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A single injection of a sustained-release prostacyclin analog (ONO-1301MS) suppresses airway inflammation and remodeling in a chronic house dust mite-induced asthma model

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ABSTRACT

ONO-1301, a novel prostacyclin agonist with thromboxane A2 synthase inhibitory activity, is a useful agent for ameliorating airway allergic inflammation; however, its short-action feature implies a requirement for the frequent administration of this drug. Therefore, we investigated the effects of ONO-1301-loaded poly (D,L-lactic-co-glycolic acid) microspheres (ONO-1301MS; to release ONO-1301 for 3 weeks) on the airway inflammation and remodeling in chronic house dust mite (HDM)-induced model. Balb/c mice were exposed to an HDM extract intranasally for 5 days/week for 5 consecutive weeks. The mice received a single subcutaneous injection of ONO-1301MS or vehicle after 3 weeks of HDM exposure, followed by 2 additional weeks of HDM exposure. Forty-eight hours after the last HDM exposure, airway hyperresponsiveness to methacholine was assessed and bronchoalveolar lavage was performed. Lung specimens were excised and stained to check for goblet cell metaplasia, airway smooth muscle hypertrophy, and submucosal fibrosis. Mice receiving ONO-1301MS showed significantly lower airway hyperresponsiveness, airway eosinophilia, and induced T helper 2 cytokine production compared with mice receiving the vehicle. Histological findings such as goblet cell metaplasia, airway smooth muscle hypertrophy, and submucosal fibrosis were decreased in ONO-1301MS-treated mice compared with vehicle-treated mice. A single administration of ONO-1301MS achieved sustained elevation of its circulating level for 3 weeks. These data suggest that a single administration of ONO-1301MS may suppress airway hyperresponsiveness, airway allergic inflammation, and development of airway remodeling in chronic HDM-induced asthma model. This agent may be effective as an anti-inflammatory and remodeling drug in the practical treatment of asthma.

1. Introduction

Bronchial asthma is characterized by recurrent episodes of airway obstruction, airway hyperresponsiveness to environmental stimuli, acute-on-chronic airway inflammation, and structural changes in the airway walls (Busse and Lemanske, 2001). Basically, environmental allergens, such as pollen and house dust mite (HDM), penetrate into the airway wall, encounter antigen-presenting cells, and cause the development of airway allergic inflammation characterized by the elevation of the levels of eosinophilia and T helper 2 (Th2) cytokines in airways; this results in wheezing, breathlessness, chest tightness, and cough (Bousquet et al., 2000).

Traditionally, ovalbumin has been used as an antigen for the development of rodent models of allergic inflammation. Although ovalbumin-induced airway inflammation shares many features observed in asthmatic individuals, it is not a naturally existing allergen in humans; moreover, chronic exposure without systemic sensitization leads to tolerance (Swirski et al., 2002; Koya et al., 2006; Swirski et al., 2006). Therefore, the use of chronic animal models related to common environmental allergen, such as house dust mite (HDM) (Johnson et al., 2004) and ragweed (Cates et al., 2003), may lead to the elucidation of the mechanisms underlying

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airway inflammation and remodeling and provide better test systems for the evaluation of novel therapeutic approaches.

ONO-1301 is a synthetic prostacyclin agonist that lacks the typical prostanoid structure of a 5-membered ring and allylic alcohol, which renders its metabolization more difficult. This feature enhances stability with long-lasting prostacyclin activity when the drug is administered *in vivo*. ONO-1301 also has thromboxane synthase inhibitory activity because of the presence of a 3-pyridine radical. We had previously demonstrated that ONO-1301 prevents acute airway inflammation in mouse models of allergic asthma via the inhibition of lung dendritic cell functions (Hayashi et al., 2010) and in a chronic airway remodeling model by prolonged exposure to ovalbumin (Yamabayashi et al., 2012).

Although the half-life of plasma ONO-1301 is longer than that of any other prostacyclin analog, ONO-1301 still needs to be administered subcutaneously twice a day to achieve significant improvement in bronchial asthma. For patients with asthma, the development of a long-acting, sustained-release prostacyclin analog would be beneficial in terms of quality of life. To overcome these problems, we prepared a novel sustained-release prostacyclin analog polymerized with poly (D,L-lactic-co-glycolic acid) (PLGA) microspheres (ONO-1301MS). PLGA microspheres, which are biodegradable and biocompatible compounds, have been used as a controlled delivery system for proteins and drugs (Alonso et al., 1994; Mullerad et al., 2003; Roullin et al., 2003).

In the present study, we investigated whether a single administration of ONO-1301MS had effects on the airway hyperresponsiveness and airway remodeling in a murine model of chronic HDM-induced airway disease. We also compared its effects with those of repeated treatment with ONO-1301.

2. Materials and methods

2.1. Animals

Eight-week-old female BALB/c mice free of murine-specific pathogens were purchased from CLEA Japan Inc. (Tokyo, Japan). Animals were housed under specific pathogen-free conditions and a 12:12 h light:dark cycle. All experiments were conducted under a protocol approved by the Niigata University ethics committee for animal experiments.

2.2. Preparation of ONO-1301MS

ONO-1301MS was obtained from the polymerization of ONO-1301 with PLGA microspheres. ONO-1301 and PLGA (polylactic acid to glycolic acid ratio, 50:50) were dissolved in dichloromethane. The dissolved polymer was added to a polyvinyl alcohol aqueous solution to form an oil-in-water emulsion. Subsequently, the dichloromethane was evaporated by stirring. After centrifugation and washing, ONO-1301MS was isolated by lyophilization. The content of ONO-1301 in the ONO-1301MS was 16.5%.

2.3. HDM-induced asthma model and treatment protocol

Mice were exposed to a purified HDM extract (Greer Laboratories, Lenoir, NC) intranasally (25 μg of protein in 10 μl of saline) without immunization 5 days/week for 5 consecutive weeks. Forty-eight hours after the last HDM challenge, airway hyperresponsiveness was assessed and bronchoalveolar lavage (BAL) fluid, serum, and lungs were obtained for further analyses. To investigate the degree of airway hyperresponsiveness and allergic airway inflammation before the start of drug administration, some mice were sacrificed after 3-week HDM challenges (Fig. 1).

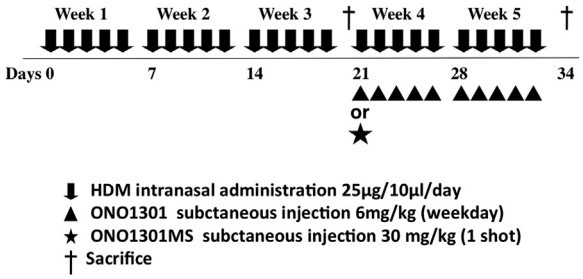

Fig. 1. Experimental protocols. Mice were exposed to a purified house dust mite (HDM) extract intranasally (25 μg of protein in 10 μl of saline) without immunization for 5 days/week for 5 consecutive weeks. Forty-eight hours after the last HDM challenge, airway hyperresponsiveness was assessed and BAL fluid, serum, and lungs were obtained for further analyses. Some of the mice were sacrificed after 3 weeks of HDM challenges.

ONO-1301MS was suspended in NaOH/saline solution. The mice received a single subcutaneous injection of 30 mg/kg of ONO-1301MS on the first day of the fourth week of HDM exposure. ONO-1301 also dissolved in NaOH/saline was administered subcutaneously (6 mg/kg/day, divided into 2 injections per day) to some mice during the last 2 weeks of HDM exposure (Fig. 1). As a control, some mice were treated with the vehicle during the last 2 weeks or with PLGA alone with a single injection.

2.4. Determination of airway hyperresponsiveness

Airway hyperresponsiveness was assessed by measuring the changes in respiratory resistance using the flexiVent system (SCIREQ; Montreal, Quebec, Canada) in response to increasing doses of inhaled methacholine, as reported previously (Hayashi et al., 2010).

2.5. Measurement of cytokine levels in bronchoalveolar lavage (BAL) fluid

Immediately after the measurement of airway hyperresponsiveness, BAL was performed via a tracheal tube, as described previously (Koya et al., 2007).

The supernatants from BAL fluid were used for the measurement of cytokines and growth factors using commercially available enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturers' instructions. ELISA kits for the detection of IL-4 and IL-5 in the supernatants from BAL fluid were obtained from BD Pharmingen (San Diego, CA, USA). The IL-13, TGF-β1, and platelet-derived growth factor (PDGF)-AA ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA).

2.6. Assay of the plasma levels of ONO-1301

To measure the plasma levels of ONO-1301, blood was collected from the mice ($n=6$) on the day before and at 15 min, 30 min, 1 h, 3 h, 24 h, 48 h, 7 days, 14 days, 21 days, and 28 days after the single subcutaneous administration of ONO-1301MS (30 mg/kg). To compare the kinetics of ONO-1301MS with ONO-1301, blood was also collected from the mice administered ONO-1301 subcutaneously (3 mg/kg) on the day before and at 15 min, 30 min, 1 h, 3 h, 8 h, 12 h, and 24 h after single subcutaneous administration. Plasma ONO-1301 levels were measured by using a liquid chromatography tandem mass spectrometry assay, as reported previously (Obata et al., 2008).

2.7. Histology and immunohistochemical staining

Left lungs were fixed in 10% formalin and immersed in paraffin. After deparaffinization, samples were stained with periodic acid-Schiff (PAS) and Masson's trichrome for histological analysis. Right lungs were fixed at 4 °C in periodate-lysine-paraformaldehyde and embedded in OCT compound, frozen in dry ice/acetone, and cut on a cryostat for the detection of α-smooth muscle actin (α-SMA) expression, as reported previously (Yamabayashi et al., 2012). Histological analyses were performed as described previously (Koya et al., 2006).

2.8. Statistical analyses

Mann–Whitney *U* tests were used to determine the levels of differences among all groups. Comparisons of all pairs were performed using the Kruskal–Wallis test. Significance was assumed at P values <0.05 for all tests. The values for all measurements were expressed as means ± S.E.M.

3. Results

3.1. Effect of ONO-1301MS on airway hyperresponsiveness, airway inflammation, and airway remodeling

Mice that were exposed to HDM for 3 weeks showed obvious airway hyperresponsiveness to methacholine, airway eosinophilia, and Th2-type cytokine elevation in the BAL fluid compared with animals that were exposed to saline. Airway hyperresponsiveness and allergic airway inflammation were augmented in mice exposed to HDM for 5 weeks (Fig. 2A–C). Mice that were administered ONO-1301MS showed obviously suppressed airway hyperresponsiveness across the methacholine dose-response curve compared

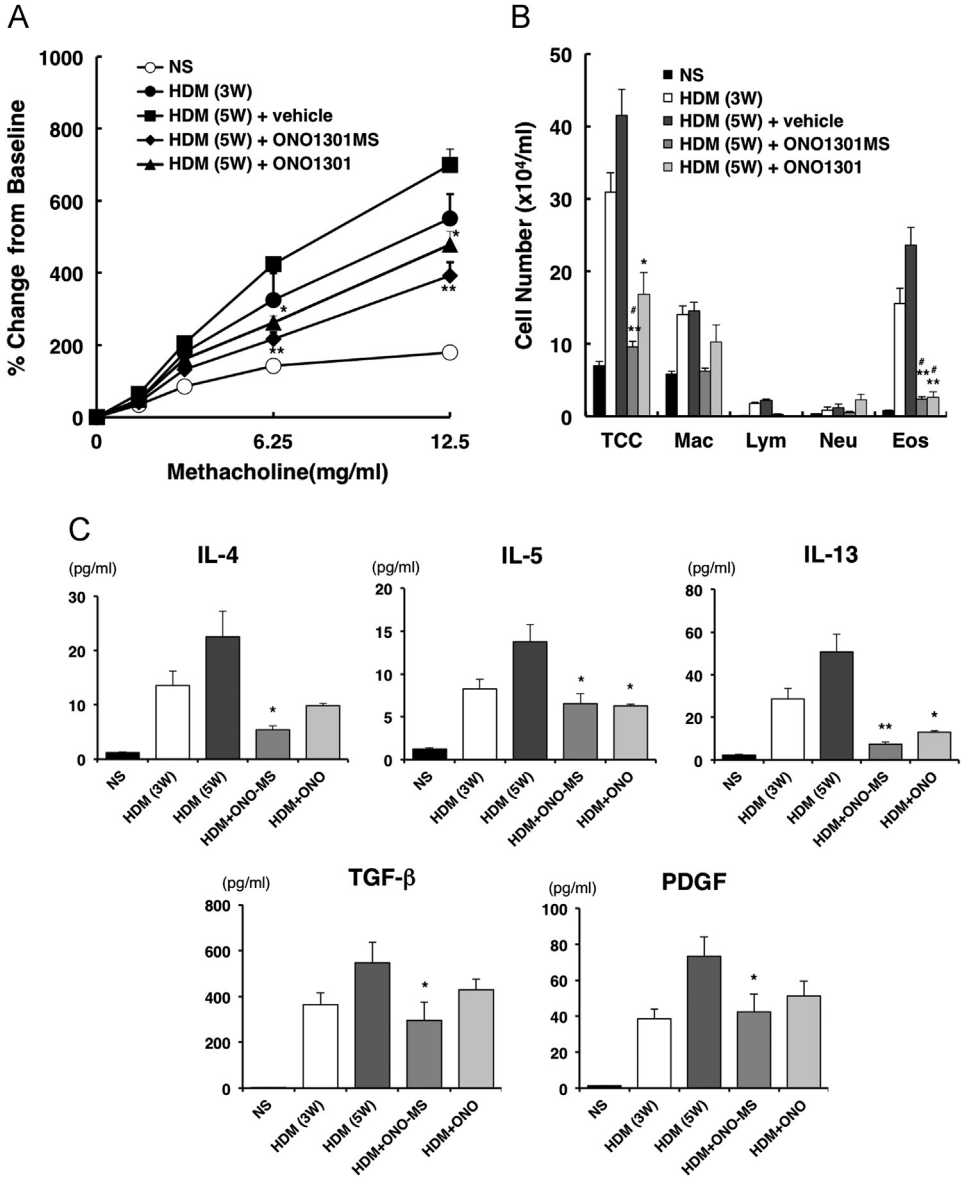

Fig. 2. Comparison of the effects of ONO-1301MS on the development of airway hyperresponsiveness and inflammation. Airway hyperresponsiveness and airway inflammation in mice exposed to saline intranasally for 5 weeks (NS); mice exposed to house dust mite (HDM) extract intranasally for 3 weeks (HDM (3W)); mice exposed to HDM for 5 weeks (HDM (5W)+vehicle) and ONO-1301MS 30 mg/kg or ONO-1301 6 mg/kg for 2 weeks (HDM (5W)+ONO-1301MS or HDM (5W)+ONO-1301, respectively). Refer to the "Material and Methods" section regarding the details of the protocol. (A) Changes in airway resistance with increased concentrations of nebulized methacholine. (B) Changes in cell composition in the BAL fluid. (C) Changes in cytokine levels (IL-4, IL-5, IL-13, TGF-β, and PDGF) in the BAL fluid. TCC, total cell count; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils. $^{*}P<0.05$ or $^{**}P<0.01$ compared with the HDM (5W)+vehicle group. $^{\#}p<0.05$ compared with the HDM (3W) group. Data represent the mean ± S.E.M. for $n=12$ per group.

with animals that were administered the vehicle (Fig. 2A). Mice that were given ONO-1301 also showed significantly lower airway hyperresponsiveness at higher doses of methacholine (6.25 and 12.5 mg/ml) compared with animals that were given the vehicle (Fig. 2A). The number of inflammatory cells in the BAL fluid was determined 24 h after the last allergen challenge. The administration of ONO-1301MS and ONO-1301 significantly attenuated the increase in lymphocyte and eosinophil counts in the BAL fluid compared with the results for the administration of the vehicle (Fig. 2B). Airway hyperresponsiveness and cell composition in BAL fluid for mice administered PLGA alone were similar to those of vehicle-treated mice (data not shown). The levels of IL-4, IL-5, IL-13, and PDGF-AA in the BAL fluid significantly decreased in ONO-1301MS- and ONO-1301-treated mice. The levels of TGF-β and PDGF-AA in the BAL fluid from mice treated with ONO-1301MS were significantly lower compared with those observed in vehicle-treated groups (Fig. 2C).

The presence of goblet cell metaplasia, which is characteristic of chronic asthma, can be easily identified by PAS staining of mucus glycoproteins. Mice exposed to HDM for 3 weeks developed obvious goblet cell metaplasia, which was augmented after 5 weeks of HDM exposure. Mice treated with ONO-1301MS and ONO-1301 showed decreased goblet cell metaplasia. In particular, the ONO-1301MS-treated group exhibited a significant decrease of this condition compared with mice that were exposed to HDM for 3 weeks (Fig. 3A and D).

In addition to goblet cell metaplasia, mice that were exposed to HDM for 5 weeks showed increase in bronchial submucosal collagen deposition, as assessed via staining with Masson's trichrome, and smooth muscle thickness, as assessed via staining with an anti-α-SMA antibody. ONO-1301MS and ONO-1301 dramatically reduced airway smooth muscle hypertrophy and collagen deposition in the bronchial submucosal area compared with

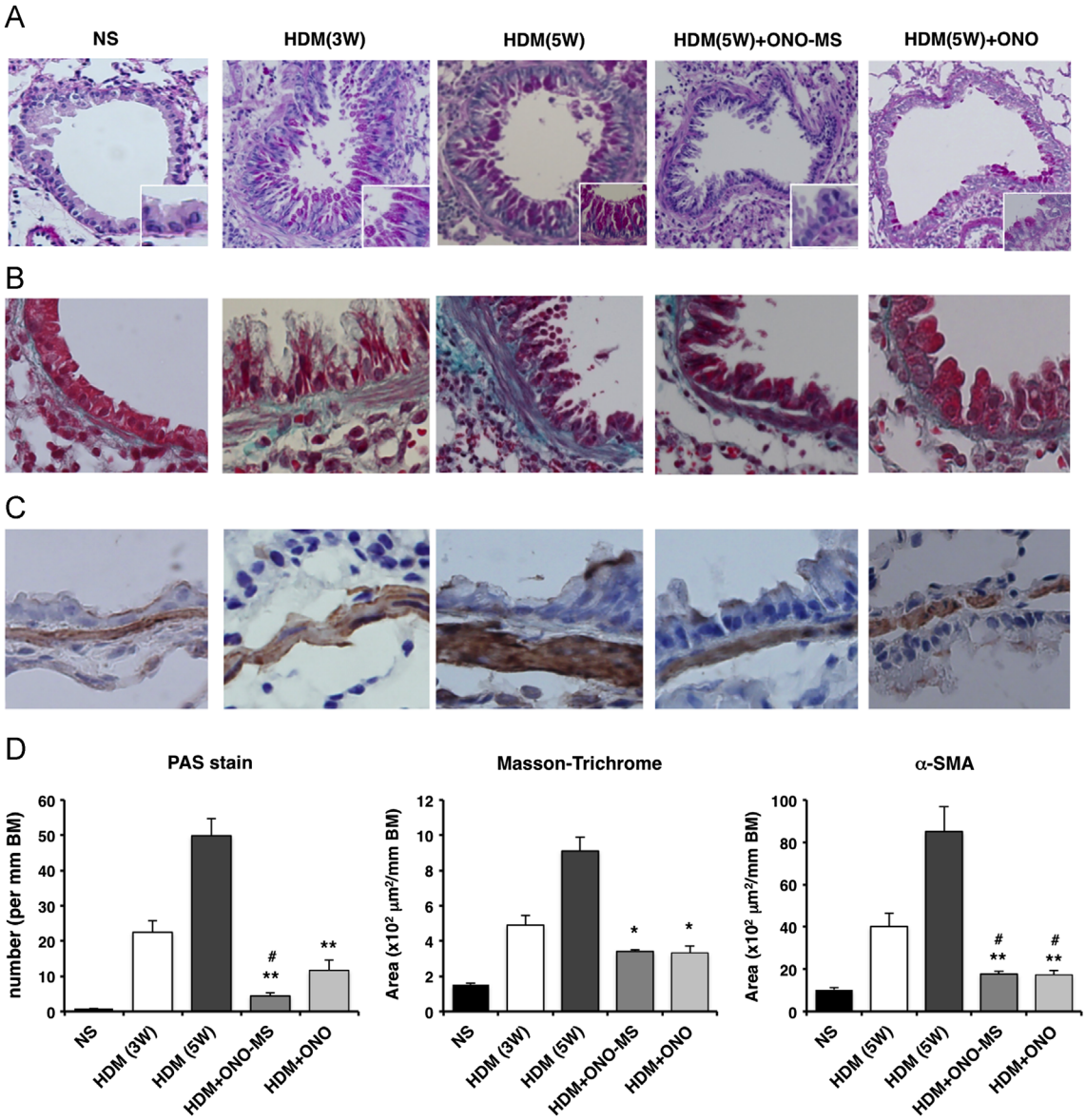

Fig. 3. Comparison of the effects of the drugs on features of airway remodeling. Representative periodic acid-Schiff (PAS) staining (A), Masson's trichrome staining (B), and immunohistochemical staining of the airway smooth muscle cells for α smooth muscle actin (α-SMA) (C) performed in lung sections 24 h after the last challenge (original magnification: 100×; insets: 400× for PAS staining; 400× for Masson's trichrome staining and α-SMA immunostaining). NS, mice exposed to saline intranasally for 5 weeks; HDM(3W), mice exposed to HDM for 3 weeks; HDM(5W), mice exposed to HDM for 5 weeks + treated with vehicle; HDM(5W)+ONO-MS, mice exposed to HDM for 5 weeks + treated with ONO-1301 MS 30 mg/kg; HDM(5W)-ONO, mice exposed to HDM for 5 weeks + treated with ONO-1301 6 mg/kg for 2 weeks. (D) Quantitative analysis of PAS-positive cells, peribronchiolar collagen deposition in bronchial tissues, and α-smooth muscle actin layer in bronchial tissue. Data represent means ± S.E.M. from 3 independent experiments ($n=12$). $^{*}P<0.05$ or $^{**}P<0.01$ compared with HDM (5W). $\#P<0.05$ compared with HDM (3W). BM, basement membrane.

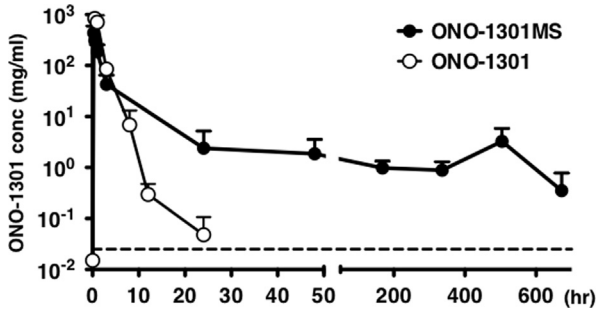

Fig. 4. Time-course changes in plasma ONO-1301 levels. Plasma ONO-1301 concentration after a single subcutaneous administration of ONO-1301MS or ONO-1301. The dotted line in the figure indicates the limit of quantification for ONO-1301 (0.025 ng/ml). Data represent means ± S.E.M. values ($n=6$).

the results for the vehicle-treated group. In particular, mice treated with ONO-1301MS showed decrease in airway smooth muscle hypertrophy compared with mice that were exposed to HDM for 3 weeks (Fig. 3B–D).

These data indicate that ONO-1301MS has the potential not only to suppress the development of allergic airway inflammation and airway remodeling but also to reverse fibrotic lesions because the exposure to HDM for 3 weeks before the drug was administered had already established airway inflammation and airway remodeling to some extent.

3.2. ONO-1301MS sustains the plasma levels of ONO-1301

To investigate whether a single subcutaneous administration of ONO-1301MS produces long-lasting prostacyclin activity in mice, we measured the plasma levels of ONO-1301 after ONO-1301MS injection (30 mg/kg). ONO-1301 was detected in the plasma even at 3 weeks after administration of ONO-1301MS, whereas the plasma level of ONO-1301 in 24 h after subcutaneous administration of ONO-1301 (3 mg/kg) went down to the detection limit (0.025 ng/ml) (Fig. 4). From the data for the plasma levels, Cmax and T1/2 of ONO-1301 were 1541.2 ng/ml, and 2.5 h respectively, whereas the Cmax of ONO-1301MS was 436.5 ng/ml. The T1/2 of ONO-1301MS could not be determined because the elimination phase could not be confirmed.

4. Discussion

In the present study, we demonstrated that a single injection of a novel sustained-release prostacyclin agonist polymerized with PLGA microspheres (ONO-1301MS) was able to interfere with the development of increased levels of airway hyperresponsiveness, airway allergic inflammation, and airway remodeling after repetitive HDM exposure in an experimental model of chronic asthma.

Conventional prostacyclin and its analogs require continuous infusion or frequent administration because of their short duration of action. Previously, Kataoka et al. reported a new type of prostacyclin agonist, ONO-1301, which has long-lasting prostacyclin activity and an inhibitory effect on thromboxane synthase (Kataoka et al., 2005). Although ONO-1301 exhibits such interesting features, its administration twice a day was still required to achieve significant reduction in airway hyperresponsiveness and airway remodeling in a chronic murine asthma model (Hayashi et al., 2010). To overcome this problem, here we investigated the effects of a single administration of ONO-1301MS.

PLGA microspheres have been used as a controlled delivery system for bioactive agents (Shive and Anderson, 1997). The release of bioactive agents from PLGA microspheres occurs via the hydrolytic degradation of the polymeric matrix. Importantly, PLGA has been used in humans. PLGA microspheres containing leuprorelin, a potent luteinizing-hormone-releasing hormone analog, have been administered to patients with prostate and breast cancer via subcutaneous injection (Fornara and Jocham, 1996; Schmid et al., 2007). The rate of release of the contents of PLGA microspheres can be changed by varying the factors affecting the hydrolytic degradation behavior of PLGA, such as lactate acid to glycolic acid ratio, average molecular weight of PLGA, and particle size (Shive and Anderson, 1997). In this study, we used the degradation rate from a previous report that described the sustained release of ONO-1301 for 3 weeks (Obata et al., 2008). We administered ONO-1301MS via injection on the first day of the fourth week of HDM exposure. We observed that the plasma concentration of ONO-1301 was sustained at 3 weeks after admission of ONO-1301MS (Fig. 4). Although the concentration of ONO-1301 was lower than the data previously reported in rat model (Obata et al., 2008), subcutaneous administration of ONO-1301MS in mice showed sustained levels of ONO-1301 compared to subcutaneous administration of ONO-1301 injection.

Prostacyclin is associated with suppression of airway remodeling in experimental asthma models and is a modulator of Th2-mediated inflammation. A deficiency of IP, which is a specific prostacyclin-binding receptor, has been reported to enhance and sustain allergic inflammation through repeated challenges for 3 weeks, and result in airway remodeling (Nagao et al., 2003; Takahashi et al., 2002). Several mechanisms, such as the inhibition of cytokine production (Zhou et al., 2007a), Th2 cell recruitment in the lung (Jaffar et al., 2007), or alteration of dendritic cell functions (Idzko et al., 2007; Zhou et al., 2007b), have been implicated in the efficacy of prostacyclin observed in murine asthma models. In our previous studies, ONO-1301 induced the hepatocyte growth factor (HGF) in the lung, and the inhibition of HGF diminished the inhibitory effects of ONO-1301 (Yamabayashi et al., 2012). Other reports based on different models also described the association between the efficacy of ONO-1301 and the induction of HGF (Xu et al., 2012; Hirata et al., 2012; Nasu et al., 2012). However, the mechanism underlying the inhibition of airway remodeling induced by ONO-1301 administration remains unclear.

In the current study, we investigated the effects of ONO-1301MS using the model with repeated exposure of HDM, which is one of the most common environmental allergens associated with asthma. Previous data from other investigators have shown that the HDM-induced mouse asthma model without systemic sensitization exhibits significant airway allergic inflammation, including airway eosinophilia, Th2 cytokine elevation in the airway, and airway remodeling (Johnson et al., 2004). Our data revealed that exposure to HDM over 3 consecutive weeks yielded prominent airway hyperresponsiveness to methacholine and Th2-type predominant airway inflammation (Fig. 2). Moreover, 5-week exposure to HDM augmented airway hyperresponsiveness and airway allergic inflammation and led to airway remodeling. ONO-1301MS ameliorated not only airway hyperresponsiveness and airway inflammation, but also resolved the goblet cell metaplasia and smooth muscle hypertrophy, based on the comparison of the data (Figs. 2 and 3). Our preliminary data showed that airway hyperresponsiveness and airway inflammation were sustained, and airway remodeling was established more robustly, after 2 additional weeks (7-week exposure) of HDM exposure (data not shown). The duration of ONO-1301MS and reversibility of airway remodeling after administration of ONO-1301MS need to be elucidated in the longer exposure model.

In conclusion, ONO-1301MS, a novel sustained-release prostacyclin agonist polymerized with PLGA microspheres, suppressed airway hyperresponsiveness to inhaled methacholine and airway allergic inflammation, and reduced the features of airway remodeling, such as goblet cell metaplasia, airway smooth muscle

hypertrophy, and submucosal collagen deposition. These data suggest that ONO-1301MS has the important potential to suppress the development of airway allergic inflammation and airway remodeling. This agent may represent a useful tool in the clinical setting of asthma in the near future.

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