

Emi Kuramoto, Teruaki Nishiuma, Kazuyuki Kobayashi, Masatsugu Yamamoto, Yuko Kono, Yasuhiro Funada, Yoshikazu Kotani, Thomas H. Sisson, Richard H. Simon and Yoshihiro Nishimura

Am J Physiol Lung Cell Mol Physiol 296:337-346, 2009. First published Dec 19, 2008;
doi:10.1152/ajplung.90434.2008

You might find this additional information useful...

This article cites 37 articles, 23 of which you can access free at:

<http://ajplung.physiology.org/cgi/content/full/296/3/L337#BIBL>

Updated information and services including high-resolution figures, can be found at:

<http://ajplung.physiology.org/cgi/content/full/296/3/L337>

Additional material and information about *AJP - Lung Cellular and Molecular Physiology* can be found at:

<http://www.the-aps.org/publications/ajplung>

This information is current as of September 27, 2009 .

Inhalation of urokinase-type plasminogen activator reduces airway remodeling in a murine asthma model

Emi Kuramoto,¹ Teruaki Nishiuma,¹ Kazuyuki Kobayashi,¹ Masatsugu Yamamoto,¹ Yuko Kono,¹ Yasuhiro Funada,¹ Yoshikazu Kotani,¹ Thomas H. Sisson,² Richard H. Simon,² and Yoshihiro Nishimura¹

¹Division of Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan; and ²Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan

Submitted 7 August 2008; accepted in final form 12 December 2008

Kuramoto E, Nishiuma T, Kobayashi K, Yamamoto M, Kono Y, Funada Y, Kotani Y, Sisson TH, Simon RH, Nishimura Y. Inhalation of urokinase-type plasminogen activator reduces airway remodeling in a murine asthma model. *Am J Physiol Lung Cell Mol Physiol* 296: L337–L346, 2009. First published December 19, 2008; doi:10.1152/ajplung.90434.2008.—The airway remodeling that occurs in asthma is characterized by an excess of extracellular matrix deposition in the submucosa, hyperplasia/hypertrophy of smooth muscle, goblet cell metaplasia, and accumulation of fibroblasts/myofibroblasts. The urokinase-type plasminogen activator (uPA)/plasmin system participates in pericellular proteolysis and is capable of directly degrading matrix components, activating latent proteinases, and activating growth factors. In a mouse ovalbumin (OVA) asthma model, we increased plasminogen activator activity in the lung by administering exogenous uPA or by using mice genetically deficient in the uPA inhibitor plasminogen activator inhibitor-1 (PAI-1) to assess the role of this system in asthma pathogenesis. After intraperitoneal OVA sensitization, mice inhaled OVA plus uPA (500 IU/mouse) or saline by ultrasonic nebulization for 3 wk. When studied 24 h after the final exposure, the groups with upregulated plasmin activity had significantly reduced subepithelial fibrosis within the airway walls and had decreased airway hyperresponsiveness (AHR) to methacholine. Morphometric analysis showed that subepithelial wall thickening of the bronchi (subepithelial area ratio) was also reduced, as were collagen and α -smooth muscle actin. Upregulation of plasmin activity also increased the level of hepatocyte growth factor activity in bronchoalveolar lavage fluid, whereas the release of transforming growth factor- β was decreased. The administration of uPA 1 wk after the last OVA inhalation also significantly reduced lung hydroxyproline content and AHR. These results show that enhancing uPA/plasmin activity lessens the airway remodeling in a murine asthma model.

urokinase; plasmin; bronchial asthma; subepithelial fibrosis

AIRWAY REMODELING is a prominent feature of asthma, and its extent is related to the severity of the disease. The manifestations of airway remodeling include subepithelial fibrosis, extracellular matrix (ECM) deposition, smooth muscle hypertrophy, and goblet cell hyperplasia in the airways (2, 12). In the pathogenesis of this airway remodeling, tissue injury from inflammation leads to the extravascular leakage of plasma (17) as well as the release of tissue factor from monocytes and the endothelium (1). Tissue factor initiates thrombin generation and fibrin formation. Thrombin also augments the inflammatory response. Ultimately, activation of the coagulation cascade

contributes to the accumulation of fibrin clot, ECM deposition, and fibrosis (3, 13).

The urokinase-type plasminogen activator (PA)/plasmin system has been implicated in many physiological and pathophysiological processes, including angiogenesis, cell migration, tumor metastasis, and fibrosis (31). There are two endogenous PAs, tissue-type PA (tPA) and urokinase-type PA (uPA), that convert plasminogen to the active protease plasmin (35). The major site of tPA activity appears to be within the intravascular compartment, while uPA activity is more important extravascularly. uPA binds to a high-affinity receptor on cell surfaces, where it can activate plasmin in the pericellular environment. Plasmin can degrade ECM components directly or indirectly through the activation of latent proteinases including matrix metalloproteinases (4). The major inhibitors of the PA system are plasminogen activator inhibitor-1 (PAI-1) and α_2 -antiplasmin (5).

Previous studies demonstrated that activation of the uPA/plasmin system can limit pulmonary fibrosis. For example, wild-type mice administered uPA and mice transgenically deficient in PAI-1 are both resistant to pulmonary fibrosis after lung injury (7, 9). Although the mechanism by which increased uPA/plasmin limits fibrogenesis is unclear, this system regulates the activity of hepatocyte growth factor (HGF) by cleaving latent HGF and by releasing it from its ECM reservoir. Importantly, increased levels of HGF have been shown to attenuate fibrotic processes in the lung (6, 10). Furthermore, it is well known that HGF can attenuate the profibrotic action of transforming growth factor (TGF)- β (21).

In the present work, we tested the hypothesis that enhancement of uPA/plasmin activity would reduce airway remodeling in a mouse model of asthma. We also measured changes in HGF induced by increased uPA/plasmin activity and correlated these findings with changes in airway remodeling.

MATERIALS AND METHODS

Animals. Genetically deficient PAI-1 (PAI-1^{-/-}) mice on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME). Wild-type C57BL/6J (PAI-1^{+/+}) mice were purchased from CLEA Japan (Tokyo, Japan). All animal experiments were undertaken according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine. Our research was approved by the Institutional Animal Care and Use Committee

Address for reprint requests and other correspondence: Y. Nishimura, Div. of Respiratory Medicine, Dept. of Internal Medicine, Kobe Univ. Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan (e-mail: nishiy@med.kobe-u.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

and carried out according to the Kobe University Animal Experimentation Regulations.

Agents. The following drugs and chemicals were purchased commercially: ovalbumin (OVA; Sigma-Aldrich Japan, Tokyo, Japan), hydroxy-L-proline (Wako Pure Chemical Industries, Osaka, Japan), human thrombin (Sigma-Aldrich), and plasminogen-depleted human plasma fibrinogen (Calbiochem, San Diego, CA). Human urokinase purified from urine was kindly provided by Mochida Pharmaceutical (Tokyo, Japan).

Sensitization and antigen challenge. We employed both acute (*experiment 1*) and chronic (*experiment 2*) asthmatic mouse models, as previously described (20, 25) (Figs. 1A and 2A, respectively). Six- to eight-week-old female mice were sensitized with intraperitoneal injections of 50 μ g of OVA with 1 mg of alum on days -20, -13, and -6. Starting on day 1, they were exposed to 1% (wt/vol) OVA diluted in sterile phosphate-buffered saline (PBS) for 30 min on 3 consecutive days (*experiment 1*) or for 3 wk (6 days/wk, *experiment 2*) with an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan). As a negative control, animals were exposed to PBS instead of OVA inhalation. uPA (500 IU/animal) diluted in sterile PBS was adminis-

tered by nebulization on days 1–3 (*experiment 1*) or days 15–21 (*experiment 2*) after OVA challenge. To test the effect of delayed uPA administration, mice that received 3 wk of nebulized OVA were treated with PBS or uPA (500 IU/animal) on days 29–35 (*experiment 3*; see Fig. 6A).

Measurement of airway hyperresponsiveness. Twenty-four hours after the final aerosol challenge, airway hyperresponsiveness (AHR) was assessed in conscious and unrestrained mice by means of whole body plethysmography (Buxco Research Systems, Wilmington, NC). Each mouse was placed in a plastic chamber and exposed to aerosolized PBS, which was followed by increasing concentrations of aerosolized methacholine solutions (1.56, 3.13, 6.25, 12.5, 25, 50 μ g/ml; Sigma-Aldrich) for 3 min each. Bronchoconstriction was recorded for an additional 5 min for each increased dose of methacholine. The highest enhanced pause (Penh) value obtained during each methacholine challenge was expressed as a percentage of the basal Penh value in response to the PBS challenge.

Histopathology. Mouse lungs were intratracheally instilled with 10% buffered formalin at a pressure of 20 cmH₂O for 24 h and then

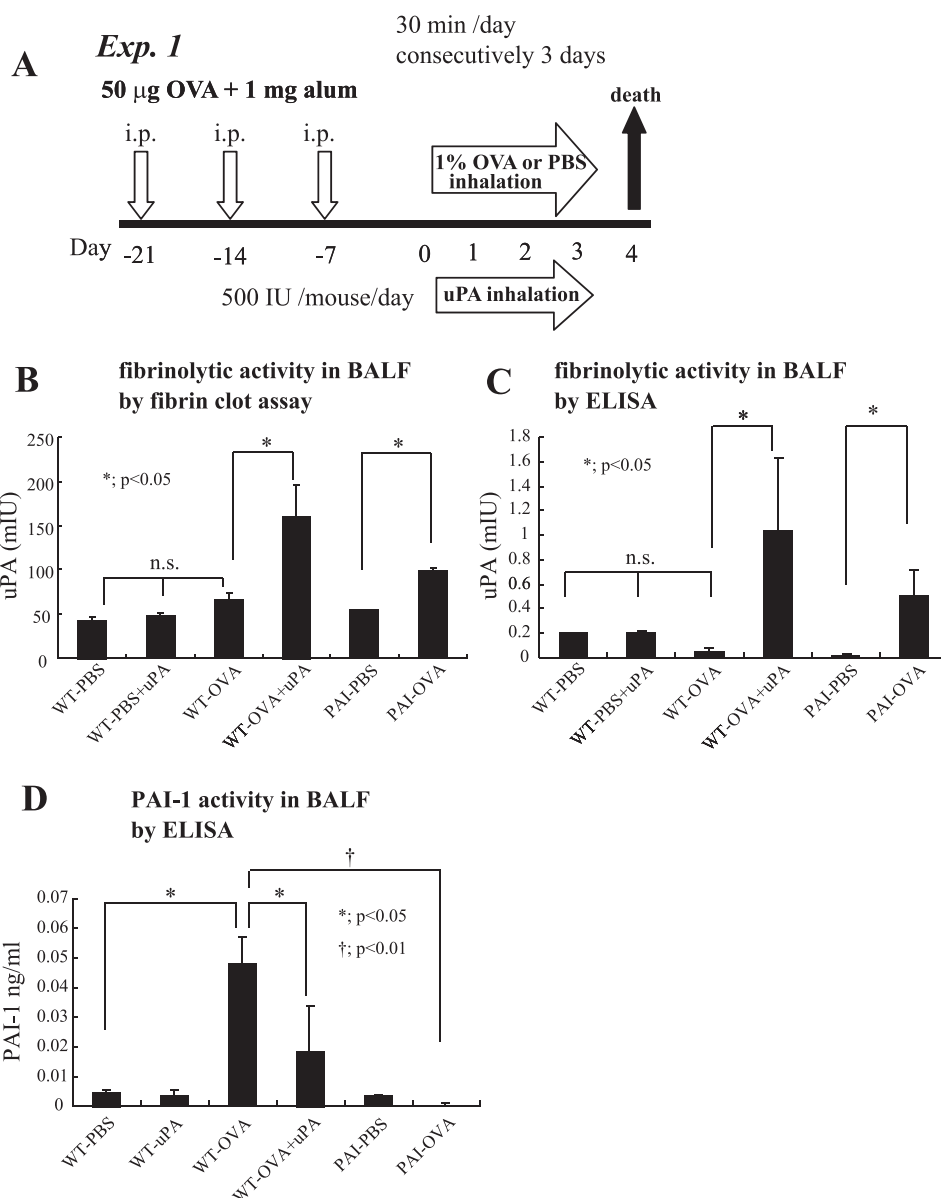


Fig. 1. A: sensitization and challenge protocols of acute asthma model (*experiment 1*). After 3 intraperitoneal (ip) injections of 50 μ g of ovalbumin (OVA) and 1 mg of alum on days -21, -14, and -7, C57BL/6J and plasminogen activator inhibitor-1 (PAI-1)-null mice were treated with an ultrasonic nebulizer with 1% OVA in phosphate-buffered saline (PBS) or PBS alone for 3 consecutive days. For the treatment group, 500 IU/ml of urokinase-type plasminogen activator (uPA) in PBS was nebulized before OVA inhalation from day 1 to day 3. After the third inhalation in *experiment 1*, bronchoalveolar lavage fluids (BALF) were obtained from PBS-exposed wild-type (WT-PBS), PBS-exposed uPA-treated wild-type (WT-PBS+uPA), OVA-exposed wild-type (WT-OVA), OVA-exposed uPA-treated wild-type (WT-OVA+uPA), PBS-exposed PAI-1-deficient (PAI-PBS), and OVA-exposed PAI-1-deficient (PAI-OVA) mice. B and C: plasminogen activator activity in BALF by fibrin clot assay (B) and ELISA (C). D: PAI-1 activity in BALF fluid by ELISA. Data are expressed as means $\pm$ SE; $n \geq 6$. n.s., Not significant.

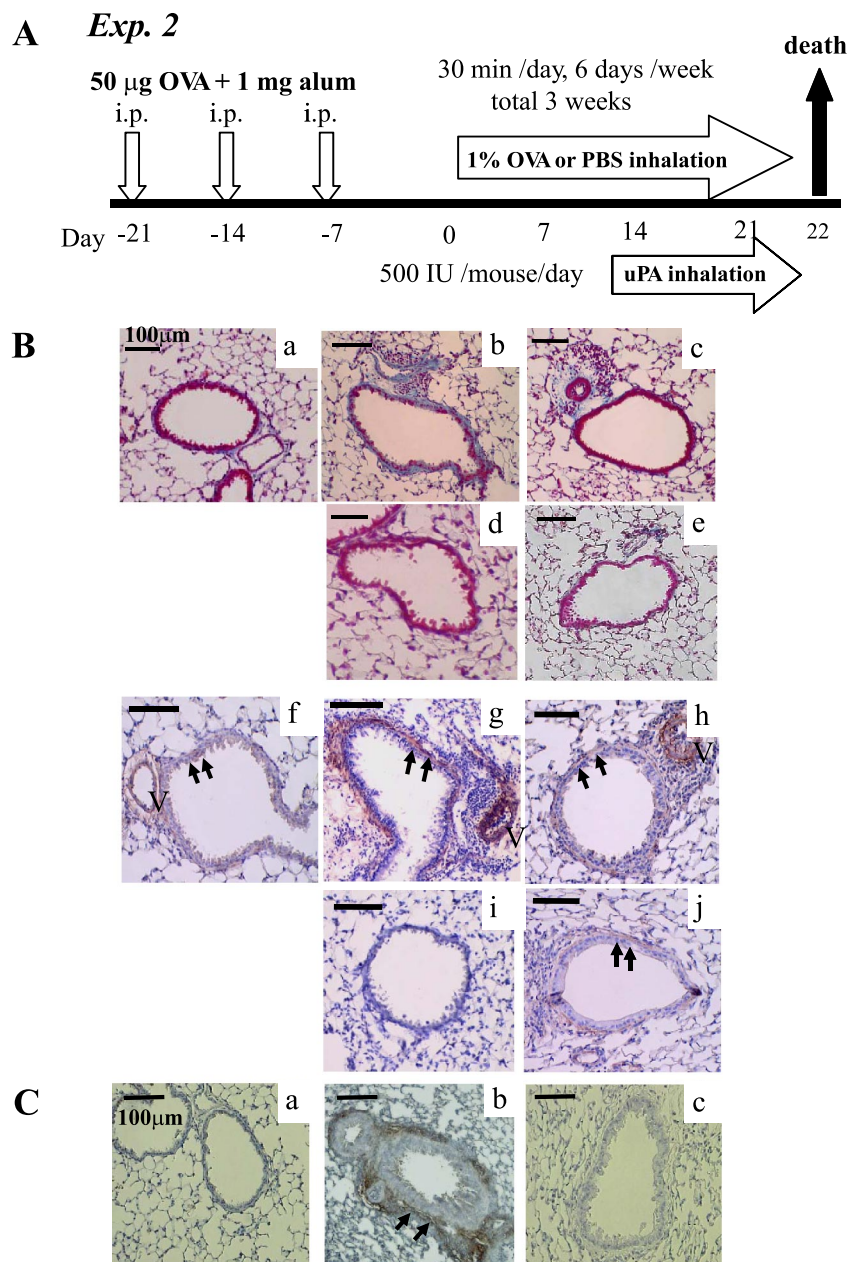


Fig. 2. A: sensitization and challenge protocols of chronic asthma model (*experiment 2*). After 3 intraperitoneal injections of 50 µg of OVA and 1 mg of alum on days -21, -14, and -7, C57BL/6J and PAI-1-deficient mice were treated with an ultrasonic nebulizer with 1% OVA in PBS or PBS alone for 3 consecutive weeks. For the treatment group, 500 IU/ml of uPA in PBS was nebulized before OVA inhalation from day 15 to day 21. B: histological findings in the chronic asthma model. Subepithelial wall fibrosis was evaluated by Masson trichrome staining (a–e) and immunohistological staining with an anti-α-smooth muscle actin (SMA) antibody (f–j) 24 h after the final challenge. a and f: Representative sections of WT-PBS. b and g: WT-OVA. c and h: WT-OVA+uPA. d and i: PAI-PBS. e and j: PAI-OVA. Immunostaining showed that α-SMA was localized to the mesenchyme (arrows) surrounding the bronchioles and vessels (V). C: lung tissues were immunostained with anti-PAI-1 polyclonal antibody: WT-PBS (a), WT-OVA (b), WT-OVA+uPA (c). Original magnification: $\times 200$.

embedded in paraffin. Serial 4-µm sections were taken every 100 µm and stained with Masson's trichrome.

Sections of lung tissue were stained immunohistochemically to detect α-smooth muscle actin (SMA), phospho-c-Met, and PAI-1. After deparaffinization, tissue sections were pretreated with 3% hydrogen peroxide for 10 min and blocked with normal goat serum for 30 min. They were then incubated for 1 h with mouse monoclonal anti-α-SMA (1:50) (Sigma-Aldrich, St. Louis, MO), rabbit polyclonal anti-phospho-Met (1:100) (Upstate), or rabbit polyclonal anti-PAI-1 (1:100) (Molecular Innovations) as a primary antibody. Normal mouse or rabbit serum was used as a negative control. After being washed with PBS, the sections were incubated with biotinylated secondary antibodies, washed with PBS, and incubated with Vectastain ABC Regent (Vector Laboratories, Burlingame, CA) for 30 min. When mouse monoclonal antibody was used, the Vector M.O.M. Immunodetection Kit (Vector Laboratories) was used according to the manufacturer's protocol. Diaminobenzidine was used as a substrate

for the immunoperoxidase reaction. Sections were lightly counterstained with hematoxylin and analyzed by bright-field microscopy.

Morphometric analysis of structural changes. In Masson's trichrome-stained lung sections, a minimum of five bronchi measuring 150–350 µm in luminal diameter were analyzed per mouse. Photomicrograph images were captured with a FUJIX digital camera (HC-300Z, Fujifilm, Tokyo, Japan), and calibration was performed with a reference micrometer slide. ImageJ software (Image Processing and Analysis in Java) was used to measure the total subepithelial cross-sectional area and the area occupied by smooth muscles (34). The ratio of smooth muscle area to total area (subepithelial area) was used as a measure of airway remodeling.

Hydroxyproline analysis of lung collagen. Whole collagen content of the right lung was evaluated by determining hydroxyproline content with a method described previously (37). Briefly, after recovery of bronchoalveolar lavage (BAL) fluid, the right lungs were removed, frozen in liquid nitrogen, and kept at -80°C until analysis. The lung

tissue was hydrolyzed in 6 N HCl for 8 h at 120°C. The samples were mixed and incubated with chloramine T solution at room temperature for 20 min. Next, 1.0 ml of Ehrlich solution was added, the mixture was incubated at 60°C for 20 min, and the absorbance at 550 nm was measured.

Bronchoalveolar lavage. Twenty-four hours after last exposure, BAL was performed by instilling and aspirating three 1.0-ml aliquots of PBS (recovery >85%). BAL fluid was centrifuged at 3,000 g for 5 min (at 4°C), and the supernatants were stored at -80°C.

Measurement of plasminogen activator activity. To measure PA activity, a fibrin clot lysis assay was used as previously described (26). Briefly, 50 µl of BAL fluid was mixed in the wells of a 96-well plate with plasminogen-depleted human plasma fibrinogen and human glu-plasminogen. After the addition of human thrombin, light absorbance at 405 nm was monitored. Reagent human uPA was used to generate a standard curve with the rate of fibrinolysis recorded as the time required for 50% of the maximum turbidity to be lost.

Enzyme-linked immunosorbent assay. Levels of uPA, PAI-1, HGF, and total TGF-β1 were determined in BAL fluid with enzyme-linked immunosorbent assay (ELISA) kits. The uPA activity kit was from Innovative Research (Novi, MI), the PAI-1 activity kit was from Molecular Innovations, the HGF kit was from the Institute of Immunology (Tokyo, Japan), and the total TGF-β1 kit was from Biosource International (Camarillo, CA). The sensitivities of these kits for uPA activity, PAI-1, HGF, and total TGF-β1 were 0.004 ng/ml, 0.02 ng/ml, 0.2 ng/ml, and 15.6 pg/ml, respectively. The absorbance of each sample was measured at 450 nm with a Multiscan JX microplate reader (Thermo Labsystems, Thermo Bio-analysis, Tokyo, Japan).

Cell culture. The human fetal lung fibroblast cell line WI-38 was obtained from Riken Cell Bank (Tsukuba, Japan). Cells were grown and maintained in six-well dishes with MF medium containing 1% fetal calf serum (FCS) (Toyobo, Osaka, Japan). Eighty to ninety percent confluent cells were serum starved for 24 h and then stimulated with TGF-β1 for the indicated time. Cells were pretreated with plasmin-specific inhibitors for 30 min before TGF-β1 stimulation.

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza Walkersville (Walkersville, MD). Cells were grown as monolayers in collagen-coated dishes at 100% humidity and 5% CO₂ at 37°C, in serum-free modified LHC-9 medium (Clonetics) supplemented as described previously (14). The cells were grown to 80% confluence and then incubated in growth factor-free medium overnight. These confluent cells were stimulated with various concentrations of TGF-β1 overnight.

Western blot analysis. Cells were harvested and lysed in a buffer containing (in mM) 50 Tris·HCl (pH 7.4), 150 NaCl, 15 NaF, 5 EDTA, and 1 sodium orthovanadate, with 1% Triton X-100 and protease inhibitor cocktails (Sigma-Aldrich). Cell lysates were prepared in SDS sample buffer and boiled at 95°C for 3 min. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and immunostained with antibodies against α-SMA and β-tubulin (Upstate). Bands were visualized by the enhanced chemiluminescence method, and their intensities were quantified with MultiGauge software (Fujifilm).

For the detection of HGF, cell lysate or 500 µl of BAL fluid was applied to 40 µl of heparin agarose beads (Sigma-Aldrich) and rotated overnight at 4°C. After being washed three times with 1 ml of PBS, beads were suspended in SDS sample buffer under reducing conditions and the supernatant was separated on SDS-PAGE on 10% acrylamide gels. Anti-rat HGF α-chain monoclonal antibody (Institute of Immunology), which cross-reacts with mouse HGF, was used as a primary antibody.

Quantitative real time PCR. Total RNA from NHBE cells was isolated with the ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 1 µg of total RNA with ExScript RT Reagent Kits (Takara, Otsu, Japan) and random hexamer primers. Quantitative PCR was performed with real-time SYBR Green PCR technology and an ABI PRISM 7500 Sequence Detection

System (Applied Biosystems, Foster City, CA). The primers used were as follows: human uPA: forward (F): 5'-GCT GCT GAC CCA CAG TGG AA-3', reverse (R): 5'-TCG TGT AGA CGC CTG GCT TG-3'; human PAI-1: F: 5'-GAC AGG CAG CTC GGA TTC AAC-3', R: 5'-CCT GGG CTC AAG CAA TCC TC-3'; human GAPDH: F: 5'-GGC CTC CAA GGA GTA AGA CC-3', R: 5'-AGG GGT CTA CAT GGC AAT G-3'. Amplification reactions were performed in duplicate with SYBR Premix Ex Taq under a manufactured protocol (Takara). uPA and PAI-1 expressions were normalized to GAPDH mRNA expression.

Statistical analysis. Results are expressed as means ± SE. Statistical significance between groups was assessed by one-way ANOVA or the Kruskal-Wallis test with appropriate pairwise post hoc analyses (Scheffé) with Statcel software (OMS Publishing, Saitama, Japan). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Fibrinolytic activity in asthmatic models. To determine whether increased fibrinolytic activity ameliorated asthmatic responses, we prepared asthmatic mouse models using C57BL/6J wild-type and PAI-1^{-/-} mice. In a subset of wild-type mice, uPA was administered by an ultrasonic nebulizer after OVA

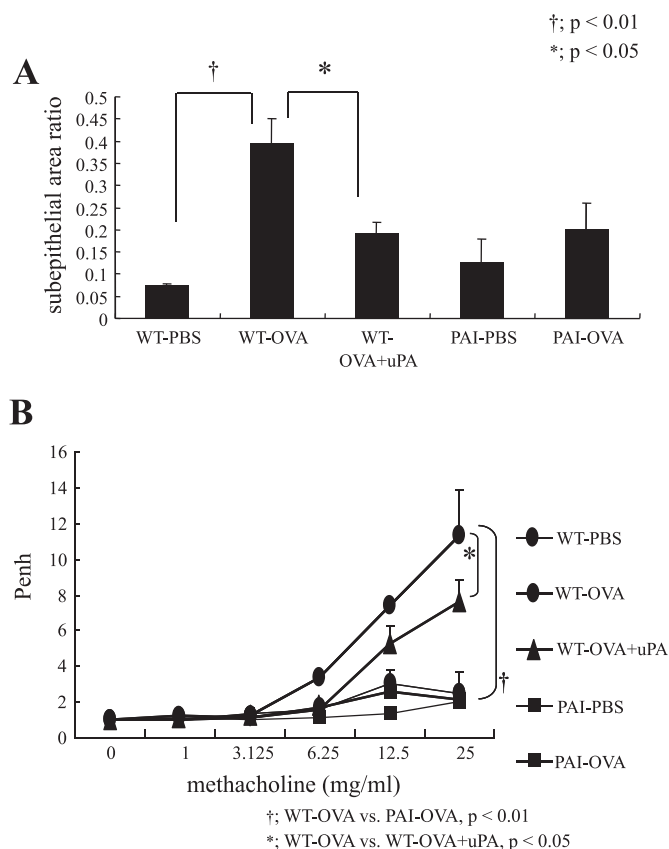


Fig. 3. Morphometric analysis and airway hyperresponsiveness (AHR) in chronic asthma model (*experiment 2*). **A:** subepithelial area ratio was calculated from histological sections and evaluated as an index of airway remodeling ($n \geq 5$). **B:** dose-response curves of methacholine challenge-induced bronchoconstriction were measured by body plethysmography 24 h after final aerosol challenge. Data are enhanced pause (Penh) change from baseline (PBS only) of individual mice: WT-PBS, WT-OVA, and WT-OVA+uPA, PAI-PBS, and PAI-OVA. Values are expressed as fold increase in Penh for each concentration of methacholine and compared with Penh value after PBS challenge ($n = 5/\text{group}$). Data are means ± SE.

treatment each day. Twenty-four hours after the last exposure, BAL fluid was collected and analyzed for fibrinolytic activity.

In the acute asthmatic model, the fibrinolytic activity of BAL fluid measured by fibrin clot lysis (Fig. 1B) or ELISA for active uPA (Fig. 1C) was not statistically different between wild-type mice receiving OVA treatment (fibrinolytic activity: WT-OVA 65.5 ± 7.6 mIU) and wild-type mice receiving PBS as a control condition (fibrinolytic activity: WT-PBS 42.2 ± 4.5 mIU). Similar to our previous observations (26), the net fibrinolytic activity in BAL fluid was uPA specific and was not due to tPA. OVA inhalation significantly increased fibrinolytic activity in PAI-1^{-/-} mice (PAI-OVA 98.6 ± 3.7 mIU) compared with saline treatment (PAI-PBS 54.7 ± 0.2 mIU; $P < 0.05$), and this latter group was not significantly different from saline-treated wild-type animals. Furthermore, uPA-treated wild-type mice (WT-OVA+uPA 160.4 ± 36.1 mIU; $P < 0.05$) showed a significant increase in fibrinolytic activity compared with the WT-PBS and WT-OVA groups. Fibrinolytic activity in BAL fluid was not detected in this assay under plasminogen-free conditions.

We also examined the fibrinolytic activity of BAL fluid by using an ELISA for uPA (Fig. 1C). This kit detects only active uPA by using PAI-1-coated microtiter plates. The WT-OVA animals had levels of active uPA similar to those of the WT-PBS and WT-PBS+uPA groups, whereas the WT-OVA+uPA animals showed a marked increase (WT-PBS 0.197 ± 0.009 , WT-PBS+uPA 0.203 ± 0.014 , WT-OVA 0.043 ± 0.028 vs. WT-OVA+uPA 1.027 ± 0.602 ng/ml; $P < 0.05$). In PAI-1^{-/-} mice, OVA treatment significantly induced uPA levels compared with control mice (PAI-OVA 0.505 ± 0.208 , PAI-PBS 0.020 ± 0.021 ng/ml; $P < 0.05$).

We also measured fibrinolytic activity in the chronic asthmatic model (*experiment 2*) and found similar trends compared with the acute approach (data not shown).

To evaluate the major endogenous inhibitor of uPA, we measured active PAI-1 content in BAL fluid by ELISA. Active PAI-1 in the WT+OVA group (0.048 ± 0.010 ng/ml) was significantly increased compared with control groups (WT-PBS 0.004 ± 0.001 , WT-PBS+uPA 0.003 ± 0.002 ng/ml; $P < 0.05$), and this increase was significantly suppressed by

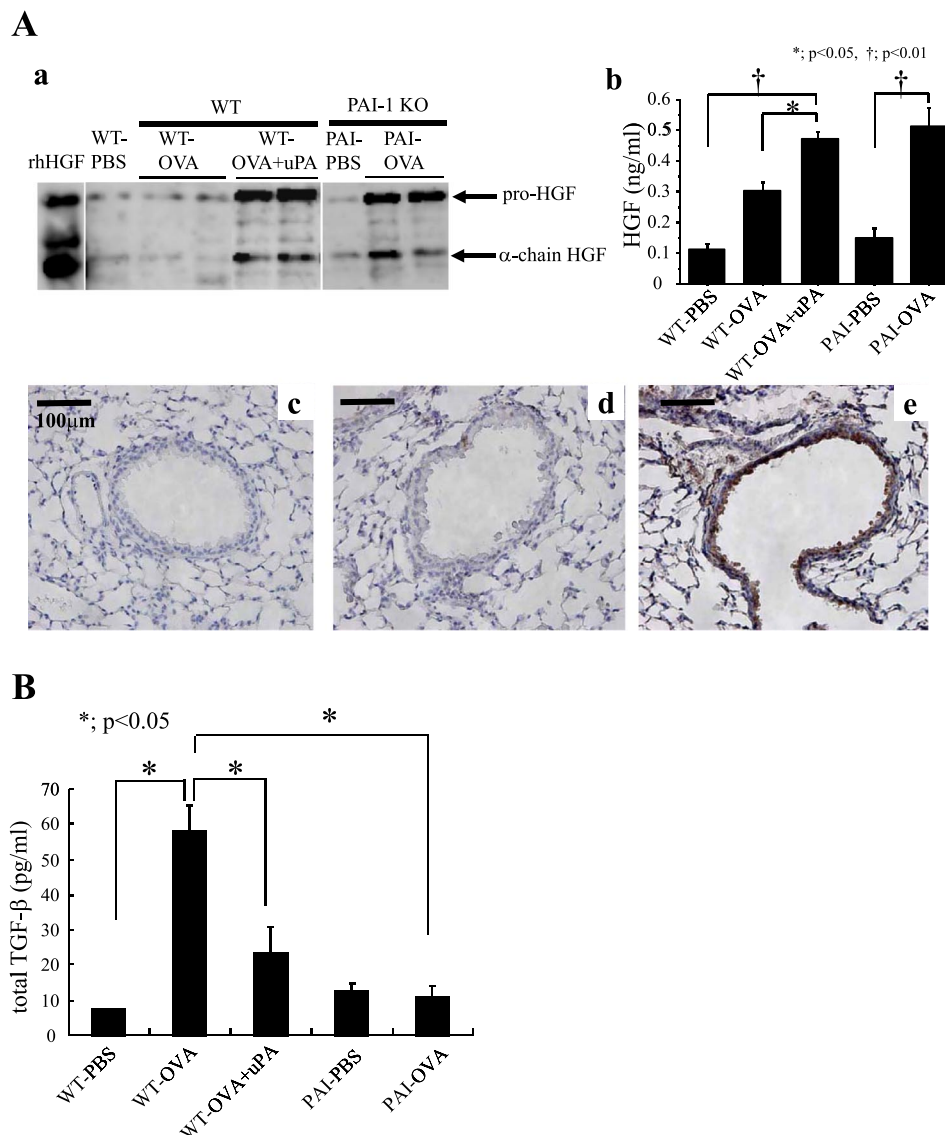


Fig. 4. Hepatocyte growth factor (HGF), c-Met, and transforming growth factor (TGF)- β 1 expression. A: HGF expression in BALF from the chronic asthma model (*experiment 2*) was examined by Western blotting analysis (a) and ELISA (b). Data are means $\pm$ SE; $n \geq 6$. rh-HGF, recombinant human HGF; KO, knockout. c–e: Lung tissues were immunostained with anti-c-Met polyclonal antibody: WT-PBS (c), WT-OVA (d), WT-OVA+uPA (e). Original magnification: $\times 200$. B: total TGF- β 1 expression in BALF from chronic asthma model was examined by ELISA.

uPA administration (WT-OVA+uPA 0.019 ± 0.015 ng/ml; $P < 0.05$). As anticipated, there was no measurable PAI-1 in the PAI-1^{-/-} mice (Fig. 1D).

Subepithelial fibrosis and bronchial hyperresponsiveness. Using the chronic asthma model (experiment 2), we evaluated the effect of the uPA/plasmin system on subepithelial fibrosis by analyzing histological sections from the different treatment groups. Figure 2B, a–e, shows representative sections of each experimental group stained with Masson's trichrome. The WT-OVA group (Fig. 2Bb) displayed an increase in the fibrotic area beneath the basement membrane of the bronchi compared with the WT-PBS mice (Fig. 2Ba). This deposition of connective tissue was clearly reduced by the enhanced uPA activity in the WT-OVA+uPA group (Fig. 2Bc) and the PAI-OVA group (Fig. 2Be).

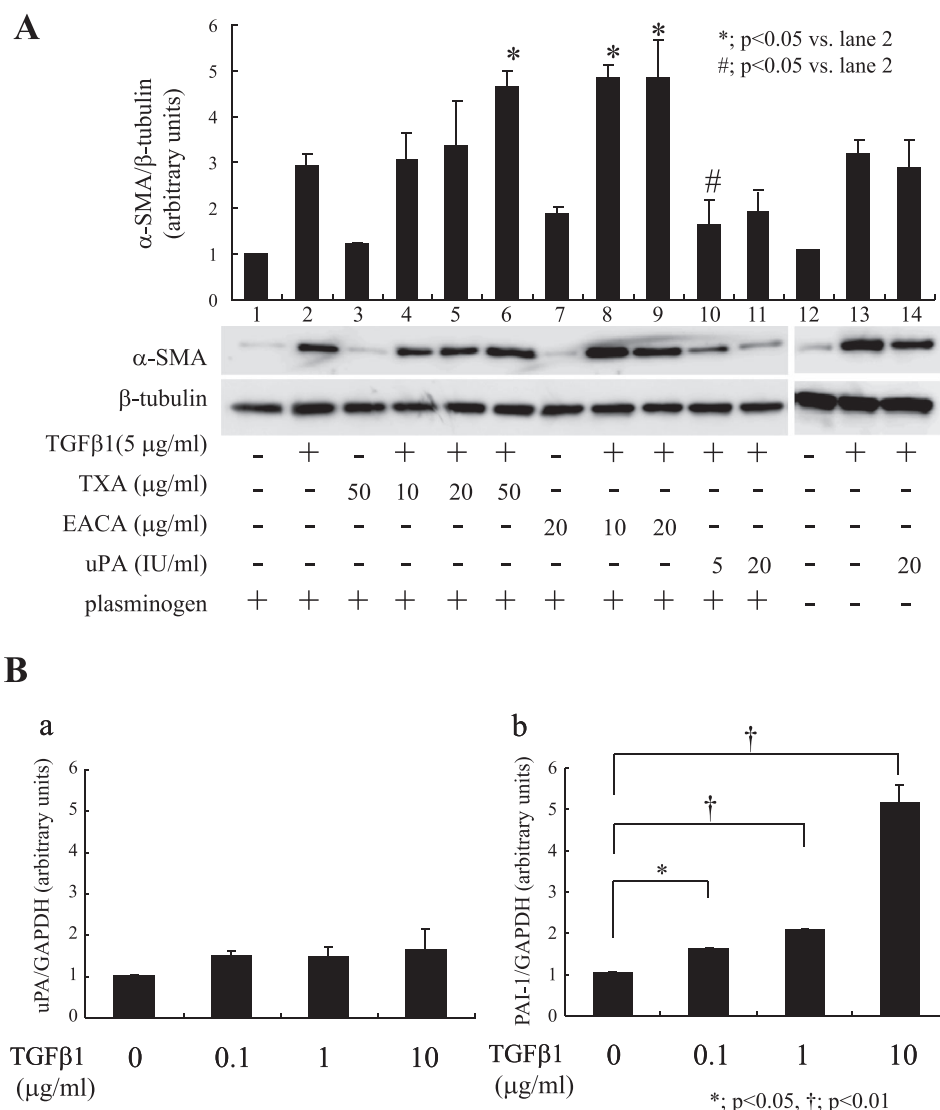
Differentiation of lung fibroblasts to myofibroblasts is characterized by the expression of α -SMA. To assess the myofibroblast response to alterations in uPA/plasmin activity in the chronic asthma model, we performed immunohistochemical staining of lung sections with an anti- α -SMA antibody (Fig. 2B, f–j). In the control group, α -SMA was immunolocalized to

the mesenchyme surrounding the bronchioles and vessels (Fig. 2Bf). In the WT-OVA group, α -SMA expression was abundant in peribronchiole and perivascular lesions compared with control animals (Fig. 2Bg). In conjunction with the decrease in subepithelial fibrosis, the WT-OVA+uPA and PAI-OVA groups showed decreased α -SMA staining compared with WT-OVA animals (Fig. 2B, h and j).

We also used immunohistochemistry to localize PAI-1 expression and found that it was increased by OVA treatment in peribronchial areas similar to α -SMA (Fig. 2Cb). This expression was diminished by uPA treatment (Fig. 2Cc).

We quantified subepithelial changes with a morphometric analysis (34). The subepithelial area ratio (A_e ratio) was defined as the ratio of subepithelial wall thickness to the diameter of bronchi. The A_e ratio was increased in WT-OVA-treated mice compared with control mice (WT-PBS 0.07 ± 0.01 , WT-OVA 0.39 ± 0.06 ; $P < 0.01$), and treatment with uPA significantly decreased the A_e ratio (WT-OVA+uPA 0.19 ± 0.03 ; $P < 0.05$) (Fig. 3A). In PAI-1^{-/-} mice, OVA treatment did not significantly change the A_e ratio from the baseline measurement.

Fig. 5. A: α -SMA expression in human lung fibroblasts. WI-38 human lung fibroblasts were stimulated in the presence or absence of plasminogen with 5 μ g/ml TGF- β 1 30 min after pretreatment with the indicated concentrations of uPA, tranexamic acid (TXA), or ϵ -aminocaproic acid (EACA). After 24 h, cell lysates were subjected to Western blotting analysis against α -SMA and β -tubulin. Each band was quantified, and the α -SMA bands were normalized to β -tubulin ($n \geq 4$ /group). B: uPA (a) and PAI-1 (b) mRNA expression in normal human bronchial epithelial (NHBE) cells. NHBE cells were stimulated with TGF- β 1 at indicated concentrations. mRNA expressions were quantified with real-time PCR, and the results were normalized to GAPDH ($n = 6$ /group).



Next, to determine whether differences in fibrinolytic activity influence the physiological response to OVA challenge, we performed methacholine-induced AHR by body plethysmography 24 h after the final aerosol challenge (Fig. 3B). Results are expressed as relative increase in Penh from baseline. The groups with upregulated fibrinolytic activity (WT-OVA+uPA and PAI-OVA) demonstrated significantly reduced AHR to methacholine induced by OVA inhalation for 21 days. The difference of Penh was significant at a dose of 25 mg/ml of methacholine (WT-OVA+uPA 7.56 ± 1.32 , PAI-OVA 2.17 ± 0.49 vs. WT-OVA 11.32 ± 2.56 ; $P < 0.05$, $P < 0.01$, respectively).

HGF, phosphorylated c-Met, and TGF- β 1 expression. To evaluate whether the enhancement of fibrinolytic activity affects HGF levels, we analyzed BAL fluid obtained from wild-type and PAI-1^{-/-} mice in the chronic asthmatic model (experiment 2). The heparin-binding fractions of BAL fluid were prepared and immunoblotted with an anti-HGF α -chain antibody under reducing conditions. Bands corresponding in molecular mass to single chain of HGF (97 kDa), α -chain of HGF (69 kDa), and nonglycosylated α -chain (54 kDa) were detected. As shown in Fig. 4Aa, both single-chain (precursor) and α -chain (active) HGF bands were increased in the WT-OVA+uPA and PAI-OVA groups compared with WT-OVA mice.

We also measured HGF concentration in BAL fluid by ELISA (Fig. 4Ab). HGF concentrations in the mouse groups with upregulated fibrinolytic activity (WT-OVA+uPA and PAI-OVA 0.471 ± 0.023 and 0.511 ± 0.062 ng/ml, respectively) were significantly higher compared with the other groups (WT-OVA 0.304 ± 0.028 , $P < 0.05$; WT-PBS $0.114 \pm$

0.016 , PAI-PBS 0.149 ± 0.031 ng/ml, $P < 0.01$). In the acute asthma models (experiment 1), the expression of HGF was relatively lower than in the chronic model (experiment 2).

We then examined the localization of phosphorylated c-Met as a marker of HGF activity. In WT-PBS and WT-OVA groups, phosphorylated c-Met was not detected (Fig. 4A, c and d). On the other hand, in WT-OVA+uPA mice phosphorylated c-Met was strongly expressed in the cytoplasm of bronchial epithelial cells (Fig. 4Ae). In PAI-1^{-/-} mice, phosphorylated c-Met was also induced compared with WT-OVA mice (data not shown).

Next, we examined TGF- β 1 release in BAL fluid by ELISA (Fig. 4B). Total TGF- β 1 concentration from the mouse groups with upregulated fibrinolytic activity (WT-OVA+uPA and PAI-OVA) was significantly lower compared with WT-OVA mice (Fig. 4B; WT-OVA+uPA 23.47 ± 7.22 , PAI-OVA 11.08 ± 3.04 , WT-OVA 57.86 ± 3.26 pg/ml; $P < 0.05$). We also measured the concentrations of active TGF- β 1 in BAL fluids by ELISA; however, the levels in all groups were below the lower limit of detection of the assay (data not shown).

Effect of plasmin activity on TGF- β -mediated myofibroblast differentiation in cultured lung fibroblasts. Myofibroblast differentiation is a hallmark of the tissue fibrosis that occurs in the subepithelium of asthma-induced airway remodeling. To examine the role of the uPA/plasmin system in myofibroblast differentiation, we studied the human lung fibroblast cell line WI-38. The cells were pretreated for 30 min with two different plasmin-specific inhibitors, tranexamic acid (TXA) and ϵ -aminocaproic acid (EACA), in the presence of plasminogen before TGF- β 1 stimulation. As shown in Fig. 5A, TGF- β 1 upregulated α -SMA protein expression (in arbitrary units) (lane 2;

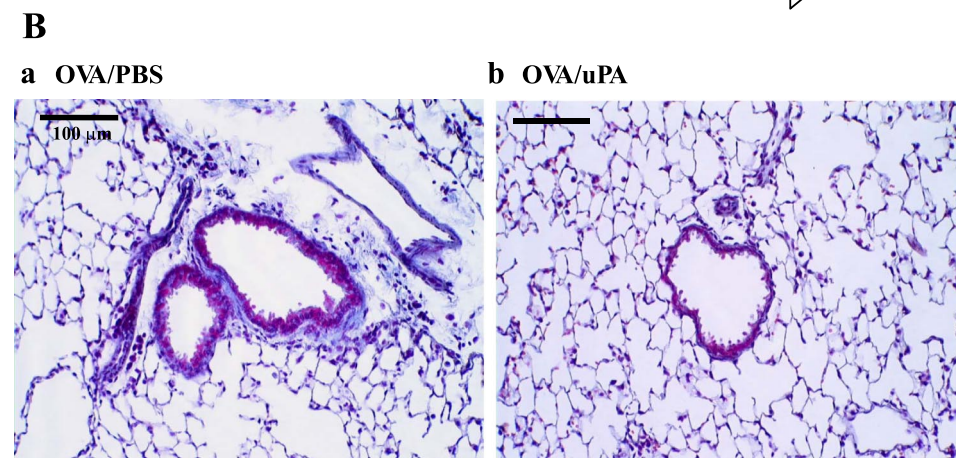
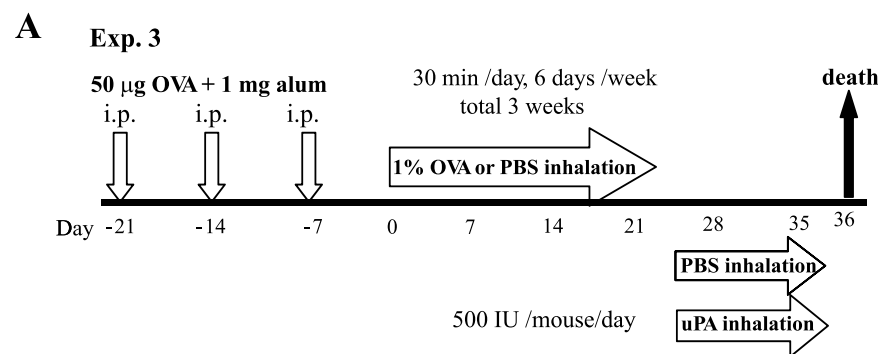


Fig. 6. A: therapeutic experimental protocol (experiment 3). B: Masson trichrome-stained lung sections in wild-type mice treated with PBS (a) or uPA (b) after OVA challenge. Original magnification: $\times 200$.

2.90 $\pm$ 0.29). Both plasmin inhibitors, TXA and EACA, caused a significant increase of α -SMA expression [lane 6 4.64 $\pm$ 0.36, lane 8 4.84 $\pm$ 0.29, lane 9 4.84 $\pm$ 0.82 (P < 0.05) vs. lane 2]. Treatment with only EACA (lane 7) or TXA (lane 3) did not show any changes. On the other hand, uPA pretreatment (5 IU/ml) resulted in a significant inhibition of α -SMA expression [lane 10 1.63 $\pm$ 0.54 (P < 0.05) vs. lane 2]. Higher concentrations of uPA (20 IU/ml) pretreatment had a tendency to reduce α -SMA expression (lane 11 1.95 $\pm$ 0.34; P = 0.058). In the absence of plasminogen, TGF- β -induced α -SMA expression was not significantly reduced by uPA pretreatment (lanes 12–14).

uPA and PAI-1 expression induced by TGF- β in normal human bronchial epithelial cells. To examine the effect of TGF- β on bronchial epithelial cells, we cultured NHBE cells and measured uPA and PAI-1 expression by real-time PCR. TGF- β 1 stimulation increased PAI-1 mRNA expression (arbitrary units) in a dose-dependent manner [0.1 μ g/ml TGF- β 1 1.54 $\pm$ 0.03 (P < 0.05), 1.0 μ g/ml TGF- β 1 1.99 $\pm$ 0.03, and 10 μ g/ml TGF- β 1 4.90 $\pm$ 0.42 (P < 0.01) vs. control] (Fig. 5Bb), whereas uPA mRNA expression was not changed (Fig. 5Ba).

Effect of nebulized uPA on airway remodeling and subepithelial fibrosis in established asthma. We next investigated the effect of uPA nebulization on the fate of established asthma (Fig. 6A, experiment 3). After 3 wk of daily OVA treatment, uPA or PBS was administered for 1 wk. Masson trichrome staining showed that fibrotic changes were significantly attenuated by treatment with uPA in wild-type mice (OVA/uPA, Fig. 6Ba) compared with PBS treatment (OVA/PBS, Fig. 6Bb). The A_e ratio was increased in OVA/PBS mice compared with PBS control mice, and treatment with uPA reduced the A_e ratio (PBS 0.07 $\pm$ 0.01, OVA/PBS 0.20 $\pm$ 0.04, OVA/uPA 0.07 $\pm$ 0.01; P < 0.05) (Fig. 7A). Similarly, the lung hydroxyproline content of OVA/uPA mice was significantly reduced compared with the OVA/PBS group (PBS 106.2 $\pm$ 38.8, OVA/PBS 201.3 $\pm$ 21.7, OVA/uPA 142.6 $\pm$ 16.6 μ g/lung; P < 0.05) (Fig. 7B). Finally, uPA treatment reduced methacholine-induced AHR in OVA-challenged mice at a dose of 50 mg/ml methacholine (OVA/uPA 2.63 $\pm$ 0.41, OVA/PBS 6.87 $\pm$ 1.56; P < 0.05) (Fig. 7C).

DISCUSSION

Several reports have demonstrated that the activity of the uPA/plasmin system affects the development of lung fibrosis. For example, mice with impaired fibrinolysis caused either by overexpression of a PAI-1 transgene (7) or by targeted deletion of the plasminogen gene (33) develop increased fibrosis after lung injury. In contrast, increasing the activity of the uPA/plasmin system by the targeted deletion of the PAI-1 gene or by upregulating uPA protects against lung fibrosis following a pulmonary insult (7, 32). Only a few studies have investigated the role of the uPA/plasmin system in the development of chronic asthma. In this study we have demonstrated that exogenous uPA treatment with an ultrasonic nebulizer can reduce multiple features of the asthmatic response.

Our previous study (26) revealed that uPA activity was strongly expressed in bronchial epithelial cells by in situ zymography. We also determined that basal uPA activity in the lung was not different between wild-type and PAI-1^{-/-} mice. After bleomycin treatment, however, PAI-1 was significantly

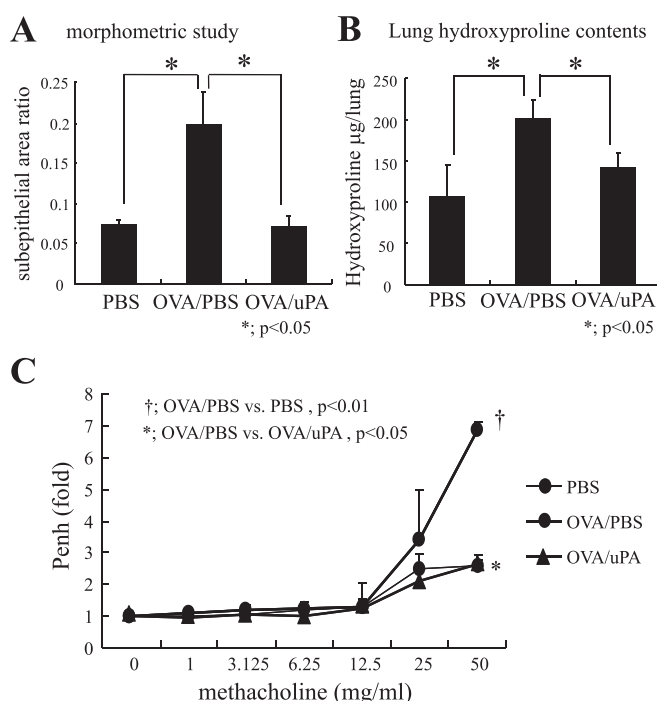


Fig. 7. Subepithelial area fibrosis and bronchial hyperresponsiveness in therapeutic experimental protocol. A: subepithelial area ratio in uPA-treated mice after OVA challenge (OVA/uPA) compared with PBS treatment (OVA/PBS) (n = 6/group). B: hydroxyproline content of lung tissue after OVA challenge in uPA-treated (OVA/uPA) compared with PBS-treated (OVA/PBS) mice (n = 6/group). C: dose-response curves of methacholine-induced bronchoconstriction in PBS-, OVA/PBS-, and OVA/uPA-treated animals. Values are expressed as fold increase in Penh for each concentration of methacholine and compared with Penh value after PBS challenge (n = 6/group).

induced in wild-type animals, and this resulted in a complete abrogation of fibrinolytic activity in the lung. In the same way, OVA treatment increased PAI-1 expression, especially around bronchial walls in wild-type mice. We found that fibrinolytic activity was not significantly altered in OVA-challenged wild-type mice compared with PBS-treated animals. However, in PAI-1^{-/-} mice, OVA treatment did lead to an increase in fibrinolysis compared with the PBS-treated PAI-1 group. To explain these findings, we hypothesize that in the wild-type animals a concurrent OVA-induced upregulation of PAI-1 and uPA within the bronchial wall resulted in no net change in fibrinolytic activity. In PAI-1^{-/-} mice, however, there is no PAI-1 to counteract the OVA-induced increase in uPA, and therefore fibrinolytic activity is enhanced. It is clear that through the nebulization of uPA we were able to change the uPA/PAI-1 balance in the wild-type animals, and the modification of fibrinolytic activity in the airways reduced airway remodeling.

In our chronic asthmatic model, we found that enhanced fibrinolytic activity reduced airway fibrosis. It has been reported that the Th2 response was downregulated and the immune response shifted from a Th2-dominant to a Th1-dominant reaction in PAI-1^{-/-} mice (30). The reduction of airway inflammation that occurs with increased fibrinolysis may be one mechanism by which the uPA/plasmin system reduces airway remodeling and fibrogenic factors such as TGF- β . Both inflammatory cell infiltration and airway remod-

eling can contribute to AHR, but airway fibrosis may lead to the greatest increase in AHR (22).

Hattori et al. (11) reported that an important mechanism by which uPA protects the lung from bleomycin-induced fibrosis is its effects on HGF. The major sources of HGF in the alveolar space are thought to be alveolar macrophages and interstitial fibroblasts (36), and fibroblasts are another potential source of plasminogen activation (35a). Another report demonstrated that fibroblasts use the uPA/plasmin system to cleave pro-HGF to its active two-chain form and to release it from the ECM. Therefore, the plasminogen activation system may reduce lung fibrosis by enhancing HGF activity (24).

We noted that HGF levels in the BAL fluid of mice exposed to OVA were increased over those of control mice. This increase is likely the net effect of multiple complex interactions that affect HGF synthesis, release, activation, and degradation. Although increased PAI-1 by itself would be expected to reduce the release of HGF from ECM, the observed increase in BAL HGF following antigen challenge is undoubtedly driven by other mechanisms. Similar increases in HGF content in BAL fluid have been seen after bleomycin injury [Hattori et al. (11)] and OVA inhalation [Ito et al. (16)]. Relevant to the present study, our data clearly demonstrate that PA activity modulates HGF level in BAL fluid. In particular, HGF levels in BAL fluid increase when PA activity is increased either by administration of exogenous uPA or by ablation of PAI-1 (PAI-1-null mice).

TGF- β can induce multiple cellular responses and has been implicated in the development of bronchial asthma (23). As demonstrated by Redington et al. (29) that allergen challenge increased TGF- β expression in BAL fluid from atopic asthmatic subjects, our asthma model showed that OVA inhalation caused a significant increase of total TGF- β in BAL fluid. This increase was suppressed by upregulated uPA activity. Reduction of TGF- β production and control of TGF- β effects would be beneficial in the development of therapeutic interventions for airway remodeling in chronic asthma (23). Ito et al. (15) also reported that the modulation of HGF controls the expressions of TGF- β and Th2 cytokines in BAL fluid in a murine model. In our experiments, although OVA inhalation caused TGF- β release and airway remodeling despite increase of HGF expression, a further increase of HGF and a decrease of TGF- β by uPA treatment protected against airway remodeling. Since the reduction of TGF- β activity and/or supplementation of HGF is believed to reduce fibrotic processes (21), our data also support the hypothesis that upregulated uPA activity can modify the balance of HGF and TGF- β , and thereby decrease airway remodeling.

Accumulation of myofibroblasts in fibrotic lesions increases ECM deposition and collagen synthetic capacity, resulting in structural remodeling and destruction of capillary/alveolar units after lung injury. Myofibroblasts are commonly identified by their expression of α -SMA and by phenotypic features that are intermediate between smooth muscle cells and fibroblasts (27). Kelly et al. (18) reported that the gene expression of α -SMA, TGF- β 1, and PAI-1 in microdissected airway walls were significantly increased 2 wk after final chronic allergen exposure compared with saline control. In the present study, we examined α -SMA expression in lung tissues by immunohistochemistry after 3 wk of OVA challenge in order to assess the presence of myofibroblasts in regions of subepithelial

fibrosis. Immunohistochemical analysis showed that α -SMA expression was increased after OVA challenge, but the upregulation of fibrinolytic activity significantly attenuated α -SMA staining. In vitro studies also showed that α -SMA protein levels were upregulated by TGF- β 1 stimulation. This expression was further increased by the plasmin inhibitor, and these changes were reversed by uPA treatment. These results demonstrate that uPA activation affects myofibroblast differentiation in the airway remodeling process.

Moreover, we also revealed that PAI-1 is strongly expressed around peribronchial areas by OVA inhalation. As shown in a previous report (8), PAI-1 was significantly induced by TGF- β stimulation in a dose-dependent manner, whereas uPA expression was not changed. These observations suggested that the uPA/PAI-1 system has a tight connection with TGF- β activity, which caused airway remodeling.

There are limitations in the animal models used to study chronic asthma. Previous reports showed that the development of AHR and airway remodeling are more marked when animals are challenged for longer periods (6–12 wk) (19). The most striking change in the chronic asthmatic model was an increase in subepithelial collagen. We used a shorter period (3 wk) of challenge for our chronic asthma protocol. The subepithelial wall thickness was relatively similar by morphometric study to our previous studies in which we used a longer challenge protocol (6–8 wk) (34).

In our experiments, we administered 500 IU of uPA (20,000 IU/kg) to each mouse, which was approximately fourfold higher per body weight than the dose of uPA used in a previously reported rabbit lung fibrosis study (9). However, in the rabbit model treatment was administered with a tight-fitting mask. Therefore, to compensate for our inability to deliver nebulized uPA to the mice by this mechanism, we administered a larger dose. No side effects such as bleeding were noted in these studies. Further investigation is required to determine the optimal dose, delivery method, and time course of uPA administration to best modulate the physiological, inflammatory, and airway remodeling alterations that occur in asthma.

In summary, we have shown that the uPA/plasmin system plays a significant role in the pathogenesis of airway remodeling in asthma and that the influence of the fibrinolytic system on asthma may be mediated through the activation of growth factors. Our results suggest that a therapeutic strategy of exogenous uPA administration may provide an effective means for the treatment of chronic asthma.

ACKNOWLEDGMENTS

We thank Dr. Takaaki Tsuchiya (Div. of Respiratory Medicine, Kobe University Graduate School of Medicine) for research technical advice.

REFERENCES

- Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, Steingrub JS, Garber GE, Helterbrand JD, Ely EW, Fisher CJ Jr. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med* 344: 699–709, 2001.
- Bousquet J, Jeffery PK, Busse WB, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 161: 1720–1745, 2000.
- Chambers RC, Leoni O, Blanc-Brude OP, Wembridge DE, Laurent GJ. Thrombin is a potent inducer of connective tissue growth factor production via proteolytic activation of protease-activated receptor-1. *J Biol Chem* 275: 35584–35591, 2000.

4. Cho SH, Tam SW, Demissie-Sanders S, Filler SA, Oh CK. Production of plasminogen activator inhibitor-1 by human mast cells and its possible role in asthma. *J Immunol* 165: 3154–3161, 2000.
5. Collen D. The plasminogen (fibrinolytic) system. *Thromb Haemost* 82: 259–270, 1999.
6. Dohi M, Hasegawa T, Yamamoto K, Marshall BC. Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. *Am J Respir Crit Care Med* 162: 2302–2307, 2000.
7. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, Simon RH. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest* 97: 232–237, 1996.
8. Gerwin BI, Keski-Oja J, Seddon M, Lechner JF, Harris CC. TGF- β 1 modulation of urokinase and PAI-1 expression in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 259: L262–L269, 1990.
9. Günther A, Lübke N, Ermert M, Schermuly RT, Weissmann N, Breithacker A, Markart P, Ruppert C, Quanz K, Ermert L, Grimminger F, Seeger W. Prevention of bleomycin-induced lung fibrosis by aerosolization of heparin or urokinase in rabbits. *Am J Respir Crit Care Med* 168: 1358–1365, 2003.
10. Hattori N, Degen JL, Sisson TH, Liu H, Moore BB, Pandrangi RG, Simon RH, Drew AF. Bleomycin-induced pulmonary fibrosis in fibrinogen-null mice. *J Clin Invest* 106: 1341–1350, 2000.
11. Hattori N, Mizuno S, Yoshida Y, Chin K, Mishima M, Sisson TH, Simon RH, Nakamura T, Miyake M. The plasminogen activation system reduces fibrosis in the lung by a hepatocyte growth factor-dependent mechanism. *Am J Pathol* 164: 1091–1098, 2004.
12. Holgate ST. Airway remodelling. *Eur Respir Rev* 8: 1007–1011, 1998.
13. Howell DC, Goldsack NR, Marshall RP, McAnulty RJ, Starke R, Purdy G, Laurent GJ, Chambers RC. Direct thrombin inhibition reduces lung collagen accumulation and connective tissue growth factor mRNA levels in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 159: 1383–1395, 2001.
14. Hozumi A, Nishimura Y, Nishiuma T, Kotani Y, Yokoyama M. Induction of MMP-9 in normal human bronchial epithelial cells by TNF- α via NF- κ B-mediated pathway. *Am J Physiol Lung Cell Mol Physiol* 281: L1444–L1452, 2001.
15. Ito W, Kanehiro A, Matsumoto K, Hirano A, Ono K, Maruyama H, Kataoka M, Nakamura T, Gelfand EW, Tanimoto M. Hepatocyte growth factor attenuates airway hyperresponsiveness, inflammation, and remodeling. *Am J Respir Cell Mol Biol* 32: 268–280, 2005.
16. Ito W, Tanimoto M, Ono K, Mizuno S, Yoshida A, Koga H, Fuchimoto Y, Kondo N, Tanimoto Y, Kiura K, Matsumoto K, Kataoka M, Nakamura T, Gelfand EW, Kanehiro A. Growth factors temporally associate with airway responsiveness and inflammation in allergen-exposed mice. *Int Arch Allergy Immunol* 145: 324–339, 2008.
17. Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol* 28: 233–236, 2001.
18. Kelly MM, Leigh R, Bonniaud P, Ellis R, Wattie J, Smith MJ, Martin G, Panju M, Inman MD, Gauldie J. Epithelial expression of profibrotic mediators in a model of allergen-induced airway remodeling. *Am J Respir Cell Mol Biol* 32: 99–107, 2005.
19. Kenyon NJ, Ward RW, Last JA. Airway fibrosis in a mouse model of airway inflammation. *Toxicol Appl Pharmacol* 186: 90–100, 2003.
20. Komai M, Tanaka H, Masuda T, Nagao K, Ishizaki M, Sawada M, Nagai H. Role of Th2 responses in the development of allergen-induced airway remodeling in a murine model of allergic asthma. *Br J Pharmacol* 138: 912–920, 2003.
21. Liu Y. Hepatocyte growth factor in kidney fibrosis: therapeutic potential and mechanisms of action. *Am J Physiol Renal Physiol* 287: F7–F16, 2004.
22. Locke NR, Royce SG, Wainwright JS, Samuel CS, Tang ML. Comparison of airway remodeling in acute, subacute, and chronic models of allergic airways disease. *Am J Respir Cell Mol Biol* 36: 625–632, 2007.
23. Makinde T, Murphy RF, Agrawal DK. The regulatory role of TGF- β in airway remodeling in asthma. *Immunol Cell Biol* 85: 348–356, 2007.
24. Matsuoka H, Sisson TH, Nishiuma T, Simon RH. Plasminogen-mediated activation and release of hepatocyte growth factor from extracellular matrix. *Am J Respir Cell Mol Biol* 35: 705–713, 2006.
25. Nagao K, Tanaka H, Komai M, Masuda T, Narumiya S, Nagai H. Role of prostaglandin I_2 in airway remodeling induced by repeated allergen challenge in mice. *Am J Respir Cell Mol Biol* 29: 314–320, 2003.
26. Nishiuma T, Sisson TH, Subbotina N, Simon RH. Localization of plasminogen activator activity within normal and injured lungs by in situ zymography. *Am J Respir Cell Mol Biol* 31: 552–558, 2004.
27. Phan SH. The myofibroblast in pulmonary fibrosis. *Chest* 122: 286–289, 2002.
28. Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, Howarth PH. Transforming growth factor- β 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 156: 642–647, 1997.
29. Sejima T, Madoiwa S, Mimuro J, Sugo T, Okada K, Ueshima S, Matsuo O, Ishida T, Ichimura K, Sakata Y. Protection of plasminogen activator inhibitor-1-deficient mice from nasal allergy. *J Immunol* 174: 8135–8143, 2005.
30. Sillaber C, Baghestanian M, Bevec D, Willheim M, Agis H, Kapiotis S, Füreder W, Bankl HC, Kiener HP, Speiser W, Binder BR, Lechner K, Valent P. The mast cell as site of tissue-type plasminogen activator expression and fibrinolysis. *J Immunol* 162: 1032–1041, 1999.
31. Sisson TH, Simon RH. The plasminogen activation system in lung diseases. *Curr Drug Targets* 8: 1016–1029, 2007.
32. Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA. The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system. *Am J Pathol* 157: 177–187, 2000.
33. Tsuchiya T, Nishimura Y, Nishiuma T, Kotani Y, Funada Y, Yoshimura S, Yokoyama M. Airway remodeling of murine chronic antigen exposure model. *J Asthma* 40: 935–944, 2003.
34. Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. *J Clin Invest* 88: 1067–1072, 1991.
35. Vayalil PK, Olman M, Murphy-Ullrich JE, Postlethwait EM, Liu RM. Glutathione restores collagen degradation in TGF- β -treated fibroblasts by blocking plasminogen activator inhibitor-1 expression and activating plasminogen. *Am J Physiol Lung Cell Mol Physiol* 289: L937–L945, 2005.
36. Ware LB, Matthay MA. Keratinocyte and hepatocyte growth factors in the lung: roles in lung development, inflammation, and repair. *Am J Physiol Lung Cell Mol Physiol* 282: L924–L940, 2002.
37. Yoshimura S, Nishimura Y, Nishiuma T, Yamashita T, Kobayashi K, Yokoyama M. Overexpression of nitric oxide synthase by the endothelium attenuates bleomycin-induced lung fibrosis and impairs MMP-9/TIMP-1 balance. *Respirology* 11: 546–556, 2006.