

Imbalance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in toluene diisocyanate-induced asthma

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Summary

Background Toluene diisocyanate (TDI)-induced asthma is an inflammatory disease of the airways characterized by airway remodelling due, at least in part, to an excess of extracellular matrix deposition in the airway wall. 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.

Objective We determined whether an imbalance of the MMP-9:TIMP-1 molar ratio is present before and/or after challenge with TDI.

Methods We used a murine model of TDI-induced asthma to evaluate the MMP-9 and TIMP-1 balance in the lung.

Results The expression of MMP-9 and TIMP-1 mRNAs and proteins in the lungs increased at 7 h after TDI inhalation and continued for up to 72 h. Immunohistochemical and immunocytological analyses in the lungs of TDI-exposed mice revealed increases of immunoreactive MMP-9 and TIMP-1. There were significant correlations between the levels of MMP-9 or TIMP-1 and the number of neutrophils, lymphocytes, or eosinophils. The molar ratio of MMP-9/TIMP-1 significantly decreased at 7 h after TDI inhalation and continued up to 72 h.

Conclusion These data suggest that TDI-induced asthma may be associated with an imbalance between MMP-9 and TIMP-1, which could be useful as a marker of airway inflammation and airway remodelling in this disease.

Keywords airway remodelling, matrix metalloproteinase-9, neutrophils, occupational asthma, tissue inhibitor of metalloproteinase-1

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Introduction

An imbalance between the expression of proteinases and proteinase inhibitors, particularly of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs), is believed to generate tissue destruction or abnormal tissue repair, such as fibrosis, in some pulmonary inflammatory diseases [1–4]. MMPs are a large family of zinc- and calcium-dependent endopeptidases with distinct substrate specificities that can degrade most components of the extracellular matrix (ECM) [5]. MMPs thus play an important role in physiologic and pathologic processes, including ECM turnover, tissue degradation and repair, cell migration, and inflammation [6, 7]. Of the MMP family, MMP-9 is involved in inflammation by inducing the migration of eosinophils, lymphocytes, and neutrophils across basement membranes [7–9]. It is also known that MMP-9 is one of the major proteinases that induces

bronchial remodelling in asthma and is associated and secreted with TIMP-1 [10, 11]. In addition, all the events involved in remodelling the airways occur in the context of the variety of structural and functional molecules contained within the airway ECM. Moreover, it is known that airway remodelling is partly due to an excess of ECM deposition in the airway wall [12]. Therefore, the imbalance between MMP-9 and TIMP-1 expression may play a role in airway inflammation in asthma.

Occupational asthma in developed countries is commonly caused by toluene diisocyanate (TDI), which is a low-molecular-weight compound used widely in the production of polyurethane foams, automobile paints, varnishes, and related products [13, 14]. Virtually all subjects who are exposed to high concentrations of TDI develop irritation of the eyes, nose, and pharynx, but 5–10% of the workers also develop occupational asthma [15]. For the development of occupational asthma, it is not necessarily due to the concentration of TDI or duration of exposure *per se* but pathogenic consequences caused by both factors appear to be important [16]. It has been suggested that multiple mechanisms may be involved in the induction of isocyanate-induced asthma, including immunologic, inflammatory, genetic,

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pharmacologic, and neurogenic ones [17–19]. TDI-induced asthma is a chronic inflammatory process of the airways, which is associated with the accumulation of activated inflammatory cells, including eosinophils, lymphocytes, and neutrophils [20, 21].

Recently, we reported that the release of MMP-9 was up-regulated in association with the accumulation of inflammatory cells to airways in a murine model of TDI-induced asthma [22]. This TDI-induced up-regulation of MMP-9 may lead to an alteration of the physiological balance between MMP-9 and TIMP-1. In the present study, we determined whether an imbalance of the MMP-9:TIMP-1 molar ratio is present before and/or after challenge with TDI. We examined the time course of the expression of MMP-9 and TIMP-1 mRNAs and proteins in the lung using a murine model of TDI-induced asthma. We determined whether an imbalance of the MMP-9:TIMP-1 molar ratio is present before and/or after challenge with TDI.

Materials and methods

Generation of a murine TDI-induced asthma model

Specific pathogen-free female BALB/c mice were purchased from the Korean Research Institute of Chemistry Technology (Daejeon, Korea). Mice were housed throughout the experiments in a laminar flow cabinet, and maintained on standard laboratory chow *ad libitum*. All mice were 8–10 weeks of age. This study was approved by the Ethics Committee for Animal Experiments of the Chonbuk National University Medical School. Mice were sensitized by intranasal administration of 20 μ L of 3% TDI dissolved in ethyl acetate:olive oil (1:4) under light anaesthesia (sodium pentobarbitone, 30 mg/kg, intraperitoneally) once daily for 5 consecutive days, as previously described [22–24] (Fig. 1). After 3 weeks, these animals were further sensitized with the same reagent for 5 consecutive days. On day 7 after the second sensitization (day 38), mice were individually placed in a horizontal cylindrical chamber and challenged via the airways with 1% TDI dissolved in ethyl acetate:olive oil (1:4) by ultrasonic nebulization (NE-U12, Omron, Tokyo, Japan). As a control, mice were sensitized and challenged by the same procedure but using only the solvent, ethyl acetate:olive oil (1:4).

Sampling and cellular analysis of bronchoalveolar lavage (BAL)

BAL was performed at 0, 0.5, 7, 24, 48, and 72 h after the 1% TDI challenge. At the time of lavage, the mice were killed with an overdose of sodium pentobarbitone (pentobarbital

sodium, 100 mg/kg of body weight, intraperitoneally). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution (0.7 mL) was slowly infused into the lung, and approximately 0.4 mL of instilled fluid was consistently recovered from each mouse. Six BAL fluid samples from six mice were collected at each time-point and were centrifuged at 4 °C. The supernatants were kept at –70 °C until use. The total cell numbers were counted with a haemocytometer. Smears of BAL cells prepared with a Cytospin II (Shandon, Runcorn, UK) were stained with Diff-Quik solution (Dade Diagnostics of PR Inc., Aguada, PR) for differential cell counting. Parts of slides were used for immunocytochemistry of MMP-9 and TIMP-1. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. The inter-investigator variation was <5%. The mean number from the two investigators was used to estimate the cell differentials.

Measurement of airway responsiveness

Airway responsiveness was measured in mice 3 days after TDI challenge (day 41). Experiments were conducted in an unrestrained and conscious state according to the method of Hamelmann et al. [25]. Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea). Aerosolized methacholine (Mch) in increasing concentrations (2.5–50 mg/mL) was nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization. The bronchopulmonary resistance is expressed as enhanced pause (P_{enh}), calculated as: (expiratory time/relaxation time – 1) $\times$ (peak expiratory flow/ peak inspiratory flow), according to the manufacturers' protocol. The results are expressed as the percent increase in P_{enh} following challenge with each concentration of Mch, where the baseline P_{enh} (after saline challenge) is expressed as 100%.

Measurements of MMP-9 and TIMP-1 in BAL fluids

Levels of MMP-9 and TIMP-1 were quantitated by enzyme immunoassays according to the manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN, USA). The hydrolytic activity of MMP-9 was measured by gelatin zymography, as previously described [10]. Samples containing the same amount of protein (10 μ g) were mixed with 5 $\times$ sample buffer (0.4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue) and were separated by 10% SDS-PAGE containing 0.1% gelatin. After running, gels were incubated in 2.5% Triton X-100 for 1 h and further incubated in the enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM

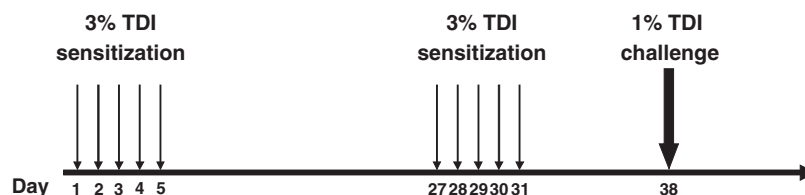


Fig. 1. Schematic diagram of the experimental protocol. Mice were sensitized twice by two courses of intranasal administration of 3% toluene diisocyanate (TDI) once a day for 5 consecutive days with a 3-week interval. Seven days later, mice were challenged via the airways with 1% TDI for 10 min by ultrasonic nebulization.

CaCl₂ and 0.02% Brij-35) for 24 h at 37 °C. The gels were stained with 0.5% Coomassie brilliant blue-250 solution and destained with several changes of 30% methanol and 10% acetic acid.

Immunohistochemistry and immunocytochemistry

At 72 h after the last challenge, lungs were removed from the mice after they were killed. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4-µm sections of fixed embedded tissues were cut using a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides and deparaffinized. For immunohistochemistry and immunocytochemistry of MMP-9 and TIMP-1, the deparaffinized 4-µm sections or the cytocentrifuge preparations of BAL cells were incubated sequentially in accordance with the RTU Vectastain Universal Quick kit instructions from Vector Laboratories (Burlingame, CA, USA). Briefly, the slides were incubated in Endo/Blocker (Biomedex, Foster City, CA, USA) for 5 min and in pepsin solution for 4 min at 40 °C. The slides were incubated in normal horse serum for 15 min at room temperature. The slides were incubated with an affinity-purified rabbit polyclonal anti-MMP-9 antibody (Chemicon International, Inc., Temecula, CA, USA) or an anti-TIMP-1 antibody (Oncogene, Cambridge, MA, USA) overnight at 4 °C and were further incubated with prediluted biotinylated pan-specific IgG for 10 min. The slides were then incubated in a streptavidin/peroxidase complex reagent for 5 min, and then in a 3-amino-9-ethylcarbazole substrate for 12 min. Controls consisted of sections of lung tissue or BAL cells from mice incubated without a primary antibody. After immunostaining, the slides were counterstained for 1 min with Gill's haematoxylin in 20% ethylene glycol, mounted with Aqueous Mounting Medium (InnoGenex, San Ramon, CA, USA) and photomicrographed (VENOX-T; Olympus, Tokyo, Japan).

Western blot analysis

Lung tissues were homogenized in a prechilled lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM NaF and protease inhibitor cocktail) with a hand-held homogenizer (Sigma, St Louis, MO, USA) in an ice bath. The homogenates were transferred to microcentrifuge tubes and centrifuged at 10 000 × g for 10 min at 4 °C. Extracts were kept at -80 °C until use. Protein concentrations in the extracts were determined using the Bradford reagent (Bio-rad, Hercules, CA, USA). Samples (30 µg protein per lane) were loaded on 12% SDS-PAGE gels and separated at 120 V for 90 min. After electrophoresis, separated proteins were transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by the wet transfer method (250 mA, 90 min). Non-specific sites were blocked with 5% non-fat milk in TBST buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h, and the blots were then incubated with an anti-MMP-9 antibody (Santa Cruz

Biotechnology, Santa Cruz, CA, USA), diluted 1 : 800, or an anti-TIMP-1 antibody (Oncogene, Cambridge, MA, USA), diluted 1 : 1000, overnight at 4 °C. Anti-rabbit horseradish peroxidase conjugated IgG was used to detect the binding of antibodies and was visualized by exposing to a photographic film after treating with enhanced chemiluminescence system reagents (Amersham Pharmacia Biotech). The membranes were stripped and reblotted with an anti-actin antibody (Sigma) to verify equal loading of protein in each lane.

RNA isolation and RT-PCR

The total RNA from frozen lung tissues was isolated using a rapid extraction method (TRI-Reagent) derived from the method of Chomczynski and Sacchi [26]. The RNA was quantified by measuring its absorption at 260 nm and was stored at -80 °C until use. Total RNA (4 µg) was reverse transcribed to cDNA in a buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl₂, 10 mM DTT, 0.5 µg random hexanucleotide primers, 2.5 mM dNTP, 40 U RNase inhibitor, and 50 U/µL SuperScript II RT (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 µL. This mixture was incubated for 50 min at 42 °C and was finally digested with 2 U/µL *Escherichia coli* RNase H for 20 min at 37 °C to further purify the cDNA that was obtained. The first-strand cDNAs were used for the PCR amplification of MMP-9, TIMP-1, or the housekeeping gene, GAPDH. PCR amplification was performed by mixing 3 µL of RT reaction with 57 µL of buffer containing 2.5 U of Taq DNA polymerase (Promega, Madison, WI, USA) and 30 pmol of specific primer pairs for mouse cDNAs encoding MMP-9, TIMP-1, or GAPDH, designed from published mouse gene sequences. The primers used were as follows: MMP-9 (predicted 420 length base pairs) sense: 5'-GTATGGTCGTGGCTCTA AGC-3', antisense: 5'-AAAACCCCTCTGGTCTGCGG-3'; TIMP-1 (predicted 333 length base pairs) sense: 5'-GAC CACCTTATACCAGCGTT-3', antisense: 5'-GTCACCTCTC CAGTTTGCAAG-3'; and GAPDH (predicted 609 length base pairs) sense: 5'-GCCATCAACGACCCCTTCATT GAC-3', antisense: 5'-ACGGAAGGCCATGCCAGTGTAG CTT-3'. PCR reactions were performed in a thermocycler (GeneAmp[®] PCR System 2400, Foster City, CA, USA) using the following reaction conditions: after an initial incubation for 2 min at 95 °C, samples were subjected to 35 cycles of 1 min at 94 °C, 2 min at 54 °C (MMP-9, GAPDH), or 58 °C (TIMP-1), and 1 min at 72 °C. A final extension step at 72 °C for 7 min was performed. The RT-PCR products were electrophoretically fractionated on 1% agarose gel stained with ethidium bromide. DNA bands were visualized after exposure to ultraviolet light.

Densitometric analyses and statistics

All signals were visualized and analysed by densitometric scanning (LAS-5000, Fuji Film, Tokyo, Japan). Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using one-way ANOVA followed by Fisher's test. Significant differences between groups were determined using the unpaired Student's *t*-test. Correlations were calculated using Spearman's rank test. Statistical significance was set at *P* < 0.05.

Results

Effect of TDI challenge on airway responsiveness in TDI-sensitized and -challenged mice

Airway responsiveness was assessed as a percent increase of enhanced pause (P_{enh}) in response to increasing doses of Mch. In TDI-sensitized and -challenged mice, the dose-response curve of percent P_{enh} shifted to the left side compared with control mice (Fig. 2). In addition, the percent P_{enh} produced by Mch (from 2.5 to 50 mg/mL) increased significantly in the TDI-sensitized and -challenged mice compared with controls. Thus, mice sensitized and challenged with TDI exhibit airway hyper-responsiveness.

Total and differential cell counts in BAL fluids

The total cell counts and differential cell counts before and after challenge with TDI are shown in Fig. 3. Macrophages were the major cellular component in the BAL fluids obtained during the prechallenge period, but their numbers did not change significantly after TDI inhalation. On the other hand, the numbers of neutrophils and lymphocytes were markedly increased in the BAL fluids at 7 h after TDI inhalation. Their numbers continued to increase up to 72 h after TDI inhalation. The numbers of eosinophils were increased significantly from 24 to 72 h after TDI inhalation. Overall, the total numbers of cells were significantly greater from 7 to 72 h after TDI inhalation. In contrast, no significant changes in the cellular components were observed after inhalation of the control agent, ethyl acetate:olive oil.

MMP-9 expression in lung tissues of mice sensitized and challenged with TDI

Western blot analysis revealed that levels of MMP-9 protein in lung tissues increased approximately 2.7-, 5.7-, 5.7-, and 5.8-fold at 7, 24, 48, and 72 h after TDI inhalation, respectively, compared with the control group. In contrast, no significant changes in MMP-9 protein levels were observed after inhalation of the vehicle control (Figs 4a and b). The expression of MMP-9 mRNA in the lung tissues increased

approximately 3.1-, 5.9-, 5.9-, and 5.9-fold at 7, 24, 48, and 72 h after TDI inhalation, respectively, compared with the control group. In contrast, no significant change in MMP-9 mRNA expression was observed after inhalation of the vehicle control (Figs 4c and d).

TIMP-1 expression in lung tissues of mice sensitized and challenged with TDI

Western blot analysis revealed that TIMP-1 protein levels in the lung tissues increased approximately 6.9-, 10.4-, 10.6-, and 10.7-fold at 7, 24, 48, and 72 h after TDI inhalation, respectively, compared with the control group. In contrast, no significant changes in TIMP-1 protein levels were observed after inhalation of the vehicle control (Figs 5a and b). TIMP-1 mRNA expression in the lung tissues increased approximately 5.0-, 10.6-, 10.6-, and 11.2-fold at 7, 24, 48, and 72 h after TDI inhalation, respectively, compared with the control group. In contrast, no significant change in TIMP-1 mRNA expression was observed after inhalation of the vehicle control (Figs 5c and d).

MMP-9 and TIMP-1 levels in the BAL fluids of TDI-sensitized and -challenged mice

Gelatin zymography revealed that proMMP-9 levels in the BAL fluids were markedly increased at 7, 24, 48, and 72 h after TDI inhalation compared with levels before inhalation or in the control group. In contrast, no significant change in the proMMP-9 level was observed after inhalation of the vehicle control (Fig. 6a). Enzyme immunoassay revealed that the concentration of MMP-9 was increased at 7, 24, 48, and 72 h after TDI inhalation (86.8 ± 5.9 , 153.2 ± 7.6 , 157.5 ± 10.0 , and 159.7 ± 10.9 pg/mL, respectively) compared with the control group (33.5 ± 2.0 pg/mL). The concentration of TIMP-1 was increased at 7, 24, 48, and 72 h after TDI inhalation (228.8 ± 11.4 , 366.5 ± 11.1 , 374.3 ± 14.8 , and 384.5 ± 10.9 pg/mL, respectively) compared with the control group (50.8 ± 5.5 pg/mL). In contrast, there were no significant changes in the levels of MMP-9 or TIMP-1 after inhalation of the control agent, ethyl acetate:

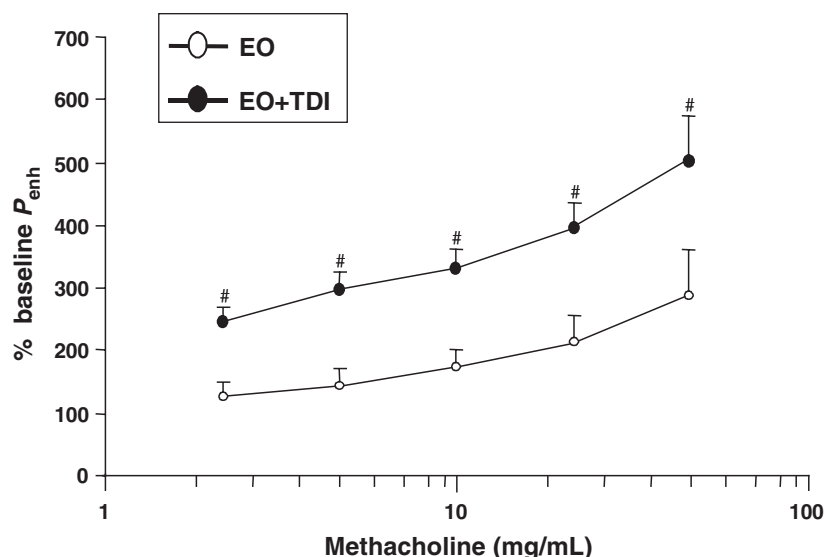


Fig. 2 Effect of toluene diisocyanate (TDI) challenge on airway responsiveness in TDI-sensitized and -challenged mice. Mice were treated as described in Fig. 1, and airway responsiveness was measured at 72 h after treatment with vehicle only (EO) or EO plus TDI. Airway responsiveness to aerosolized methacholine (Mch) was measured in unrestrained, conscious mice. Mice were placed into the main chamber and were nebulized first with saline, then with increasing doses (2.5–50 mg/mL) of Mch for 3 min for each nebulization. Readings of breathing parameters were taken for 3 min after each nebulization during which P_{enh} -values were determined. Six mice in each group were tested. $\#P < 0.05$ vs. EO.

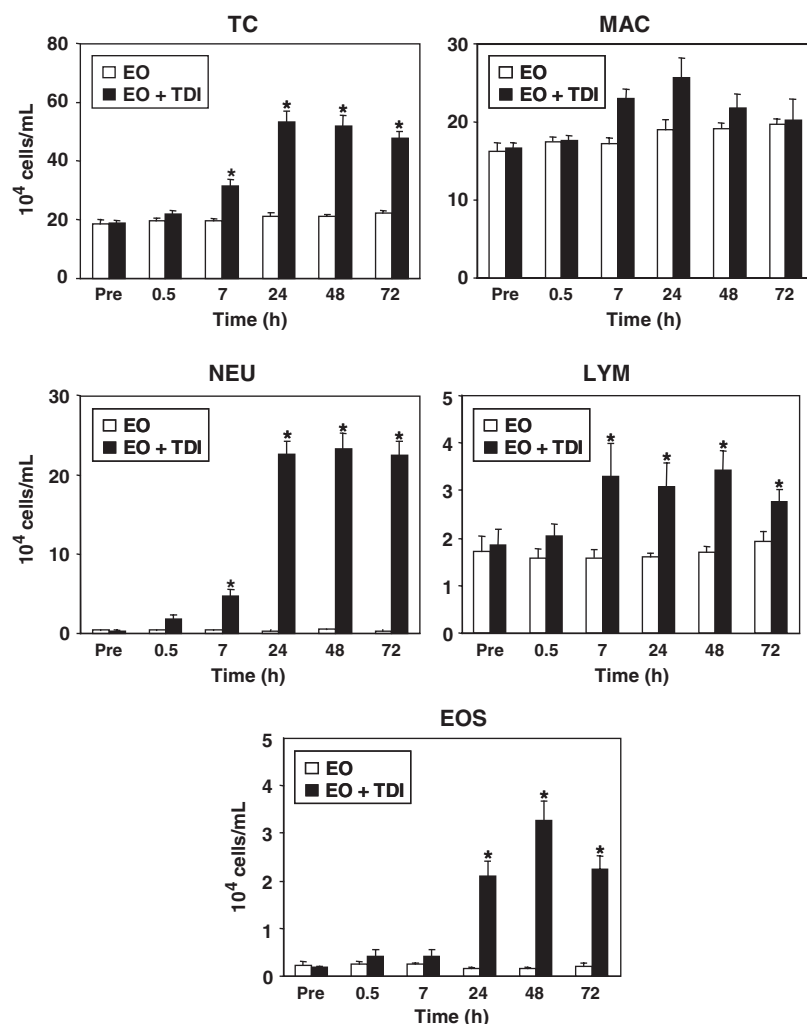


Fig. 3. Effect of toluene diisocyanate (TDI) challenge on total and differential cellular components in bronchoalveolar lavage (BAL) fluids. In BAL fluids from TDI-sensitized and -challenged mice (EO+TDI) or from vehicle control mice (EO), the numbers of cells were counted. Six BAL fluid samples from six mice were analysed at each time-point. Pre, before challenge; 0.5, 7, 24, 48, and 72 h after challenge. TC, total cell; MAC, macrophage; NEU, neutrophil; LYM, lymphocyte; EOS, eosinophil. *significantly different from preinhalation and vehicle control mice ($P < 0.05$).

olive oil (Figs 6b and c). We also counted the lymphocytes, neutrophils, and eosinophils in the BAL fluids at the same time-points. There were significant correlations between the levels of MMP-9 or TIMP-1 and the numbers of lymphocytes, neutrophils, and eosinophils (data not shown).

Ratio of MMP-9/TIMP-1 in mice sensitized and challenged with TDI

In TDI-exposed mice, the molar ratio of MMP-9/TIMP-1 was significantly decreased at 7 h after TDI inhalation and continued at almost the same level up to 72 h (Fig. 7). In contrast, no significant changes of the molar ratio of MMP-9/TIMP-1 were observed in control mice.

Expression of MMP-9 and TIMP-1 in the airway

Immunohistochemical analyses of lung tissues of TDI-exposed mice revealed immunoreactive MMP-9 on inflammatory cells and in the ECM around bronchioles, and on inflammatory cells and debris in the airway lumen (Fig. 8b). In the control mice, positive MMP-9 staining was seen only in the airway epithelium (Fig. 8a). Immunocytologic analyses in the BAL fluids of TDI-exposed mice revealed that the

immunoreactive MMP-9 was localized on BAL cells, including cells that resembled macrophages, neutrophils, or eosinophils (Fig. 8d). In the control mice, almost no MMP-9 positive cells were detected (Fig. 8c). Similar results were obtained with respect to TIMP-1. In the TDI-exposed mice, positive TIMP-1 staining was seen on inflammatory cells and in the ECM around bronchioles and on inflammatory cells and debris in the airway lumen (Fig. 8f). However, in the control mice, positive TIMP-1 staining was seen only in the airway epithelium (Fig. 8e). Immunocytologic analyses of BAL fluids showed abundant TIMP-1-positive cells in the TDI-exposed mice, but not in the control mice (Figs 8g and h).

Discussion

Airway inflammation is an important pathophysiologic feature, and structural changes and remodelling of ECM components are believed to be important causative factors of airflow obstruction in asthma [27]. In this study, we found that induction of asthma by inhalation of TDI increased the expression of MMP-9 and TIMP-1 mRNAs and proteins,

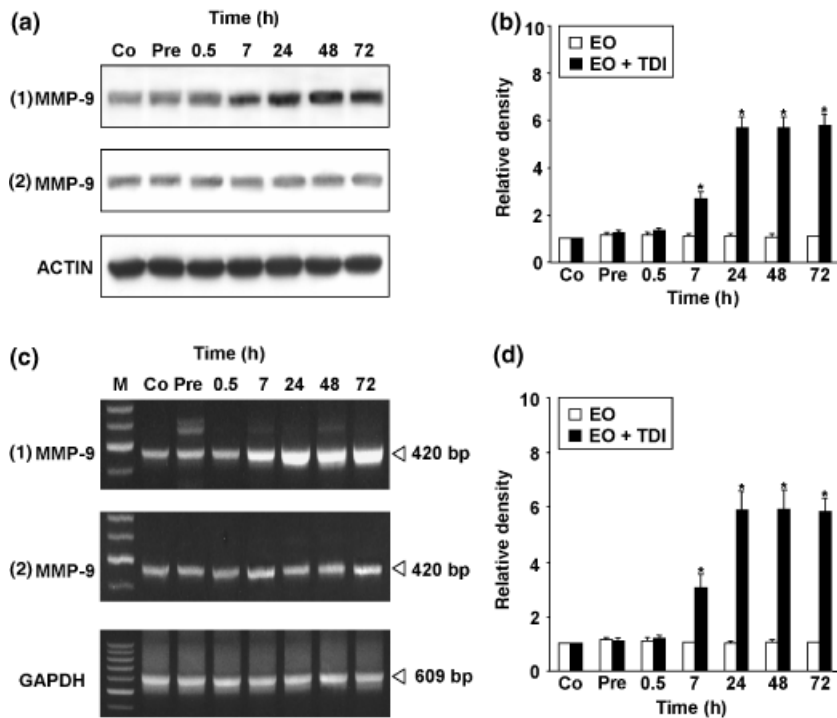


Fig. 4. Effect of toluene diisocyanate (TDI) challenge on matrix metalloproteinase-9 (MMP-9) expression in lung tissues. (a) Western blotting of MMP-9. (1) EO+TDI; (2) EO, vehicle control; Co, no treatment; Pre, before challenge; 0.5, 7, 24, 48, and 72 h after challenge. (b) Densitometric analyses are presented as the relative ratio of MMP-9 to actin. The relative ratio of MMP-9 in the lung tissues of control mice (Co) is arbitrarily presented as 1. (c) RT-PCR of MMP-9. (d) Densitometric analyses are presented as the relative ratio of MMP-9 mRNA to GAPDH mRNA. The relative ratio of MMP-9 mRNA in the lung tissues of control mice (Co) is arbitrarily presented as 1. Six mice at each time-point were tested. * $P < 0.05$ vs. Pre; # $P < 0.05$ vs. EO.

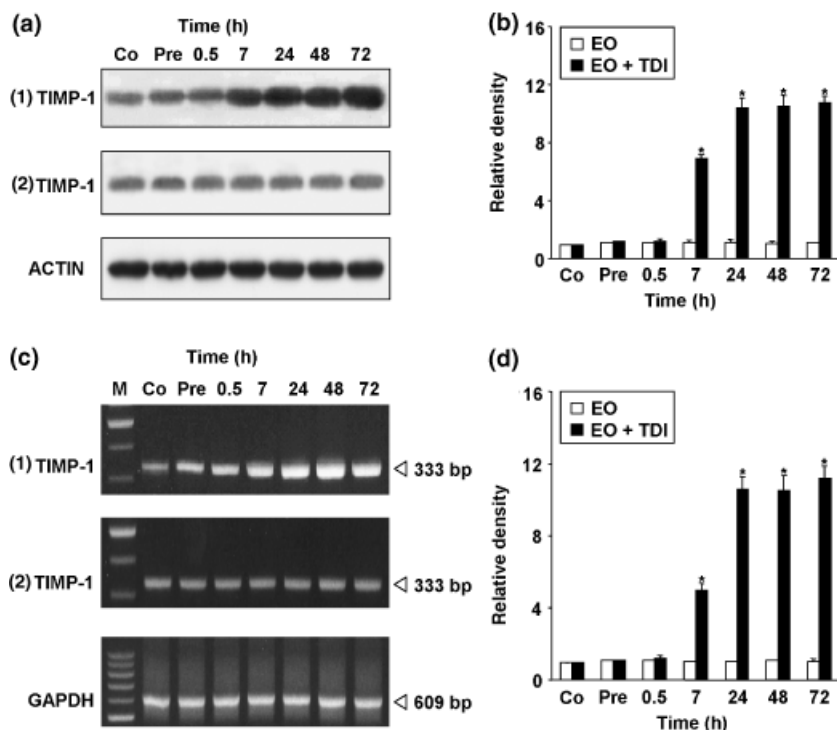


Fig. 5. Effect of toluene diisocyanate (TDI) challenge on tissue inhibitor of metalloproteinase-1 (TIMP-1) expression in lung tissues. (a) Western blotting of TIMP-1. (1) EO+TDI; (2) EO, vehicle control; Co, no treatment; Pre, before challenge; 0.5, 7, 24, 48, and 72 h after challenge. (b) Densitometric analyses are presented as the relative ratio of TIMP-1 to actin. The relative ratio of TIMP-1 in the lung tissues of control mice (Co) is arbitrarily presented as 1. (c) RT-PCR of TIMP-1. (d) Densitometric analyses are presented as the relative ratio of TIMP-1 mRNA to GAPDH mRNA. The relative ratio of TIMP-1 mRNA in the lung tissues of control mice (Co) is arbitrarily presented as 1. Six mice at each time-point were tested. * $P < 0.05$ vs. Pre; # $P < 0.05$ vs. EO.

and that there were significant correlations between the levels of MMP-9 or TIMP-1 and the numbers of neutrophils, lymphocytes, or eosinophils. The molar ratio of MMP-9/TIMP-1 decreased significantly in TDI-exposed mice.

TDI is widely used compound for the production of many chemical products and is well known to cause occupational asthma [13, 14]. Although considerable controversy remains regarding its pathogenesis, TDI-induced asthma is characterized by airway inflammation and hyper-responsiveness to

TDI [14, 20]. Our present study showed that the expression of MMP-9 and TIMP-1 mRNAs and proteins was up-regulated in association with the accumulation of inflammatory cells in the lung in a murine model of TDI-induced asthma. Although sources of MMP-9 and TIMP-1 detected in BAL fluids after TDI inhalation were not determined in this study, an increase in the expression of MMP-9 and TIMP-1 in BAL cells suggests that inflammatory cells are potential sources of these proteins. Supporting this notion, several reports also indicate

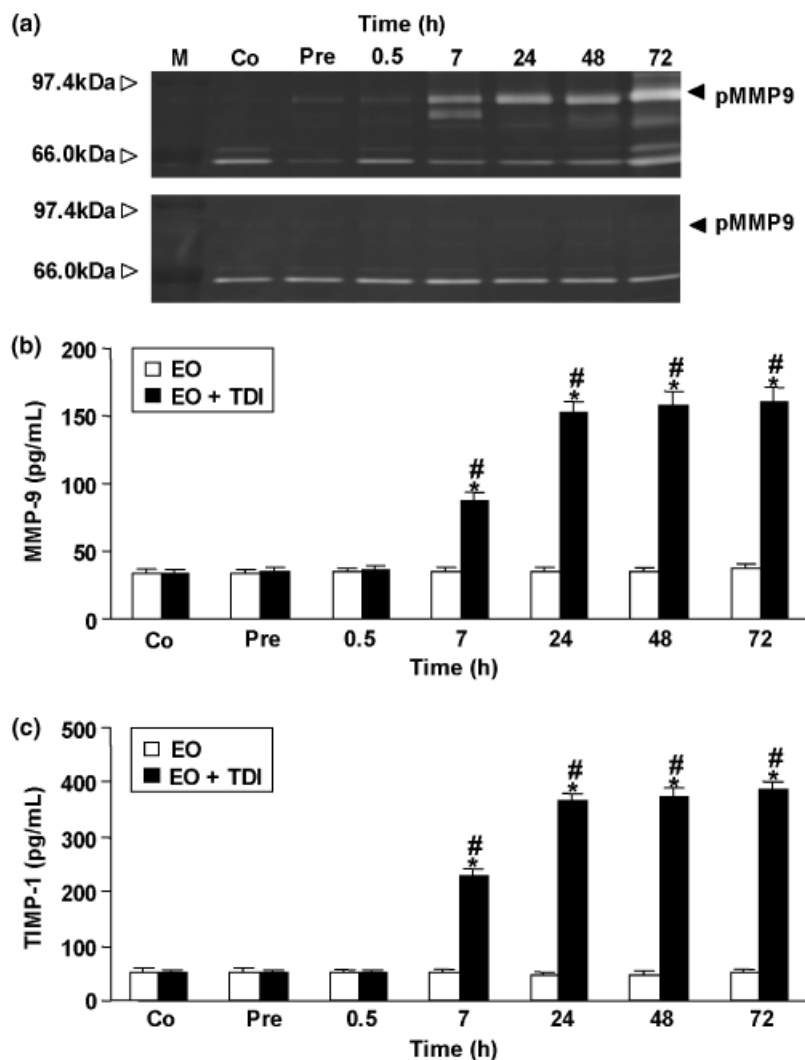


Fig. 6. Effect of toluene diisocyanate (TDI) challenge on matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) secretions in bronchoalveolar lavage (BAL) fluids. Mice were treated as described in Fig. 1. (a) Gelatin zymography. In each lane, 10 µg protein from each BAL fluid was loaded. The animals were challenged with TDI in ethyl acetate (upper panel) or vehicle control (EO) alone (lower panel). Molecular weight markers (open arrowheads on the left) were used to estimate molecular masses. pMMP9, the proform of MMP-9; Co, no treatment; Pre, before challenge; 0.5, 7, 24, 48, and 72 h after challenge. (b) Enzyme immunoassay of MMP-9. (c) Enzyme immunoassay of TIMP-1. Six mice at each time-point were tested. * $P < 0.05$ vs. Pre; # $P < 0.05$ vs. EO.

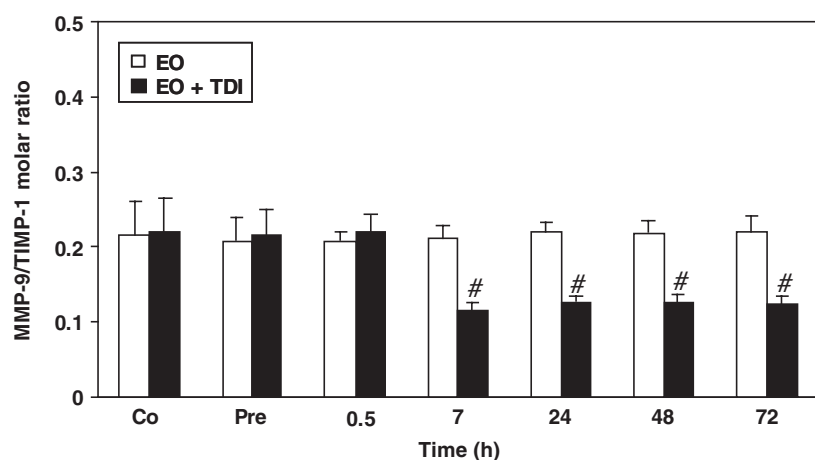


Fig. 7. Effect of toluene diisocyanate (TDI) challenge on matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) molar ratio in bronchoalveolar lavage (BAL) fluids. Mice were treated as described in Fig. 1. Six mice at each time-point were tested. EO, vehicle control; Co, no treatment; Pre, before the challenge; 0.5, 7, 24, 48, and 72 h after challenge. # $P < 0.05$ vs. Pre and EO.

that MMP-9 and TIMP-1 were observed in macrophages, eosinophils, and neutrophils in asthma [10, 28, 29]. Recently, we demonstrated that TDI-induced occupational asthma is associated with the induction of MMP-9 in inflammatory cells, and the administration of the MMP inhibitor reduces the migration of inflammatory cells through the

endothelial and epithelial basement membranes [22]. Consistent with these observations, we found that the levels of MMP-9 in the BAL fluids were significantly increased after TDI inhalation. The expression of MMP-9 mRNA and protein was also increased in the lung tissues after TDI inhalation.

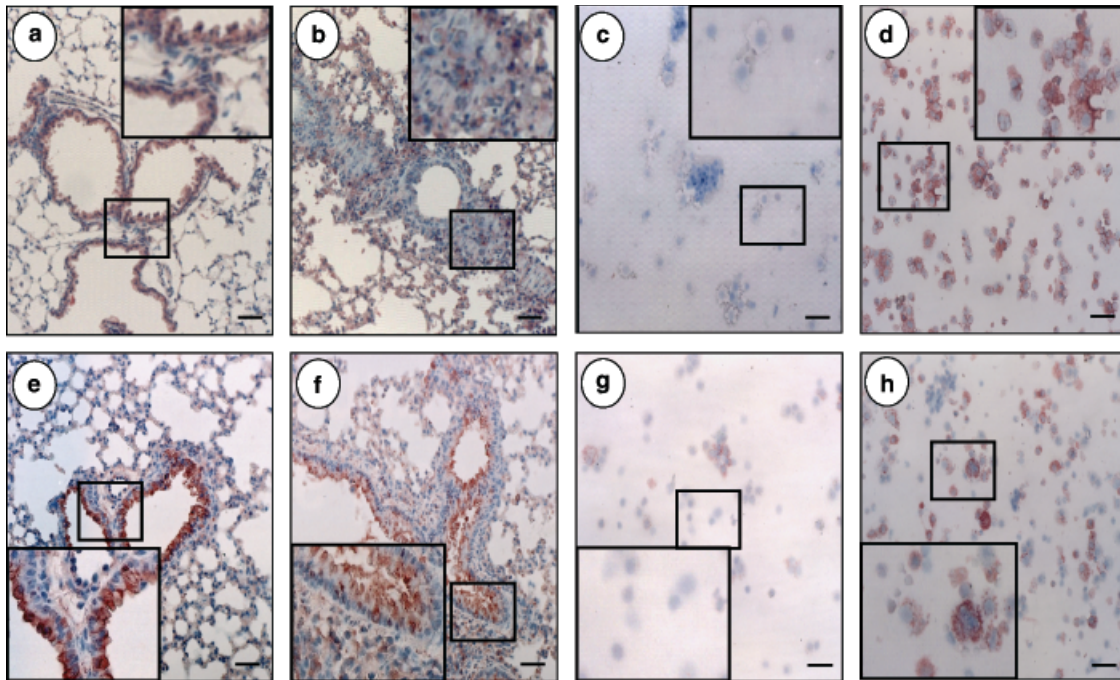


Fig. 8. Effect of toluene diisocyanate (TDI) challenge on matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression in lung tissues and bronchoalveolar lavage (BAL) fluids of TDI-sensitized and challenged mice. Mice were treated as described in Fig. 1, and sampling was performed at 72 h after treatment with vehicle only (a, c, e, and g), vehicle plus TDI (b, d, f, and h). Representative light microscopy of MMP-9-positive cells in the bronchioles (a and b); the dark brown colour indicates MMP-9-positive cells. Representative light microscopy of TIMP-1-positive cells in the bronchioles (e and f); the dark brown colour indicates TIMP-1-positive cells. Representative light microscopy of MMP-9-positive cells in the BAL fluids (c and d); the brownish pink colour indicates MMP-9-positive cells. Representative light microscopy of TIMP-1-positive cells in the BAL fluids (g and h); the brownish pink colour indicates TIMP-1-positive cells. Bars indicate a scale of 50 μ m.

TIMP-1 binds non-covalently in a 1:1 proportion with MMP-9 or proMMP-9 and inhibits their enzymatic activity [30–32]. Our results show that the levels of TIMP-1 mRNA and protein were significantly up-regulated in the BAL fluids as well as in the lung tissues after TDI inhalation compared with the levels before inhalation or in the control group. The molar concentrations of TIMP-1 were much higher than that of MMP-9, indicating that TDI inhalation may alter the balance of the protein ratio. Since TIMP-1 inhibits MMP-9 activity that is involved in the degradation of ECM and subepithelial fibrosis is one of the general pathologic features, the decreased ratio of MMP-9/TIMP-1 by TDI inhalation may cause the deposition of ECM that leads to structural changes of the airway wall during the airway tissue destruction and repair processes in TDI-induced asthma. In addition, the induction of TIMP-1 by TDI inhalation is probably caused by the several mediators released during the development of airway inflammation. It has been observed that transforming growth factor- β increases the production of TIMPs [33], and that the expression of this protein is increased in the airways of TDI-induced asthmatic patients [34].

In conclusion, we used a murine model of TDI-induced asthma. Murine TDI-induced asthma shows typical pathophysiologic features, such as airway hyper-responsiveness, airway inflammation, and increased expression of MMP-9 and TIMP-1 mRNAs and proteins in the lungs. We also found that the ratio of MMP-9/TIMP-1 was significantly decreased in this TDI-induced asthma model. These findings suggest that the ratio of MMP-9/TIMP-1 could be used as a marker of airway inflammation in TDI-induced asthma.

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