

The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma

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Summary

Background Bronchial asthma is an inflammatory disease of the airway characterized by airway remodelling, and is due at least in part to an excess of extracellular matrix (ECM) deposition in the airway wall, which leads to subepithelial collagen deposition. Matrix metalloproteinase-9 (MMP-9) is the major proteolytic enzyme that induces bronchial remodelling in asthma. MMP-9 is also important in the migration of inflammatory cells through basement membrane components.

Objectives We evaluated whether airway inflammatory cells correlated with levels of MMP-9 in acute asthma and we examined the time course of sputum levels of MMP-9 activity in patients with spontaneous asthma exacerbation.

Methods We performed zymographic analysis and checked levels of MMP-9 by means of enzyme immunoassay. MMP-9 levels were also evaluated during a spontaneous attack of asthma.

Results Pro-MMP-9 activities and concentrations of MMP-9 in asthmatic patients significantly exceeded those of control subjects ($P < 0.01$). The activities of pro-MMP-9 were significantly higher in acute asthmatic patients than in stable asthmatic patients ($P < 0.01$). The elevated MMP-9 activities significantly decreased after 7 and 28 days of therapy. In acute asthmatic patients, the levels of sputum MMP-9 significantly correlated with the total macrophage + neutrophil + eosinophil cell numbers.

Conclusion These data suggest that airway inflammation after asthma exacerbation correlates with the overproduction of MMP-9, which then leads to airway remodelling.

Keywords: airway inflammation, asthma, matrix metalloproteinase-9, remodelling, extracellular matrix

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Introduction

Bronchial asthma is characterized by hyper-responsiveness and inflammation of airways. This inflammation is associated with the accumulation of activated inflammatory cells, including eosinophils, lymphocytes and mast cells/basophils, in airway lumens and walls. Airway remodelling is a well-recognized feature in patients with asthma that arises as a consequence of inflammatory processes [1,2].

Airway remodelling is due at least in part to an excess of extracellular matrix deposition in the airway wall that leads to subepithelial collagen deposition. The extracellular matrix is a dynamic structure, and an equilibrium between the synthesis and the degradation of extracellular matrix components is required for the maintenance of its homeostasis.

The matrix metalloproteinases (MMPs) are a large family of zinc- and calcium-dependent endopeptidases with distinct substrate specificities that can degrade most components of the extracellular matrix. The MMPs play an important role in physiological and pathological processes,

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including extracellular matrix turnover, tissue degradation and repair, cell migration and inflammation. Of the MMP family, MMP-9 is the major proteinase that induces bronchial remodelling [3]. In addition, MMP-9 induces the migration of eosinophils and neutrophils across basement membranes [4,5]. Recently, MMPs have been reported to play a crucial role in the infiltration of ovalbumin-induced airway inflammatory cells and the induction of airway hyper-responsiveness in a murine model of asthma [6]. Mautino *et al.* reported that MMP-9 content in bronchoalveolar lavage (BAL) fluids from untreated asthmatics was increased compared with controls [7].

In the present study, we evaluated whether airway inflammatory cells correlated with the levels of MMP-9 in acute asthma and we examined the time course of sputum levels of MMP-9 activity in patients with spontaneous asthma exacerbation.

Materials and methods

Subjects

Ten asymptomatic patients with stable asthma, five women and five men, 36–71 years of age (mean 47.4 years), and 10 acute asthmatic patients, five women and five men, 30–70 years of age (mean 49.9 years), were recruited from the Chonbuk National University Hospital. Asthma was diagnosed according to the criteria of the American Thoracic Society [8]. Of these 20 patients, eight were non-atopic, as determined by their clinical history and by a skin prick test performed with common aeroallergens generally used on the skin. Ten normal healthy subjects, five women and five men, 28–72 years of age (mean 47.4 years), were also selected for the study. None of the subjects was a current or previous smoker. FEV₁ was recorded using a computerized pulmonary function sensor (Model 2200; Sensor Medics Co., Yorba Linda, CA, USA).

Ten patients were asymptomatic and had stable asthma, as defined by no fall in FEV₁ < 80% for at least 6 months and absence of symptoms such as episodic wheezing, chest tightness and dyspnoea over a period of 4 weeks. None of the subjects had received any inhaled or oral corticosteroids or any other anti-inflammatory drugs in the previous 6 months. The other 10 subjects were acute asthmatic patients who were admitted to the hospital for more than 7 days because of severe or moderate asthma exacerbation and were treated under American Thoracic Society recommendations [9]. That treatment consisted of an infusion of methylprednisolone (1–2 mg/kg/day), salbutamol inhalation (5–20 µg/kg/day) and continuous theophylline drip infusion (400–600 mg/day).

This study was approved by the Ethics Committee of

Chonbuk National University, Medical School and fully informed, written consent was obtained from each subject.

Sputum collection and processing

Spontaneous sputum samples were obtained during acute asthma. If spontaneous sputum could not be obtained, induced sputum was obtained by nebulized hypertonic saline solution. Sputum samples in patients with acute asthma were collected before (day 1) and during (days 7 and 28) treatment. Sputum induction and processing were performed according to the method of Fahy *et al.* [10] with slight modifications [11]. Patients were exposed to an aerosol of 3% hypertonic saline solution for 20 min early in the morning in a fasting condition. The aerosol was administered by an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan) with an output of 1 mL/min and a particle size of 4.5 µm aerodynamic mass median diameter.

The volume of the induced sputum sample was determined and an equal volume of dithiothreitol (Sigma Chemical Co., St Louis, MO, USA), diluted with saline solution to obtain 0.1% concentration, was added. The samples were then mixed gently with a vortex mixer. The homogenized sputum samples were centrifuged at 800 g for 10 min and the cell pellets were resuspended in saline solution. The total cell count and cell viability of each sample were assessed using a standard haemocytometer and by trypan blue exclusion, respectively. The cells were then cytocentrifuged (Cytospin 2; Shandon Instruments, Runcorn, UK) and stained by Diff-Quik staining (International Reagents, Kobe, Japan) to perform the differential cell counts. Two independent investigators counted the cells in a double-blind fashion using a microscope. Approximately 400 cells were counted in each of four different random locations. The number of squamous cells was subtracted from the total cell counts and the differential cell counts were expressed as corrected percentages. The supernatants were aspirated and frozen at –70 °C for MMP-9 analysis, as detailed below.

Measurements of MMP-9 in sputum

Levels of MMP-9 were quantified by enzyme immunoassays according to the manufacturer's protocol (Fuji Chemical Industries, Toyama, Japan). The hydrolytic activities of MMP-9 were measured by gelatin-zymography, performed as previously described [12]. Samples containing similar amounts of protein were mixed with 5X sample buffer (0.4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue) and were separated on a 10% SDS-PAGE gel containing 0.15% fibrin or 0.1% gelatin. Reference standards were MMP-2 and MMP-9 (Chemicon International Inc., Temecula, CA, USA). The

gels were subsequently incubated in 2.5% Triton X-100 for 1 h and were then incubated in the enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM CaCl₂ and 0.02% Brij-35) for 24 h at 37 °C. The gels were stained with 0.5% Coomassie brilliant blue-250 and de-stained with several changes of 30% methanol and 10% acetic acid. Gelatinolytic activity was detected as clear bands against a blue background. The intensities of the bands in the inverted image zymogram were estimated using densitometry (LAS-1000, Fuji Film, Tokyo, Japan).

Statistical analysis

All values are reported as means $\pm$ SD unless otherwise stated. The Kruskal–Wallis analysis of variance was used to examine the significance of intergroup differences and, if significant, the Mann–Whitney *U*-test was used for between-group comparisons. Spearman's rank correlation was used to assess the correlation between data. Comparisons of the time course of sputum MMP-9 were made through an ANOVA and Scheffes's test. Statistical significance was assumed for *P*-values lower than 0.05.

Results

Characteristics of subjects and cell counts in sputum

The mean ages of patients with stable asthma and acute asthma and of control subjects were similar. FEV₁ values of acute asthmatic patients (mean $\pm$ SD, 31.1 $\pm$ 7.3% of predicted values) were significantly lower than subjects with stable asthma and controls (means $\pm$ SD, 91.3 $\pm$ 7.1 and 98.8 $\pm$ 6.6% of predicted values, respectively). The percentages of squamous cells were not significantly

different in sputum samples obtained from control subjects, stable asthmatic patients or acute asthmatic patients (Table 1). The total cell counts were significantly greater in acute asthmatic patients (day 1) than in control subjects and in stable asthmatic patients (*P* < 0.01 and *P* < 0.01, respectively). The percentages of eosinophils and lymphocytes were significantly higher in stable asthmatic patients than in control subjects (*P* < 0.01). The percentages of neutrophils and eosinophils were greater in acute asthmatic patients (day 1) than in stable asthmatic patients (*P* < 0.01).

Zymographic analysis

Profiles of MMP-9 in the sputum samples were assayed semiquantitatively by gelatin zymography. Zymographic analysis showed the presence of the 92-kDa pro-MMP-9 band (Fig. 1a). The levels of pro-MMP-9 were significantly increased in stable asthmatic patients and were even higher in acute asthmatic patients compared with controls (*P* < 0.01). The levels of pro-MMP-9 were significantly higher in acute asthmatic patients than in stable asthmatic patients (*P* < 0.01) (Fig. 1b). The time course of pro-MMP-9 levels in acute asthmatic patients before and during treatment is shown in Fig. 2. The elevated pro-MMP-9 levels on day 1 had significantly decreased by day 7 and by day 28 (*P* < 0.05 and *P* < 0.01, respectively) (Fig. 2b).

MMP-9 levels in sputum

Consistent with the results obtained from the zymographic analysis, the levels of MMP-9 assessed by enzyme assay were significantly increased in stable asthmatic patients and

Table 1. Cell analysis of sputum samples

	Control subjects	Stable asthmatics	Acute asthma		
			Day 1	Day 7	Day 28
Squamous cell (%)	24.8 $\pm$ 5.9	21.1 $\pm$ 5.7	21.8 $\pm$ 7.0	19.8 $\pm$ 5.3	20.9 $\pm$ 6.7
Total cell count (10 ⁶ /mL)	1.6 $\pm$ 0.3	1.7 $\pm$ 0.3	2.6 $\pm$ 0.8*†	2.1 $\pm$ 0.4	1.8 $\pm$ 0.3
Corrected cell count (10 ⁶ /mL)	1.2 $\pm$ 0.3	1.4 $\pm$ 0.2	2.1 $\pm$ 0.7*†	1.8 $\pm$ 0.3	1.4 $\pm$ 0.3
Differential cell count (%)					
Macrophages	85.3 $\pm$ 3.7	80.8 $\pm$ 3.5	54.4 $\pm$ 12.1*†	71.4 $\pm$ 5.8	81.9 $\pm$ 2.6
Neutrophils	12.5 $\pm$ 3.7	11.8 $\pm$ 3.2	31.8 $\pm$ 9.2*†	20.2 $\pm$ 4.3	12.5 $\pm$ 2.7
Lymphocytes	0.4 $\pm$ 0.6	1.6 $\pm$ 1.5‡	2.3 $\pm$ 0.9*	1.1 $\pm$ 0.8	1.0 $\pm$ 0.8
Eosinophils	0	4.4 $\pm$ 1.1‡	10.6 $\pm$ 4.2*†	6.7 $\pm$ 1.6	3.9 $\pm$ 1.0
Epithelial cells	1.8 $\pm$ 2.0	1.4 $\pm$ 0.8	0.9 $\pm$ 0.8	0.7 $\pm$ 0.8	0.7 $\pm$ 0.8

**P* $\leq$ 0.01, control vs. acute asthma (day 1); †*P* $\leq$ 0.01, stable asthma vs. acute asthma (day 1); ‡*P* $\leq$ 0.01, control vs. stable asthma.

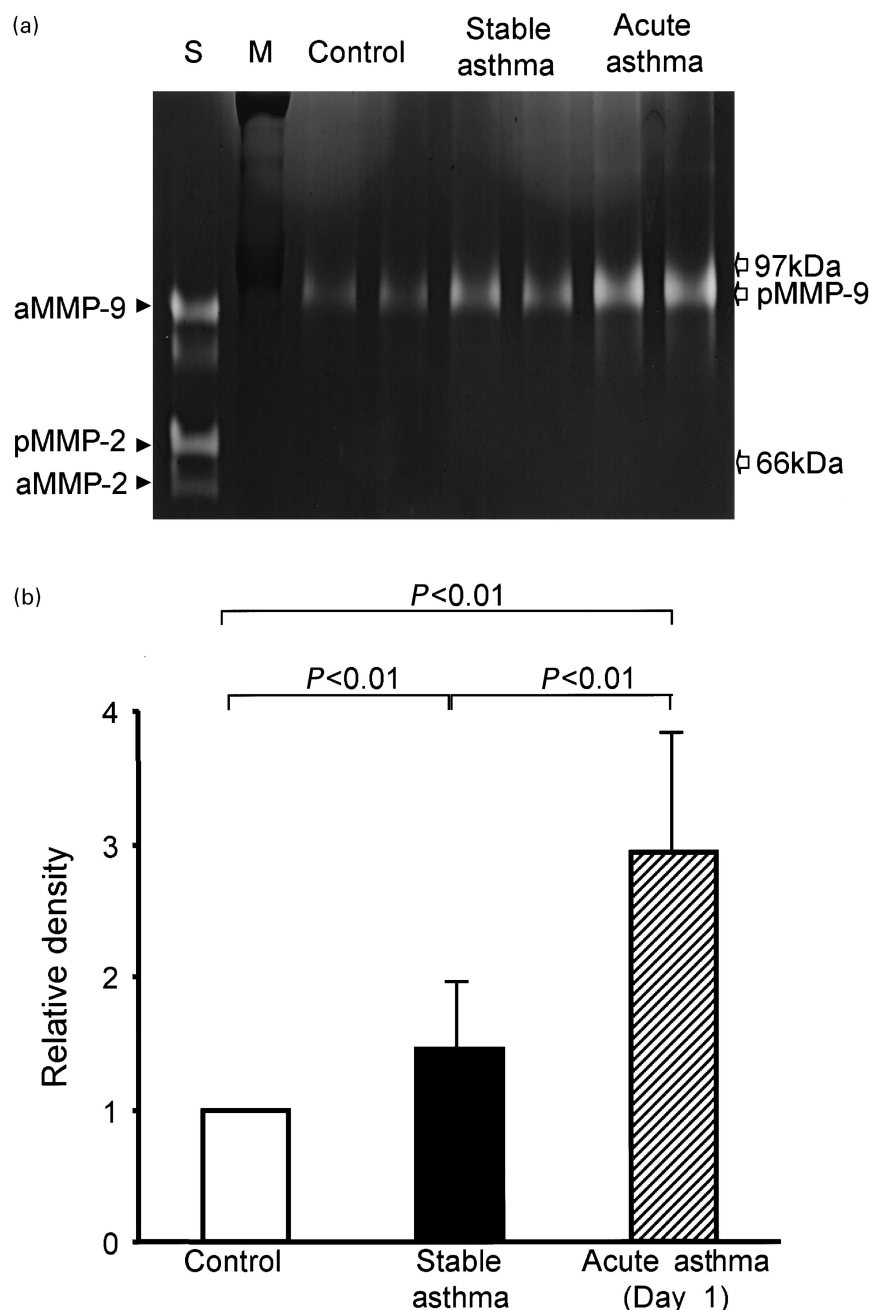


Fig. 1. Analysis of sputum by gelatin zymography. (a) A representative zymographic analysis of sputum supernatant samples obtained from two controls (lanes 3, 4), two stable asthma patients (lanes 5, 6), and two acute asthma patients (lanes 7, 8). Gelatin zymography revealed only a major band at 92 kDa in all groups. Lane S contains standards: the active form of MMP-9 (aMMP-9), the pro-form of MMP-2 (pMMP-2), and the active form of MMP-2 (aMMP-2). Molecular weight markers (lane 2) were used to estimate molecular masses. (b) Densitometric analyses of the zymographs are presented as the relative ratio of the pro-form of MMP-9 (pMMP-9) activities. The pMMP-9 in the sputum of control subjects is arbitrarily presented as 1; all data are expressed as means $\pm$ SD ($n = 10$).

in acute asthmatic patients compared with controls ($P < 0.01$ and $P < 0.01$, respectively). The levels of MMP-9 were significantly higher in acute asthmatic patients compared with stable asthmatic patients ($P < 0.01$) (Fig. 3). The time course of MMP-9 levels in acute asthmatic patients is shown in Fig. 4. The elevated MMP-9 levels on day 1 had significantly decreased by day 7 and even further by day 28 ($P < 0.05$ and $P < 0.01$, respectively). In acute asthmatic patients, the levels of sputum MMP-9 were significantly correlated with the total

macrophage + neutrophil + eosinophil cell numbers ($P < 0.01$) (Fig. 5).

Discussion

In this study, we found that the levels of MMP-9 in sputum were significantly higher in acute asthmatic patients than in stable patients and in control subjects. Further, sputum MMP-9 levels during asthma exacerbation were significantly higher compared with remission days, and they

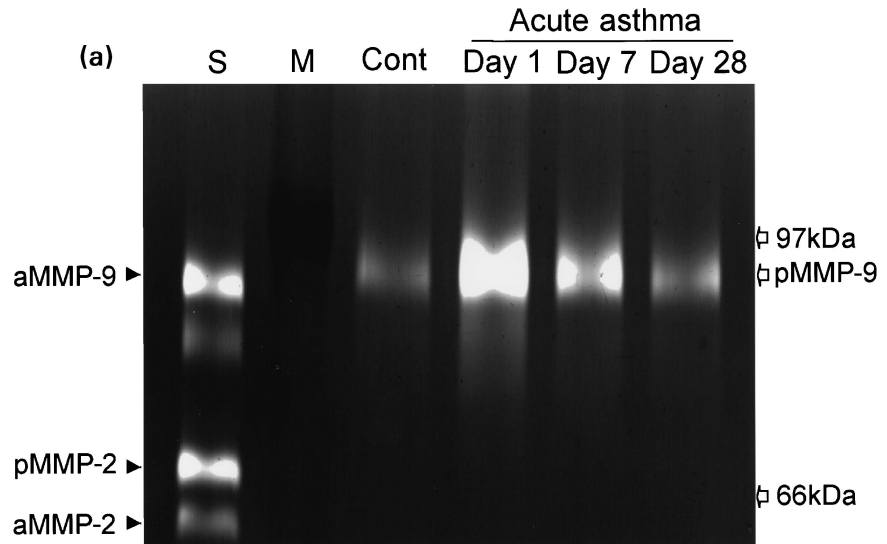
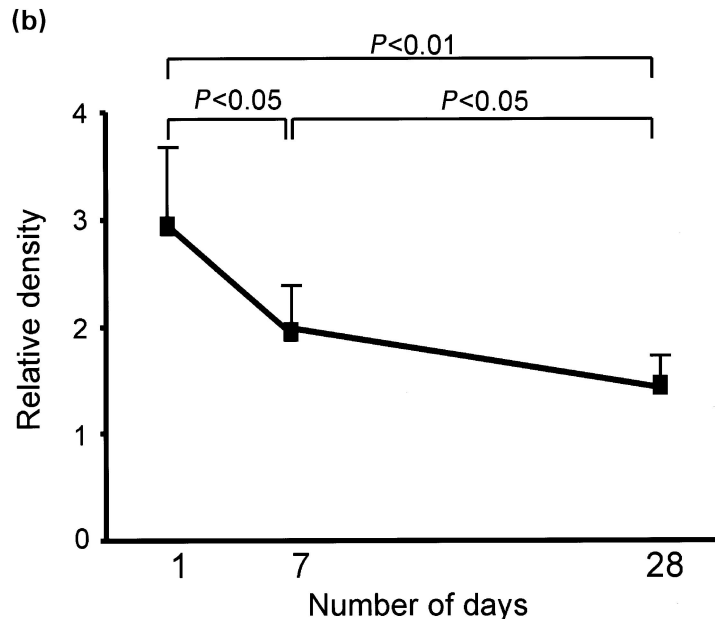


Fig. 2. Analysis of sputum by gelatin zymography in patients undergoing treatment for acute asthma. (a) A representative zymographic analysis of sputum supernatant samples obtained from one control (lane 3). Lane 4, sample obtained at day 1 of exacerbation prior to therapy; lane 5, from day 7 of therapy; lane 6, from day 28 of therapy. Gelatin zymography revealed only a major band at 92 kDa in all groups. Lane S contains standards as detailed for Fig. 1. Molecular weight markers (lane 2) were used to estimate molecular masses. (b) Densitometric analyses of the zymographs are presented as the relative ratio of the pro-form of MMP-9 (pMMP-9) activities. The pMMP-9 in sputum of control subjects is arbitrarily presented as 1; all data are expressed as means $\pm$ SD ($n = 10$).



decreased after asthma therapy. In acute asthmatic patients, the levels of sputum MMP-9 were significantly correlated with the total macrophage + neutrophil + eosinophil cell numbers.

The extracellular matrix is a dynamic structure and an equilibrium between the synthesis and the degradation of extracellular matrix components is required for the maintenance of its homeostasis. A balance between the expression of proteases and anti-proteases, particularly the MMPs and their inhibitors, maintains this homeostasis under normal conditions. The expression of these same proteases and anti-proteases is critical in tissue repair and remodelling in some pulmonary inflammatory diseases

[13–16]. Extracellular matrix homeostasis plays an important role in the breakdown and deposition of matrix components in the airway walls of the lungs. In asthma, the mechanisms of remodelling of the bronchial tree due to extracellular matrix deposition are well characterized. Of the MMP family, MMP-9 is the major proteinase that induces bronchial remodelling in asthma [12,17]. The up-regulation of MMP-9 release observed in this study is consistent with previous reports that MMP-9 content in BAL fluids from untreated asthmatics was increased compared with controls and that sputum levels of MMP-9 during asthma exacerbation were significantly higher than on remission days [7,18]. In our study, the elevated MMP-9

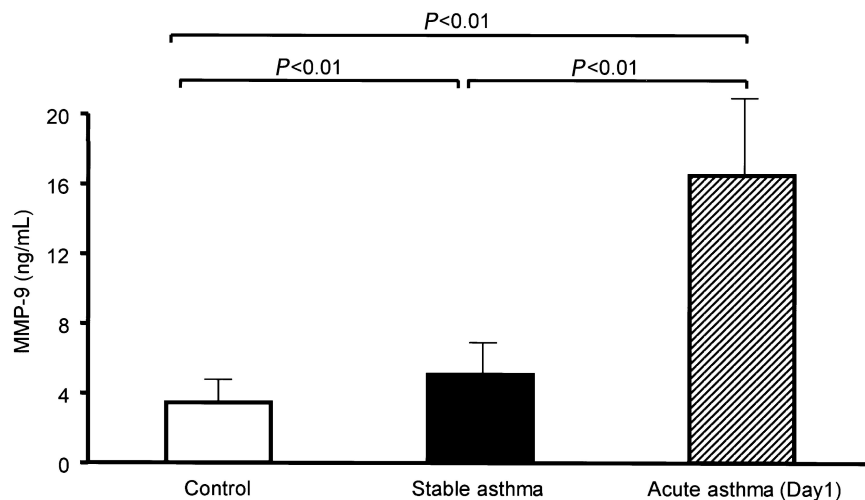


Fig. 3. Concentrations of MMP-9 in sputum obtained from control, stable asthmatic and acute asthmatic subjects. Enzyme activities were measured as detailed in the methods section and are reported as means $\pm$ SD.

levels on day 1 of exacerbation prior to treatment decreased on day 7 and day 28 during treatment. The results of our study suggest that extracellular matrix turnover by MMPs occurs in the early phase of asthma exacerbation, and suggest that airway wall remodelling must be accompanied by degradation of the extracellular matrix in addition to the synthesis and deposition of new matrix in asthma.

The major sources of MMP-9 in the airways are macrophages and eosinophils [19,20], and MMP-9 may also be released by neutrophils. Vinola *et al.* [11] reported a significant correlation between the percentage of neutrophils and the concentrations of free elastase in the induced sputum of asthmatic patients. In several lung diseases the content of MMP-9 has also been found to correlate with the percentage of neutrophils. In addition, MMP-9 induces the migration of eosinophils and neutrophils across basement

membranes [4,5]. Therefore, our study is consistent with previous reports and suggests that MMP-9 release depends on the extent of airway inflammation. It further suggests that inflammatory cells, including neutrophils, have the potential to destroy the extracellular matrix and to determine airway injury in asthma.

MMP-9 is the major metalloproteinase detectable in sputum and its activity is higher in stable and acute asthmatic patients than in control subjects. However, we were unable to find the activated form of MMP-9 using zymography. In addition, detection of the active form of MMP-9 is difficult because of the lack of commercially available antibodies recognizing it. Although the mechanisms of MMP activation still remain unknown, it is likely that MMP-9 activation occurs in inflamed airways owing to the presence of several inflammatory mediators and also

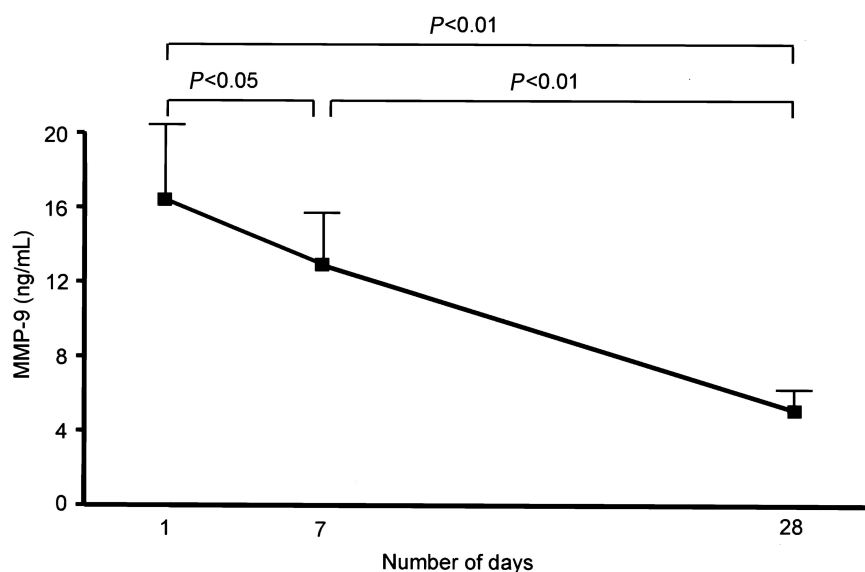


Fig. 4. Time course of MMP-9 in sputum obtained from acute asthmatic subjects. Samples obtained at day 1 of exacerbation, from day 7, and from day 28. Data were obtained and are reported as described for Fig. 3.

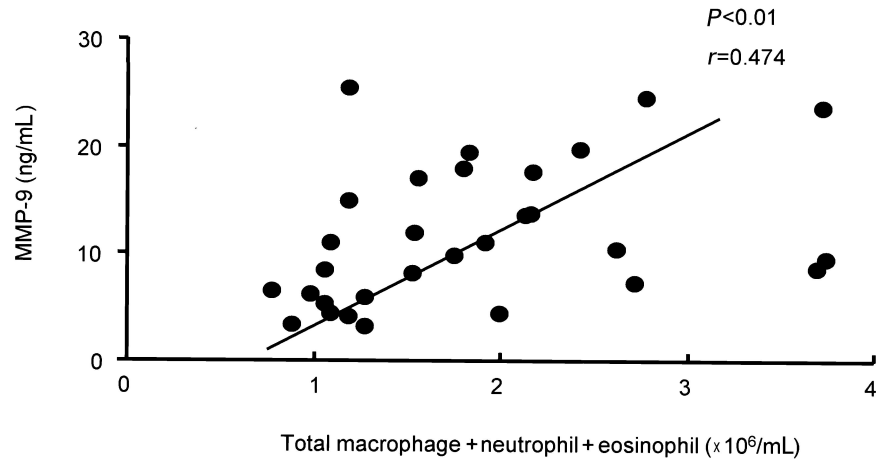


Fig. 5. Correlation between the total macrophage + neutrophil + eosinophil cell numbers and the concentrations of MMP-9 in induced sputum of acute asthmatic patients.

occurs on the surface of inflammatory cells without being released into the extracellular *milieu* [21].

In this study, overproduction of MMP-9 in acute asthma was inhibited by asthma therapy, including corticosteroids in acute asthma. Recently, Hoshino *et al.* [17] reported that corticosteroid treatment of asthma can reduce subepithelial collagen deposition by down-regulating MMP-9 expression. However, the effects of corticosteroid on MMP-9 production have not yet been demonstrated.

In conclusion, our study has shown that production of MMP-9 is increased in acute asthmatic patients, and that it decreased after asthma therapy. Further, we showed that the levels of sputum MMP-9 correlated with the total macrophage + neutrophil + eosinophil cell numbers. We therefore suggest that airway inflammation after asthma exacerbation is associated with the overproduction of MMP-9, which might lead to airway remodelling.

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