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Disease-modifying effect of ASP3258, a novel phosphodiesterase type 4 inhibitor, on subchronic cigarette smoke exposure-induced lung injury in guinea pigs

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ABSTRACT

ASP3258 is a novel, orally active, selective phosphodiesterase (PDE) 4 inhibitor which has an improved therapeutic window over second generation compounds such as roflumilast and cilomilast. Here, we investigated the effect of ASP3258 on cigarette smoke exposure-induced lung injury in guinea pigs, a well-defined model for chronic obstructive pulmonary disease (COPD). COPD-like lung injury was induced by repeated cigarette smoke exposure (10 cigarettes/day, 5 days/week, for 4 weeks). Orally administered ASP3258 (0.3, 1, and 3 mg/kg) dose-dependently suppressed pulmonary accumulation of mononuclear cells and neutrophils, and the inhibitory effect of ASP3258 (1 mg/kg) was almost the same as that of roflumilast (1 mg/kg). In contrast, a glucocorticoid prednisolone (10 mg/kg, p.o.) did not show any effect. Histological examination revealed that ASP3258 treatment significantly inhibited infiltration of neutrophils and macrophages into either or both alveolar or peribronchiolar areas, as well as hyperplastic and squamous metaplastic changes of epithelium in the bronchi. Decreasing trends in histological scores for accumulation of lymphocytes in the alveoli and alveolar wall thickening were also observed in ASP3258-treated animals. Further, ASP3258 attenuated augmentation of matrix metalloproteinase-9 activity in the bronchoalveolar lavage fluid. These findings suggest that ASP3258 has therapeutic potential for treating COPD not only through inhibition of pulmonary cellular accumulation but also by preventing lung structural alterations initiated by repeated cigarette smoke exposure. To our knowledge, this is the first paper demonstrating that PDE4 inhibitors exert significant inhibitory effects on subchronic cigarette smoke exposure-induced lung injury in guinea pigs.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a common and debilitating chronic inflammatory disease characterized by progressive and largely irreversible airflow limitation and is predicted to become the third leading cause of death worldwide by 2020. Cigarette smoking is a major causal factor for ongoing pulmonary inflammation in the airways and lung parenchyma, and the degree of inflammation is correlated with the severity of airflow limitation. Macrophages, neutrophils, and $CD8^+$ lymphocytes are predominantly involved in the inflammatory response, and various inflammatory mediators derived from these cells, such as cytokines, chemokines, and proteases contribute to COPD development (Pauwels et al., 2001; Barnes et al., 2003; Saetta et al., 2001).

Suppression of the inflammatory responses is thus a rational approach to treating COPD. Although glucocorticoids are the most effective agents in the treatment of inflammatory diseases such as asthma, they have been found to be largely ineffective in attenuating inflammation in COPD patients (Barnes et al., 2003). For this reason, developing novel anti-inflammatory agents with mechanisms of action differing from those of glucocorticoids is an urgent issue in this area. Phosphodiesterase 4 (PDE4) inhibitor, which has potent anti-inflammatory activity, is one possible candidate. PDE4 is the main cAMP-metabolising enzyme in immune and inflammatory cells and airway smooth muscle, and its inhibition suppresses the recruitment and activation of several inflammatory cells, including macrophages, neutrophils, and $CD8^+$ lymphocytes (Souness et al., 2000; Giembycz, 2000). Previous clinical studies have found that second generation PDE4 inhibitors cilomilast and roflumilast significantly improve lung function and reduce the rate of exacerbation in patients with COPD (Rennard et al., 2006; Calverley et al., 2007), and more recently, roflumilast has been approved by the European Medicines Agency for the treatment of COPD (Fabbri et al., 2010). Although cilomilast and roflumilast have shown improved safety margins over the prototype, rolipram, between anti-inflammatory activities and class-specific side effects such as nausea and diarrhea, their therapeutic use is still likely to be limited due to the compounds' adverse effects (Rennard et al., 2006; Calverley et al., 2007).

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We recently discovered a novel orally active PDE4 inhibitor ASP3258 which has a wider therapeutic window than second generation PDE4 inhibitors cilomilast and roflumilast (Kobayashi et al., in press). Here, we investigated the effects of ASP3258, roflumilast, and prednisolone on repeated cigarette smoke exposure-induced lung injury in guinea pigs, a well-defined experimental model for COPD (Wright and Churg, 2002; Kubo et al., 2005). Further, using this model, we investigated the detailed mechanism involved in the therapeutic effects of PDE4 inhibitors on COPD, which remain largely unknown compared to asthma.

2. Materials and methods

2.1. Animals

Male Hartley strain guinea pigs were purchased from SLC (Shizuoka, Japan). The animals were maintained in ordinary animal cages, with food and water available *ad libitum*. Animals aged 7 weeks and weighing 400–600 g at the start of the experiments were used. All experiments were performed in accordance with the regulations of the corporate Animal Ethical Committee.

2.2. Drugs

ASP3258 (3-[4-(3-chlorophenyl)-1-ethyl-7-methyl-2-oxo-1,2-dihydro-1,8-naphthyridin-3-yl]propanoic acid) and roflumilast were synthesized at Astellas Pharma Inc. (Tsukuba, Japan). Prednisolone was purchased from Nacalai Tesque (Kyoto, Japan). These compounds were suspended in 0.5% (w/v) methylcellulose (Shin-Etsu Chemical Co., Tokyo, Japan) solution and orally administered at 3 ml/kg. The normal and control groups were treated with vehicle (0.5% methylcellulose).

2.3. Cigarette smoke exposure-induced lung injury model

Cigarette smoke exposure was performed using commercially available non-filter cigarettes (Peace brand cigarettes; Japan Tobacco Inc., Tokyo, Japan) and a cigarette smoke generator SG-200 and inhalation apparatus made up of 20 individual chambers (Sibata Scientific Technology Ltd., Tokyo, Japan) as described previously (Kubo et al., 2005). Briefly, each animal was placed into an individual chamber and exposed to diluted cigarette smoke in a conscious and restrained state. Animals were repeatedly exposed to the smoke of 10 cigarettes/day, 5 consecutive days/week, for 4 weeks. Each cigarette was puffed 15 times for 3 min at a rate of 5 puffs/min. One puff meant drawing 35 ml of cigarette smoke into a 50 ml syringe, and then blowing this cigarette smoke, which was diluted to 4% with air, into the apparatus. Fresh room air inhalation was performed for 1 min every 3 min of cigarette smoke exposure. According to the manufacturer's specifications, each cigarette contained 2.4 mg of nicotine and 24 mg of tar. All drugs and vehicle were orally administered 1 h before the start of each cigarette smoke exposure session. Age-matched non-smoke-exposed and vehicle-administered animals were used as normal group animals.

2.4. Bronchoalveolar lavage

Guinea pigs were sacrificed under urethane anesthesia (1.2 g/kg, i.p.), after which their tracheas were cannulated. The lungs were lavaged with 5 ml of ice-cold saline containing 1 U/ml heparin five times via the cannula. Bronchoalveolar lavage (BAL) fluid was centrifuged at 400×*g* for 10 min at 4 °C. The resultant cell pellet was resuspended in 2 ml of ice-cold heparinised saline to measure total cell count, and the supernatant was stored at −80 °C until use. BAL was performed 4–6 h after the final cigarette smoke exposure. The total number of leukocytes in the BAL fluid was counted using an automated cell counter (Celltac-α; Nihon Kohden, Tokyo, Japan), and differential cell count was performed using a cytospin preparation stained with Diff-Quik (Sysmex International Reagent Co., Ltd., Kobe, Japan). A minimum of 300 cells were identified and differentiated as mononuclear cells, neutrophils, or eosinophils using the standard morphological criteria.

2.5. Histological evaluation

To avoid possible traumatic damage due to BAL, histological assessment of the lung tissue was performed in separate animals. Animals to be evaluated were sacrificed 4–6 h after the final cigarette smoke exposure, after which the lungs were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 2-μm sections, and stained with hematoxylin and eosin. The bronchus and parenchyma sections were analyzed in a blind fashion, and the degrees of observed pathological changes including hyperplasia/squamous metaplasia of epithelium; infiltration of neutrophils, macrophages, and lymphocytes into the bronchiole or the alveolus; and thickening of the alveolar wall were scored as follows: 0, none; 1, slight; 2, mild; 3, moderate; and 4, severe.

2.6. Gelatin zymography

BAL fluid supernatant samples were concentrated 5-fold using Centricon YM-3 filters (Millipore, Billerica, MA, USA). The concentrated samples and positive controls (purified human metalloproteinase (MMP)-2 and -9; Chemicon, Temecula, CA, USA) were loaded onto 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 1 mg/ml gelatin under nonreducing conditions. After electrophoresis, the gel was washed for 2 h in 50 mM Tris–HCl (pH 7.5) containing 0.1 M NaCl and 2.5% Triton X-100 to remove the SDS. The gel was then rinsed in water and incubated overnight at 37 °C in development buffer (50 mM Tris–HCl [pH 7.5] containing 20 mM $CaCl_2$). After development, the gel was stained with staining solution (0.25% Coomassie blue R250 in 45% methanol/10% acetic acid/45% H_2O) and subsequently washed with destaining solution (30% methanol/10% acetic acid/60% H_2O). Gelatinolytic activities were detected as clear bands of gelatin lysis against a blue background stain. Band density was quantified using ImageQuant software (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.7. Statistical analysis

All statistical analyses were conducted using the SAS system (SAS Institute Inc., Cary, NC, USA). Data were expressed as means ± S.E.M. The statistical significance of differences between groups was determined using Student's t-test, Dunnett's multiple range test, or Wilcoxon rank sum test. Values of $P<0.05$ were considered significant.

3. Results

3.1. Effects of ASP3258, roflumilast, and prednisolone on pulmonary leukocyte infiltration in lungs after subchronic cigarette smoke exposure

Repeated exposure of guinea pigs to cigarette smoke (10 cigarettes/day, 5 days/week) for 4 weeks resulted in a significant increase in leukocytes counts in the BAL fluid. Differential cell count analysis revealed that the increased cells were composed mainly of mononuclear cells (particularly macrophages) and neutrophils. Orally administered ASP3258 (1 mg/kg, q.d.) markedly inhibited this increase in numbers of total leukocytes, mononuclear cells, and neutrophils in the BAL fluid. The reference compound roflumilast (1 mg/kg, p.o., q.d.), which is the first PDE4 inhibitor approved for the treatment of COPD, similarly attenuated increases in BAL cell counts. The inhibitory activities of ASP3258 and roflumilast at 1 mg/kg were almost identical (Fig. 1).

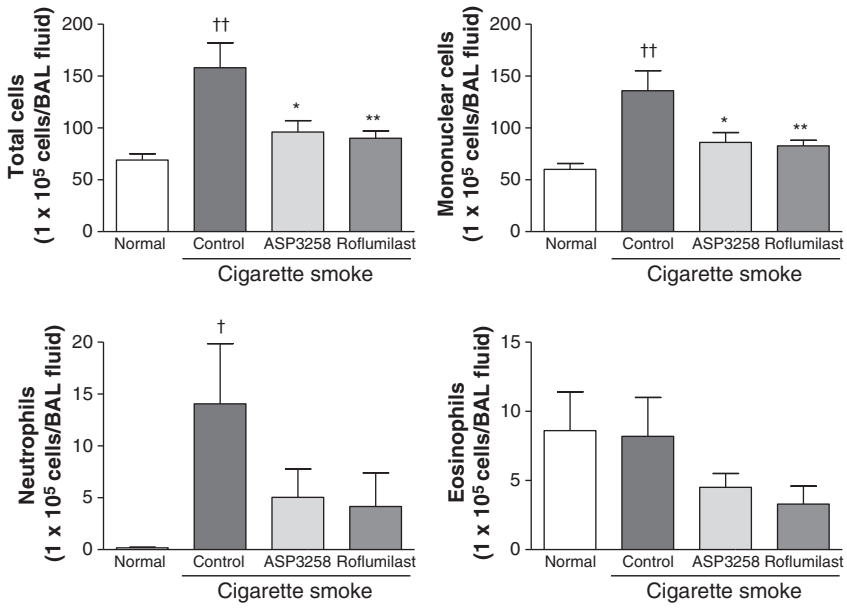

Fig. 1. Effects of ASP3258 and roflumilast on subchronic cigarette smoke exposure-induced pulmonary cellular accumulation in guinea pigs. Guinea pigs were repeatedly exposed to cigarette smoke (10 cigarettes/day, 5 days/week) for 4 weeks. Age-matched non-smoke-exposed animals were used as normal group. Test compounds (1 mg/kg) or vehicle was orally administered 1 h prior to every exposure session. Data are expressed as the mean ± S.E.M. of 9 to 12 animals. $^{\dagger\dagger}P<0.01$, $^{\dagger}P<0.05$, significantly different from normal group (Student's t-test). $^{**}P<0.01$, $^{*}P<0.05$, significantly different from control group (Student's t-test).

A dose–response study with ASP3258 (0.3, 1, and 3 mg/kg) showed a dose-dependent inhibition of increased numbers of BAL cells. In addition, ASP3258 tended to reduce the eosinophil counts to below the basal (normal) level at doses of 1 and 3 mg/kg (Fig. 2). This tendency toward a decrease in residential eosinophil counts was also observed in roflumilast-treated animals (Fig. 1). In contrast, oral administration of the glucocorticoid prednisolone (q.d.) showed no inhibitory effects on the numbers of the total or differential cells at a dose of 10 mg/kg, the dose at which significant inhibitory effects have been demonstrated in asthmatic pulmonary inflammation models in the same species (Aoki et al., 2001; Llupiá et al., 1991). Taken together, these results suggest that PDE4 inhibitors, but not prednisolone, exert marked inhibitory effects on macrophagic and neutrophilic pulmonary inflammation induced by subchronic exposure to cigarette smoke, and that ASP3258 has potent therapeutic activity comparable to that of roflumilast.

3.2. Effect of ASP3258 on subchronic cigarette smoke exposure-induced histological changes in the lung

To better understand the effect of ASP3258 on cigarette smoke-induced lung injury at the pathological level, we histologically analyzed the lung tissues. Results showed significant hyperplastic and squamous metaplastic changes to the bronchus epithelium of lungs removed from guinea pigs repeatedly exposed to cigarette smoke for 4 weeks (Fig. 3A vs. B and Fig. 4A). In the lung parenchyma sections, marked infiltration of macrophages into the alveoli and neutrophils into the alveoli and small airways were noted in cigarette smoke-exposed control animals compared to normal animals (Fig. 3D vs. E and Fig. 4B, C, and D). In some cigarette smoke-exposed control animals, infiltration of lymphocytes into the alveoli was also observed (Fig. 3E and Fig. 4E). In addition, evidence of significant alveolar wall thickening was noted in the cigarette smoke-exposed control animals (Fig. 3E and Fig. 4F).

Treatment with ASP3258 (1 mg/kg, p.o., q.d.) significantly reduced not only the accumulation of macrophages in the alveoli and neutrophils in the alveoli and small airways (Fig. 3E vs. F and Fig. 4B, C, and D) but also relieved epithelial hyperplasia and squamous metaplasia in the bronchus (Fig. 3B vs. C and Fig. 4A). Further, ASP3258-treated animals showed decreasing trends in histological scores for the lymphocyte infiltration as well as the alveolar wall thickening (Fig. 3E vs. F and Fig. 4E and F).

3.3. Effect of ASP3258 on gelatinolytic activity in BAL fluid of guinea pigs subchronically exposed to cigarette smoke

We evaluated gelatinolytic activity in BAL fluid supernatant samples by performing gelatin zymography. Gelatinolytic activity in the 72-kD band, which was regarded as MMP-2, was observed at almost identical levels in the BAL fluid of normal and control animals (Fig. 5A). In contrast, activity at 92 kD, corresponding to MMP-9, was markedly increased in the BAL fluid from cigarette smoke-exposed control animals compared to normal animals. Treatment with ASP3258 (1 mg/kg, p.o., q.d.) significantly reduced this increased level of MMP-9 activity but did not affect the basal level of MMP-2 activity (Fig. 5A and B).

4. Discussion

Here, we demonstrated that ASP3258, a novel, orally active PDE4 inhibitor, exerts a potent inhibitory effect on subchronic cigarette smoke exposure-induced lung injury in guinea pigs. As previously demonstrated (Kubo et al., 2005), repeated exposure of guinea pigs to cigarette smoke for 4 weeks caused marked accumulation of macrophages and neutrophils in the lung, induced structural alterations of lung tissue, and augmented MMP-9 activity in the BAL fluid, changes similar to the pathological features observed in patients with COPD. Given these findings, this guinea pig model is therefore deemed adequate for predicting the efficacy of potential disease-modifying agents for COPD.

A large number of studies have demonstrated that macrophages and neutrophils play crucial roles in the inflammatory condition of COPD (Pauwels et al., 2001; Barnes et al., 2003; Saetta et al., 2001). In BAL cell analyses in the present study, oral administration of ASP3258 markedly and dose-dependently attenuated increases in mononuclear cells (particularly macrophages) and neutrophils counts induced by subchronic cigarette smoke exposure. Roflumilast, the first PDE4 inhibitor approved for the treatment of COPD, also induced an inhibitory effect.

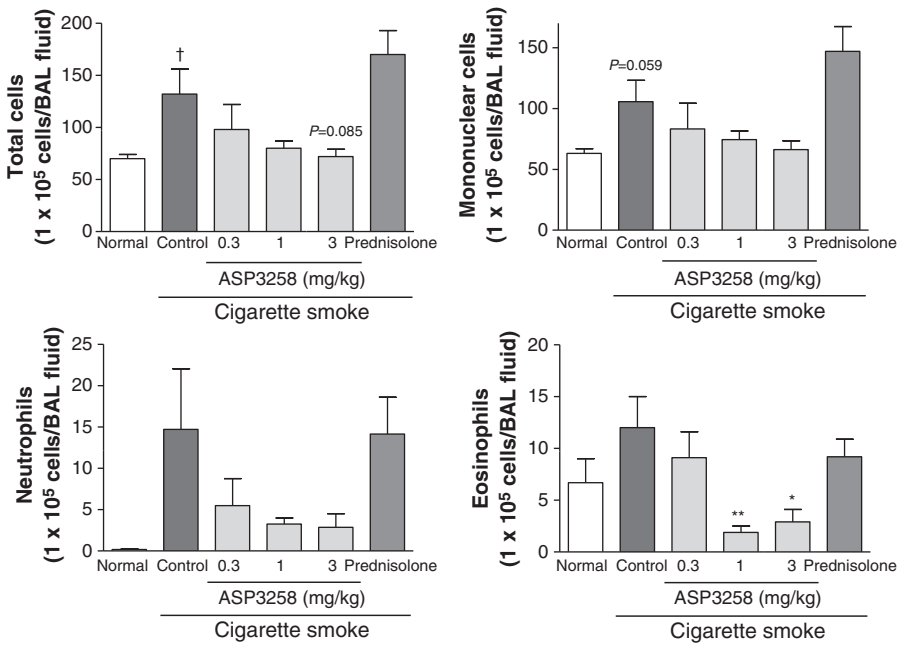

Fig. 2. Effects of ASP3258 (dose–response study) and prednisolone on subchronic cigarette smoke exposure-induced pulmonary cellular accumulation in guinea pigs. Guinea pigs were repeatedly exposed to cigarette smoke (10 cigarettes/day, 5 days/week) for 4 weeks. Age-matched non-smoke-exposed animals were used as normal group. ASP3258 (0.3, 1, and 3 mg/kg), prednisolone (10 mg/kg), or vehicle was orally administered 1 h prior to every exposure session. Data are expressed as the mean ± S.E.M. of 8 to 12 animals. $^{\dagger}P<0.05$, significantly different from normal group (Student's t-test). $^{**}P<0.01$, $^{*}P<0.05$, significantly different from control group (Dunnett's multiple range test).

Given that the chemical structures of ASP3258 and roflumilast differ quite significantly, these similar suppressing effects are considered to be a consequence of PDE4 inhibitory action and not some off-target mechanism. These results suggest that ASP3258 may have an important pharmacological role as a therapeutic agent for treating COPD.

Although subchronic exposure of guinea pigs to cigarette smoke did not significantly affect eosinophil numbers in the BAL fluid in the experimental conditions of the present study, a decreasing trend in the basal number of eosinophils was observed in both ASP3258- and roflumilast-treated animals. This finding raises the possibility that

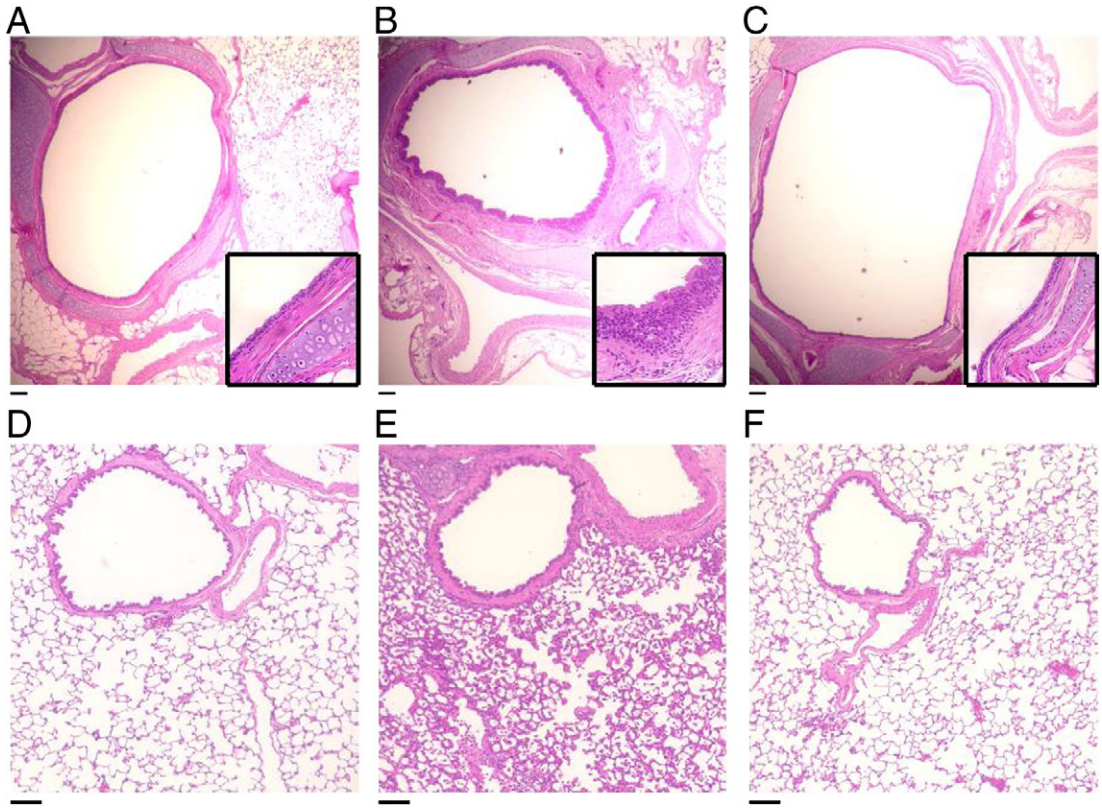

Fig. 3. Representative micrographs of lung tissue stained with hematoxylin and eosin. A, B, and C show sections of bronchus, and D, E, and F show sections of parenchyma from normal (A and D), cigarette smoke-exposed (B and E), and cigarette smoke-exposed and ASP3258 (1 mg/kg)-treated (C and F) animals, respectively. Scale bars represent 100 μm.

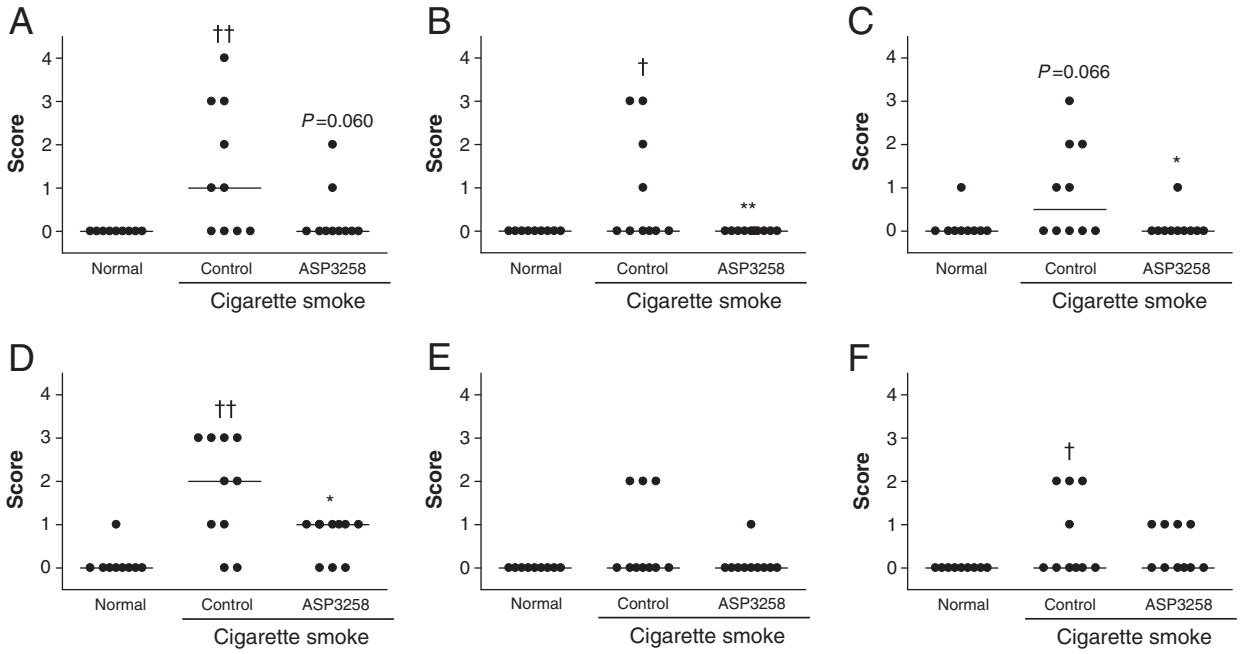

Fig. 4. Quantitative assessment of histological scores in the lung from normal, control, and ASP3258 (1 mg/kg)-treated guinea pigs. Histological signs observed in bronchus (A) and parenchymal (B to F) areas were scored. A) Hyperplasia/squamous metaplasia of epithelium in the bronchus, B) infiltration of neutrophils into the bronchiole in the parenchyma, C) infiltration of neutrophils into the alveolus in the parenchyma, D) infiltration of macrophages into the alveolus in the parenchyma, E) infiltration of lymphocytes into the alveolus in the parenchyma, and F) thickening of the alveolar wall. Each symbol represents an individual's data point. Horizontal lines indicate medians. $^{††}P<0.01$, $^{†}P<0.05$, significantly different from normal group (Wilcoxon rank sum test). $^{**}P<0.01$, $^{*}P<0.05$, significantly different from control group (Wilcoxon rank sum test).

PDE4 inhibitors attenuate the steady-state turnover of residential eosinophils in the lung. Unlike in asthma, eosinophils are not considered to play a dominant role in the pathogenesis of COPD; however, their involvement during the exacerbation phase is indicated. Evidence has shown that roflumilast and cilomilast induce a significant reduction in the mean number of COPD exacerbations per patient in clinical studies (Rennard et al., 2006; Calverley et al., 2007). This reducing effect of PDE4 inhibitors on residential eosinophils may contribute to the subsequent reduction of COPD exacerbation, at least in part.

Of particular note is the fact that prednisolone (10 mg/kg, p.o.) exerted no inhibitory effect on the increase in BAL cell counts in this cigarette smoke model. Although guinea pigs are known to exhibit a kind of glucocorticoid resistance with high circulating levels of cortisol and a low-affinity glucocorticoid receptor (Keightley and Fuller, 1996) oral prednisolone at 10 mg/kg has exerted significant inhibitory effects in asthmatic pulmonary inflammation models in this species (Aoki et al., 2001; Llupiá et al., 1991). Our model appears to well reflect the limited efficacy of glucocorticoids in patients with COPD (Barnes et al., 2003). In human studies, the lack of effectiveness of glucocorticoids in treating COPD is assumed to be due in part to an active resistance mechanism linked to a reduction in histone deacetylase (HDAC)-2 expression, which is required by glucocorticoids to switch off activated inflammatory genes (Barnes, 2006). Future studies should conduct expression analysis of HDAC-2 in this guinea pig cigarette smoke model in order to clarify the reason for the observed ineffectiveness of prednisolone.

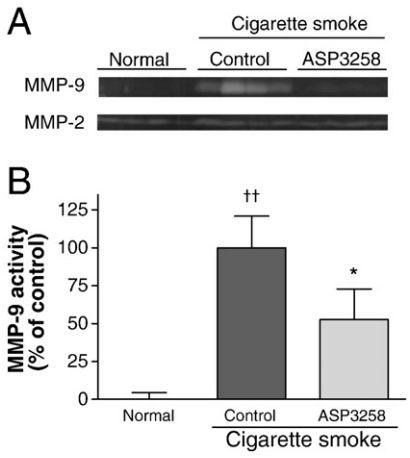

Fig. 5. Effect of ASP3258 on MMP-2 and MMP-9 activities in the BAL fluid. Gelatin zymography was performed with the BAL fluid supernatant samples obtained from normal, control, and ASP3258 (1 mg/kg)-treated guinea pigs. A) Representative zymograms for MMP-2 and MMP-9. B) The bands of MMP-9 activities were quantified by densitometric scanning. Results are expressed as the mean ± S.E.M. of 8 animals. $^{††}P<0.01$, significantly different from normal group (Student's t-test). $^{*}P<0.05$, significantly different from control group (Student's t-test).

Histological examination in the cigarette smoke-exposed control animals in the present study showed not only pulmonary cellular accumulation but also structural alterations of lung tissue such as hyperplastic and squamous metaplastic changes of airway epithelium and alveolar wall thickening. Consistent with BAL cell analyses, treatment with ASP3258 significantly reduced histological scores for infiltration of neutrophils and macrophages into alveolar and peribronchiolar areas. Further, the ASP3258-treated group also showed decreased scores for hyperplasia and squamous metaplasia of bronchial epithelia and alveolar wall thickening. These findings strongly suggest that pulmonary structural alterations in cigarette smoke-related diseases are primarily due to sustained inflammatory responses and not the direct irritative effect of cigarette smoke. These progressive pulmonary structural alterations in COPD may therefore be controllable with anti-inflammatory drugs such as PDE4 inhibitors.

While emphysematous airspace enlargement, a characteristic feature of COPD, was not observed in this cigarette smoke model, a marked increase in MMP-9 activity in the BAL fluid was detected. MMP-9, which can degrade both elastin and collagen, is predominantly produced by macrophages and also found in neutrophil granules (Belvisi and Bottomley, 2003). Previous studies have noted

increased MMP-9 but not MMP-2 activity in alveolar macrophages in patients with emphysema (Finlay et al., 1997) or COPD (Russell et al., 2002). In addition, a recent study using MMP-9 transgenic mice demonstrated the direct involvement of MMP-9 in the induction of pulmonary emphysema (Foronjy et al., 2008). These findings suggest that MMP-9 is an important protease involved in the development of emphysema. Given our finding that ASP3258 significantly reduced the level of MMP-9 activity in the BAL fluid, ASP3258 is likely to attenuate the development or progression of cigarette smoke-related emphysematous lesions by improving the protease–antiprotease imbalance. A previous study in mice showed that roflumilast fully prevented development of pulmonary emphysema induced by seven months' exposure to cigarette smoke (Martorana et al., 2005).

In summary, we report here on the disease-modifying effect of the novel PDE4 inhibitor ASP3258 in a subchronic cigarette smoke exposure-induced COPD model in guinea pigs. Given its improved therapeutic window over roflumilast and cilomilast (Kobayashi et al., in press), ASP3258 is a promising candidate for use in treating COPD. In addition, the data obtained here will help provide new insight into the mechanisms responsible for the anti-COPD activity of PDE4 inhibitors.

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