

Sputum matrix metalloproteinase-9: Tissue inhibitor of metalloproteinase-1 ratio in acute asthma

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Background: The ratio of matrix metalloproteinase-9 (MMP-9) and its inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1) may be a marker of the balance between airway tissue destruction and repair. TIMP-1 may potentially contribute to the pathogenesis of increased submucosal extracellular matrix deposition in asthma.

Objective: Our purpose was to assess the variation in sputum MMP-9 and TIMP-1 during acute asthma.

Methods: We evaluated the MMP-9 and TIMP-1 balance in sputa of 16 asthmatic patients admitted with spontaneous exacerbation, conducting measurement before (day 1) and after methylprednisolone infusion therapy (days 2, 3, 5, and 7), and on remission days.

Results: Peak expiratory flow and eosinophilic cationic protein levels were significantly ($P < .05$) improved within 7 days in all patients. Sputum MMP-9 levels on day 2 tended to be lower than on day 1, but not significantly. Zymography revealed that the main enzyme was identified immunologically as MMP-9, and gelatinase activity on day 1 had a tendency to decrease for the following 7 days. The TIMP-1 levels gradually increased until day 5, were significantly ($P < .05$) high on day 5, and decreased on day 7. The MMP-9/TIMP-1 molar ratios were significantly ($P < .05$) decreased on days 2, 3, 5, and 7 compared with day 1. Sputum levels of MMP-9 and TIMP-1 and the MMP-9/TIMP-1 molar ratios on day 1 were significantly higher ($P < .02$) than those on remission days.

Conclusions: An imbalance between MMP-9 and TIMP-1 was present in acute asthma, with an excess of MMP-9 resulting in a high ratio of MMP-9/TIMP-1 before treatment, and over time with glucocorticosteroid the TIMP-1 levels rose, dropping the ratio of MMP-9/TIMP-1. It was suggested that overproduction of MMP-9 and TIMP-1 after asthma exacerbation might contribute significantly to airway tissue remodeling and that TIMP-1 production in acute asthma might not be suppressed by glucocorticosteroid. (*J Allergy Clin Immunol* 2000;105:900-905.)

Key words: Matrix metalloproteinase, tissue inhibitor of metalloproteinase, acute asthma, sputum, glucocorticosteroid

Abbreviations used

BAL:	Bronchoalveolar lavage
BDP:	Beclomethasone dipropionate
ECM:	Extracellular matrix
ECP:	Eosinophil cationic protein
MMP:	Matrix metalloproteinase
PEF:	Peak expiratory flow
TIMP:	Tissue inhibitor of metalloproteinase

The matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes that degrade the proteins of the extracellular matrix (ECM). MMPs may be essential for the invasion of malignant cells or the migration of inflammatory cells into tissues.¹⁻⁴ MMP-9 (92-kd gelatinase) contributes to the migration of inflammatory cells through the ECM, basement membrane, and the endothelial layer in asthma. T-cell transmigration through basement membrane in vitro is mediated by MMP-2 and MMP-9⁵ and in the case of eosinophil by MMP-9.⁶ Ohno et al⁷ reported that the eosinophil was a major cellular source of MMP-9 in bronchial tissue in asthmatic patients. On the other hand, it was reported that MMP-9 during status asthmaticus originated from activated neutrophils and epithelium.^{8,9} MMP-9 is most prominent among MMPs found in bronchoalveolar lavage (BAL) from untreated asthmatic patients.¹⁰

The major physiologic inhibitors of MMPs are α_2 -macroglobulin and their specific tissue inhibitors of metalloproteinases (TIMPs).¹¹ TIMPs play a significant role in the regulation of MMP activation in vivo.¹² TIMP-1 is a sialoglycoprotein with a molecular weight of 28 kd that inhibits MMPs such as interstitial collagenase (MMP-1), gelatinase (MMP-2 and MMP-9), and stromelysin (MMP-3), all of which can degrade the ECM. TIMP-1 is synthesized and secreted by many connective tissue cells that also produce MMPs, including fibroblasts, inflammatory cells, and endothelial cells.¹¹ TIMP-1 is secreted in association with MMP-9. TIMP-1 binds noncovalently in a 1:1 proportion with MMP-9 or proMMP-9 and inhibits their enzymatic activity.^{13,14} TIMP-1 may potentially contribute to the pathogenesis of the increased thickness of the basement membrane in asthma¹⁵ and of the ECM deposition in the airway wall.¹⁶

An imbalance between MMPs and TIMPs occurs in a number of chronic inflammatory conditions, such as rheumatoid arthritis.¹ Sputum MMP-9/TIMP-1 imbal-

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ance may be critical in bronchial tissue destruction and repair, including deposition of ECM in the airway wall in patients with cystic fibrosis.² In chronic asthma a decreased MMP-9/TIMP-1 ratio may have important consequences for airway obstruction, probably because of collagen deposition and the increase of airway wall thickness.^{17,18} Recently, Bossé et al¹⁹ reported that the serum MMP-9/TIMP-1 ratio could predict the response to oral corticosteroid therapy in severe asthma; a low MMP-9/TIMP-1 ratio was observed in patients having little or no FEV₁ improvement with corticosteroids. Vignola et al²⁰ reported that the sputum MMP-9/TIMP-1 ratio positively correlated with the FEV₁ in asthmatic patients, and Mautino et al²¹ noted that TIMP-1 levels in BAL were higher in untreated asthmatic subjects than in a glucocorticoid-treated group. Treatment with inhaled corticosteroids may prevent airway remodeling. However, the effects of corticosteroid on MMPs and TIMPs are unknown.

We hypothesized that asthma exacerbation might contribute to airway remodeling by changing the balance of the MMP-9/TIMP-1 ratio in airways. The kinetics of sputum MMP-9 and TIMP-1 in acute asthma have been little investigated. In this study we examined the time course of sputum levels of MMP-9, TIMP-1, and MMP-9 enzymatic activity in acute asthma and determined whether an imbalance of the MMP-9/TIMP-1 molar ratio is present before and after the treatment with corticosteroids in spontaneous asthma exacerbation.

METHODS

Patients

Twenty-seven asthmatic patients, 23 women and 4 men (aged 53 ± 5 years [mean ± SEM]) were recruited from Sapporo Medical University Hospital and Sapporo Hospital of Hokkaido Railway Company. Asthma was diagnosed according to the American Thoracic Society guidelines for the diagnosis of asthma.²² None of the subjects was a current or previous smoker. Atopy was defined by one or more positive specific serum IgE tested by a commercial kit (MAST systems, Hitachi Kasei, Tokyo, Japan). The MAST system includes 16 antigens (house dust, *Dermatophagoides pteronyssinus*, ragweed, orchard grass and 2 other grasses, Japanese cedar, *Alternaria*, another 4 molds, cat, dog, egg, and soy bean). All patients were treated with oral theophylline (400 mg/d), inhaled β₂-agonist when required, and inhaled beclomethasone dipropionate (BDP), ranging from 200 to 1200 μg per day, according to the international guidelines.²³ They were admitted to these hospitals for more than 7 days because of severe or moderate asthma exacerbation and were treated under American Thoracic Society recommendations.²⁴ The treatment consisted of infusion of methylprednisolone (1–2 mg/kg/d), salbutamol inhalation (5–20 μg/kg/d), and continuous theophylline drip infusion (400–600 mg/d). All patients gave informed consent before this study.

Study design

We measured morning peak expiratory flow (PEF) and sputum. Sputum levels of eosinophil cationic protein (ECP), MMP-9, and TIMP-1 were measured before (day 1) and after treatment (days 2, 3, 5, and 7). In remission days 3 months or more after this acute exacerbation, we collected sputum sample and measured MMP-9 and TIMP-1.

Sputum collection

Spontaneous sputum samples were obtained in the morning during acute asthma. In remission days, if spontaneous sputum could not be obtained, induced sputum was obtained by nebulized hypertonic saline solution. The induction procedure described by Pin et al²⁵ was followed but was made safer by beginning with an aerosol of β₂-agonist followed by a 3% saline solution generated by an ultrasonic nebulizer (NE-U10B, Omron, Tokyo, Japan) with an output of 1 mL/min and particle size of 4.5 μm aerodynamic mass median diameter. Patients were asked to rinse the mouth, swallow the water, and blow the nose to minimize contamination with saliva and postnasal drip. They were instructed to cough sputum into a sterile plastic container whenever they felt that sputum might be present. These procedures were repeated for up to a total of 20 minutes of inhalation until approximately 1 mL of sputum sample was obtained.

The volumes of the sputum samples were determined, and a 4-fold volume of saline solution was added. The samples were then mixed gently by touch mixer (MT31, Yamato Scientific, Tokyo, Japan) for 2 minutes. The homogenized sputum samples were centrifuged at 2000 revolutions/min for 15 minutes. The supernatants were aspirated and frozen at –80°C for MMP-9, TIMP-1, and ECP analysis.

Zymography

MMPs present in the sputum sample were detected by their capacity to degrade gelatin²⁶ according to a method previously described.¹⁰ Briefly, each supernatant of sputum was diluted (1:3) in nonreducing sample buffer, and 12 μL of the sample was subjected to electrophoresis on 10% polyacrylamide gels containing 1 mg/mL gelatin in the presence of SDS under nonreducing conditions. After electrophoresis, gels were washed in 10 mmol/L TRIS, pH 8.0, 2.5% Triton X-100 for 1 hour, rinsed for 30 minutes, and incubated at 37°C for 24 hours in buffer containing 50 mmol/L TRIS–hydrochloric acid, pH 8.0, 0.5 mmol/L calcium chloride, and 10^{–6} mol/L zinc chloride. After incubation the gels were stained with Coomassie brilliant blue R250 and then destained in a solution of 5% acetic acid with 10% methanol. Zones of enzymatic activity were indicated by negative staining: areas of proteolysis appeared as clear bands against a blue background. The molecular weight of the gelatinolytic bands was estimated relative to prestained low-range molecular weight markers (Bio-Rad Laboratories, Hercules, Calif). To identify the gelatinolytic activities, samples were subjected to immunoprecipitation with mouse mAb antihuman MMP-9 (Fuji Chemical Industries, Toyama, Japan), as described previously,¹⁰ before being subjected to electrophoresis.

MMP-9 and TIMP-1 assay

Determinations of total MMP-9 and TIMP-1 in induced sputum were performed by commercially available BIOTRAK ELISA kits (Amersham, Little Chalfont, UK). The MMP-9 kit detects proMMP-9, and proMMP-9–TIMP-1 complex, and it has <3% cross-reactivity with proMMP-1, proMMP-2, proMMP-3, and active MMP-9. The TIMP-1 kit detects the total TIMP-1, free TIMP-1, and MMPs–TIMP-1 complex and does not cross-react with TIMP-2. The detection limit was 4 μg/mL for MMP-9 and 3.13 μg/mL for TIMP-1.

ECP assay

ECP in sputum was determined by means of a specific and sensitive double-antibody RIA by a Pharmacia ECP RIA kit (Pharmacia Diagnostica, Uppsala, Sweden). Reproducibility of the assay was good; coefficient of variations of intra-assay and interassay were 8.9% and 7.5%, respectively. The detection limit of the assay was 2 ng/mL.

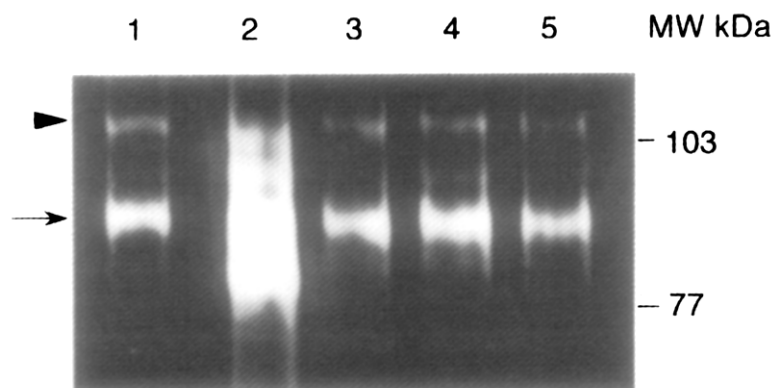


FIG 1. Analysis of MMPs in sputum by zymography. A representative zymographic analysis of sputum supernatant samples obtained from one patient. Sputum sample on remission days was submitted to immunoprecipitation with an anti-MMP-9 antibody before zymographic analysis (*lane 1*). Arrow, Position of 92-kd MMP-9; arrowhead, complexed MMP-9. *Lane 2*, Sample obtained at day 1 of exacerbation; *lane 3*, from day 2; *lane 4*, from day 3; *lane 5*, from day 7. MW, Molecular weight.

TABLE I. Characteristics of patients

No.	Age (y)	Sex	Atopy	Severity*	Remission days		Treatment (mg/d)
					FEV ₁ (L)	% FEV ₁ predicted†	
1	63	F	Yes	2	1.68	105.0	BDP, 0.2
2	33	F	No	2	2.05	76.5	BDP, 0.2
3	68	M	Yes	3	ND	ND	BDP, 0.6
4	64	F	Yes	4	1.29	66.8	BDP, 0.8; methylprednisolone, 4
5	52	F	No	4	1.29	61.1	BDP, 1.2
6	70	F	No	3	0.82	57.3	BDP, 0.9
7	43	F	No	2	ND	ND	BDP, 0.4
8	51	M	Yes	2	ND	ND	BDP, 0.8
9	49	M	Yes	3	3.44	101.2	BDP, 1.2
10	73	F	No	3	ND	ND	BDP, 1.2
11	46	F	Yes	2	2.04	83.3	BDP, 0.4
12	64	F	No	4	2.03	115.3	BDP, 1.2; prednisolone, 7.5
13	27	F	Yes	3	2.42	77.8	BDP, 0.8
14	25	F	Yes	3	2.49	85.9	BDP, 0.8
15	70	F	No	2	1.17	73.1	BDP, 0.4
16	43	F	Yes	3	2.43	95.7	BDP, 0.8

ND, Not determined.

*Clinical severity was based on step system of Global Initiative for Asthma.

†Percentage of the predicted value.

Statistical analysis

Data on PEF and sputum levels of MMP-9, TIMP-1, and ECP are presented as the mean $\pm$ SEM. Comparisons of the time course of PEF, sputum MMP-9, TIMP-1, and ECP levels were made through an ANOVA and Scheffe's test. The paired *t* test was used to compare the sputum levels of MMP-9, TIMP-1, and the MMP-9/TIMP ratio between day 1 and remission days. Data on subgroups of subjects were analyzed with a nonparametric test (Mann-Whitney *U* test). The Pearson correlation and the Fisher test was used to calculate *P* values for the correlation. Statistical significance was assumed for *P* values lower than .05.

RESULTS

Of 27 patients, 7 had infection accompanied with purulent sputum, 3 had incomplete sample collection, and 1 needed tracheal intubation and mechanical ventila-

tion. These 11 subjects were eliminated from this study. The characteristics of the remaining 16 patients are shown in Table I.

Zymographic analysis of the sputum samples is shown in Fig 1. The figure shows a representative zymographic analysis of sputum supernatant samples obtained from one patient. This patient's sputum sample on remission days was submitted to immunoprecipitation with an anti-MMP-9 antibody before zymographic analysis (*lane 1*). The arrow indicates the position of 92-kd MMP-9, and the arrowhead shows the MMP-9/TIMP-1 complex. *Lane 2* is the sample obtained on day 1 of exacerbation, *lane 3* is from day 2, *lane 4* from day 3, and *lane 5* from day 7. The main enzyme was identified as MMP-9 in each lane.

The time course of PEFs and sputum ECP levels is

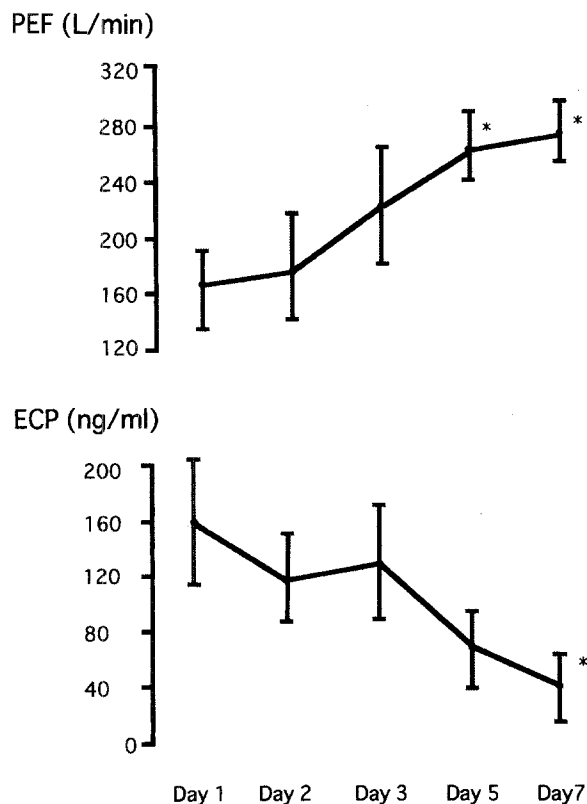


FIG 2. Time course of PEF and sputum ECP before (day 1) and after methylprednisolone infusion therapy (days 2-7). Asterisk, $P < .05$ versus day 1, ANOVA.

shown in Fig 2, and all patients improved during the 7 days. As shown in Fig 3, the elevated MMP-9 levels on day 1 tended to decrease in the following 7 days, but not significantly. The TIMP-1 levels gradually increased until day 5, at which point they were significantly high ($P < .05$), and then decreased on day 7. The MMP-9/TIMP-1 molar ratios on day 1 were variable from 0.05 to 1.8, and the percent change of MMP-9/TIMP-1 ratios (the ratio of day 1 is as 100% in each patient) is shown in Fig 3. The percent changes in the MMP-9/TIMP-1 molar ratios of days 2, 5, and 7 ($P < .01$) and day 3 ($P < .05$) were significantly lower than those of day 1 (Fig 3). There was no relationship between sputum ECP and TIMP-1, ECP and MMP-9, and TIMP-1 and MMP-9 on day 1 of exacerbation.

We were able to obtain sputum samples on remission days from 9 of 16 patients (patients 1, 2, 4, 5, 9, 11, 12, 13, and 14 in Table I), with more than 80% of PEF in each patient's personal best, and there had been no asthma exacerbation or upper respiratory infection within 1 month. Sputum levels of MMP-9, TIMP-1, and the MMP-9/TIMP-1 ratio were significantly ($P < .02$) higher on day 1 than those in the remission period (Fig 4). There were no differences between induced ($n = 5$) and spontaneous sputum ($n = 4$) on remission days in the levels of TIMP-1 and MMP-9.

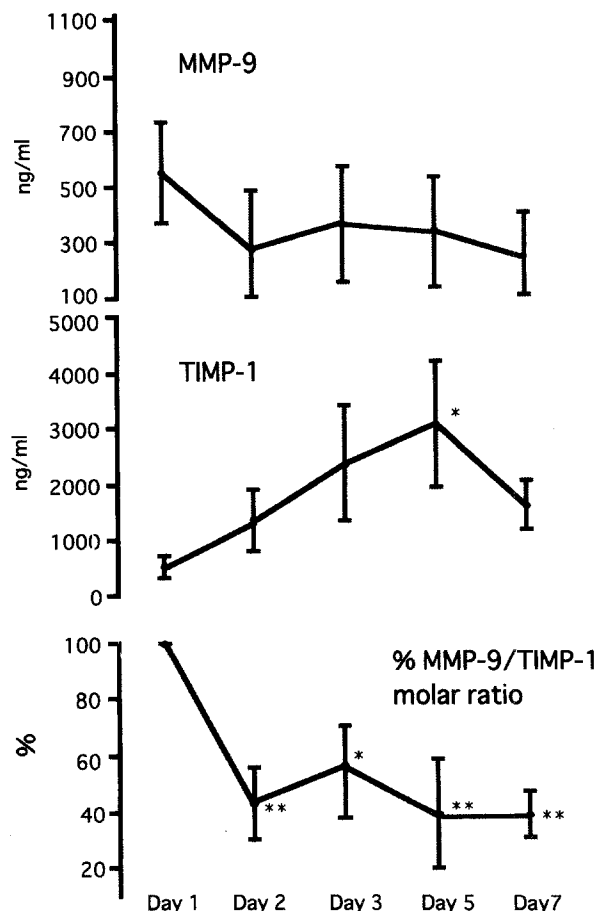


FIG 3. Time course of MMP-9, TIMP-1, and percent MMP-9/TIMP-1 molar ratio in sputum before (day 1) and after methylprednisolone infusion therapy (days 2-7). Percent MMP-9/TIMP-1 molar ratios present the ratio of day 1 as being 100% in each patient. Asterisk, $P < .05$; two asterisks, $P < .01$ versus day 1, ANOVA.

DISCUSSION

We found that sputum MMP-9/TIMP-1 ratios on asthma exacerbation were significantly high compared with those on remission days, and they quickly decreased after asthma therapy, including corticosteroids. From these results it was suggested that the ECM might have been destroyed by inflammatory processes before treatment. Warner et al²⁷ reported that levels of MMPs but not TIMP-1 were increased in BAL fluid of patients with mild asthma 18 hours after endobronchial antigen challenge. Shute et al²⁸ reported that urinary glycosaminoglycan levels, degradative products of proteoglycans by heparanases and proteinases, including neutrophil elastase and MMPs, were increased in acute severe asthma. In patients with chronic obstructive lung disease, sputum MMP-9 levels in exacerbation were also significantly higher than in pre-exacerbation.²⁹ In our study sputum TIMP-1 levels, MMP-9 levels, and gelatinolytic activity on day 1 were higher than on remission days; therefore our results suggested that the ECM and basement mem-

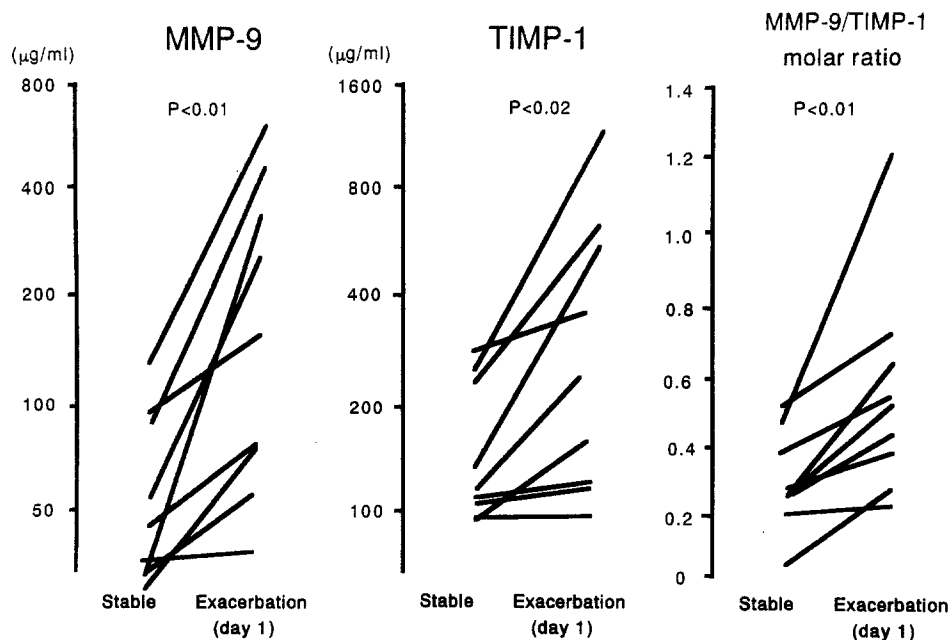


FIG 4. Comparison of sputum levels of MMP-9, TIMP-1, and MMP-9/TIMP-1 molar ratio between remission period and asthma exacerbation (day 1).

brane degeneration by MMPs and other proteinases occurred in the early phase of asthma exacerbation and that TIMP-1 production might have accompanied or followed the increase of MMP-9 to inhibit these processes.

Airway wall remodeling in asthma must be accompanied by degradation of the ECM in addition to synthesis and deposition of new matrix. The results of our study support this concept and MMP-9 is the major enzyme detected in sputum in acute asthma and on remission days. We could not identify the active form of MMP-9 (84 kd) immunologically because of the lack of commercially available antibodies recognizing the active form of the enzyme. However, zymography on day 1 showed MMP-9, MMP-9/TIMP-1 complex, and many other bands, including the 84-kd band, which suggested the presence of active MMP-9. Interestingly, the numerous bands except for MMP-9 and MMP-9/TIMP-1 complex disappeared quickly after glucocorticosteroid therapy. It was suggested that glucocorticosteroid therapy might decrease sputum gelatinolytic activity in acute asthma.

This study demonstrated that sputum TIMP-1 levels on day 1 were higher than on remission days, gradually increased until day 5, and declined on day 7. From these observations, it was suggested that an overproduction of TIMP-1 production might have occurred during days 1 to 5, followed by a decrease on day 7. These findings have important implications because high levels of TIMP-1 can lead to the deposition of ECM components and increased thickness of the basement membrane in asthma and may increase myofibroblast proliferation through its cell growth-promoting activity.^{15,30}

In our study overproduction of TIMP-1 was not inhibited by asthma therapy, including corticosteroid in acute

asthma. Treatment with inhaled corticosteroids may prevent airway remodeling. However, the effects of corticosteroid on MMPs and TIMPs are unknown. Recently Hoshino et al³¹ reported that 6-month BDP treatment increased submucosal TIMP-1 expression and decreased both submucosal MMP-9 expression and collagen type III deposition, suggesting that corticosteroid treatment of asthma can reduce subepithelial collagen deposition by down-regulation of MMP-9 expression and up-regulation of TIMP-1 expression. It was also reported that a 4-week treatment with inhaled budesonide (1600 $\mu\text{g/d}$) did not affect TIMP-1 levels in the BAL fluid of patients with mild asthma.³² On the other hand, dexamethasone inhibits TIMP transcription in alveolar macrophages *in vitro*.³³ Mautino et al²¹ reported that the content of TIMP-1 was approximately 4-fold higher than that of MMP-9 in BAL fluid from untreated asthma, whereas it was about 2-fold higher in glucocorticoid-treated asthmatic subjects. However, direct *in vivo* effects of glucocorticoid on TIMP-1 production have not yet been demonstrated, and indirect effects by other mediators or cytokines in acute asthma, which are capable of increasing TIMP-1 production, should be considered (ie, transforming growth factor- β and IL-6).^{34,35}

MMP-9 binds to TIMP-1 in a 1:1 proportion and inhibits its enzymatic activity. Sputum gelatinolytic activity in zymography decreased after steroid therapy; activity of free MMP-9 might possibly decrease by binding to TIMP-1. Unexpectedly, sputum MMP-9 levels declined, although not significantly, in our study. In the assay system for MMP-9 we measured the total of MMP-9, and we could not distinguish free MMP-9 from the MMP-9/TIMP-1 complex. We detected the band of

MMP-9/TIMP-1 complex in zymography. A possible reason why sputum MMP-9 levels did not significantly decrease during the 7 days may be that we measured both free MMP-9 and inactive MMP-9/TIMP-1 complex. Indeed, this is the limitation of this study. The assay kit for TIMP-1 was also incapable of distinguishing free forms of TIMP-1 from the MMP-9/TIMP-1 complex. The absolute levels of TIMP-1 were 6- to 10-fold higher than the MMP-9 levels in our study; thus the influence of the complex on our results for TIMP-1 levels might be less than on those for MMP-9. Therefore the increased levels of TIMP-1 in this study seem to have newly generated TIMP-1 during the period of asthma attack.

In conclusion, we found that an imbalance between MMPs and TIMPs was present in acute asthma, with an excess of MMP-9 implying a high ratio of MMP-9/TIMP-1 on exacerbation before treatment, meaning that airway tissue destruction and, over time with corticosteroid treatment, the TIMP-1 levels rose, dropping the ratio of MMP-9/TIMP-1. It was suggested that overproduction of MMP-9 and TIMP-1 in acute asthma might contribute to airway tissue remodeling and that TIMP-1 production might not be suppressed by glucocorticosteroids.

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