s (14). PAI-1 was shown to be upregulated in the lungs of both acute (30) and chronic murine asthma models (19). In addition, PAI-1 deficiency in mice was reported to reduce the degree of upper airway eosinophilic inflammation and AHR in an upper airway allergic model (25) and suppress airway remodeling in a chronic asthma model (19). Furthermore, in the present study, we demonstrated that PAI-1 levels in induced sputum from asthmatic subjects were associated with asthma duration and were correlated with TGF-β levels. Based on the previous observations that asthma duration (1, 13) and TGF-β (7, 17, 29) were related to airway...
remodeling, our findings suggest that PAI-1 is also involved in the development of airway remodeling.

In the present study, using PAI-1-deficient mice, we showed that PAI-1 was associated with the development of eosinophilic airway inflammation and AHR in an acute asthma model and the development of airway remodeling in a chronic asthma model. PAI-1 involvement in eosinophilic airway inflammation and AHR in these asthma models may be considered compatible with the previous observations shown with an upper airways allergy model (25). Our new findings further demonstrate the possibility that PAI-1 plays an important role in the pathogenesis of bronchial asthma.

The most important finding in the present study was that downregulation of PAI-1 in the airways limited the development and the progression of bronchial asthma in a murine model. Upregulation of fibrinolytic activity in the airways by administering aerosols of tPA or uPA into mice was previously reported to diminish the development and progression of allergic airway inflammation, AHR, and airway remodeling in murine models of acute and chronic asthma (16, 30). The results of the present study also demonstrated the feasibility that knockdown of PAI-1 by siRNA could be a therapeutic strategy for treating bronchial asthma.

To knock down PAI-1 in the airways, we administered siRNA that targeted PAI-1 into mouse lungs without using a viral vector or a transfection agent. Because previous studies reported that intra-airway administration of siRNAs that targeted tumor necrosis factor-related apoptosis-inducing ligand (31) or signal transducers and activators of transcription-6 (4) suppressed allergic airway inflammation and AHR in murine models of acute asthma, the possibility of this approach as a therapeutic intervention for asthma was already demonstrated.

In the present study, using rhodamine-labeled PAI-1-siRNA, we confirmed that siRNA could be primarily incorporated into the bronchial epithelium, where PAI-1 was shown to be upregulated in a murine asthma model or in asthmatic patients following a single intranasal instillation. This also indicates the feasibility of suppressing PAI-1 in the airways by intra-airway administration of PAI-1-siRNA as a therapeutic strategy for bronchial asthma. In contrast to the strategy to upregulate
fibrinolytic activity in the airways by administering aerosols of uPA or tPA, intra-airway administration of PAI-1-siRNA may be a more preferable strategy, because the preparation of siRNA might be much easier compared with that for recombinant plasminogen activators.

In the murine model of acute asthma, the absence or knockdown of PAI-1 decreased the degree of eosinophilic airway inflammation and AHR. Indeed, previous studies reported a strong link between the absence of PAI-1 and Th1-type immune responses. Renckens et al. (23) demonstrated that PAI-1 deficiency was significantly associated with enhanced IFN-γ production in lipopolysaccharide and staphylococcal enterotoxin B-induced inflammation. Sejima et al. (25) reported that, in an OVA-induced upper airways allergy model generated in PAI-1-deficient mice, the immune response shifted from a Th2 to a Th1 type. These observations suggest that PAI-1 is associated with a Th2-type immune response, which may lead to eosinophilic airway inflammation in an asthma model. In addition, the absence or knockdown of PAI-1 was shown to elevate HGF levels in BALFs from mice for the acute asthma model. In murine acute asthma models, HGF has been reported to attenuate eosinophilic airway inflammation and AHR by inhibiting Th2 cytokine production (12) or suppressing the antigen-presenting capacity of dendritic cells (20). HGF is also known to be downregulated by PAI-1 (3). These observations suggest that HGF elevation in the airway resulted from the absence or knockdown of PAI-1 and also plays a role in the inhibition of eosinophilic airway inflammation and AHR. The presence of eosinophilic airway inflammation is believed to enhance AHR (8), and the deposition of fibrin on the airway surface resulting from overexpression of PAI-1 in the airway is reported to enhance AHR (30). A previous study demonstrated that the degree of fibrin deposition in the airway is reduced by the absence of PAI-1 in a murine asthma model (19). Judging from these observations, we can speculate that the absence or knockdown of PAI-1 reduced fibrin deposition in the airway and decreased AHR in the murine model of acute asthma.

In the murine model of chronic asthma, the absence or knockdown of PAI-1 decreased the degree of airway remodeling. The relationship between PAI-1 and tissue remodeling, includ-
ing pulmonary fibrosis, has been firmly established (6, 9, 10, 16, 19, 26), and our results confirmed the findings of previous reports. Interestingly, we found that the levels of TGF-β, a highly potent profibrotic cytokine, in BALFs from PAI-1-deficient mice or WT mice administered PAI-1-siRNA were significantly reduced compared with WT mice or WT mice administered NS-siRNA in a chronic asthma model. Previous studies demonstrated the involvement of TGF-β in the development of airway remodeling in bronchial asthma (7, 17, 29); therefore, our findings strongly suggest that the levels of PAI-1 in the airways have an influence on the effects of TGF-β for the development of airway remodeling.

In conclusion, the results of the present study indicate the strong involvement of PAI-1 in the pathogenesis of bronchial asthma and the feasibility of PAI-1 knockdown in the airways as a therapeutic strategy for bronchial asthma. Using PAI-1-deficient mice, PAI-1 deficiency was shown to reduce the degrees of eosinophilic airway inflammation and AHR in an acute asthma model and limit airway remodeling in a chronic asthma model. Interestingly, knockdown of PAI-1 in the airways by repeated intra-airway administrations of PAI-1-siRNA showed nearly identical effects in both asthma models. These findings strongly suggest that the intra-airway administration of siRNA that targets PAI-1 may be able to become a new therapeutic approach to suppress the development and the progression of bronchial asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.M. and T.S. performed experiments; S.M., T.S., T.H., T.K., and T.S. analyzed the data; S.M. and T.S. wrote the manuscript; S.M., T.S., T.K., and T.S. approved the final version of the manuscript.

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