

Increase in the Expression of Matrix Metalloproteinase-12 in the Airways of Rats with Allergic Bronchial Asthma

Yoshihiko CHIBA,* Yingyan YU, Hiroyasu SAKAI, and Miwa MISAWA

Department of Pharmacology, School of Pharmacy, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan. Received October 5, 2006; accepted November 6, 2006; published online November 13, 2006

Although an involvement of matrix metalloproteinase (MMP)-12 in the development of chronic obstructive pulmonary disease (COPD) and airway inflammation has been suggested, its detailed role in the airways is not well known now. In the present study, the changes in the expression and localization of MMP-12 in airways of repeatedly antigen-challenged rats were investigated to show an association of MMP-12 with allergic bronchial asthma. Rats sensitized by dinitrophenylated *Ascaris* antigen were 3 times repeatedly challenged with aerosolized antigen solution to induce an asthmatic reaction. Twenty-four hours after the last antigen challenge, marked airway inflammation and bronchial smooth muscle hyperresponsiveness were observed. In this animal model of allergic bronchial asthma, a significant increase in the expression/activity of MMP-12 was found: the peak was observed at 12 h after the last antigen challenge. Furthermore, mRNA expression of MMP-12 was also increased at the early phase (1–3 h) after the last antigen challenge. Immunohistochemical studies revealed that MMP-12 was mainly expressed in airway epithelia and alveolar macrophages. These findings suggest that MMP-12 is up-regulated after the induction of asthmatic reaction. MMP-12 might be a new target for the therapy against allergic bronchial asthma.

Key words matrix metalloproteinase-12; airway inflammation; allergic bronchial asthma

Matrix metalloproteinases (MMPs) are a family of proteinases which have zinc dependent structure centre, widely degrade extracellular matrix components and cleave non-matrix proteins including growth factors, chemoattractants and cell surface receptors.¹⁾ Most of the MMPs contain pro-domain which is accordant, through binding of the cysteine of pro-domain and histidines of the catalytic domain, to the metallo cation Zn^{2+} . MMPs are activated by cleavage to set the catalytic domain free to act on substrates.

MMP-12, which is also called metalloelastase and macrophage elastase, was firstly detected in mouse peritoneal macrophage.²⁾ Subsequently, it was also found in alveolar macrophages,^{3,4)} bronchial epithelial cells⁵⁾ and airway smooth muscle cells.⁶⁾ Like other MMPs, MMP-12 consists of a pro-domain, a catalytic domain and a hemopexin domain, and is activated by taking off the pro-domain and then the hemopexin domain remaining the catalytic domain. It has been reported that the rat MMP-12 catalytic domain can degrade collagen-V and partially degrade collagen-I⁷⁾ and other extracellular matrix proteins.⁸⁾

Although the role of MMP-12 in airways is not fully understood, an involvement of MMP-12 has been reported in cigarette smoking-associated airway inflammatory diseases, such as chronic obstructive pulmonary disease (COPD).⁹⁾ MMP-12 has been reported to be associated with cigarette smoke-induced emphysema¹⁰⁾ and macrophage migration.¹¹⁾ In patients with COPD, MMP-12-positive macrophages were increased in airway epithelial layers and bronchoalveolar lavage fluids when compared with normal subjects.¹²⁾ On the other hand, an upregulation of MMP-12 was also found in a mouse model of airway inflammation and remodeling induced by IL-13.¹³⁾ Furthermore, an increased expression of MMP-12 was reported in a mouse model of allergic airway inflammation: the MMP-12 upregulation was not observed in IL-13-knockout mice.¹⁴⁾ It is thus possible that MMP-12 is also relating to airway diseases mediated by Th2 cytokines such as allergic bronchial asthma. Indeed, MMP-12 has been

proposed as one of the asthma candidate genes.¹⁵⁾ In the present study, the changes in the expression and localization of MMP-12 in airways of repeatedly antigen-challenged rats were investigated to reveal whether or not MMP-12 has relevance to allergic bronchial asthma.

MATERIALS AND METHODS

Sensitization and Antigenic Challenge Male Wistar rats (6 weeks of age, specific pathogen-free, 170–190 g; Charles River Japan, Inc.) were sensitized and repeatedly challenged with 2,4-dinitrophenylated *Ascaris suum* antigen (DNP-Asc) by the method described in the previous papers.^{16–24)} In brief, the rats were sensitized with DNP-Asc together with *Bordetella pertussis* and were boosted 5 d later. Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc for 40 min under conscious state. Then the animals were subjected to totally three times repeated antigen challenge every 48 h with the same inhalational challenge method. One, 3, 12 or 24 h after the last antigen challenge, animals were sacrificed as describe below. Age-matched non-sensitized normal and/or sensitized (saline inhalation instead of antigen challenge) animals were used as controls. All experiments were approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

Functional Study The isometrical contraction of the circular smooth muscle of the main bronchus was measured as described previously.^{16–21)} In brief, the rats were sacrificed by exsanguinations from abdominal aorta under chloral hydrate (400 mg/kg, i.p.) anesthesia. Then the airway tissues below the larynx to lungs were immediately removed. About 4 mm length (3 mm diameter) of the left main bronchus was isolated (8–9 cartilages) and the resultant tissue ring preparation was then suspended in an organ bath at a resting tension of 1 g. The organ bath contained modified Krebs–Henseleit solution with the following composition (mM); NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$

* To whom correspondence should be addressed. e-mail: chiba@hoshi.ac.jp

25.0, KH_2PO_4 1.2 and glucose 10.0 (pH 7.4). The isometrical contraction of the circular smooth muscle was measured with a force-displacement transducer (TB-612T, Nihon Kohden, Tokyo, Japan). During an equilibration period, the tissues were washed three or four times at 15–20-min intervals and were equilibrated slowly to a baseline tension of 1 g. After the equilibration period, the concentration–response curve to acetylcholine (ACh; 10^{-7} – 10^{-3} M in final concentration) was constructed cumulatively. A higher concentration of ACh was successively added after attainment of a plateau response to the previous concentration.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Airway tissues below the main bronchi were removed under anesthesia, quick-frozen in liquid nitrogen, crushed to powders by using CryopressTM (CP-100W; Microtec, Co., Ltd., Chiba, Japan: 15 s $\times$ 3), and total RNA was isolated from each frozen sample with a one-step guanidium–phenol–chloroform extraction procedure using TRI ReagentTM (Sigma, St. Louis, MO, U.S.A.). The mRNA levels of MMP-12 were examined by RT-PCR. Briefly, cDNAs were prepared from the total RNA (1.0 μg) by using RevertAid First Strand cDNA Synthesis Kit (Fermentas Inc., Hanover, MD, U.S.A.) in a total volume of 50 μl reaction buffer containing 50 mM Tris–HCl, pH 8.3, 50 mM KCl, 4 mM MgCl_2 , 1 mM dNTP mixture, 1 U/ μl RNase inhibitor, 10 ng/ μl random 6mers, and 200 U/ μl M-MuLV reverse transcriptase. The reaction mixture was incubated for 10 min at 25 °C followed by 60 min at 42 °C to initiate the synthesis of cDNAs. Reverse transcriptase was inactivated at 70 °C for 5 min. Then the RT reaction mixture (1 μl) was subjected to PCR (0.1 μM forward and reverse primers, 0.025 U/l *Taq* DNA polymerase, 2 mM MgCl_2 , 0.2 mM dNTPs) in a final volume of 5 μl . The PCR primer sets used were: 5'-AGGTCAAGATGGATGAAGCGG-3' (sense) and 5'-GAAGTAATGTTGGTGGCTGGACTC-3' (antisense) for MMP-12, 5'-CCATCACTGCCACTCAGAAGAC-3' (sense) and 5'-TACTCCTTGAGGCCATGTAGG-3' (antisense) for GAPDH. The thermal cycle profile used was 1) denaturing for 15 s at 95 °C, 2) annealing primers for 15 s at 55 °C, 3) extending the primers for 60 s at 72 °C. The PCR amplification was performed at 30 cycles according to the preliminary cycle dependence experiment. The PCR products were subjected to electrophoresis on 1.2% agarose gel and visualized by ethidium bromide staining. The band intensity was quantified by a densitometer (Atto Densitograph; Atto Co., Tokyo, Japan). The ratio of the MMP-12/GAPDH was calculated for the relative expression of MMP-12 mRNA.

Protein Extraction and Western Blots Total protein was extracted from an aliquot of the tissue powder described above by using T-RERTM (Pierce, Rockford, IL, U.S.A.) in the presence of proteinase inhibitors according to the manufacturer's instructions. The protein samples were mixed with SDS sample buffer and heated at 100 °C for 4 min. The samples (10 μg per lane) were subjected to 10% SDS-PAGE. Proteins were then electrophoretically transferred for 2 h onto PVDF membranes (Hybond-P, Amersham, U.K.) in transfer buffer (20% methanol containing 25 mM Tris and 192 mM glycine). After repeatedly washing with Tris buffer (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TTBS), the membranes were incubated with blocking buffer (5% skim milk in TTBS) for 3 h at room temperature. Then

the membranes were incubated with primary rabbit anti-MMP-12 (hinge region; 1 : 1000 dilution; Sigma, St. Louis, MO, U.S.A.) in antibody buffer (2% bovine serum albumin in TTBS) for 12 h at room temperature. The membranes were then washed with TTBS for 10 min 6 times. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1 : 5000 dilution; Amersham, U.K.) for 1.5 h at room temperature, and were washed with TTBS for 10 min 6 times. The blots were detected with an enhanced chemiluminescent method (ECL System; Amersham, U.K.) and quantitated by densitometry system (Atto Densitograph; Atto Co., Tokyo, Japan). To normalize the MMP-12 contents by an internal control protein, GAPDH, immunoblotting was also performed on the same gel by using monoclonal mouse anti-GAPDH (1 : 5000 dilution; Chemicon, Temecula, CA, U.S.A.) and goat anti-mouse IgG (1 : 5000 dilution; Amersham, U.K.).

α -Casein Zymography To measure the activity of MMP-12, α -casein zymography was performed using protein extraction described above. In brief, protein samples were mixed with SDS sample buffer without heating and then loaded onto 10%-polyacrylamide gel containing 1 mg/ml of α -casein (USB Corp., Cleveland, OH, U.S.A.). Electrophoresis was performed at 4 °C and the gels were then incubated for 30 min in fixation buffer (50% methanol, 10% acetic acid). Gels were then washed with 2.5% Triton X-100 for 15 min 4 times to remove SDS and equilibrated in incubation buffer (50 mM Tris–HCl, 5 mM CaCl_2 , 0.02% NaN_3 , pH 8.5) for 30 min at room temperature. Gels were then incubated in incubation buffer at 37 °C for 4 d. After incubation, the gels were stained with 2.5% CBB and destained with destaining buffer (30% methanol, 10% acetic acid). The caseinolytic bands were detected and quantitated by densitometry system (Atto Densitograph; Atto Co., Tokyo, Japan).

Histochemistry and Immunohistochemistry Lungs were fixed in 10% formaldehyde and embedded in Paraplast X-TRATM paraffin (Fisher Healthcare, Houston, TX, U.S.A.). Four-micrometers sections were obtained from blocks and mounted on silane-coated glass slides, deparaffinized with xylene and graded ethanol. For the histochemical examination, the sections were stained with hematoxylin and eosin (HE) by a standard technique. For the immunohistochemical examination, the sections were incubated with rabbit anti-MMP-12 as the primary antibody by using a streptavidin–biotin immunoperoxidase method as described previously²⁵⁾ with minor modification. In brief, before immunostaining, rehydrated sections were pretreated by incubation in 0.5% Triton X-100 in PBS for 10 min for permeabilization and were immersed in 0.3% hydrogen peroxide in 100% methanol for 30 min to remove endogenous peroxidase activity. The pretreated sections were washed with PBS and incubated in 5% skim milk in PBS for 1 h. The sections were then rinsed and incubated sequentially at room temperature in the following solutions: (1) primary antibody (diluted 1 : 500 in PBS containing 1% skim milk) overnight, (2) biotinylated goat anti-rabbit IgG (diluted 1 : 200 in PBS containing 1% skim milk) for 30 min, and (3) avidin-biotinylated peroxidase complexes (Vector Laboratory, Inc., Burlingame, CA, U.S.A.) in PBS for 30 min. The bound peroxidase activity was visualized by incubation with 0.7 mg/ml 3,3'-diaminobenzidine–0.02% H_2O_2 in 60 mM Tris buffer (pH 7.5).

Sections were rinsed in PBS after each step of immunostaining procedure. Finally, the sections were counterstained with Hematoxylin QS (Vector Laboratories, Inc., Burlingame, CA, U.S.A.), dehydrated, and mounted in permanent mounting medium. The MMP-12-positive macrophages in alveoli were counted under $\times 400$ magnification by two different investigators. The average number of five random microscopic fields from a section was defined as $N=1$.

Statistical Analyses All the data were expressed as the mean with S.E.M. Statistical significance of difference was determined by unpaired Student's *t*-test or Bonferroni/Dunn analysis. A value of $p < 0.05$ was considered as significant.

RESULTS

Phenotype of the Rat Model of Allergic Bronchial Asthma

Figure 1 shows the results of histologic examinations of lung tissues obtained from the sensitized control and 12 and 24 h after the last antigen challenge. A marked increase in the inflammatory cells infiltration was observed at 12–24 h after the antigen challenge (Figs. 1c–f) when compared with control animals (Figs. 1a, b). More serious inflammation was found at 24 h after the antigen challenge. Most of the infiltrated cells were eosinophils as determined by Diff-Quik staining of lung sections (not shown). In this animal model of allergic bronchial asthma, an augmented bronchial smooth muscle contraction was also observed (Fig. 2) as reported previously.^{16–22)}

Change in the mRNA Expression of MMP-12 The semi-quantitative analyses for mRNA expression of MMP-12 were determined by RT-PCR. Under the condition used, the PCR amplification revealed a single band corresponding to the expect size of MMP-12 mRNA (644 bp). As shown in

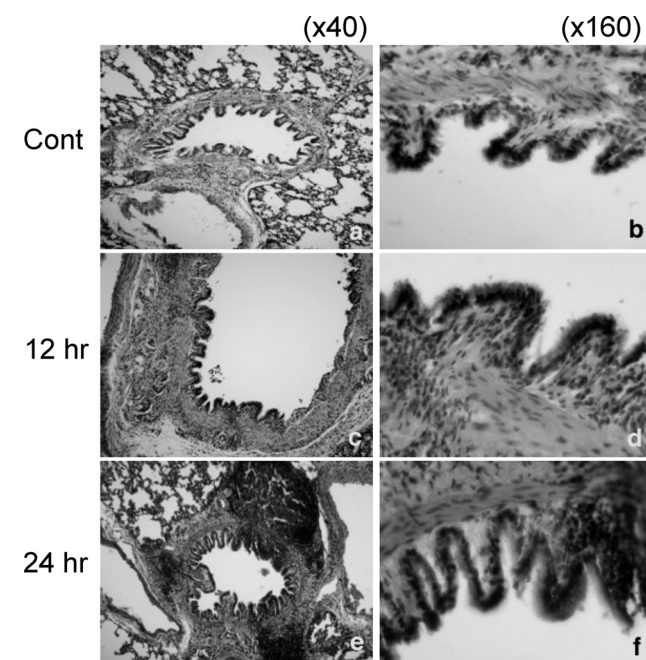


Fig. 1. Histological Change in Lungs after the Antigen Challenge in Rats

Lung sections ($4 \mu\text{m}$ thickness) of formalin-fixed tissues of the sensitized control (a and b) and 12 h (c and d) and 24 h (e and f) after the last antigen challenge were stained with hematoxylin and eosin before examination by light microscopy. Original magnification: a, c and e, $\times 40$; b, d and f, $\times 160$. Many inflammatory cells were infiltrated into the periphery of intrapulmonary bronchi in the antigen challenged groups (c–f).

Fig. 3a, the mRNA expression of MMP-12 was increased at 1–12 h after the antigen challenge when compared with control group. Figure 3b shows the summary of the relative expression of MMP-12 mRNA to GAPDH mRNA. The peak expression of MMP-12 mRNA was observed at 3 h after the last antigen challenge.

Change in the Protein Expression of MMP-12 Immunoblot analyses revealed that the antibody against MMP-12 used in the current study recognizes the proenzyme (54 kDa) and intermediate form (45 kDa) but not the active form of MMP-12 (Fig. 4a) reported in rat abdominal aortic aneurysm.²⁶⁾ When the relative expressions of proenzyme and intermediate form of MMP-12 to GAPDH were calculated respectively, no significant change in the expression was observed among the groups (Figs. 4b, c).

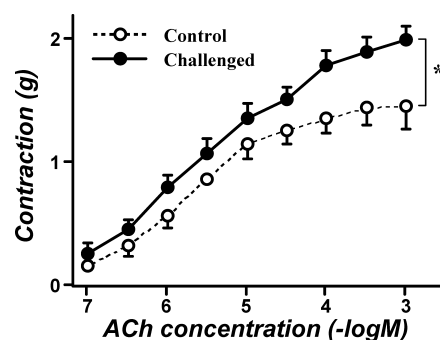


Fig. 2. Effect of Repeated Antigen Exposure on Bronchial Smooth Muscle Responsiveness to Acetylcholine (ACh) in Rats

The ring preparations (ca. 4 mm length, 3 mm diameter) were isolated from the left main bronchi 24 h after the last antigen challenge (Challenged; closed circles) or the sensitized control animals (Control; open circles). Each point represents the mean with S.E.M. from 4 different animals. The bronchial smooth muscle responsiveness to ACh was significantly augmented in the Challenged group ($*p < 0.05$ vs. Control by two-way ANOVA with *post hoc* Bonferroni/Dun).

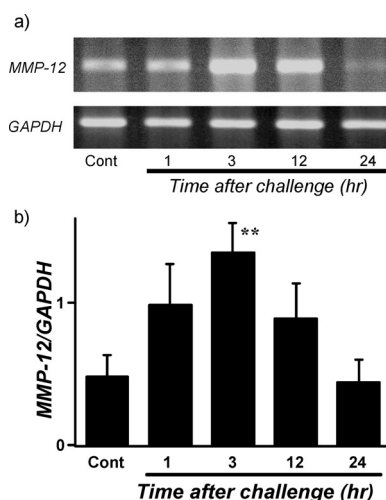


Fig. 3. Time Course of Changes in the mRNA Expression of Matrix Metalloproteinase-12 (MMP-12) after the Last Antigen Challenge in Airways of Rats

The mRNA levels were measured by semi-quantitative RT-PCR using total RNA extracted from airway tissues 1, 3, 12 and 24 h after the last antigen challenge or sensitized control animals (Cont). a) Typical photos of the PCR products of MMP-12 (upper panel) and GAPDH (lower panel). b) Summary of the mRNA levels of MMP-12. The bands of PCR products were analyzed by a densitometer and normalized by the intensity of corresponding GAPDH band. Each column represents the mean with S.E.M. from 4–5 different animals. The mRNA expression of MMP-12 was significantly increased 3 h after the last antigen challenge ($**p < 0.01$ vs. Cont by unpaired Student's *t*-test).

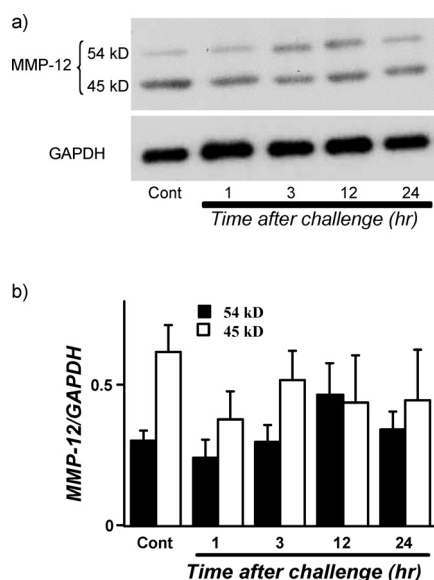


Fig. 4. Time Course of Changes in the Protein Expression of Matrix Metalloproteinase-12 (MMP-12) after the Last Antigen Challenge in Airways of Rats

The protein levels were measured by Western blots using total protein extracted from airway tissues 1, 3, 12 and 24 h after the last antigen challenge or sensitized control animals (Cont). a) Typical photographs of the bands of MMP-12 (upper panel) and GAPDH (lower panel). The bands for 54 kDa proenzyme and 45 kDa intermediate form of MMP-12 were distinctly detected. b) Summary of the expression of the proenzyme (54 kDa; closed columns) and intermediate form (45 kDa; open columns) of MMP-12. Each column represents the mean with S.E.M. from 5 different animals. No significant change in the expression of proenzyme or intermediate form of MMP-12 was observed.

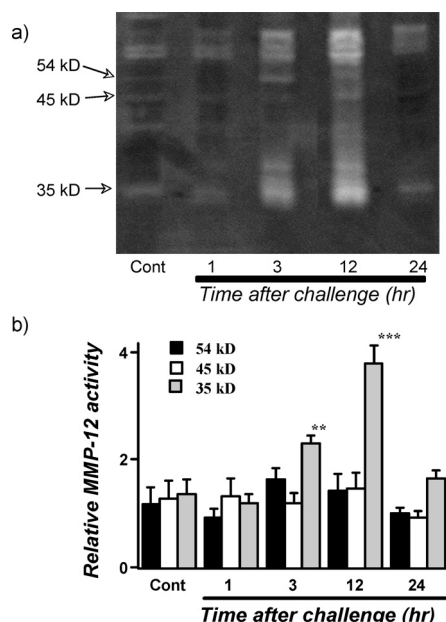


Fig. 5. Time Course of Changes in the Activities of Matrix Metalloproteinase-12 (MMP-12) after the Last Antigen Challenge in Airways of Rats

The MMP-12 activities were measured by α -casein zymography using total protein extracted from airway tissues 1, 3, 12 and 24 h after the last antigen challenge or sensitized control animals (Cont). a) Typical photos of the α -casein zymography. The caseinolytic bands corresponding to 54 kDa proenzyme and 45 kDa intermediate form of MMP-12 were distinctly detected. In addition, a *ca.* 35 kDa caseinolytic band was also found. b) Summary of the densities of caseinolytic 54 kDa (closed columns), 45 kDa (open columns) and *ca.* 35 kDa (somber columns) bands. The caseinolytic activities were expressed as density ratios to the respective bands obtained from non-sensitized normal animals. Each column represents the mean with S.E.M. from 5 different animals. The *ca.* 35 kDa caseinolytic activity was significantly increased 3 and 12 h after the last antigen challenge (** $p < 0.01$ and *** $p < 0.001$ vs. Cont by unpaired Student's *t*-test), whereas no significant change in the 54 or 45 kDa activity was observed.

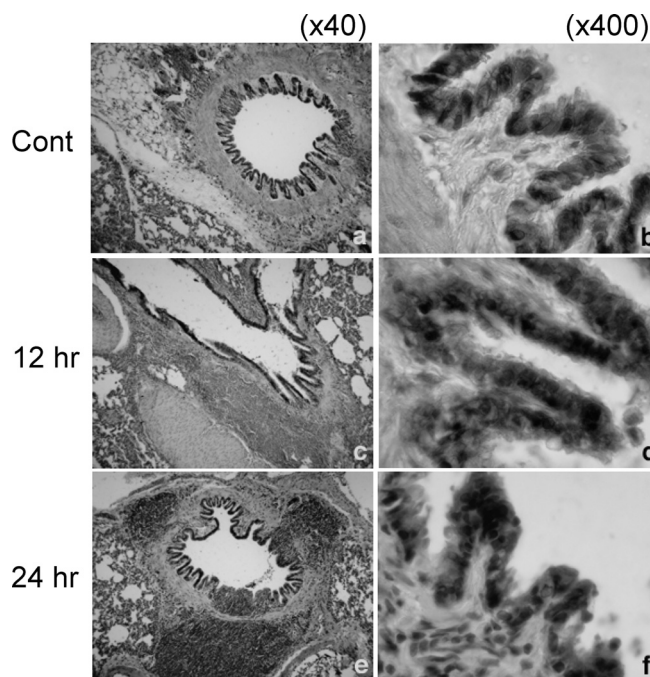


Fig. 6. Immunohistochemistry for Matrix Metalloproteinase-12 (MMP-12) in Lungs of Rats

Lung sections (4 μ m thickness) of formalin-fixed tissues of the sensitized control (a and b) and 12 h (c and d) and 24 h (e and f) after the last antigen challenge were immunostained with anti-MMP-12 antibody before examination by light microscopy. Original magnification: a, c and e, $\times 40$; b, d and f, $\times 400$. A strong immunostaining was observed in bronchial epithelia of each animal. A weak but distinct immunostaining was also observed in bronchial smooth muscle layer (c).

Change in MMP-12 Activity Determined by α -Casein Zymography

Figure 5a shows a typical photograph of α -casein zymography. In addition to the bands corresponding to the proenzyme and intermediate form of MMP-12 (54, 45 kDa, respectively), distinct caseinolytic bands were also detected at *ca.* 35 kDa. The densities of these three bands of the sensitized control and antigen-challenged rats were normalized by the corresponding bands of nonsensitized normal animals. Interestingly, the densities of caseinolytic bands of *ca.* 35 kDa were significantly increased at 3–12 h after the antigen challenge, whereas the bands corresponding to the proenzyme and intermediate form of MMP-12 were not changed (Fig. 5).

MMP-12 Immunohistochemistry of Lung Tissues Immunohistochemical examinations revealed a strong MMP-12 staining in bronchial epithelia of the sensitized control (Figs. 6a, b) and non-sensitized normal control rats (not shown). Although the MMP-12-positive staining was not found in subepithelial layers (Figs. 6a, b), a weak but distinct immunostaining was observed in bronchial smooth muscle layers (Fig. 6b) and lung alveolar cells of these animals (Fig. 7). Most of the alveolar macrophages of the control animals were also positive to the MMP-12 staining (Fig. 7a). Similar results were also found in the repeatedly antigen challenged animals (Figs. 7b, c). However, the number of the MMP-12-positive alveolar macrophages was significantly increased 12 and 24 h after the antigen challenge (Fig. 7d).

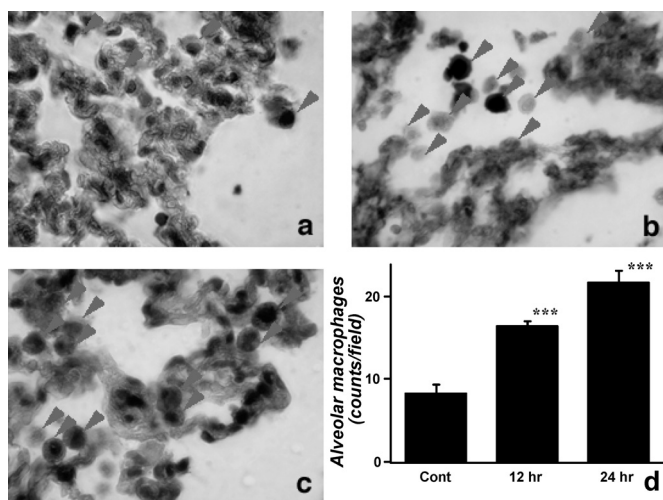


Fig. 7. Immunohistochemistry for Matrix Metalloproteinase-12 (MMP-12) in Lungs of Rats

Lung sections (4 μ m thickness) of formalin-fixed tissues of the sensitized control (a) and 12 h (b) and 24 h (c) after the last antigen challenge were immunostained with anti-MMP-12 antibody before examination by light microscopy. Original magnification: $\times 400$. A strong immunostaining was observed in alveolar macrophages of each animal (arrow heads). A weak but distinct immunostaining was also observed in lung alveolar cells. (d) Changes in the number of alveolar macrophages counted by the method described in the Materials and Methods section. Each column represents the mean with S.E.M. from 4 different animals. A significant increase in the MMP-12-positive alveolar macrophages was observed 12 and 24 h after the last antigen challenge.

DISCUSSION

In the present study, the changes in the expression of MMP-12 in the airways were investigated in a rat model of allergic bronchial asthma that has marked airway inflammation and bronchial smooth muscle hyperresponsiveness (Figs. 1, 2). Both the mRNA expression of MMP-12 and the *ca.* 35 kDa caseinolytic band were significantly increased in this animal model of asthma: the mRNA expression was increased at relatively early phase after the last antigen challenge (1–3 h; Fig. 3), while the peak expression of the caseinolytic band was observed at 12 h after the challenge (Fig. 5). Immunohistochemical examinations revealed that MMP-12 is located in airway epithelia, bronchial smooth muscles, lung alveoli and alveolar macrophages (Fig. 6): the infiltrated MMP-12-positive macrophages were increased after the repeated antigen challenge. These findings suggest that MMP-12 expression and activity might be increased in the airways of allergic bronchial asthma, probably due to the increased infiltration of the MMP-12-positive macrophages in the lungs.

Currently, the 54 and 45 kDa bands corresponding to the proenzyme and intermediate forms of MMP-12 reported previously in rat abdominal aortic aneurysms²⁶ were detected by immunoblottings (Fig. 4). However, we could not detect the 22 kDa band reported in the same paper.²⁶ This might be due to the difference in the antibody used. We used a commercially available antibody that recognizes the hinge region of MMP-12. The MMP-12 proenzyme consists of three domains, *i.e.*, pro-domain and catalytic and hemopexin domains.⁴ The 22 kDa active form of MMP-12 is generated by the loss of the N-terminal pro-domain and the C-terminal hemopexin domain.⁴ The hemopexin domain is connected to the catalytic domain by the hinge region, an autolytic site of

MMP-12.⁴ It is thus probable that the antibody used in the present study could not recognize the MMP-12 without the hemopexin domain. On the other hand, a distinct *ca.* 35 kDa caseinolytic band, a form of MMP-12 in rat alveolar macrophage,²⁷ was detected in rat airways (Fig. 5). On the basis of apparent molecular weights, the *ca.* 35 kDa MMP-12 might consist of the pro-domain and the catalytic domain (MMP-12 without the hemopexin domain). The *ca.* 35 kDa band was not recognized by the antibody used in the present study (data not shown), probably due to the reason as above. On the other hand, the casein zymography also revealed caseinolytic bands other than 54, 45 and *ca.* 35 kDa (Fig. 5a). The presence of bands with apparent molecular weights higher than 54 kDa and between 35 and 45 kDa in the casein zymography could be explained by the presence of other MMPs, such as MMP-2, MMP-9, and so on.^{28–30} It seems likely that the MMPs activities other than MMP-12 are also increased in the airways of this animal model of allergic asthma. Indeed, an increased MMP-9 activity has been reported in patients with allergic asthma.^{31–33}

Allergic bronchial asthma is characterized by upregulation of IgE and Th2 cytokine levels, infiltration of inflammatory cells such as macrophages, neutrophils and eosinophils, and airway hyperresponsiveness (AHR). It has also been reported that the gene expression of MMP-12 was upregulated in patients with asthma.⁶ Although the pathogenic role of MMP-12 in asthma is not clear now, gene knockout of MMP-12 can inhibit infiltration of inflammatory cells in a murine model of allergic asthma.³⁴ Moreover, instillation of catalytic domain of MMP-12 into mouse airways induced a recruitment of neutrophils at early phase and an increase in macrophages at later phase after the administration.³⁵ MMP-12 also has an ability to induce the release of proinflammatory cytokines/chemokines.³⁵ It is thus possible that MMP-12 works as a stimulator of airway inflammation.

In the present study, we used our well-established rat model of allergic bronchial asthma.^{16–24} In this animal model of asthma, the mRNA expression of MMP-12 was significantly increased following the process of antigen challenge (Fig. 3). Similar result has also been reported in a murine model of allergic airway inflammation.¹⁴ On the other hand, no significant change in the protein expression of proenzyme (54 kDa) or intermediate form (45 kDa) of MMP-12 detected by immunoblotting was observed between the challenged and control groups (Fig. 4). The discrepancy between the mRNA (Fig. 3) and immunodetectable protein (Fig. 4) expressions might be due to its rapid change into alternative forms of MMP-12. Indeed, the *ca.* 35 kDa caseinolytic band was significantly increased after the antigen challenge (Fig. 5).

Immunohistochemical examinations revealed a strong MMP-12 staining in bronchial epithelia and alveolar macrophages and a weak but distinct staining in bronchial smooth muscles and lung alveoli in rats (Figs. 6, 7). From the results of immunoblottings (Fig. 4), the positive immunostainings indicate the existence of 54/45 kDa of MMP-12. Interestingly, the MMP-12-positive macrophage was significantly increased after the antigen challenge (Fig. 7). Although the exact role of MMP-12 generated by these airway cells in antigen-induced airway inflammation and AHR is not clear now, an inhibitory effect of a broad MMPs inhibitor,

GM6001, on airway inflammation has been reported in a murine model of allergic asthma.³⁶⁾ Moreover, administration of the endogenous MMPs inhibitors, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, also inhibits inflammatory cells infiltration and AHR induced by ovalbumin in mice.³⁷⁾ On the other hand, intratracheal instillation of recombinant human MMP-12 can induce airway inflammation in naive mice.³⁵⁾ Thus, MMP-12 might be one of the important mediators in the pathogenesis of allergic airway inflammation.

Currently, the *ca.* 35 kDa MMP-12 activity in the lung homogenates returned to near the control level at 24 h after the last antigen challenge (Fig. 5b), whereas the MMP-12-positive macrophages were still increased (Fig. 7d). Although the detail of the discrepancy is not clear now, the MMP-12 activity in lung homogenate may mainly derive from the cells other than macrophages. The relatively small amount change in the macrophage-derived MMP-12 activity may be masked by the large amount of the activities derived from airway epithelia and so on. Indeed, the increased number of MMP-12-positive macrophages was not reflected in the results of immunoblot analyses of lung homogenates (Fig. 4) though the same anti-MMP-12 antibody was used for the immunohistochemical and immunoblot studies. Alternatively, 24 h after the last antigen challenge, the expression level of MMP-12 per one macrophage cell may be decreased though the number of MMP-12-positive macrophage was increased: the immunohistochemical examination used in the present study can not distinguish the changes in the expression levels.

In conclusion, the current study demonstrates an upregulation of MMP-12 in airways of rat model of allergic bronchial asthma. An increase in the MMP-12-positive macrophages in alveoli might be, at least in part, involved in the increased MMP-12 activity. Because a similar role of macrophage MMPs in rat acute lung inflammation and human lung disease has been suggested,²⁷⁾ MMP-12 might be a new target for the therapy against allergic bronchial asthma.

Acknowledgements We thank Satoshi Onoda and Miyuki Watanuki for their technical assistance.

REFERENCES

- McCawley L. J., Matrisian L. M., *Curr. Opin. Cell Biol.*, **13**, 534—540 (2001).
- Werb Z., Gordon S., *J. Exp. Med.*, **142**, 361—377 (1975).
- Shapiro S. D., Griffin G. L., Gilbert D. J., Jenkins N. A., Copeland N. G., Welgus H. G., Senior R. M., Ley T. J., *J. Biol. Chem.*, **267**, 4664—4671 (1992).
- Shapiro S. D., Kobayashi D. K., Ley T. J., *J. Biol. Chem.*, **268**, 23824—23829 (1993).
- Lavigne M. C., Thakker P., Gunn J., Wong A., Miyashiro J. S., Wasserman A. M., Wei S. Q., Pelker J. W., Kobayashi M., Eppihimer M. J., *Biochem. Biophys. Res. Commun.*, **324**, 534—546 (2004).
- Xie S., Issa R., Sukkar M. B., Oltmanns U., Bhavsar P. K., Papi A., Caramori G., Adcock I., Chung K. F., *Respir. Res.*, **6**, 148 (2005).
- Fu J. Y., Lyga A., Shi H., Blue M. L., Dixon B., Chen D., *Protein Expr. Purif.*, **21**, 268—274 (2001).
- Gronski T. J., Jr., Martin R. L., Kobayashi D. K., Walsh B. C., Holman M. C., Huber M., Van Wart H. E., Shapiro S. D., *J. Biol. Chem.*, **272**, 12189—12194 (1997).
- Nenan S., Boichot E., Lagente V., Bertrand C. P., *Mem. Inst. Oswaldo Cruz.*, **100** (Suppl. 1), 167—172 (2005).
- Hautamaki R. D., Kobayashi D. K., Senior R. M., Shapiro S. D., *Science*, **277**, 2002—2004 (1997).
- Belvisi M. G., Bottomley K. M., *Inflamm. Res.*, **52**, 95—100 (2003).
- Molet S., Belleguic C., Lena H., Germain N., Bertrand C. P., Shapiro S. D., Planquois J. M., Delaval P., Lagente V., *Inflamm. Res.*, **54**, 31—36 (2005).
- Lanone S., Zheng T., Zhu Z., Liu W., Lee C. G., Ma B., Chen Q., Homer R. J., Wang J., Rabach L. A., Rabach M. E., Shipley J. M., Shapiro S. D., Senior R. M., Elias J. A., *J. Clin. Invest.*, **110**, 463—474 (2002).
- Pouladi M. A., Robbins C. S., Swirski F. K., Cundall M., McKenzie A. N., Jordana M., Shapiro S. D., Stampfli M. R., *Am. J. Respir. Cell Mol. Biol.*, **30**, 84—90 (2004).
- Zimmermann N., King N. E., Laporte J., Yang M., Mishra A., Pope S. M., Muntel E. E., Witte D. P., Pegg A. A., Foster P. S., Hamid Q., Rothenberg M. E., *J. Clin. Invest.*, **111**, 1863—1874 (2003).
- Chiba Y., Misawa M., *Eur. J. Pharmacol.*, **278**, 79—82 (1995).
- Chiba Y., Misawa M., *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.*, **111**, 351—357 (1995).
- Chiba Y., Sakai H., Misawa M., *Biochem. Pharmacol.*, **61**, 921—924 (2001).
- Chiba Y., Sakai H., Misawa M., *Br. J. Pharmacol.*, **133**, 886—890 (2001).
- Chiba Y., Sakai H., Yu Y., Misawa M., *J. Biochem. (Tokyo)*, **137**, 751—756 (2005).
- Sakai H., Chiba Y., Hirano T., Misawa M., *Mol. Pharmacol.*, **68**, 145—151 (2005).
- Misawa M., Chiba Y., *Jpn. J. Pharmacol.*, **61**, 41—50 (1993).
- Chiba Y., Misawa M., *Life Sci.*, **55**, 1919—1928 (1994).
- Chiba Y., Takada Y., Miyamoto S., Mitsui-Saito M., Karaki H., Misawa M., *Br. J. Pharmacol.*, **127**, 597—600 (1999).
- Chiba Y., Kurotani R., Kusakabe T., Miura T., Link B. W., Misawa M., Kimura S., *Am. J. Respir. Crit. Care Med.*, **173**, 958—964 (2006).
- Curci J. A., Liao S., Huffman M. D., Shapiro S. D., Thompson R. W., *J. Clin. Invest.*, **102**, 1900—1910 (1998).
- Gibbs D. F., Warner R. L., Weiss S. J., Johnson K. J., Varani J., *Am. J. Respir. Cell Mol. Biol.*, **20**, 1136—1144 (1999).
- Elshaw S. R., Henderson N., Knox A. J., Watson S. A., Buttle D. J., Johnson S. R., *Br. J. Pharmacol.*, **142**, 1318—1324 (2004).
- Cataldo D. D., Gueders M., Munaut C., Rocks N., Bartsch P., Foidart J. M., Noel A., Louis R., *Lab. Invest.*, **84**, 418—424 (2004).
- Quesada A. R., Barbacid M. M., Mira E., Fernandez-Resa P., Marquez G., Aracil M., *Clin. Exp. Metastasis*, **15**, 26—32 (1997).
- Cataldo D. D., Bettiol J., Noel A., Bartsch P., Foidart J. M., Louis R., *Chest*, **122**, 1553—1559 (2002).
- Pham D. N., Chu H. W., Martin R. J., Kraft M., *Ann. Allergy Asthma Immunol.*, **90**, 72—78 (2003).
- Oshita Y., Koga T., Kamimura T., Matsuo K., Rikimaru T., Aizawa H., *Thorax*, **58**, 757—760 (2003).
- Warner R. L., Lukacs N. W., Shapiro S. D., Bhagarvathula N., Nerusu K. C., Varani J., Johnson K. J., *Am. J. Pathol.*, **165**, 1921—1930 (2004).
- Nenan S., Planquois J. M., Berna P., De Mendez I., Hitier S., Shapiro S. D., Boichot E., Lagente V., Bertrand C. P., *Int. Immunopharmacol.*, **5**, 511—524 (2005).
- Corry D. B., Rishi K., Kanellis J., Kiss A., Song L. Z., Xu J., Feng L., Werb Z., Kheradmand F., *Nat. Immunol.*, **3**, 347—353 (2002).
- Kumagai K., Ohno I., Okada S., Ohkawara Y., Suzuki K., Shinya T., Nagase H., Iwata K., Shirato K., *J. Immunol.*, **162**, 4212—4219 (1999).