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Inhibition of Matrix Metalloproteinases Prevents Allergen-Induced Airway Inflammation in a Murine Model of Asthma

Katsunori Kumagai,* Isao Ohno,* Shinji Okada,* Yuichi Ohkawara,* Ko Suzuki,[†] Takashi Shinya,[‡] Hideaki Nagase,[§] Kazushi Iwata,[‡] and Kunio Shirato^{1*}

Although matrix metalloproteinases (MMPs) have been reported to play crucial roles in the migration of inflammatory cells through basement membrane components *in vitro*, the role of MMPs in the *in vivo* accumulation of the cells to the site of inflammation in bronchial asthma is still obscure. In this study, we investigated the role of MMPs in the pathogenesis of bronchial asthma, using a murine model of allergic asthma. In this model, we observed the increase of the release of MMP-2 and MMP-9 in bronchoalveolar lavage fluids after Ag inhalation in the mice sensitized with OVA, which was accompanied by the infiltration of lymphocytes and eosinophils. Administration of tissue inhibitor of metalloproteinase-2 to airways inhibited the Ag-induced infiltration of lymphocytes and eosinophils to airway wall and lumen, reduced Ag-induced airway hyperresponsiveness, and increased the numbers of eosinophils and lymphocytes in peripheral blood. The inhibition of cellular infiltration to airway lumen was observed also with tissue inhibitor of metalloproteinase-1 and a synthetic matrix metalloproteinase inhibitor. These data suggest that MMPs, especially MMP-2 and MMP-9, are crucial for the infiltration of inflammatory cells and the induction of airway hyperresponsiveness, which are pathophysiologic features of bronchial asthma, and further raise the possibility of the inhibition of MMPs as a therapeutic strategy of bronchial asthma. *The Journal of Immunology*, 1999, 162: 4212–4219.

Airway inflammation and hyperresponsiveness are fundamental features of bronchial asthma (1). Airway inflammation in asthmatics is characterized with the accumulation of activated inflammatory cells, including eosinophils, lymphocytes, and mast cells/basophils in airway lumen and walls. These cellular infiltrates release various chemical mediators such as histamine, eosinophil granule proteins, leukotrienes (LTs),² and platelet-activating factor (PAF), which are involved in the induction of airway hyperresponsiveness (2–4). In this respect, the recruitment of these inflammatory cells from the circulation to the site of inflammation is regarded as a key event in the development and maintenance of allergic inflammation that occurs in patients with bronchial asthma. The molecular events in the infiltration of inflammatory cells through endothelium and epithelium structures have been investigated intensively in *in vivo* and *in vitro* system, showing the involvement of adhesion molecules, cytokines, chemokines, LTs, and PAF (3, 5–7). However, little attention has been given to how inflammatory cells transmigrate the basement membrane zone underlying endothelial and epithelial cell layers, a fine

meshwork consisted of type IV collagen, laminin, heparan sulfate proteoglycan, and others (8).

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases capable of proteolytically degrading many of the components of extracellular matrix (9). MMPs are produced by not only structural cells such as fibroblasts, endothelial cells, and epithelial cells (10, 11), but also inflammatory cells such as macrophages (12), lymphocytes (13), neutrophils (14), and eosinophils (15). They are secreted as latent forms followed by proteolytic processing to active forms (9), and involved in the physiologic processes such as development, angiogenesis, and wound healing, and in the pathologic conditions such as tumor invasion and inflammation (10, 16). The proteolytic activation of latent forms and enzymatic activities by active forms of MMPs is inhibited by endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) that form a 1:1 complex with MMPs (9). The balance between the levels of MMPs and TIMPs is thought to be a critical factor in regulating the breakdown of connective tissues by MMPs, which is the case in pulmonary emphysema (17). Of the MMP family, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) cleave type IV collagen, which is an important constituent of basement membrane. They are posttranslationally regulated by TIMP-1 and -2, which make complexes with active form of either MMP-2 or -9, and with latent form of MMP-9 and -2, respectively (9).

Tumor cells penetrate basement membrane by making holes with secreted MMP-2 and -9 (18). Recently, MMP-2 and -9 were reported in *in vitro* system to play a crucial role also in the transmigration of lymphocytes (13), and neutrophils (14) through basement membrane components. Furthermore, we demonstrated that MMP-9 was also important in the *in vitro* transmigration of basement membrane components by eosinophils (19), and that MMP-9 was overexpressed by eosinophils accumulating in airway walls of asthmatics (20). However, despite the results that MMP-2 and -9 are crucial for the *in vitro* transmigration of inflammatory cells and

*First Department of Internal Medicine, Tohoku University School of Medicine, Sendai, Japan; [†]Miyagi Red Cross Blood Center, Sendai, Japan; [‡]Biopharmaceutical Department, Fuji Chemical Industries, Takaoka, Japan; and [§]Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66160

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¹ Address correspondence and reprint requests to Dr. Kunio Shirato, Professor and Chairman, First Department of Internal Medicine, Tohoku University School of Medicine, 1-1, Seiryomachi, Aoba-ku, Sendai, 980-8574, Japan.

² Abbreviations used in this paper: LT, leukotriene; BAL, bronchoalveolar lavage; h, human; m, mouse; MCh, methacholine; MMP, matrix metalloproteinase; PAF, platelet-activating factor; RL, pulmonary resistance; TIMP, tissue inhibitor of metalloproteinase.

that MMPs are produced by inflammatory cells at the site of inflammation, it has not been determined yet whether they are also crucial for the *in vivo* accumulation of inflammatory cells to the airways in bronchial asthma. Thus, we hypothesized that MMP-2 and/or -9 play important roles in the *in vivo* migration of inflammatory cells to the airways in which the cells have to transverse endothelial and epithelial basement membrane. To examine the hypothesis, using a murine model of allergic asthma, we studied first the production of MMP-2 and -9 in airways after Ag challenge, and secondly the effect of MMP inhibitors, recombinant human (rh) TIMP-1 and -2, and a synthetic inhibitor of MMPs, on inflammatory cell accumulation to airways and on airway hyperresponsiveness.

Materials and Methods

Sensitization and Ag challenge protocol

Specific pathogen-free female BALB/c mice, 6 to 8 wk old, purchased from our animal facility (Institute for Experimental Animals, Tohoku University School of Medicine, Sendai, Japan), were sensitized and challenged, as previously described (21). Briefly, mice were sensitized by i.p. injections of OVA (Sigma, St. Louis, MO) (8 μ g/mouse) adsorbed with aluminum hydroxide (Wako Pure Chemical Industries, Osaka, Japan) on days 0 and 5. Ten to fourteen days after the last injection, mice were challenged with aerosolized OVA or saline as a control. At various time points, mice were anesthetized with diethylether (Wako), and blood samples were obtained by retro-orbital bleeding. Blood smears were stained with Diff-Quick solution (International Reagents, Kobe, Japan) for differential cell counting. Total white cell number in peripheral blood was counted with a hemocytometer after RBC lysis using RBC lysis buffer. Bronchoalveolar lavage (BAL) was performed and lung tissues were collected, as previously described (21). Lungs were washed twice by the injection of HBSS (Life Technologies, Grand Island, NY) (0.25 ml and 0.20 ml, respectively) through the trachea, and approximately 0.4 ml of the instilled fluid was consistently recovered from each mouse. Total cell numbers were counted with a hemocytometer. Smears of BAL cells prepared with a Cytospin II (Shandon, Runcorn, U.K.) were stained with Diff-Quick solution for differential cell counting or fixed with PLP solution (10 mM sodium *m*-periodate (Sigma) to 75 mM L-lysine monohydrochloride (Wako)-2% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) in 37.5 mM PBS) for immunocytochemistry. Counts of cells in peripheral blood and BAL fluids were performed by a person unaware of the experiment. The percentages of cell differentials were determined by counting at least 300 cells under a light microscopy. After centrifugation of BAL fluid, supernatants were stored at -20°C until for use. Tracheal tissues were fixed with PLP solution and embedded in OCT compound (Miles Laboratories, Naperville, IL) with liquid nitrogen. Five-micrometer-thick sections were used for the counting of tissue eosinophils after staining with Diff-Quick solution and for that of CD3-positive cells after immunohistochemical staining. The numbers of eosinophils and CD3-positive cells were counted in whole areas of submucosae and expressed as a number/mm of basement membrane of epithelium. The length of the basement membrane was measured with MCID image analyzer (Imaging Research, St. Catharines, Ontario, Canada). An average of data in three sections was used as the result for each mouse. These studies had been approved by the Ethics Committee for Animal Experiments of the Tohoku University School of Medicine.

Administration of TIMPs and a MMP inhibitor

rhTIMP-1 (Fuji Chemical Industries, Toyama, Japan) (25 μ g in 25 μ l of saline/mouse), rhTIMP-2 (Fuji) (25 μ g in 25 μ l of saline/mouse), or saline alone (25 μ l/mouse), each of which contained <0.005 endotoxin units (EU) of endotoxin, was instilled intranasally six times, in some experiments fourteen times, with 12-h interval, beginning at 1 h before OVA inhalation. A synthetic inhibitor of MMPs, R-94138 (IC_{50} on MMP-9; 1.2 nM, MMP-2; 38 nM, MMP-3; 120 nM, MMP-7; 23 μ M, MMP-13; 38 nM) (compound 31f in Ref. 22) (kindly provided by Dr. K. Tanzawa, Sankyo, Tokyo, Japan), was injected i.p. (30 mg/kg body weight) three times with 24-h interval, beginning at 30 min before OVA inhalation. Three days after the OVA inhalation, mice were sacrificed and samples were collected, as mentioned above.

Zymography

Gelatin zymography was performed as described previously (23). A total of 10 μ l of BAL fluid underwent electrophoresis in 7.5% polyacrylamide gels containing 2.8 mg/ml gelatin (Sigma), in the presence of SDS (Wako) under nonreducing conditions. After electrophoresis, gels were washed three times each for 15 min in 50 mM Tris-HCl (Sigma) (pH 7.5) containing 2.5% Triton X-100 (Sigma), 10 mM CaCl_2 (Wako), and 1 μ M ZnCl_2 (Wako), and then incubated in 1% Triton X-100 at 37°C for 24 h in the presence or absence of EDTA (Dojin Chemical Laboratories, Kumamoto, Japan) (20 mM), 1,10-phenanthroline (Sigma) (5 mM), or PMSF (Sigma) (2 mM). Following incubation, the gels were stained with Coomassie brilliant blue R 250 (Fluka Chemie AG, Buchs, Switzerland) and destained in a solution of 1% formic acid with 30% methanol. Gelatinolytic activity was detected as clear bands against a blue background. The intensity of the bands in inverted image of zymogram was estimated using densitometry with National Institute of Health image 1.61. hMMP-2 and hMMP-9 from HT1080 cells were used as positive controls in gelatin zymography.

Immunoprecipitation

To determine types of MMPs to which intranasally administered rhTIMP-2 bound, BAL fluids from rhTIMP-2-administered mice after OVA inhalation were immunoprecipitated with a mouse monoclonal anti-hTIMP-2 IgG₁, 68-6H4 (Fuji), which recognizes free TIMP-2, TIMP-2 binding to active MMP-2 and -9, and pro-MMP-2 (24). A total of 300 μ l of BAL fluids was incubated with anti-hTIMP-2 Ab at the concentration of 70 μ g/ml in a 1.5-ml Eppendorf tube. After overnight incubation at 4°C , samples were added with 30 μ l of packed protein G-Sepharose (4FF; Amersham, Buckinghamshire, U.K.), and further incubated for 90 min. The immunoprecipitates were collected as pellets after centrifugation. After the supernatants were removed, the pellets were washed and suspended with 300 μ l of electrophoresis sample buffer. Then the immunoprecipitates and the supernatants were subjected to gelatin zymography, as mentioned above.

Western blot analysis of BAL fluids

Immunoblotting was performed to certify the gelatinolytic activity as MMP-9. BAL fluids concentrated five times by Microcon 10 (Amicon, Beverly, MA) and purified hMMP-9 were loaded to SDS-PAGE under nonreducing conditions, and were transferred to a membrane (Immobilon PVDF; Millipore, Bedford, MA). After blocking with 22% skim milk, the blots were incubated overnight with fibronectin-adsorbed polyclonal anti-hMMP-9 sheep IgG (25). After several washes and blocking again, rabbit IgG anti-sheep IgG conjugated with horseradish peroxidase (ICN Pharmaceuticals, Costa Mesa, CA) were applied to the blots. After several washes, blots were soaked in enhanced chemoluminescence (ECL) solution (Amersham Pharmacia Biotech, Uppsala, Sweden) and were exposed to x-ray films. Western blotting was performed also with fibronectin-adsorbed polyclonal anti-hMMP-2 rabbit IgG and anti-hMMP-1 and -3 sheep serum (25).

Immunostaining

CD3-positive cells in sections of tracheal tissues were investigated with anti-mouse CD3e hamster IgG (PharMingen, San Diego, CA) or normal hamster IgG (PharMingen) as a control Ab, using biotin-labeled anti-hamster IgG (PharMingen) and alkaline phosphatase-conjugated avidin-biotin complex (Strept ABCComplex/AP; Dako, Carpinteria, CA), and visualized with a Fast Red Substrate System (Dako), followed by counterstaining with hematoxylin. Immunoreactivity of MMP-9 in BAL cells was examined with fibronectin-adsorbed polyclonal anti-hMMP-9 sheep IgG or normal sheep IgG (ICN) as a control Ab, and rabbit IgG anti-sheep IgG conjugated with horseradish peroxidase, and was visualized with diaminobenzidine, followed by counterstaining with methyl green.

Measurement of IL-5 in BAL fluids

The content of IL-5 in BAL fluids was measured with a specific mouse (m) IL-5 ELISA kit (Amersham). The detection limit of the IL-5 assay was 5 pg/ml, and the assay is said by the manufacturer to be specific for IL-5.

Airway hyperresponsiveness

Airway responsiveness of mice 3 days after OVA inhalation, followed by intranasal administration of rhTIMP-2 or saline as mentioned above, was assessed. As a control, sensitized mice were inhaled with saline, and then intranasally administered with saline in the same way. Airway responsiveness was assessed as a change in pulmonary resistance (*R*_L) after injections of increasing dose of methacholine (MCh, acetyl- β -methylcholine chloride) (Wako) (0.1–30 mg/kg) in 50- μ l volumes. Mice were anesthetized by

i.p. injection of pentobarbital sodium (Wako) (50 mg/kg), and were tracheostomized. Air flow rate at airway opening during spontaneous breathing was monitored by a pneumotachogram (8430B; Hans Rudolph, Kansas City, MO) combined with a differential pressure transducer (LCVR, 0–2 cm H₂O; Celesco, Canoga Park, CA). Esophageal pressure monitored by a water-filled tube and a pressure transducer (Ohmeda, Singapore) was used as transpulmonary pressure, because the pressure difference generated by the pneumotachogram connected to tracheal tube was very small (more than 100 times) compared with the amplitude of esophageal pressure. R_I was calculated by the subtraction method of Mead and Whittenberger (26). An average R_I of three breaths at 3 min after each injection of MCh was calculated, and expressed as a percentage of baseline R_I that was measured and calculated in the same way after the injection of saline used as a diluent of MCh.

Data analysis

All data are presented as means \pm SEM. Significant differences in counts of cells in BAL fluids and in tracheal walls during the time course after OVA or saline inhalation were determined using one-way analysis of variance with post hoc analysis of Fisher's Protected Least Significance. Significant differences between groups in the others were determined using unpaired Student's *t* test. These analyses were performed using StatView 4.11 (Abacus Concepts, Berkeley, CA) for Macintosh. A *p* value of less than 0.05 was taken as significant.

Results

Cellular changes in BAL fluids after OVA inhalation

Total cell numbers in BAL fluids were significantly increased at 3 to 14 days after OVA inhalation compared with either those before the inhalation or those after saline inhalation (Fig. 1, A and B). The increase of total cell numbers was associated with significant increase of eosinophils and lymphocytes at 3 to 7 days, that of macrophages at 3 days, and that of neutrophils at 5 days after OVA inhalation. In contrast, no significant changes in cell numbers were observed after saline inhalation (Fig. 1B).

The accumulation of eosinophils in tracheae after OVA inhalation

Eosinophils in tracheal walls significantly increased at 12 h after OVA inhalation and remained increased until day 5 compared with those before the inhalation (Fig. 1C). In contrast, only a few increases of eosinophils were observed after saline inhalation (data not shown).

Gelatinolytic activities in BAL fluids

Gelatin zymography of BAL fluids showed the constitutive expression of 65- and 60-kDa gelatinase activities and the induction of 105-kDa gelatinase activity after OVA inhalation (Fig. 2, A and B). The gelatinolytic activities in BAL fluids at 3 days after OVA inhalation, as well as those of purified hMMP-2 and -9, were inhibited by EDTA or by 1,10-phenanthroline, but not by PMSF (data not shown). The gelatinolytic activity with a molecular mass of 105 kDa is consistent with being the zymogen of mMMP-9 (27). The identity of pro-MMP-9 was further confirmed by Western blotting analysis (Fig. 3). Western analysis of concentrated BAL fluids from OVA-inhaled mice with anti-hMMP-9 Ab also showed the presence of a 95-kDa band (Fig. 3), which corresponds to the active mMMP-9 (27). The 65- and 60-kDa gelatinolytic activities correspond to a pro- and active forms of mMMP-2, respectively (28). Anti-hMMP-2 could not detect any band in blots of BAL fluids. We also performed Western blot analysis of BAL fluids with anti-hMMP-1 and -3 Abs, but these MMPs were not detected. Semiquantitative analysis of gelatinolytic activities revealed that pro-MMP-9 activities were significantly higher on days 3 and 5 in OVA-inhaled mice than in saline-inhaled mice (Fig. 2C), and that pro-MMP-2 and active MMP-2 activities were significantly higher on day 3 (Fig. 2, D and E).

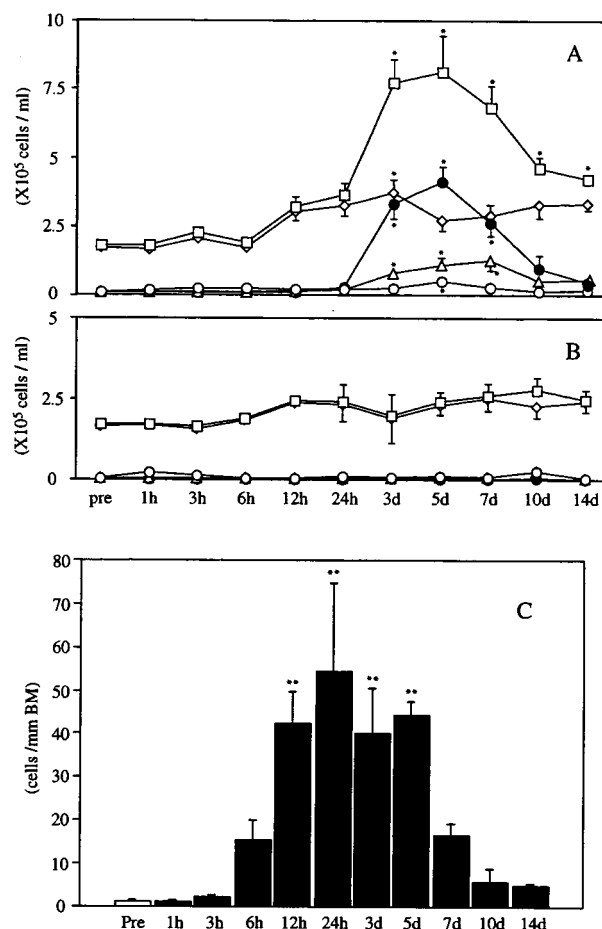


FIGURE 1. Cellular infiltration to airways in sensitized and challenged mice. In BAL fluids from sensitized mice challenged with OVA (A) or saline (B), the numbers of total cells (open squares), macrophages (open rhomboids), eosinophils (closed circles), lymphocytes (open triangles), and neutrophils (open circles) were counted ($n = 4-6$). Data marked with asterisks were significantly different from both those of preinhalation and those of saline challenge. The number of eosinophils in tracheal walls from sensitized mice challenged with OVA was counted ($n = 4-6$) (C). **, $p < 0.01$, compared with preinhalation. BM, basement membrane.

Immunolocalization of MMP-9

Immunocytochemistry with anti-hMMP-9 Ab used in Western blotting analysis showed the localization of immunoreactive MMP-9 on BAL cells, including macrophage-, lymphocyte-, and eosinophil-like cells, from sensitized mice at 3 days after OVA inhalation (Fig. 4B). In contrast, no immunoreactivity was detected on BAL cells from those at 3 days after saline inhalation (Fig. 4A). No positive staining was detected with control Ab in BAL cells from either group of mice (data not shown).

The effects of TIMP-1, -2, and a synthetic MMP inhibitor on cell counts in BAL fluids

Intranasal administration of rhTIMP-2 ($n = 6$) significantly inhibited the enhanced leukocyte accumulation in the airway lumen at 3 days after OVA inhalation, compared with intranasal administration of saline ($n = 4$), as shown by total cell counts (3.35 ± 0.37 versus $7.50 \pm 0.70 \times 10^5/\text{ml}$, $p < 0.05$) and differential cell counts including macrophages (2.21 ± 0.19 versus 3.34 ± 0.36 , $p < 0.05$), eosinophils (0.80 ± 0.14 versus 3.37 ± 0.43 , $p < 0.01$), and lymphocytes (0.19 ± 0.03 versus 0.61 ± 0.05 , $p < 0.05$) (Fig. 5A). The inhibitory effect of rhTIMP-2 was observed also at 7 days after

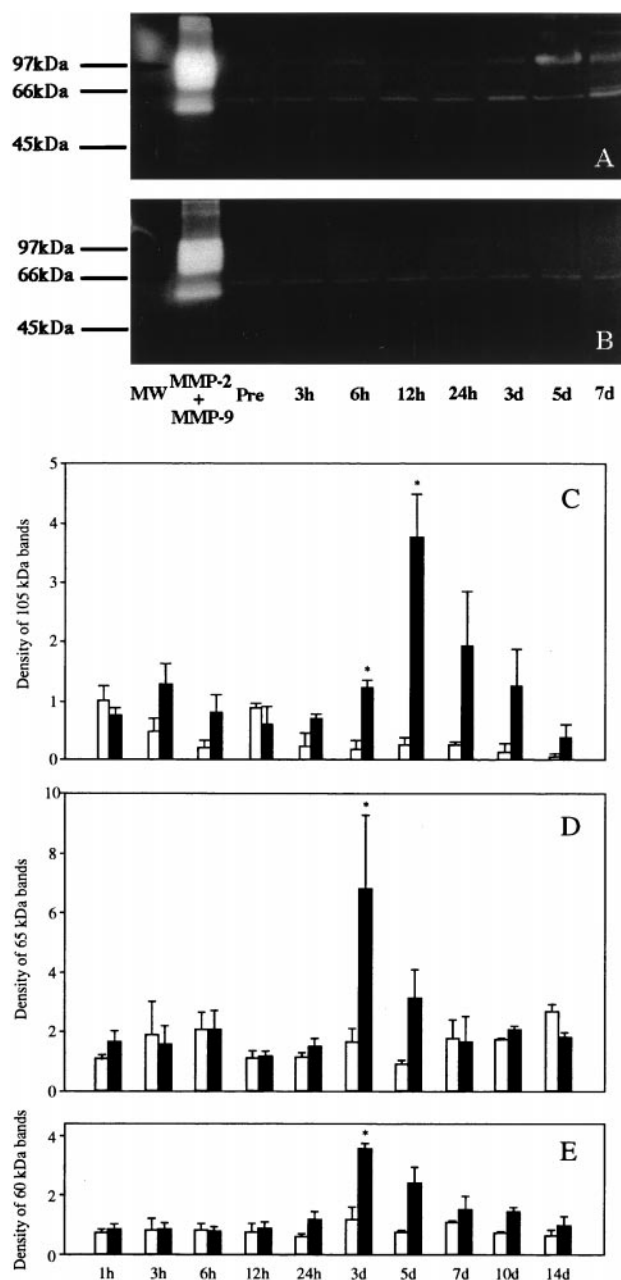


FIGURE 2. Analysis of BAL fluids by gelatin zymography. BAL fluids from sensitized mice inhaled with OVA (A) or saline (B) were analyzed with gelatin zymography. The positions of m.w. standards (MW) are marked on the left. C–E show the data of semiquantification of gelatinolytic activities in BAL fluids from sensitized mice inhaled with OVA (closed columns) or saline (open columns) ($n = 4–6$). *, $p < 0.05$, compared with saline inhalation.

OVA inhalation (data not shown). There was no significant difference in both total and differential cell counts between rhTIMP-2- and saline-administered mice after saline inhalation (data not shown). Similarly, either rhTIMP-1 (Fig. 5B) or R-94138, a synthetic MMP inhibitor (Fig. 5C), inhibited leukocyte infiltration after OVA inhalation. The MMP inhibitors used in this study had no effect on neutrophil number, which was not significantly increased after OVA inhalation.

The effect of TIMP-2 on IL-5 content in BAL fluids

The concentration of IL-5 in BAL fluids was increased at 3 days after OVA inhalation (173.8 ± 47.1 pg/ml, $n = 5$) compared with

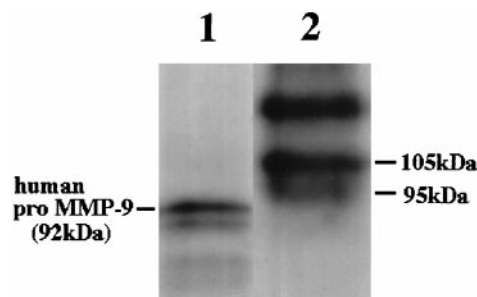


FIGURE 3. Western blot analysis with anti-hMMP-9 of BAL fluids from sensitized mice inhaled with OVA. Purified hMMP-9 (lane 1) and BAL fluids from sensitized mice 3 days after OVA inhalation (lane 2) were analyzed with Western blot using anti-hMMP-9 Ab. This picture is a representative of three independent experiments.

saline inhalation (6.4 ± 1.1 pg/ml, $n = 5$). However, the increase of IL-5 concentration was not significantly affected by the administration of rhTIMP-2 (144.9 ± 36.2 pg/ml, $n = 5$).

The effect of TIMP-2 on the accumulation of eosinophils and CD3-positive cells in tracheal walls

The increased numbers of eosinophils and CD3-positive cells in tracheal walls of sensitized mice at 3 days after OVA inhalation were significantly reduced (eosinophils; 38.5 ± 6.4 ($n = 6$) versus 18.7 ± 2.8 ($n = 6$) cells/mm, $p < 0.05$, CD3-positive cells; 11 ± 2.1 ($n = 4$) versus 4.3 ± 1.3 ($n = 4$) cells/mm, $p < 0.05$) by the intranasal administration of rhTIMP-2 (Fig. 6).

The effect of TIMP-2 on leukocyte counts in peripheral blood

In contrast to the reduction of cell counts in airway lumen and tracheal walls, total leukocyte, eosinophil, and lymphocyte counts in peripheral blood were significantly increased in mice administered with rhTIMP-2 ($n = 6$) compared with those administered with saline ($n = 4$) after OVA inhalation ($17,080 \pm 1,336.6$ versus $10,920 \pm 1,555$, $p < 0.05$; $1,076.3 \pm 163.2$ versus 516.1 ± 95 , $p < 0.05$; and $13,655.6 \pm 1,112.2$ versus $8,942.1 \pm 1,323.7$ 10^3 /ml, $p < 0.05$, respectively) (Fig. 7).

The effect of TIMP-2 on airway hyperresponsiveness

Baseline RI of saline-challenged and saline-administered, OVA-challenged and saline-administered, and OVA-challenged and

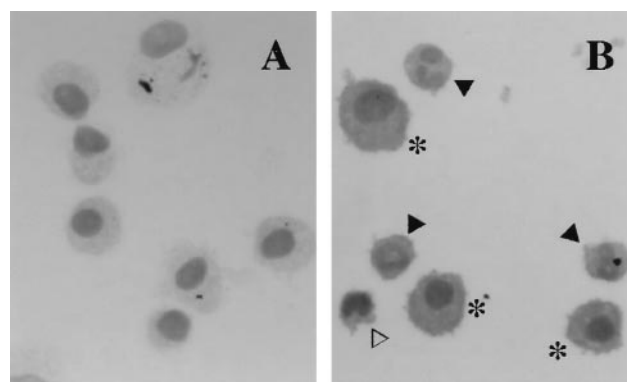


FIGURE 4. The localization of immunoreactive MMP-9 on BAL cells from sensitized mice inhaled with OVA. Immunolocalization of MMP-9 on BAL cells from sensitized mice 3 days after saline (A) or OVA (B) inhalation was examined with anti-hMMP-9 Ab. Positive staining was detected on macrophage (asterisks)-, eosinophil (closed triangles)-, and lymphocyte (open triangles)-like cells after OVA inhalation. $\times 200$.

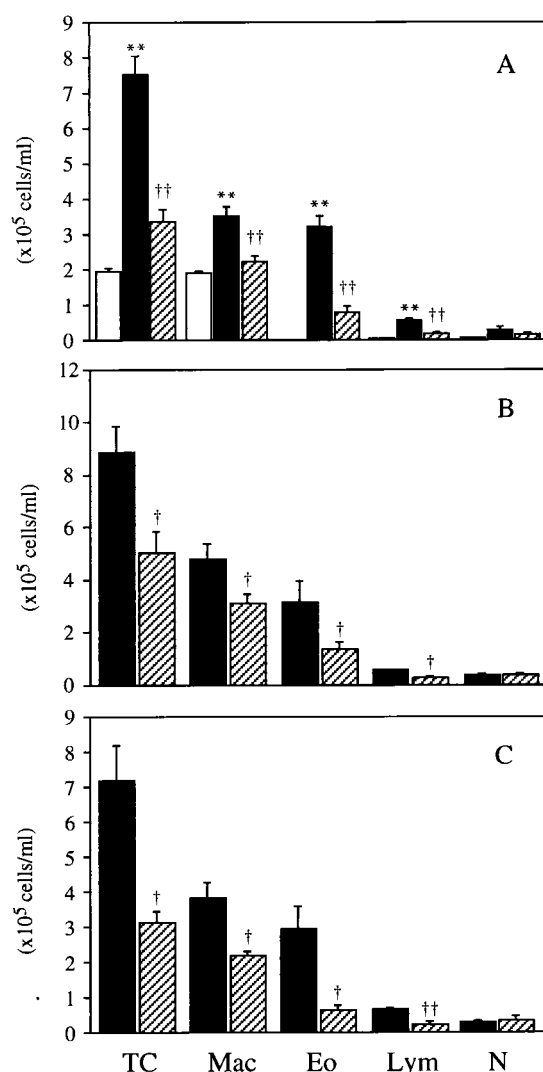


FIGURE 5. The effects of MMP inhibitors on cell counts in BAL fluids from sensitized mice. The numbers of total cells (TC), macrophages (Mac), eosinophils (Eo), lymphocytes (Lym), and neutrophils (N) in BAL fluids from saline-inhaled mice with the administration of saline (open columns), OVA-inhaled mice with the administration of saline (closed columns), and OVA-inhaled mice with the administration of MMP inhibitors (hatched columns) were counted ($n = 4-6$). MMP inhibitors used were rhTIMP-2 (A), rhTIMP-1 (B), and a synthetic MMP inhibitor, R-94138 (C). **, $p < 0.01$, compared with saline inhalation group (open columns), and †, $p < 0.05$ and ††, $p < 0.01$, compared with OVA inhalation group (closed columns).

rhTIMP-2-administered groups were 0.29 ± 0.11 ($n = 4$), 0.26 ± 0.06 ($n = 6$), and 0.33 ± 0.11 ($n = 4$) $\text{cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{sec}$, respectively, and there was no significant difference between three groups. Airway responsiveness assessed as percentage of increase in R_l in response to increasing dose of MCh was increased after the OVA challenge (Fig. 8). Compared with the saline-challenged group, dose-response curve of percentage of R_l shifted to left side, and percentage of R_l significantly increased at 10 mg/kg of MCh in the OVA-challenged group ($717 \pm 153\%$, $p < 0.05$). Administration of rhTIMP-2 reduced airway hyperresponsiveness induced by OVA challenge. In the OVA-challenged group with rhTIMP-2 administration, percentage of R_l at 10 mg/kg of MCh was significantly reduced ($212 \pm 94\%$, $p < 0.05$) compared with the OVA-challenged group with saline administration, and was almost as same as the saline-challenged group ($207 \pm 31\%$). The

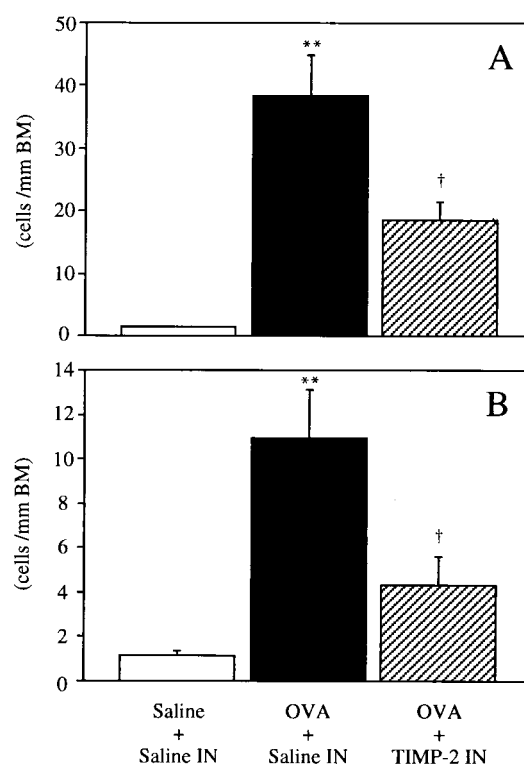


FIGURE 6. The effect of rhTIMP-2 on the numbers of eosinophils and CD3-positive cells in tracheal walls. The numbers of eosinophils (A) and CD3-positive cells (B) in tracheal tissues from saline-inhaled mice with the administration of saline (Saline + Saline IN), OVA-inhaled mice with the administration of saline (OVA + Saline IN), and OVA-inhaled mice with the administration of rhTIMP-2 (OVA + TIMP-2 IN) were counted ($n = 4-6$). **, $p < 0.01$, compared with Saline + Saline IN group, and †, $p < 0.05$, compared with OVA + Saline IN group. BM, basement membrane.

inhibitory effect of rhTIMP-2 on airway hyperresponsiveness was observed also at 7 days after OVA inhalation (data not shown).

Characterization of MMPs binding to rhTIMP-2

There was no significant difference in gelatinolytic activities detected by zymography in BAL fluids between rhTIMP-2- and saline-administered mice after OVA inhalation (data not shown).

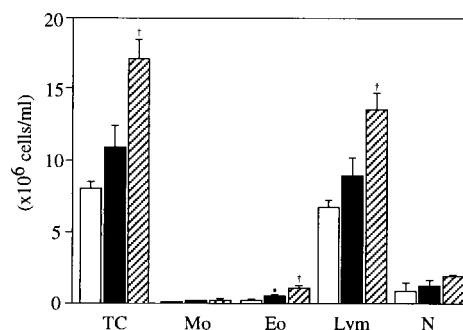


FIGURE 7. The effect of rhTIMP-2 on leukocyte counts in peripheral blood. The numbers of total cells (TC), monocytes (Mo), eosinophils (Eo), lymphocytes (Lym), and neutrophils (N) in peripheral blood from saline-inhaled mice with the administration of saline (open columns), OVA-inhaled mice with the administration of saline (closed columns), and OVA-inhaled mice with the administration of rhTIMP-2 (hatched columns) were counted ($n = 4-6$). *, $p < 0.05$, compared with saline inhalation group (open columns), and †, $p < 0.05$, compared with OVA inhalation group (closed columns).

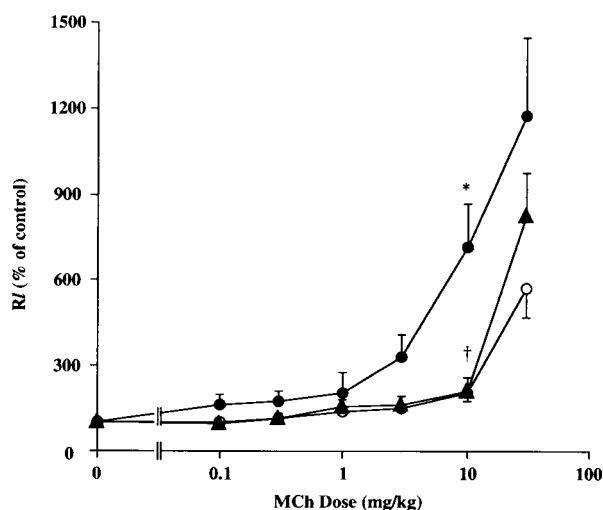


FIGURE 8. Pulmonary resistance (R_l) in sensitized and challenged mice. R_l values in saline-challenged mice with the administration of saline (open circles), OVA-challenged mice with the administration of saline (closed circles), and OVA-challenged mice with the administration of rhTIMP-2 (closed triangles) were obtained in response to increasing concentrations of MCh ($n = 4-6$). *, $p < 0.05$, compared with saline-challenged and saline-administered group (open circles), and †, $p < 0.05$, compared with OVA-challenged and saline-administered group (closed circles).

Gelatin zymography of immunoprecipitates with anti-hTIMP-2 Ab showed that rhTIMP-2 intranasally administered had made complexes with, at least, active and pro-MMP-2 in mice with OVA inhalation, while not with pro-MMP-9, as expected (Fig. 9, lane 3).

Discussion

In this study using a murine model of allergic asthma, we demonstrated for the first time that the release of MMP-2 and -9 was up-regulated in association with the accumulation of inflammatory cells to airways after Ag challenge, and that the inhibition of the MMPs prevented the cellular infiltration and the induction of airway hyperresponsiveness.

The up-regulation of MMP-9 release observed in this study is consistent with the previous reports that MMP-9 content in BAL fluids from untreated asthmatics was increased compared with that from control subjects including steroid-treated asthmatics (12), and that Ag challenge increased enzyme activity mainly due to MMP-9 in BAL fluids from asthmatics (29). In this study, we could not detect MMP-2 in BAL fluids by Western blotting analysis using anti-hMMP-2 antiserum that cross-reacts with mouse MMP-2 (25). A reason for this failure is most likely due to low sensitivity of the assay. MMP-1 and -3 were also not detected either by zymography or Western blotting. However, because of the same reason as in the

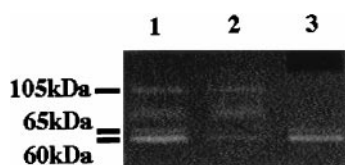


FIGURE 9. Immunoprecipitation of BAL fluids with anti-hTIMP-2 Ab. BAL fluids from OVA-challenged mice with the administration of rhTIMP-2 were precipitated with anti-hTIMP-2 Ab. The original BAL fluids (lane 1), nonprecipitates (lane 2), and precipitates (lane 3) were analyzed with gelatin zymography. This picture is a representative of three independent experiments.

case of MMP-2, i.e., the limitation of the assay sensitivity, the possibility of the presence of these and other types of MMPs could not be ruled out.

Sources of MMP-2 and -9 detected in BAL fluids after OVA inhalation were not determined in this study. However, the result of immunostaining of BAL cells with anti-MMP-9 Ab suggests that macrophages, lymphocytes, and eosinophils in airways are potential sources of the MMPs, and this is supported by the studies that have been done on human leukocytes (12, 13, 20). It has also been demonstrated that bronchial and alveolar epithelial cells constitutively express MMP-2 and -9, and that the expression is increased by the stimulation with TNF- α and IL-1 β (11), which are increased in asthmatic inflammation (30, 31). The release of MMP-2 and -9 from bovine airway mucosa is enhanced when cocultured with eosinophils (32). These reports suggest that the release of MMP-2 and -9 from structural cells including epithelial cells during the activation by proinflammatory cytokines and eosinophils might occur in our model. In our study, the peak of MMP-9 activity was at 5 days after OVA challenge, while MMP-2 activity was maximal at 3 days. These data suggest that the expressions of MMP-2 and MMP-9 after Ag challenge are differentially regulated. More importantly, the levels of MMPs in BAL fluids were not reduced by rhTIMP-2 in OVA-challenged mice, but the cell infiltration to airway lumen was reduced. Thus, a variety of cell types and inductive mechanisms are likely to be involved in the production of MMPs during asthmatic reaction.

Our studies suggest the relationship between the release of MMP-2 and -9 and the recruitment of leukocytes to the site of inflammation. To investigate further the role of the MMPs in allergic airway inflammation, we applied two types of natural inhibitors of MMPs, TIMP-1 and -2, and a synthetic MMP inhibitor to this model. rhTIMP-2 reduced the cellular infiltrates in BAL fluids and tracheal walls that were increased after OVA inhalation. It is unlikely that rhTIMP-2 was cytotoxic to cellular infiltrates, because leukocyte counts in peripheral blood were increased in contrast to those in BAL fluids and tracheal walls. In addition, there was no change in cell counts in BAL fluids from sensitized mice treated with rhTIMP-2 after saline challenge. Therefore, the reduction of the number of inflammatory cells in BAL fluids and tracheal walls by the administration of rhTIMP-2 is likely to be due to the inhibition of the movement of leukocytes from circulation to the site of inflammation, resulting in an increase of blood leukocytes. As demonstrated in Fig. 9, rhTIMP-2 was bound to active and pro-MMP-2 in vivo. rhTIMP-1 and a synthetic MMP inhibitor that inhibit enzyme activity of MMP-2 and MMP-9 (9, 10, 22) also prevented the cellular infiltration in BAL fluids. Together with these results, the inhibitory effect of rhTIMP-2 on the cell infiltration was attributed to the inhibition of enzymatic activities of MMPs, including, at least, MMP-2 and -9 at airway lumen and submucosae, where the presence of rhTIMP-2 was demonstrated by reverse zymography of BAL fluids and by immunohistochemistry of tracheal tissues (data not shown). Alternatively, these results indicate the participation of MMPs, at least MMP-2 and -9, to the cellular infiltration in allergic airway responses.

In the processes of cellular infiltration from circulation to inflammatory sites, MMP-2 and -9 could be involved in the migration of inflammatory cells through endothelial and epithelial basement membrane, as suggested by in vitro studies demonstrating that lymphocytes, neutrophils, and eosinophils passed through the basement membrane by degrading its components with their own MMP-2 and MMP-9 (13, 14, 19). In this study, we observed the delay in eosinophil accumulation in BAL fluids compared with that in the airway walls. Previously, we observed that eosinophils

purified from peripheral blood passed through the basement membrane in vitro within 30 min upon stimulation (19), and that tissue eosinophils in airway mucosa of asthmatics synthesized MMP-9 (20). These results led us to assume that eosinophils utilize stored MMP-9 for the transmigration through the subendothelial basement membrane, and, after extravasation, start to newly synthesize MMP-9 for the transmigration through the subepithelial basement membrane in response to microenvironmental stimulation such as cytokines, LTs, and PAF.

Cytokines and chemokines are believed to play a central role in the induction of allergic airway inflammation in bronchial asthma (3, 6, 7). Among them, IL-5, whose expression is increased in BAL fluids and bronchial biopsy tissues from asthmatics (33), is considered as one of the key cytokines promoting eosinophil accumulation (34, 35). In our model, the level of IL-5 in BAL fluids increases at 3 days after OVA inhalation (21). Interestingly, the level of IL-5 in rhTIMP-2-treated mice, in which the eosinophil number in airways was significantly reduced, was similar to that in saline-treated mice after OVA inhalation. These results indicate that the inhibitory effect of rhTIMP-2 on eosinophil infiltration was not, at least, due to the down-regulation of IL-5. Although the accumulation of lymphocytes, which are main sources of IL-5 in airways (33), was reduced significantly by the administration of rhTIMP-2, the IL-5 content in BAL fluids of the rhTIMP-2-treated mice was not changed. One possible interpretation of this observation was that a small number of lymphocyte, eosinophil (36), and/or mast cells (37) residing in or infiltrated to airways might be enough to secrete the same level of IL-5 as in OVA-challenged and saline-administered mice.

The administration of rhTIMP-2 also reduced airway hyperresponsiveness, which is a pathophysiologic feature of bronchial asthma (1). Increasing pieces of evidence have demonstrated that eosinophilic airway inflammation plays a crucial role in the induction of airway hyperresponsiveness. During the activation at the site of inflammation, eosinophils release toxic granule protein such as eosinophilic cationic protein, and major basic protein, LTC₄, and PAF. These products can cause the physiologic manifestation of airway hyperresponsiveness (38). T lymphocytes are also thought to play a role in the development of airway hyperresponsiveness (39), although the mechanism is not clear. Nonetheless, our studies have demonstrated that inhibition of cell infiltration by rhTIMP-2 abolished airway hyperresponsiveness, indicating that infiltrated cells after Ag challenge, but not resident cells, are important in the development of airway hyperresponsiveness.

Although neutrophil infiltration to airways is not so prominent as eosinophils in our model, the number of neutrophils in BAL fluids was not different between rhTIMP-2-treated mice and saline-treated mice after OVA inhalation. In a separate experiment, we examined the effect of rhTIMP-2 on neutrophil accumulation into airway lumen in mice inhaled with LPS (50 µg/ml, 30 min). In this case, the accumulation of neutrophils to BAL fluids was not inhibited by rhTIMP-2 (data not shown). It is suggested that neutrophils utilize both MMP-9 and elastase in their *trans*-basement membrane migration in vitro (14), and neutrophil infiltration to airways in an animal model with LPS inhalation is inhibited by an elastase inhibitor (40). Thus, infiltration of neutrophils appears to be more dependent on elastase than MMPs, and this may be one of the reasons that neutrophils and eosinophils accumulate to the site of inflammation differently.

In summary, we have demonstrated that inhibitors of MMPs, TIMP-1, TIMP-2, and a synthetic inhibitor reduced the development of allergic airway inflammation, possibly by inhibiting MMP-2 and MMP-9, and that the administration of rhTIMP-2 itself does not affect the physiologic cell infiltration. Our studies

suggest that the inhibition of MMPs is a new therapeutic strategy for bronchial asthma, although further investigation is necessary for our clear understanding of the regulatory mechanisms of MMP expression and the exact role of different MMPs in asthmatic airways.

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