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Metalloelastase (MMP-12) induced inflammatory response in mice airways: Effects of dexamethasone, rolipram and marimastat

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Abstract

Direct instillation of a recombinant human form of MMP-12 (rhMMP-12) in mice airways elicited an early inflammatory response characterized by neutrophil influx, cytokine release and gelatinase activation followed by a delayed response, mainly characterized by macrophage recruitment. As this experimental model of lung inflammation partially mimics some features of chronic obstructive pulmonary disease (COPD), we have investigated the effects of treatment by anti-inflammatory compounds, dexamethasone and rolipram and a non-specific matrix metalloproteinase (MMP) inhibitor, marimastat. The compounds were administrated orally, 1 h before rhMMP-12 instillation (8×10^{-3} U/mouse). Total and differential cell counts were evaluated in the bronchoalveolar lavage fluids. Cytokines and MMP-9 were quantified in bronchoalveolar lavage fluids and in lung homogenate supernatants. Marimastat (100 mg/kg), dexamethasone (10 mg/kg) and rolipram (0.1 and 0.3 mg/kg) were able to decrease significantly neutrophil recruitment at 4 and 24 h after rhMMP-12 instillation, but only marimastat (30 and 100 mg/kg) was effective at decreasing the macrophage recruitment occurring at day 7. Marimastat (100 mg/kg), dexamethasone (10 mg/kg) and rolipram (0.3 mg/kg) reduced significantly IL-6, KC/CXCL1, MIP-1α/CCL3 and MMP-9 levels in bronchoalveolar lavage fluid. Similar results were obtained in lung homogenates except with rolipram. Dexamethasone and rolipram were able to inhibit the early inflammatory response but were ineffective to limit the macrophage influx. In contrast, marimastat was able to reduce early and late response. These data indicate that MMP-12 instillation in mice could highlight some of the inflammatory response seen in COPD and could be used for the pharmacological evaluation of new anti-inflammatory mechanisms of action.

Keywords: Metalloelastase; Matrix metalloproteinase; Inflammation; Chronic obstructive pulmonary disease; Dexamethasone; Rolipram

1. Introduction

Several pulmonary diseases including chronic obstructive pulmonary disease (COPD) are characterized by chronic inflammation involving the influx of cells, mainly neutrophils and mononuclear cells, into the lungs (Barnes et al., 2003). Since inflammatory cells are able to produce and release various proteases, it is generally believed that the development of tissue remodeling reflects a relative excess of cell-derived proteases that degrade connective tissue of the lung and a relative paucity of antiproteolytic defenses. This theory is often referred as the "protease-antiprotease imbalance" hypothesis and involves mainly serine protease like neutrophil elastase and matrix metalloproteinases (MMPs). Among these MMPs, MMP-12 or macrophage metalloelastase which is mainly produced by macrophages and characterized by an elastolytic activity, could play a predominant role in the pathogenesis of COPD, particularly in emphysema (Shapiro, 2000). *In vitro* studies on alveolar macrophages collected from COPD patients have

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shown their ability to degrade more elastin than macrophages collected from healthy volunteers (Russell et al., 2002). We have also previously shown MMP-12 overexpression on the bronchoalveolar lavage cells and bronchial biopsies of COPD patients, compared with controls (Molet et al., 2005). This overexpression is also associated with an increase release of MMP-12 in the bronchoalveolar lavage fluids (Molet et al., 2005) or induced sputum (Demedts et al., 2006) of patients with COPD suggesting a role for MMP-12 in the development of COPD in smokers. Moreover, studies using MMP-12 KO mice have demonstrated that macrophage recruitment in lungs and emphysema induced by long-term exposure to cigarette smoke were directly linked to MMP-12 presence (Hautamaki et al., 1997).

MMP-12 is also reported to be involved in the acute inflammatory response, mainly neutrophil influx, since MMP-12 KO mice are less sensitive than controls to exposure of cigarette smoke (Leclerc et al., 2006). These results are in agreement with another study that has also evoked inflammatory properties for MMP-12 linked to its capacity to release tumor necrosis factor (TNF)-alpha from macrophages with subsequent endothelial activation, neutrophil influx and proteolytic matrix breakdown caused by neutrophil-derived proteases (Churg et al., 2003). Moreover, in a previous study, we have shown that MMP-12 could also have a proinflammatory function. Indeed, MMP-12 instilled in mice airways initially induced a severe inflammatory response characterized by a neutrophil influx associated with a release of cytokines. This early phase was followed by severe and stable macrophage recruitment over a period of two weeks (Nenan et al. 2005).

Hence, taken together, these data suggest an involvement of MMP-12 in the early airway inflammatory process. As our model of lung inflammation induced by rhMMP-12 instilled in mice airways partially mimics some COPD features, we have tested anti-inflammatory drugs with a potential efficacy in COPD, the corticosteroid, dexamethasone, and the selective phosphodiesterase (PDE) 4 inhibitor, rolipram. We have also tested a non-specific MMP inhibitor, marimastat, on neutrophil influx associated with cytokine release and on the delayed macrophage recruitment.

2. Materials and methods

2.1. Animals and experimental protocols

Seven week-old A/J mice (Charles River laboratories, L'Arbresles, France) were used in this study according to guidelines of a local ethical committee that complied with the Interdisciplinary Principles and Guidelines for the Use of Animals in Research Marketing and Education, New York Academy of Sciences' ad Hoc Committee on Animal Research. They were instilled with either recombinant human MMP-12 (rhMMP-12) (8×10^{-3} U/mouse) or with dialysis buffer (control) (25 µl of each solution) in the airways. This tracheal instillation was performed using a microsprayer and a high-pressure syringe (Penncentury, Inc, Philadelphia, PA, USA) under etomidate (15 mg:kg, i.p.) anesthesia as previously described (Nenan et al. 2005).

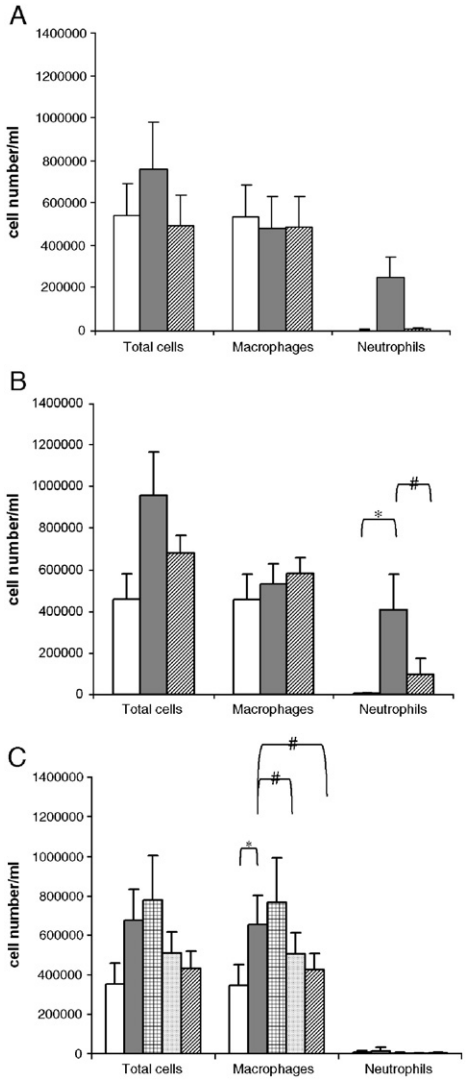

Fig. 1. Effect of marimastat, a non-selective MMP inhibitor, on cellular composition in bronchoalveolar lavage fluids 4 h (A), 24 h (B) or 7 days (C) after rhMMP-12 instillation in mice airways. Treated mice received marimastat at the dose of 100 mg kg^{-1} (hatched bar), 30 mg kg^{-1} (dotted bar) or 10 mg kg^{-1} (crossed bar) one hour before the instillation of rhMMP-12 (8×10^{-3} U/mouse). Control mice received either vehicle (open bar) or rhMMP-12 (grey bar). Results are expressed as the number of cells (mean±SD). *$P<0.05$ as compared to control mice. #$P<0.05$ as compared to mice instilled with rhMMP-12 and treated with vehicle. $N=5$ animals per group.

One hour before the rhMMP-12 instillation, mice were treated with either dexamethasone (10 mg/kg), the selective PDE type 4 inhibitor rolipram (0.1–0.3 or 1 mg/kg), or the non-selective MMPs inhibitor marimastat (10–30 or 100 mg/kg). All these compounds were administrated *per os* (20 ml/kg) diluted in a methylcellulose suspension (0.5%) in distilled water.

2.2. Bronchoalveolar lavage

Four hours, 24 h or 7 days after rhMMP-12 instillation, bronchoalveolar lavages were performed. Mice were anaesthetized with an i.p. administration (20 ml/kg) of pentobarbital sodium 0.6%. To realize bronchoalveolar lavage, airspaces were

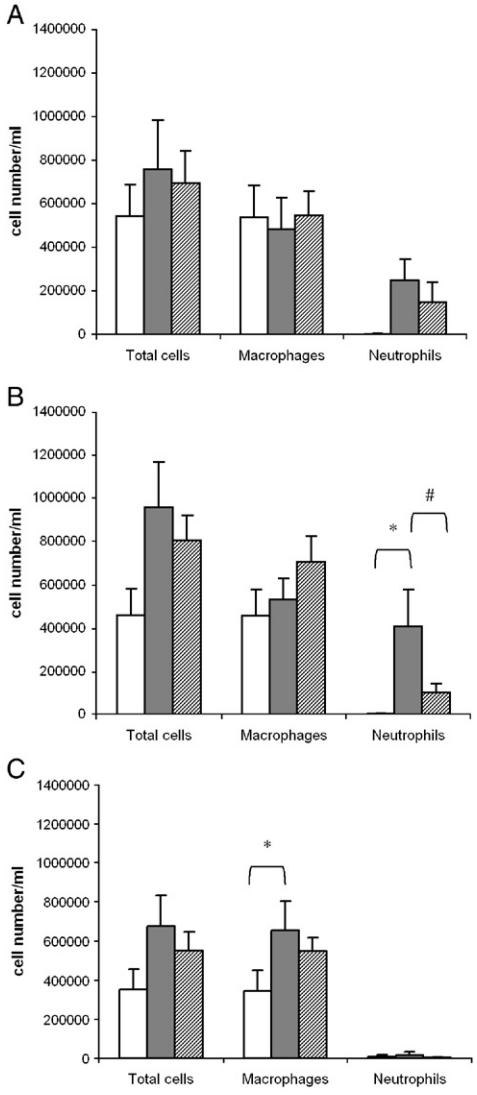

Fig. 2. Effect of dexamethasone on cellular composition in bronchoalveolar lavage fluids 4 h (A), 24 h (B) or 7 days (C) after rhMMP-12 instillation in mice airways. Treated mice received dexamethasone 10 mg kg^{-1} (hatched bar) one hour before the instillation of rhMMP-12 (8×10^{-3} U/mouse). Control mice received either vehicle (open bar) or rhMMP-12 (grey bar). Results are expressed as the number of cells (mean ± SD). *$P<0.05$ as compared to control mice. #$P<0.05$ as compared to mice instilled with rhMMP-12 and treated with vehicle. $N=5$ animals per group.

washed 6 times with 0.3 ml of phosphate buffer saline (PBS) with a 1 ml syringe. Bronchoalveolar lavage fluid was centrifuged (600 g for 10 min, 4 °C) and the supernatant of the first three fractions of bronchoalveolar lavage (0.9 ml) was aliquoted and frozen at −80 °C until analysis. Cell pellets were then pooled into the last three fractions of bronchoalveolar lavage. Total cell count was performed using a Beckman coulter Z2®. Red blood cells were eliminated by adding 3 ml of distilled water during 30 s and then 1 mL of KCl 0.6 M on cell pellets. After centrifugation (600 g for 10 min, 4 °C), supernatant was eliminated and cells were suspended in 1 ml of PBS. Cytospin preparations were performed at 700 rpm for 10 minutes (Cytospin 3®, Thermo Shandon, Ltd., Astmoor, United Kingdom). After cytocentrifugation, cells were stained using the May-Grünwald Giemsa method. Differential cell counts were made on 100 cells using standard morphological criteria.

2.3. Lung homogenate

Lungs were removed and frozen in liquid nitrogen and then stored at −80 °C until the realization of tissue homogenates. To perform lung homogenates, lung tissues were placed in 1 ml of homogenization buffer (TRIS 50 mM, $CaCl_2$ 10 mM, NaCl 150 mM) in Lysing Matrix D tubes (Ozyme, Saint Quentin en Yvelines, France). Tubes were then shaken in a Fast-Prep® FP 120 cell disrupter (Qbiogene, Inc, Illkirch, France). Following homogenization and centrifugation (3000 *g* for 15 min, 4 °C), supernatants were collected, aliquoted and frozen at −80 °C until analysis.

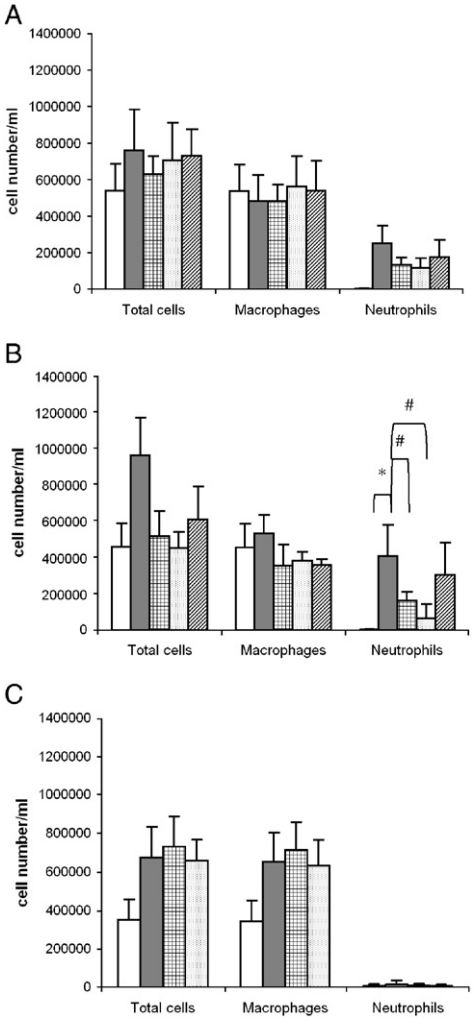

Fig. 3. Effect of rolipram, a PDE4 inhibitor, on cellular composition in bronchoalveolar lavage fluids 4 h (A), 24 h (B) or 7 days (C) after rhMMP-12 (8×10^{-3} U/mouse) instillation in mice airways. Treated mice received rolipram at the dose of 0.1 mg kg^{-1} (crossed bar), 0.3 mg kg^{-1} (dotted bar) or 1 mg kg^{-1} (hatched bar) 1 h before the instillation of rhMMP-12. Control mice received either vehicle (open bar) or rhMMP-12 (grey bar). Results are expressed as the number of cells (mean ± SD). *$P<0.05$ as compared to control mice. #$P<0.05$ as compared to mice instilled with rhMMP-12 and treated with vehicle. $N=5$ animals per group.

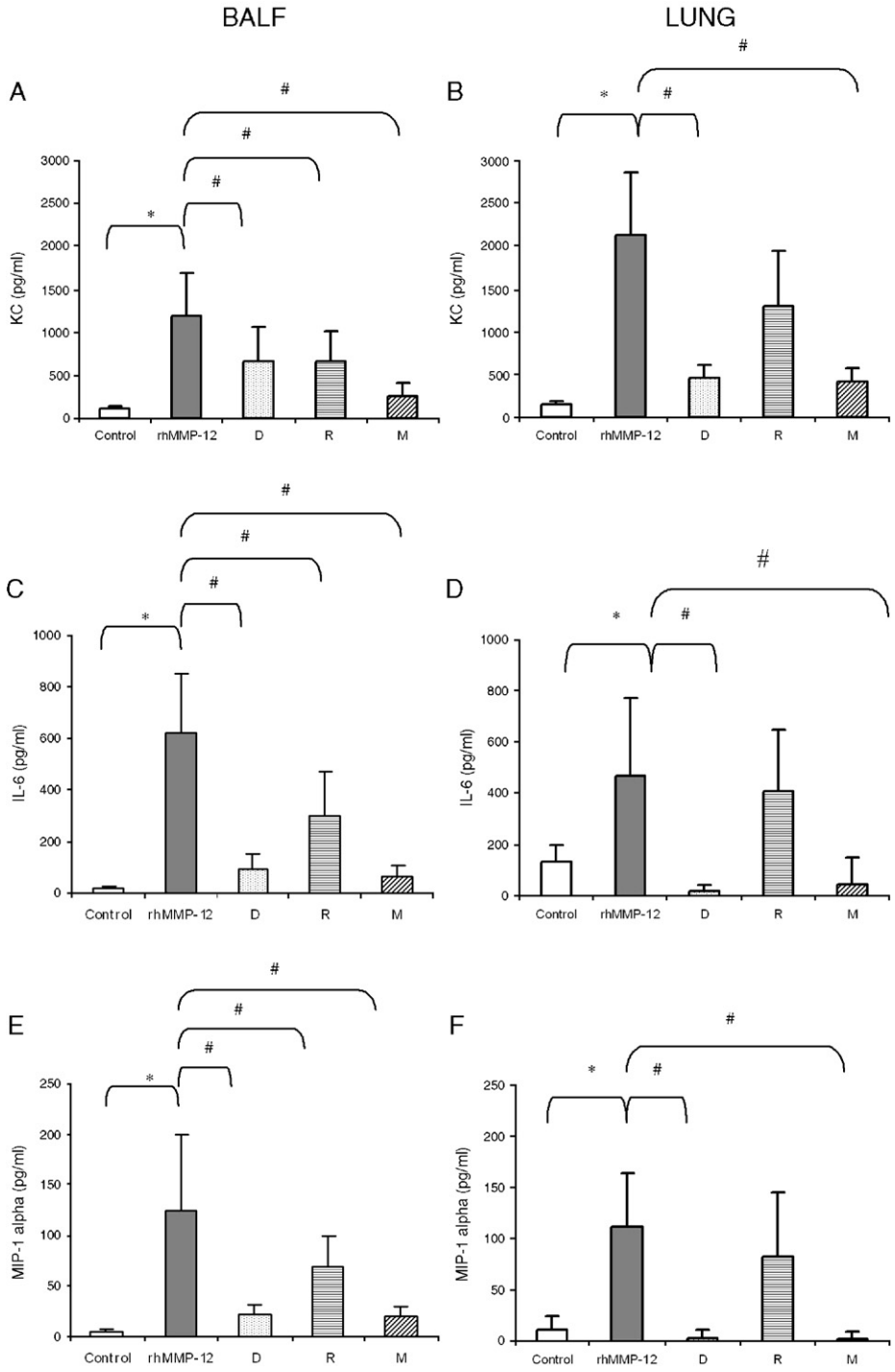

Fig. 4. Effect of 10 mg kg^{-1} dexamethasone (D), 0.3 mg kg^{-1} rolipram (R) and 100 mg kg^{-1} marimastat (M) on KC/CXCL1 release in bronchoalveolar lavage fluids (A) and in lung homogenate supernatants (B), on IL-6 release in bronchoalveolar lavage fluids (C) and in lung homogenate supernatants (D) and on macrophage inflammatory protein (MIP)-1α/CCL3 release in bronchoalveolar lavage fluids (E) and in lung homogenate supernatants (F). Results are expressed as the cytokine concentration (pg/ml) (mean ± SD). *$P<0.05$ as compared to control mice. #$P<0.05$ as compared to mice instilled with rhMMP-12 and treated with vehicle. $N=5$ animals per group.

2.4. Cytokines, chemokines and pro-MMP-9 measurements

Cytokines, chemokines (MIP-1α (Macrophage Inflammatory Protein-1α/CCL3), IL-6 (Interleukin-6) and KC/CXCL1) and pro-MMP-9 were quantified in bronchoalveolar lavage fluids and lung homogenate supernatants by enzyme link immunosorbent assay (ELISA).

2.5. Materials

Etomidate and pentobarbital sodium 0.6% were respectively purchased from Janssen-Cilag (Issy-Les-Moulineaux, France) and from Sanofi santé nutrition animale (Libourne, France). Methylcellulose (UPS SM-400 quality) was purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Marimastat and rolipram

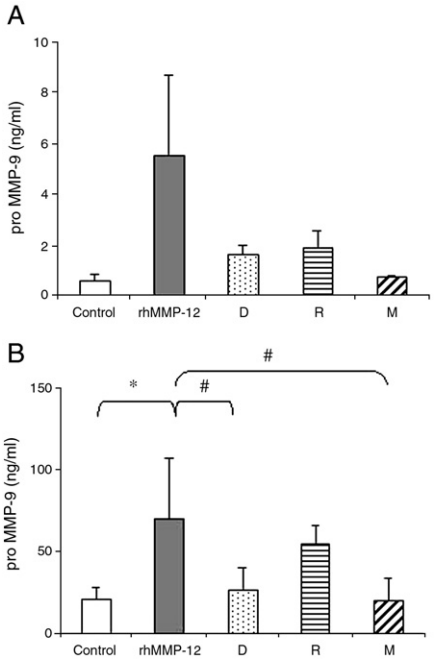

Fig. 5. Effect of 10 mg kg^{-1} dexamethasone (D), 0.3 mg kg^{-1} rolipram (R) and 100 mg kg^{-1} marimastat (M) on proMMP-9 release in bronchoalveolar lavage fluids (A) and in lung homogenate supernatants (B). Results are expressed as the proMMP-9 concentration (ng/ml) (mean ± SD). *$P<0.05$ as compared to control mice. #$P<0.05$ as compared to mice instilled with rhMMP-12 and treated with vehicle. $N=5$ animals per group.

were synthesized in-house (Pfizer Global R&D, Fresnes, France) and dexamethasone-21-phosphate disodium salt was purchased from Sigma Chemical Co (St. Louis, MO, USA). ELISA kits were obtained from R&D systems (Minneapolis, MN, USA).

2.6. Analysis and expression of results

Results are presented as mean ± SD (standard deviation) in each group. Inter-group significances were assessed by one or two-ways Analyses of Variance (ANOVA). In order to control type I error rate, the Holm multiple comparison procedure was used for all post-hoc tests. *P*-values less than 5% were considered significant.

3. Results

3.1. Effect of marimastat on cellular recruitment induced by rhMMP-12 instillation in mice airways

As previously described (Nenan et al. 2005), rhMMP-12 instillation induced an acute neutrophil influx in A/J mice airways that could be observed 4 h and 24 h after instillation (Fig. 1A and B). This neutrophil recruitment was strikingly suppressed by the treatment with marimastat (100 mg/kg) as shown in Fig. 1A and B. Marimastat has no effect on basal cellular level even if used at high doses (100 mg/kg) (data not shown). Seven days after rhMMP-12 instillation, severe macrophage recruitment could be noticed whereas no in crease in neutrophil number was observed (Fig. 1C). Marimastat (10, 30 and 100 mg/kg) elicited a dose dependant inhibition of the increase in macrophage number in bronchoalveolar lavage fluids.

3.2. Effect of dexamethasone on cellular recruitment induced by rhMMP-12 instillation in mice airways

Four and 24 h after rhMMP-12 instillation, dexamethasone (10 mg/kg) significantly inhibited neutrophil recruitment (Fig. 2A and B), whereas it had no effect on basal cellular level. In contrast, dexamethasone was ineffective in reducing macrophage influx 7 days after rhMMP-12 instillation (Fig. 2C).

3.3. Effect of rolipram on cellular recruitment induced by rhMMP-12 instillation in mice airways

Three doses of rolipram were tested (0.1, 0.3 and 1 mg kg^{-1}). No effect on basal cellular level was noted at any of the doses used (data not shown). Rolipram (0.1 and 0.3 mg/kg) was able to significantly reduce neutrophil influx at 4 h and at 24 h (Fig. 3A and B), whereas it was ineffective at decreasing the macrophage recruitment 7 days after rhMMP-12 instillation (Fig. 3C).

3.4. Effect of marimastat, dexamethasone or rolipram on cytokine release induced by rhMMP-12 instillation in mice airways

Cytokines were quantified in bronchoalveolar lavage fluids as well as in lung homogenate supernatants, 4 h after rhMMP-12 instillation. As previously described (Nenan et al. 2005), rhMMP-12-induced a significant increase in KC/CXCL-1, IL-6 and MIP-1α/CCL3 in both bronchoalveolar lavage fluids and lung homogenates in comparison with control (Fig. 4). The effect of marimastat and rolipram on cytokine release in bronchoalveolar lavage fluid and in lung tissue was evaluated only with the most efficient dose of these compounds observed on cellular recruitment, i.e., 100 mg/kg marimastat and 0.3 mg/kg rolipram.

Treatment with either dexamethasone (10 mg/kg) or marimastat (100 mg/kg) elicited a significant inhibition of the enhanced KC, IL-6 and MIP-1α levels in bronchoalveolar lavage fluids as well as in lung homogenate supernatants (Fig. 4). Rolipram (0.3 mg/kg) partially reduced the increase in KC, IL-6 and MIP-1α levels in bronchoalveolar lavage fluids whereas no modification of these cytokine levels was observed in lung homogenate supernatants (Fig. 4).

3.5. Effect of marimastat, dexamethasone or rolipram on proMMP-9 levels in bronchoalveolar lavage fluids and in lung homogenate supernatants

Pro-MMP-9 was quantified in bronchoalveolar lavage fluids and in lung homogenate supernatants 4 h after rhMMP-12 instillation. RhMMP-12 instillation induced a severe rise in pro-MMP-9 as compared to control mice instilled with dialysis buffer (Fig. 5). Dexamethasone, rolipram and marimastat were all able to reduce significantly the enhanced level of proMMP-9 in bronchoalveolar lavage fluids, whereas in lung homogenate

supernatants, only dexamethasone and marimastat elicited a decrease in proMMP-9 level.

4. Discussion

This study showed that the direct administration of rhMMP-12 in mouse airways elicited some of the inflammatory components reported in COPD, namely influx of neutrophils and macrophages, and production of cytokines and chemokines. It also showed that in mice these features could be modulated by treatment with a corticosteroid, a PDE4 inhibitor and a non-selective MMP inhibitor.

In the present study, we have shown that orally administrated dexamethasone was able to limit the early inflammatory response induced by rhMMP-12 in mice airways. Indeed, neutrophil recruitment, chemokine (KC/CXCL1, IL-6 and MIP-1α/CCL3) release and proMMP-9 level in bronchoalveolar lavage fluids and in lung homogenate supernatants were significantly decreased by this corticosteroid. Since chemokines are mainly involved in neutrophil influx, it is hypothesized that the reduction of neutrophil influx induced by MMP-12 is a consequence of the inhibition of production of these chemokines by corticosteroid. The well understood cellular mechanism of action of corticosteroids is to reduce inflammatory responses (neutrophil chemotaxis and activation) by intracellular inhibition of transcription or translation of proinflammatory cytokines and chemokines (Scheinman et al., 1995). It is also known that corticosteroids inhibit the production of MMPs by *in vitro* cultured macrophages, inhibit MMP-9 release in the bronchoalveolar lavage fluid of asthmatic patients, and markedly reduce the release of MMP-9 in bronchoalveolar lavage fluid of mice exposed to lipopolysaccharide (LPS) (Corbel et al., 1999; Mautino et al., 1997; Shapiro et al., 1991). However, the lack of efficacy of dexamethasone to decrease the number of macrophages 7 d after MMP-12 instillation is unclear. Nevertheless, some differences may exist in the sensitivity of macrophages to corticosteroids. Indeed, *in vitro* release of IL-8, TNF-α and MMP-9 by macrophages from normal subjects and normal smokers are inhibited by corticosteroids, whereas corticosteroids are ineffective in macrophages from patients with COPD (Culpitt et al., 2003). The smoking status of COPD patients could be an explanation for the failure of glucocorticoids to reduce inflammation (Barnes, 2006). Indeed, corticosteroid suppression of inflammatory genes requires the recruitment of the histone deacetylase, HDAC2, and a direct inhibition of a molecular complex involved in the histone acetylation, inducing the repression of gene transcription. However, cigarette smoking, the main risk factor in COPD, is associated with a reduction in HDAC2 expression and activity in macrophages and bronchial biopsies (Barnes, 2006).

The poor efficacy of glucocorticoids in COPD suggests that novel types of anti-inflammatory compounds must be developed for the treatment of this pathology. Among these molecules, the most promising are the phosphodiesterase 4 (PDE4) inhibitors which have an inhibitory effect on key inflammatory cells involved in COPD, namely macrophages and neutrophils (Lagente et al., 2005). PDE4 inhibition induces an intracellular accumulation of cyclic AMP with many consequences, including smooth muscle relaxation, and reduction in inflammatory cell chemotaxis and activation. In our study, we have shown that rolipram was ineffective in decreasing macrophage recruitment whereas it was able to elicit a significant decrease in neutrophil numbers, cytokine levels and pro-MMP-9 release in bronchoalveolar lavage fluids. The reduced neutrophil number and the inhibition of cytokine levels have been previously described (Spond et al., 2001; Toward and Broadley, 2002; Verghese et al., 1995). However, we previously reported that alveolar macrophages are not completely sensitive to PDE4 inhibitors (Germain et al., 1998), suggesting the resistance of certain components of the inflammatory response in the lungs to the PDE4 inhibition as we have seen it with the macrophage recruitment in the present study. It has also previously been shown that selective PDE4 inhibitors reduced MMP-9 activity in bronchoalveolar lavage fluids of LPS-exposed mice, that is closely associated to the reduction of neutrophil recruitment (Corbel et al., 2002). These results suggest that PDE4 inhibitors could also modulate the remodeling process associated with lung inflammation in pathologies such as COPD.

Our results also clearly demonstrate that marimastat markedly inhibits the MMP-12-induced early inflammatory reaction, including neutrophil influx, cytokine level and MMP-9 release, as well as the delayed reaction, mainly characterized by macrophage influx in bronchoalveolar lavage fluids. These results differ from those obtained following LPS aerosol exposure in rodents where either batimastat, a structural chemically analog of marimastat (Corbel et al., 2001) or a Bayer broad spectrum MMP inhibitor (Birrell et al., 2006) were ineffective to diminish inflammatory cell recruitment in bronchoalveolar lavage fluids. These results are also consistent with the absence of reduction of neutrophil influx in bronchoalveolar lavage fluids of MMP-12 knock out mice exposed to LPS (Leclerc et al., 2006). This indicates that the mechanisms involved in cell recruitment induced by LPS and by rhMMP-12 are different and could explain the poor efficacy of dexamethasone and rolipram in our model on macrophage recruitment. This also highlights that MMP-12 could induce cell recruitment by an indirect route, independently of well known described inflammatory processes. This hypothesis has already been evoked by Hautamaki et al. (1997). Indeed, MMP-12 is a potent elastase and can generate *in situ* fragments of elastin, that are able to induce monocyte/macrophage recruitment to the lung (Houghton et al., 2006). However, it has been recently demonstrated that dexamethasone and rolipram were able to reduce the elastolytic activity in bronchoalveolar lavage fluids of guinea-pigs exposed to LPS (Johnson et al., 2005). However, this indicates that compounds which inhibit recruitment and/or activation of inflammatory cells, mainly neutrophils and macrophages, are also able to reduce elastase release and/or production by these cells. Moreover, whether or not elastolytic activity produced by neutrophil influx after LPS exposure is different from that of MMP-12-induced macrophage recruitment remains to be clarified.

In summary, this study shows that the instillation of rhMMP-12 in mouse airways elicited some of the inflammatory components seen in COPD. It also showed that a corticosteroid and a PDE4 inhibitor were able to reverse part of the inflammatory process at an early stage but not macrophage recruitment. Taken together, these data indicate that this model could exhibit some characteristics of the inflammatory response seen in COPD and could be used for the pharmacological evaluation of new compounds such as MMP inhibitors.

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