

Enhancement of gelatinase activity during development of subepithelial fibrosis in a murine model of asthma

M. Corbel, S. Caulet-Maugendre*, N. Germain, V. Lagente and E. Boichot

INSERM U 456, Faculté des Sciences Pharmaceutiques et Biologiques, Université de Rennes 1 and *Anatomie Pathologie B, CHR Pontchaillou, Rennes, France

Summary

Background Chronic asthma is characterized by inflammatory cell infiltration and tissue remodeling leading to subepithelial fibrosis. Metalloproteinases (MMPs) are involved in degradation of extracellular matrix in most chronic inflammatory diseases.

Objective The aim of this study was to investigate the expression of MMPs in the development of inflammatory processes associated or not with the concomitant development of subepithelial fibrosis in an experimental model of asthma.

Methods Sensitized BP2 mice were challenged with ovalbumin (OA) every 2 weeks during 8 months. Several mice were removed once a month and bronchoalveolar lavages (BAL) or lung biopsies were performed.

Results Lung sections stained with picrosirius and hydroxyproline measurements showed a significant collagen deposition after 16 weeks of OA challenge, demonstrating the development of subepithelial fibrosis. Pulmonary inflammation was present from the first OA challenge and was consistent throughout the 8 months of the study. Moreover, an up-regulation and activation of MMP-9 and, to a less extent, MMP-2 were observed in BAL fluid from challenged mice. The level of tissue inhibitor of metalloproteinases (TIMP)-1 increased after 12 weeks of OA challenge vs. control mice.

Conclusion This study reveals that a decrease in the activation of the MMP-9 due to the increase in TIMP-1, could contribute to excessive collagen deposition following repeated antigen challenge in sensitized mice.

Keywords fibrosis, metalloproteinase, mice, ovalbumin, subepithelial, tissue inhibitor of matrix metalloproteinase

Submitted 17 December 2001; revised 2 August 2002; accepted 27 September 2002

Introduction

Chronic airway inflammation plays a major role in the pathogenesis of asthma. Bronchial asthma is featured by airways infiltration of inflammatory cells, namely eosinophils and T lymphocytes [1]. These cells are thought to contribute to the structural changes that characterize asthma. Moreover, an inadequate bronchial mucosa repair process initiated by chronic inflammation and resulting in mucosal structural changes plays an important role in the pathogenesis of asthma [2]. For many years, pathologists have described a basement membrane thickening as a feature of asthmatic airways [3]. It now appears that the basement membrane is actually normal in asthmatic airways but that in fact the thickening concerns the reticular lamina and is the result of a subepithelial fibrotic response characterized by an increase in type-I, type-III and type-V collagen deposition [4]. Several studies have highlighted the importance of myofibroblast hyperplasia and hypertrophy,

and epithelial alterations. A correlation has been found between the extent of subepithelial collagen with the number of myofibroblasts [5] and the severity of asthma [6]. More recently, Vignola et al. [7] confirmed the correlation between the number of subepithelial myofibroblasts and the degree of subepithelial thickening in patients with asthma, suggesting that fibroblasts play a role in exacerbating the deposition of extracellular collagen in the airway wall of asthmatics.

Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent endopeptidases, are involved in tissue remodelling through their capacity to degrade almost all components of the extracellular matrix (ECM). These enzymes are synthesized as zymogens that require activation to achieve their full catalytic competence. Latency is maintained by interaction between a sulphhydryl group in the prodomain and a Zn²⁺ atom at the catalytic site. The disruption of this interaction results in activation [8, 9]. The major physiological inhibitors of MMPs are the broad-spectrum serum inhibitor α 2-macroglobulin and a special class of tissue inhibitors of metalloproteinases (TIMPs) [10]. Among MMPs, two gelatinases have been particularly studied in asthma. MMP-2 is preferentially secreted by fibroblasts and epithelial cells [10, 11], whereas MMP-9 is preferentially secreted by inflammatory cells, including macrophages [12], lymphocytes [13], neutrophils [14] and

Correspondence: Vincent Lagente, INSERM U 456, Faculté des Sciences Pharmaceutiques et Biologiques, Université de Rennes 1, 2 Avenue du Prof Léon Bernard, 35043 Rennes cedex, France.
E-mail: vincent.lagente@rennes.inserm.fr

eosinophils [15]. These gelatinases show a catalytic activity towards denatured fibrillar collagens (gelatin), and degrade native collagens (type-IV collagen as well as type-I, -III, -V and -XI collagens) and other structural proteins (fibronectin, laminin, elastin and proteoglycan core protein) [16, 17]. Among TIMPs, which exert their inhibitory activity through a high-affinity binding to active MMPs, TIMP-1 and TIMP-2 act mainly on MMP-9 and MMP-2, respectively. In addition, TIMP-1 and TIMP-2 can be also associated with latent MMP-9 and MMP-2, respectively [8].

With regard to lung diseases, MMPs have been implicated in the pathophysiology of acute and chronic inflammatory disorders, including acute respiratory distress syndrome (ARDS), interstitial lung fibrosis and cystic fibrosis [16]. The possible implication of MMPs in asthma is suggested by morphological studies where matrix remodelling, lung tissue damage, bronchial and alveolar structure alteration, or abnormal repair have been described [4, 5, 18]. Moreover, increased levels of MMP-9 have been found in bronchoalveolar lavage (BAL) fluids, in bronchial biopsies from asthmatic patients [15, 19] and in alveolar macrophages from asthmatic patients [12, 20–22]. Recently, an increased production of TIMP-1 has been observed in human alveolar macrophages from asthmatic patients [20]. These data suggest that the balance between MMP and TIMP biological activities may represent an important determinant of the nature of the remodelling process.

In order to investigate the expression of MMPs and TIMPs in the airway remodelling associated with inflammatory processes leading to pulmonary fibrosis, we developed a murine experimental model of allergic asthma that may reproduce the pathological features of remodelled asthmatic airways. We also investigated the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in the generation of airway injury and tissue repair during chronic (long time course) ovalbumin challenge in sensitized mice.

Materials and methods

Materials

Ovalbumin, gelatin, ethylene diamine tetraacetic acid (EDTA), triton X-100, hydroxyproline, phenanthroline, N-ethylmaleimide (NEM) and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma (St Louis, MO, USA). May-Grünwald and Giemsa stains were from RAL (Paris, France). Sodium pentobarbital was from Sanofi santé animale (Libourne, France). Acrylamide was from ICN (Aurora, OH, USA) and Coomassie blue was from Biorad (München, Germany). RPMI 1640, penicillin, streptomycin, L-glutamine, sodium dodecyl sulphate (SDS) and Tris solution were from Eurobio (Les Ulis, France). TGF- β and IL-5 antibodies were provided by R & D Systems (Minneapolis, MN, USA). TGF- β antibodies (MAB1835) recognize mouse TGF- β 1 and TGF- β 2. TIMP-1 and TIMP-2 antibodies were purchased from Chemicon International (Temecula, CA, USA) and SantaCruz Biotechnology (Santa Cruz, CA, USA), respectively.

Animal models

We followed sensitization and challenge protocols that have been previously described [23]. Briefly, 6-week-old male BP2 mice (CERJ, Le Genest Saint Isle, France) were sensitized by

a subcutaneous injection in the cervical area of 100 μ g OA adsorbed with aluminium hydroxide. One week later the mice received a similar injection. After an additional week, mice were slightly anaesthetized with ether and challenged intranasally every 2 weeks with 10 μ g OA or saline solution alone as control. Forty-eight hours after the first challenge and then once a month, 48 h after antigen challenge, groups of control and OA exposed-mice were analysed.

Mice were intraperitoneally anaesthetized with sodium pentobarbital (60 mg/kg) in order to perform BAL or to remove the lungs.

Bronchoalveolar lavage

After semiexcision of the trachea, a plastic cannula was inserted and air spaces were washed with 0.5 mL of 0.9% NaCl containing 2.6 mM EDTA using a 1-mL syringe. This operation was carried out 10 times and the recovery of the total lavage volume (5 mL) exceeded 95%. After centrifugation (600 g for 10 min, 4 °C), the fluid phase of the first millilitre of BAL fluid was aliquoted and frozen at –80 °C for cytokine and metalloproteinase activity measurements. The cell pellets of the first part of the BAL were added to the rest of the BAL fluid. After lysis of red blood cells with distilled water, BAL cell pellets were resuspended in 500 μ L of RPMI 1640 medium supplemented with 50 UI/mL penicillin, 50 μ g/mL streptomycin and 2 mM L-glutamine.

Total cell count was evaluated using an haemocytometer chamber and the viability was determined by the trypan blue exclusion method. BAL cells were adjusted to a concentration of 5×10^5 cells/mL in supplemented RPMI. After cytocentrifugation (Cytopro 7620 WESCOR) at 700 r.p.m. for 10 min, the cells were stained with May-Grünwald-Giemsa. Differential counts on 200 cells were made using standard morphological criteria.

Zymographic analysis of MMPs

Using zymography, MMPs were detected in BAL by their capacity to degrade gelatin. Defined volumes of BAL fluid were subjected to electrophoresis onto a 4.5% acrylamide stacking gel/7.5% acrylamide separating gel containing 1 mg/mL gelatin, in the presence of sodium dodecyl sulphate (SDS) under non-reducing conditions as previously described [24]. After electrophoresis, gels were washed twice with 2.5% triton X-100, rinsed with water and incubated at 37 °C overnight in 50 mM Tris, 5 mM CaCl_2 , 1 mM ZnCl_2 , pH 8. Then, the gels were stained with Coomassie Brilliant Blue and destained in a solution of 25% ethanol and 10% acetic acid. Gelatinase activities appeared as clear bands against a blue background. To determine the inhibition profile of the enzyme activities, we also incubated gels in the presence of one of the following inhibitors in the activation buffer: 10 mM EDTA, 10 mM Phenanthroline (two inhibitors of MMPs) and 1 mM PMSF, 2 mM NEM (two inhibitors of serine protease). BAL fluids from LPS-exposed mice [25] were used as positive control and were run in parallel to confirm that gelatinases observed in BAL fluid samples from OA-exposed mice were MMP-2 and MMP-9. Moreover, molecular weights of gelatinolytic bands were estimated using recombinant protein molecular weight marker (10 to 225 kDa). The gels were scanned and the black-white image was inverted. Enzyme amount was quantified by measuring the intensity of the

negative bands using a densitometric analyser ('Densylab' software package; Bioprobe Systems, les Ulis, France) that quantifies both surface and intensity of lysis bands. Results were expressed as a percentage of a band of migration of an internal standard loaded onto each gel in order to allow comparison between gels.

Histological preparations and hydroxyproline measurement

For each mouse, two lobes of lung were removed and fixed in 10% formalin and then embedded in paraffin; 5 µm sections were stained using Picro-sirius haematoxyline dye. The other lobes were frozen in cold isopentane before storage at -80°C .

The extent of pulmonary fibrosis was evaluated by an estimation of total lung collagen deposition as reflected by the measurement of the hydroxyproline (OHproline) content in control and antigen-exposed mice. Frozen lung tissue was lyophilized, weighed, ground to a fine powder in a mortar and pestle and homogenized in potassium phosphate buffer (PBS), pH 7.4. The OHproline content of duplicate aliquots of the homogenates was assessed as previously described [26]. The homogenates were centrifuged and the supernatants were aliquoted and frozen at -80°C for TGF- β analysis.

Measurement of cytokine release in BAL fluids

IL-5 and TGF- β were evaluated by Western blot. The same amounts of proteins (2 µg) recovered from BAL fluids or supernatants of tissue homogenates were loaded onto SDS-PAGE under reducing conditions, and were transferred electrophoretically onto nitrocellulose membranes (Hybond-ECL, Amersham Life Science, Amersham, UK). After blocking the blots with 3% Bovine Serum Albumin (BSA) in Tris-buffered saline (TBS), they were incubated with anti-mouse IL-5 or TGF- β monoclonal antibodies (R & D Systems) diluted in TBS containing 0.1% BSA and 0.3% NP-40. The blots were then washed three times for 10 min in TBS and incubated with peroxidase-conjugate IgG. All incubations were performed at room temperature (RT) for 2 h. After repeated lavages, the membranes were incubated in equal volumes of detection reagents 1 and 2 (ECL Western blotting detection reagents from Amersham, UK) for 1 min at RT and immediately exposed to X-ray film.

IL-5 level was quantified by measuring the intensity of the signals using a densitometric analyser ('Densylab' software package; Bioprobe Systems). Results were expressed as a percentage of an internal standard loaded onto blots in order to allow comparison between filters. The quantification of IL-5 was performed throughout the study, whereas TGF- β was analysed after one OA challenge and after 36 weeks of repeated

OA challenges. Under reducing conditions, the antibody for TGF- β detects the active TGF- β homodimer (25 kDa).

Western blot analysis of TIMPs

In a preliminary experiment using Western blotting, we were not able to observe TIMPs expression in individual BAL fluids of several mice. Therefore, BAL fluids from mice killed in the same experiment were pooled ($n = 5$) and concentrated ($\times 30$). The samples were loaded to SDS-PAGE under reducing conditions, and were transferred electrophoretically onto nitrocellulose membranes (Hybond-ECL, Amersham Life Science). After blocking the blots with 5% non-fat milk in Tris-buffered saline/0.1% Tween 20 (TBS-T), they were incubated overnight at 4°C with rabbit anti-human TIMP-1 or goat anti-human TIMP-2 polyclonal antibodies in TBS-T containing 1% BSA. The blots were then washed three times for 15 min in TBS-T and incubated for 2 h at RT with the appropriate peroxidase-conjugate IgG. Membranes were washed four times in TBS-T for 15 min, and specific bands were visualized using the ECL system from Amersham according to the manufacturer's instructions.

Expression of the results and statistical analysis

The results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed with Statview software from Abacus concepts Inc. (Berkeley, CA, USA) on an Apple computer. Analysis between the different groups was performed by ANOVA and Fischer's test. For each analysis, $P < 0.05$ was considered statistically significant.

Results

Total number of cells and cellular composition in BAL

As the values obtained in control mice were constant at each time of our kinetic study and present non-significant variation (data not shown), we decided to average these values for each parameter studied. This is illustrated by a single point named 'C' on Figs 1, 2, 3, 6 and 8.

A significant increase in the total number of BAL cells from OA-challenged mice was observed after 8 weeks in comparison with saline-challenged mice (Fig. 1). The increase in total cell numbers was associated with a significant increase in eosinophils (Fig. 2b), lymphocytes (Fig. 2c) and to a less extent in neutrophils (Fig. 2d) throughout the experiments. The increase in macrophages (Fig. 2a) was significant from 16 weeks of repeated OA challenge.

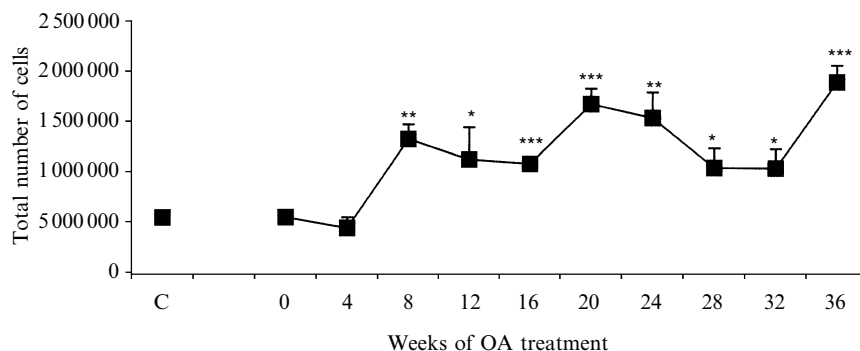


Fig. 1. Total number of cells in BAL fluids from OA-exposed mice ($n = 5$) throughout the study vs. control mice (C, $n = 29$). The results are presented as mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

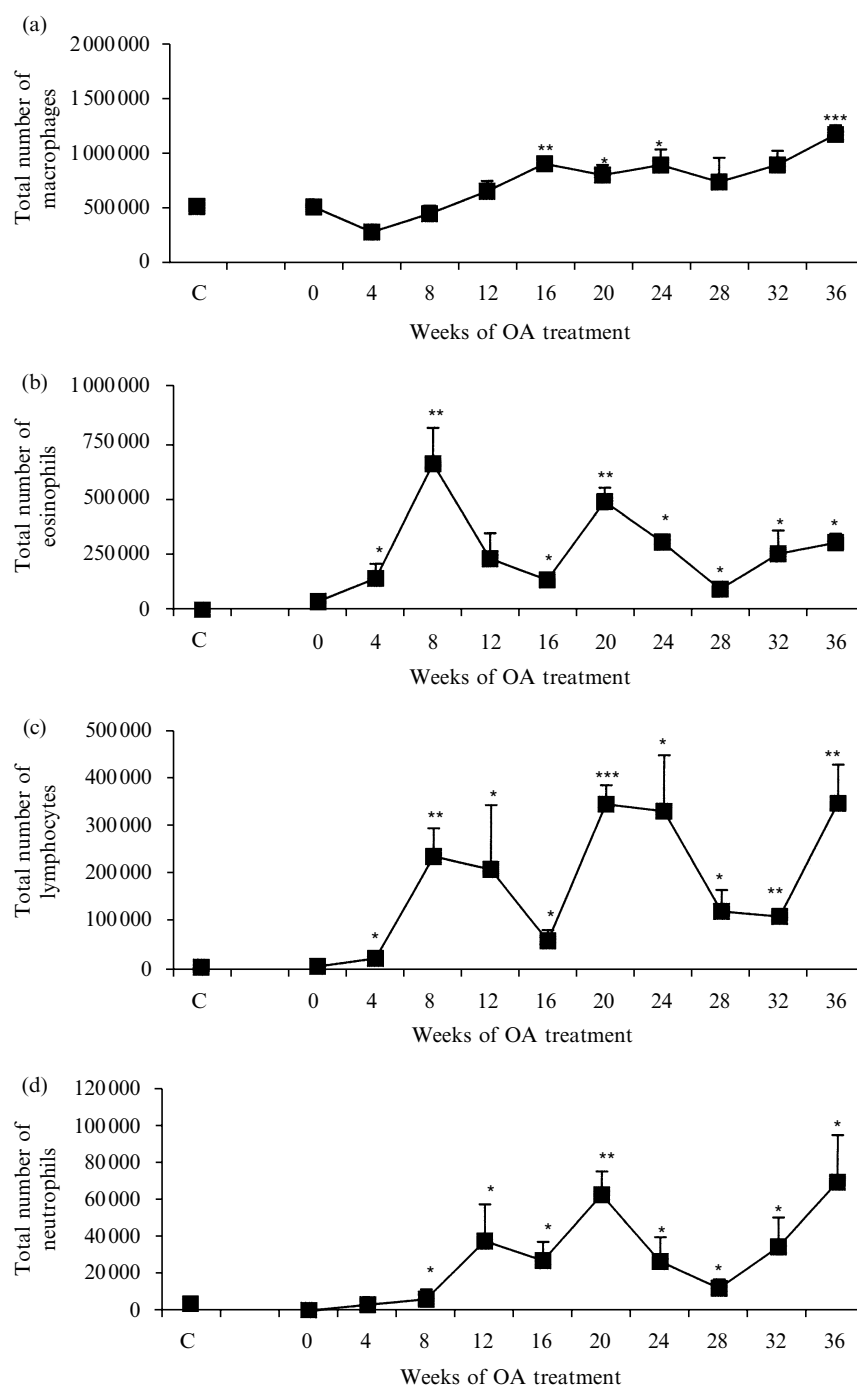


Fig. 2. Time course of macrophages (a), eosinophils (b), lymphocytes (c) and neutrophils (d) collected in BAL fluids from OA-exposed mice ($n=5$) vs. control (C, $n=29$). There were no eosinophils in BAL fluids from saline-exposed mice. The results are presented as mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Cytokine measurements

IL-5 levels were significantly increased in BAL from 8 weeks of repeated ovalbumin challenge in comparison with control mice (Fig. 3).

TGF- β levels were analysed in BAL and tissues from sensitized mice exposed to one ovalbumin challenge and after 36 weeks of repeated ovalbumin provocation (Fig. 4). No difference in TGF- β was noted between mice exposed to a single ovalbumin administration (lanes 2 to 4) and control mice (lanes 1 and 5), either in BAL or in tissues. However, we observed a strong increase in TGF- β both in BAL and tissues from

mice exposed to repeated ovalbumin challenge during 36 weeks (lanes 6 to 8).

Histological study and hydroxyproline measurement in the lungs

Histological preparations showed an intense inflammatory process in the lungs accompanied by the emergence of a subepithelial collagen deposition and smooth muscle hypertrophy/hyperplasia. Lungs from the control mice did not present any inflammatory infiltrate or any modification of the pulmonary structure at any time during our kinetic study (Fig. 5a).

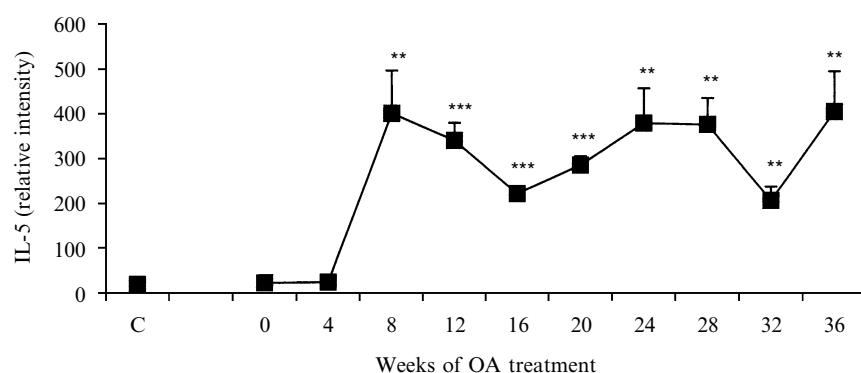


Fig. 3. Evaluation of IL-5 level in BAL fluids from OA-exposed mice ($n = 5$) throughout the study vs. control mice (C, $n = 29$). IL-5 amount was quantified by Western blot and by measuring the intensity of the signals using densitometric analysis. Results were expressed as a percentage of an internal standard loaded onto filters. The results are presented as mean $\pm$ SEM. ** $P < 0.01$; *** $P < 0.001$.

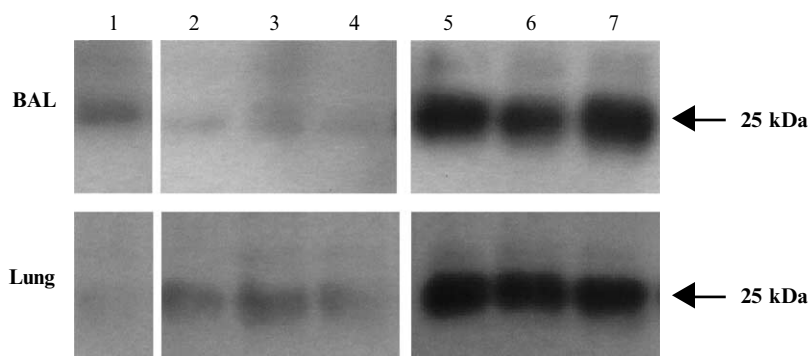


Fig. 4. Representative Western blot analysis of TGF- β level in BAL fluid and lung tissue from OA-exposed mice. Under reducing conditions, the antibody for TGF- β detects the active TGF- β homodimer (25 kDa). Lane 1: control mice (exposed to saline solution). Lanes 2 to 4: mice exposed to a single OA administration (first week of the study). Lanes 5 to 7: mice exposed to OA during 36 weeks.

A representative example of lung sections removed at 20 and 28 weeks after chronic OA challenge is presented in Fig. 5(b,c), respectively. A peribronchial cellular infiltrate including macrophages, lymphocytes, polymorphonuclear neutrophils and eosinophils, was detected throughout the study in the lungs of OA-exposed mice. This inflammatory infiltrate was mainly increased around the bronchioles but was also extended to the interstitium. In some cases lymphoid nodules were also observed. After 20 weeks of OA administration, the smooth muscle mass seems to be increased around the bronchioles, while the basement membrane appeared thickened and multi-lamellar (Fig. 5b). These features of airway remodelling remained constant until the end of the study. An increase in goblet cells was detected after 28 weeks of OA challenge but this observation remained inconstant (Fig. 5c).

Figure 6 shows the changes in total lung collagen throughout the study, as measured by the hydroxyproline content. These results demonstrated the appearance of a dramatic collagen deposition in the lungs from mice challenged with OA during 16 weeks. There was no significant modulation in the total lung collagen content in control mice at any time of the study.

Taken together these results of total lung collagen estimation and histological analysis for observation of lung collagen deposition suggest that these mice have developed a pulmonary subepithelial fibrosis in response to chronic OA challenge.

Zymography analysis

Figure 7 shows an increase in latent (72 kDa) and active (66 kDa) MMP-2 and in the active MMP-9 (88 kDa) in BAL fluids from OA-challenged mice (lanes 5 to 9) in comparison with control mice (lanes 1 to 3). We were able to detect the 62 kDa final active form of MMP-2 in some samples of BAL

fluids (lanes 7 and 8). These gelatinase activities were inhibited by 10 mM EDTA or 10 mM phenanthroline but were not modulated by 1 mM PMSF or 2 mM NEM (data not shown).

Semiquantitative analysis of gelatinolytic activities revealed that the latent MMP-9 activity (105 kDa) [27] remained weak after OA challenge in comparison with the active MMP-9 (88 kDa) (Fig. 8a). Indeed, active MMP-9 increased from 48 h after the first challenge and throughout the study (Fig. 8a). The 88 kDa MMP-9 activity was significantly correlated with the number of neutrophils in BAL fluid ($r = 0.8$, $P = 0.0066$). In comparison with control mice, OA administration led to an increase in the 66 kDa MMP-2 activity from 48 h after the first challenge and remained enhanced throughout 36 weeks of OA treatment (Fig. 8b). Regarding latent MMP-2, its activity was weaker in comparison to the active form.

Metalloproteinase inhibitors: TIMP-1 and TIMP-2

As expected, TIMP-1 signal was detected at around 30 kDa (Fig. 9). TIMP-1 was undetectable, in BAL fluids from mice exposed to repeated OA challenge during 4 weeks (lane 2) and in control mice (lane 1). TIMP-1 level reached its maximum at 12 weeks of repeated OA challenge (lane 3) and then decreased weakly after 16 weeks but remained stable until 32 weeks (lanes 4 to 6). In contrast, TIMP-2 was undetectable by Western blot in both control and OA-exposed mice even using concentrated BAL (data not shown).

Discussion

In the present report, we showed that the development of pulmonary allergic inflammation induced by repeated antigen

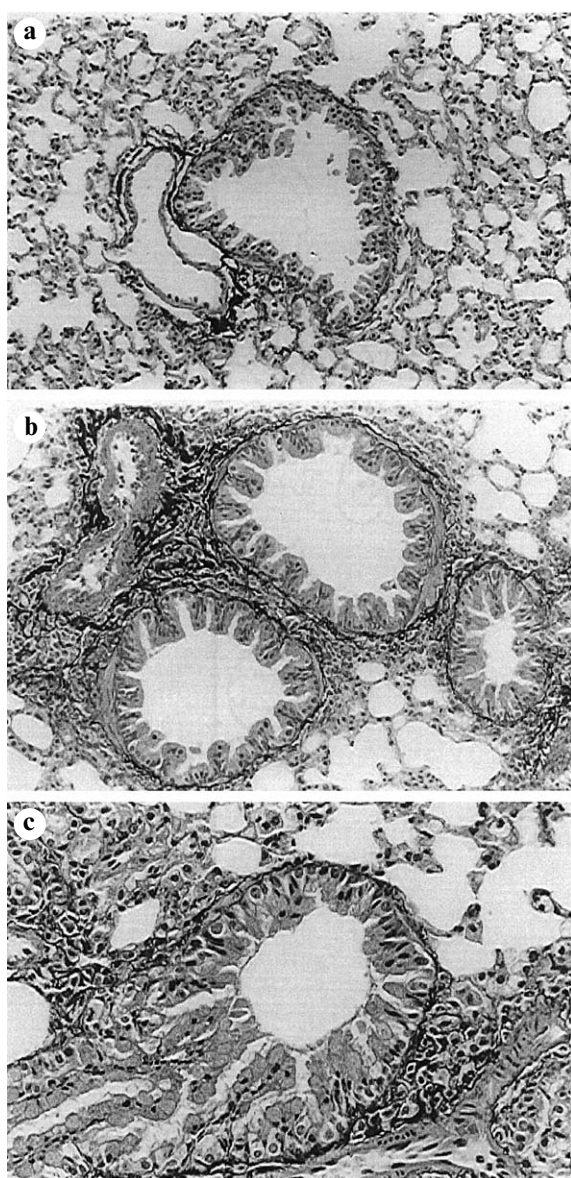


Fig. 5. Representative photomicrographs of Picro-sirius haematoxyline sections of lungs demonstrating histological changes in response to repeated OA challenges. (a) Pulmonary architecture of control lung ($\times 250$). (b) Pulmonary parenchyma from mice exposed for 20 weeks to repeated OA challenges. Peribronchial smooth muscle mass seems to be more visible around the bronchioles and peribronchial basement membrane thickened and multilamellar featuring a subepithelial fibrosis ($\times 250$). (c) Pulmonary parenchyma from mice exposed for 28 weeks to repeated OA challenges. Note the presence of goblet cells ($\times 400$).

challenge in sensitized mice is associated with an increase in the expression of matrix metalloproteinase (MMP-2 and MMP-9) and TIMP-1 activities in BAL fluid. Moreover, we demonstrated that repeated antigen exposures lead to marked structural changes in the airways with the development of subepithelial fibrosis and hypertrophy/hyperplasia of airway smooth muscle, 16 weeks after the beginning of OA challenges.

In order to investigate the relationship between allergic pulmonary inflammation and airway remodelling, we developed a murine model of chronic antigen challenge, which may mimic asthmatic reactions in humans. This model showed that repeated ovalbumin administration induced an intense inflammatory

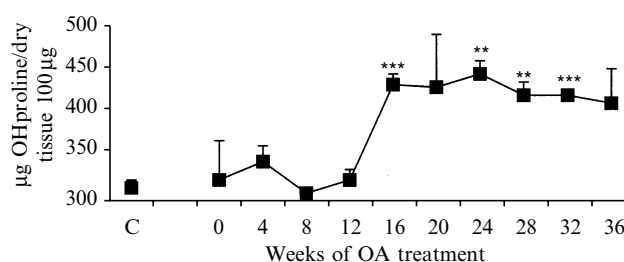


Fig. 6. Hydroxyproline content in the lung from control ($n = 15$) and OA-exposed mice ($n = 3$). The hydroxyproline (OHproline) content was measured throughout the study. No significant change in the hydroxyproline content was noted in saline-exposed mice. The results are presented as mean $\pm$ SEM. ** $P < 0.01$; *** $P < 0.001$.

reaction, as demonstrated by the marked influx of eosinophils, lymphocytes and neutrophils in BAL fluid. These results are consistent with previous studies done in various animal models of asthma [1].

Cytokines and chemokines are believed to play a central role in the induction and the perpetuation of allergic airway inflammation [1]. Among them, the Th2-like cytokine IL-5 is considered as one of key cytokines promoting eosinophil accumulation as shown in the study by Foster et al. [28]. In our study, the level of IL-5 in BAL fluids is increased 8 weeks after repeated OA challenges and appears to be correlated with the increase of BAL lymphocytes from 8 weeks of treatment. This is consistent with the studies showing that T lymphocytes are one of the main sources of IL-5 in airways [29, 30]. Our study and all these models are consistent with human studies where IL-5 has been shown to exert its effects not only on eosinophil differentiation in the bone marrow but also on eosinophil influx and survival locally in the lung [31].

We also analysed structural changes in lung tissue during 36 weeks of chronic antigen challenge in sensitized mice. In this model, the inflammatory and remodelling processes led to the development of a subepithelial fibrosis observed after 16 weeks. Indeed, an increase in collagen deposition was detected on lung sections by a picro-sirius haematoxyline staining. The measurement of hydroxyproline content confirmed that the amount of total collagen in lung tissue was increased following chronic antigen exposure. Therefore, this model reproduced many features of human asthma as shown by the infiltration of eosinophils, the development of a subepithelial fibrosis, the increase in smooth muscle mass and the increase in goblet cells. Indeed, a direct correlation has been reported between the severity of asthma and the amount of subepithelial collagen, as well as the number of myofibroblasts [4, 5].

We also found an increase in TGF- β in tissue and in BAL fluids at 36 weeks. TGF- β is a potent profibrotic mediator that stimulates fibroblasts to promote the synthesis and secretion of ECM proteins [32, 33]. Moreover, TGF- β presents immunomodulatory effects and is a potent chemoattractant for several cell types including monocytes [34] and fibroblasts [35]. A recent study in human bronchial asthma reported that the up-regulation of TGF- β expression was associated with the degree of airway fibrosis and that activated eosinophils were also a source of this cytokine [6]. The strong expression of TGF- β in our model of chronic asthma strengthened the existence of a remodelling process associated with an increase of collagen deposition and probably a fibroblast recruitment.

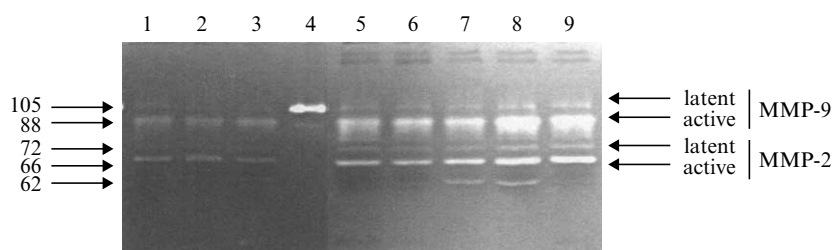


Fig. 7. Analysis of MMPs in BAL fluids by zymography. The figure shows a representative zymographic analysis of BAL fluids from control mice (lanes 1 to 3) or after 20 weeks of OA repeated instillations (lanes 5 to 9). BAL fluids from LPS-exposed mice are used as internal standard of MMP-9 migration and density (lane 4).

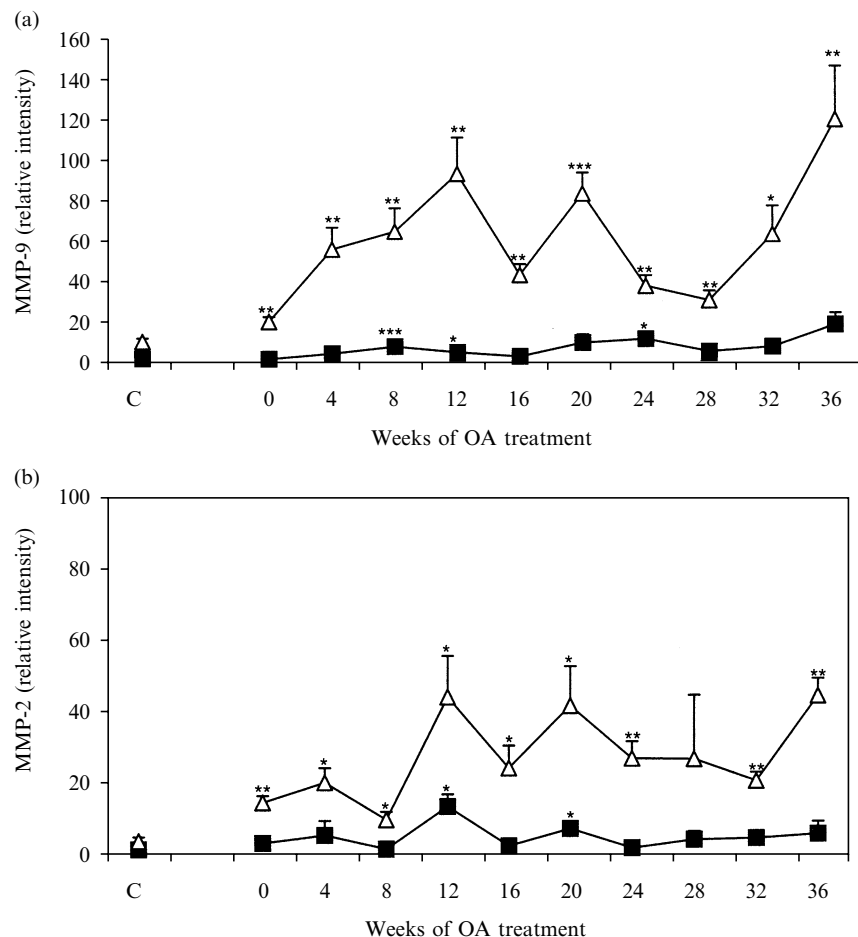


Fig. 8. MMP activity in BAL fluids from OA-exposed mice ($n = 5$) throughout the study vs. control mice (C; $n = 29$). (a) MMP-9 under its proform (105 kDa; Δ) or its active form (88 kDa; $\blacksquare$). (b) MMP-2 under its proform (72 kDa; Δ) or its active form (66 kDa; $\blacksquare$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The intensity of MMP activity was evaluated on gelatin zymography gels and measured by densitometry. Results are expressed as mean $\pm$ SEM of relative percentages of an internal control band of migration.

We also observed an enhanced expression of gelatinase (MMP-2 and MMP-9) activity in BAL fluids from chronically exposed-mice compared with control mice. These results are in agreement with a study showing the association of gelatinase activity, inflammatory cell accumulation and cytokine release in sensitized and challenged mice [36]. The up-regulation of MMP-9 release after repeated OA challenges is also consistent with previous studies reporting that (i) MMP-9 content was increased in BAL fluids from untreated asthmatics compared with steroid-treated asthmatics and control subjects [12, 22], and (ii) antigen challenge increased MMP-9 activity in BAL fluids from asthmatics [21]. Interestingly, we found a positive correlation between MMP-9 levels and neutrophil number in BAL fluids. Even though several studies suggested that MMP-9 was mainly secreted by eosinophils in human asthma [15, 19, 37], recent reports showed that neutrophils may also



Fig. 9. Representative Western blot analysis of TIMP-1 in BAL fluids from mice exposed to repeated OA challenges. TIMP-1 (30 kDa) was not present in BAL from control mice (saline exposed) (lane 1) and mice exposed to OA during 4 weeks (lane 2). At 12 weeks (lane 3), TIMP-1 was dramatically increased. Then TIMP-1 level decreased weakly but its level remained stable (lane 4, 20 weeks; lane 5, 28 weeks; lane 6, 32 weeks).

consist of a potent source of MMP-9 [38]. In addition, Lemjabbar et al. [22] suggested recently that the increase in MMP-9 levels in BAL from status asthmaticus patients may be shared between neutrophils and activated bronchial epithelial cells in response to lung injury.

In our study, the MMP-2 activity (namely the 66 kDa active form), was also increased in BAL fluids from mice exposed to repeated OA challenges especially after 12 to 16 weeks. However, MMP-2 levels were twofold lower than those of MMP-9. Although MMP-2 and MMP-9 shared some substrate specificities, these enzymes are known to be synthesized by different cell types. MMP-2 is synthesized by structural cells, including fibroblasts, endothelial and epithelial cells, whereas MMP-9 is produced mainly by inflammatory cells [12–15]. Our data and those of others suggest that MMP-9 may be linked to inflammatory process-induced tissue remodelling, whereas MMP-2 may be associated with an impaired tissue remodelling leading to pathological collagen deposition and subepithelial fibrosis. Indeed, in the well-described animal model of bleomycin-induced interstitial pulmonary fibrosis, MMP-2 activity is predominantly increased in lung tissue [39, 40] and BAL fluids [41, 42].

An appropriate regulation of MMPs and their inhibitors (TIMPs) is important for tissue healing. Although endogenous TIMPs presumably exert some protective effects in preventing ECM degradation in tissues, the amount of these antiproteases *in vivo* is low. Moreover, TIMPs have been shown to be susceptible to oxidative or proteolytic attack [43]. TIMPs are also known to promote fibroblast proliferation [44], so they may be responsible for the autocrine promotion of fibrogenesis. Recent studies demonstrated an imbalance between MMP-9 and TIMP-1 productions characterized by a low MMP-9:TIMP-1 ratio in serum [45] and sputum [46] from asthmatic subjects. An enhanced production of TIMP-1 by alveolar macrophages was reported in chronic asthma by Mautino et al. [20, 47]. In mice, TIMP-1 or TIMP-2 administration was demonstrated to be efficient in inhibiting the antigen-induced cellular infiltration in BAL fluids [36], suggesting a major role of TIMPs in development of inflammation in asthma. In our study, we detected a dramatic increase in expression in TIMP-1 in BAL fluids from mice exposed to repeated OA challenges in comparison with those of control mice where TIMP-1 was undetectable. This increase was detected after 12 weeks of OA repeated challenge and remained important until the end of the study. Our results suggest that the increased production of TIMP-1 may prevent the MMP-induced ECM protein degradation, and as a consequence TIMP-1 may participate in the increased ECM component deposition and the development of subepithelial fibrosis. Although an increase in MMP-9 activity was found, it may not be sufficient to prevent the excessive deposition of collagen observed after 16 weeks in our model. In contrast, even though we used the same experimental conditions as for TIMP-1 evaluation, we were not able to detect TIMP-2 in BAL fluids from either OA-exposed or control mice. As TIMP-2 interacts more specifically with MMP-2, this last result is in agreement with the relatively mild increase of MMP-2 observed in BAL fluids.

In conclusion, the chronic model of asthma presented in this study showed the development of a subepithelial fibrosis in mice exposed to repeated antigen challenges. We managed to describe the kinetic of both inflammatory process and the associated airway remodelling development. The marked up-regulation and activation of MMP-9 in response to repeated OA challenges and the absence of TIMP-1 regulation during the first weeks may suggest an imbalance between gelatinase and TIMP in favour of MMPs, thus promoting an extensive ECM

degradation. However, this up-regulation and activation of MMP-9 in the latter stages of this study was associated with both an increase in the production of TIMP-1 and in collagen deposition, so that TIMP-1 may be involved in the development of the subepithelial fibrosis by preventing MMP-9 enzymatic activity. Further investigations are needed to clarify our understanding of the role of the regulatory mechanisms of MMP activity and their inhibitors in the asthmatic disease.

Acknowledgements

Marianne Corbel is funded by a fellowship from Conseil Régional de Bretagne, France. The authors thank Mrs A. Denais and Mr R. Trinquart (Faculté de Médecine, Université de Rennes 1).

References

- 1 Kay AB. Asthma and inflammation. *J Allergy Clin Immunol* 1991; 87:893–910.
- 2 Vignola AM, Chanez P, Siena L, Chiappara G, Bonsignore G, Bousquet J. Airways remodelling in asthma. *Pulm Pharmacol Ther* 1998; 11:359–67.
- 3 Laitinen LA, Heino M, Laitinen A, Kava T, Haahtela T. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 1985; 131:599–606.
- 4 Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; 1:520–4.
- 5 Brewster CEP, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1990; 3:507–11.
- 6 Minshall EM, Leung DYM, Martin RJ et al. Eosinophil-associated TGF- β 1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1997; 17:326–33.
- 7 Vignola AM, Chanez P, Chiappara G et al. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1997; 156:591–9.
- 8 Nagase H. Activation mechanisms of matrix metalloproteinases. *Biol Chem* 1997; 378:151–60.
- 9 Murphy G, Stanton H, Cowell S et al. Mechanisms for pro matrix metalloproteinase activation. *APMIS* 1999; 107:38–44.
- 10 Murphy G, Docherty AJP. The matrix metalloproteinases and their inhibitors. *Am J Respir Cell Mol Biol* 1992; 7:120–5.
- 11 Yao PM, Maitre B, Delacourt C, Buhler JM, Harf A, Lafuma C. Divergent regulation of 92-kDa gelatinase and TIMP-1 by HBECs in response to IL-1 β and TNF- α . *Am J Physiol* 1997; 273: L866–74.
- 12 Mautino G, Oliver N, Chanez P, Bousquet J, Capony F. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am J Respir Cell Mol Biol* 1997; 17:583–91.
- 13 Leppert D, Hauser SL, Kishiyama JL, An S, Zeng L, Goetzl EJ. Stimulation of matrix metalloproteinase-dependent migration of T cells by eicosanoids. *FASEB J* 1995; 9:1473–81.
- 14 Delclaux C, Delacourt C, d'Ortho MP, Boyer V, Lafuma C, Harf A. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 1996; 14:288–95.
- 15 Ohno I, Ohtani H, Nitta Y et al. Eosinophils as a source of matrix metalloproteinase-9 in asthmatic airway inflammation. *Am J Respir Cell Mol Biol* 1997; 16:212–9.
- 16 O'Connor CM, FitzGerald MX. Matrix metalloproteinases and lung diseases. *Thorax* 1994; 49:602–9.

- 17 Pardo A, Selman M. Matrix metalloproteinases and lung injury. *Braz J Med Biol Res* 1996; 29:1109–15.
- 18 Bousquet J, Chanez P, Lacoste JY et al. Asthma: a disease remodeling the airways. *Allergy* 1992; 47:3–11.
- 19 Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 1998; 102:783–8.
- 20 Mautino G, Henriquet C, Gougat C et al. Increased expression of tissue inhibitor of metalloproteinase-1 and loss of correlation with matrix metalloproteinase-9 by macrophages in asthma. *Laboratory Invest* 1999; 79:39–47.
- 21 Warner JA, Julius P, Luttmann W, Kroegel C. Matrix metalloproteinases in bronchoalveolar lavage fluid following antigen challenge. *Int Arch Allergy Immunol* 1997; 113:318–20.
- 22 Lemjabbar H, Gosset P, Lamblin C et al. Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. *Am J Respir Crit Care Med* 1999; 159:1298–307.
- 23 Eum SY, Haile S, Lefort J, Huerre M, Vargaftig BB. Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper-IgE mice is accompanied by interleukin-5 dependent bronchial hyperresponsiveness. *Proc Natl Acad Sci USA* 1995; 92:12290–4.
- 24 Th  ret N, Musso O, L'Helgouach A, Cl  ment B. Activation of matrix metalloproteinase-2 from hepatic stellate cells requires interactions with hepatocytes. *Am J Pathol* 1997; 150:51–8.
- 25 Corbel M, Lagente V, Th  ret N, Germain N, Cl  ment B, Boichot E. Comparative effects of betamethasone, cyclosporin and nedocromil sodium in acute pulmonary inflammation and metalloproteinase activities in bronchoalveolar lavage fluid from mice exposed to lipopolysaccharide. *Pulm Pharmacol Ther* 1999; 12:165–71.
- 26 Reddy GK, Chukuka SE. A simplified method for the analysis of hydroxyproline in biological tissues. *Clin Biochem* 1996; 29:225–9.
- 27 Kato Y, Nakayama Y, Umeda M, Miyazaki K. Induction of 103 kDa gelatinase/type IV collagenase by acidic culture conditions in mouse metastatic melanoma cell lines. *J Biol Chem* 1992; 267:11424–30.
- 28 Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996; 183:195–201.
- 29 Hamid Q, Azzawi M, Ying S et al. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J Clin Invest* 1991; 87:1541–6.
- 30 Robinson DS, Hamid Q, Ying S et al. Evidence for a predominant 'Th2-type' bronchoalveolar lavage T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; 326:298–304.
- 31 Robinson DS, Durham SR, Kay AB. Cytokines in asthma. *Thorax* 1993; 48:845–53.
- 32 Raghu G, Masta S, Meyers D, Narayanan AS. Collagen synthesis by normal and fibrotic human lung fibroblasts and the effect of transforming growth factor-  . *Am Rev Respir Dis* 1989; 140:95–100.
- 33 Raghov R. Role of transforming growth factor-beta in repair and fibrosis. *Chest* 1991; 99:61S–65S.
- 34 Wahl SM, Hunt DA, Wakefield LM et al. Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. *Proc Natl Acad Sci USA* 1987; 84:5788–92.
- 35 Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *J Exp Med* 1987; 165:251–6.
- 36 Kumagai K, Ohno I, Okada S et al. Inhibition of matrix metalloproteinases prevents allergen-induced airway inflammation in a murine model of asthma. *J Immunol* 1999; 162:4212–9.
- 37 Okada S, Kita H, George TJ, Gleich GJ, Leiferman KM. Migration of eosinophils through basement membrane components in vitro: role of matrix metalloproteinase-9. *Am J Respir Cell Mol Biol* 1997; 17:519–28.
- 38 Halliday JL, Chu HW, Langmack E, Wenzel SE. Matrix metalloproteinase 9 (MMP-9) expression occurs in asthmatic extracellular matrix (ECM) and subbasement membrane (SBM) and is associated with severity of disease. *Am J Respir Crit Care Med* 1999; 159:A325.
- 39 Yaguchi T, Fukuda YM, Ishizaki Yamanaka N. Immunohistochemical and gelatin zymography studies for matrix metalloproteinases in bleomycin-induced pulmonary fibrosis. *Pathol Int* 1998; 48:954–63.
- 40 Swiderski RE, Dencoff JE, Floerchinger CS, Shapiro SD, Hunninghake GW. Differential expression of extracellular matrix remodeling genes in a murine model of bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1998; 152:821–8.
- 41 Bakowska J, Adamson IYR. Collagenase and gelatinase activities in bronchoalveolar lavage fluids during bleomycin-induced lung injury. *J Pathol* 1998; 185:319–23.
- 42 Corbel M, Caulet-Maugendre S, Th  ret N, Germain N, Lagente V, Cl  ment B, Boichot E. Development of pulmonary fibrosis and increased activity of matrix metalloproteinases following intranasal administration of bleomycin in BP2 Mice. *Am J Respir Crit Care Med* 1999; 159:A190.
- 43 Kramps JA, van Twisk C, Klasen EC, Dijkman JH. Interactions among stimulated human polymorphonuclear leucocytes, released elastase and bronchial antileucoprotease. *Clin Sci* 1988; 75:53–62.
- 44 Corcoran ML, Stetler-Stevenson WG. Tissue inhibitor of metalloproteinase-2 stimulates fibroblast proliferation via a camp-dependent mechanism. *J Biol Chem* 1995; 270:13453–9.
- 45 Bosse M, Chakir J, Rouabhia M, Boulet LP, Audette M, Laviolette M. Serum matrix metalloproteinase-9: tissue inhibitor of metalloproteinase-1 ratio correlates with steroid responsiveness in moderate to severe asthma. *Am J Respir Crit Care Med* 1999; 159:596–602.
- 46 Vignola AM, Riccobono L, Mirabella A et al. Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1998; 158:1945–50.
- 47 Mautino G, Henriquet C, Jaffuel D, Bousquet J, Capony F. Tissue inhibitor of metalloproteinase-1 levels in bronchoalveolar lavage fluid from asthmatic subjects. *Am J Respir Crit Care Med* 1999; 160:324–30.