

Intratracheal Delivery of Hepatocyte Growth Factor Directly Attenuates Allergic Airway Inflammation in Mice

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Key Words

Hepatocyte growth factor · Bronchial asthma · Gelatin hydrogel

Abstract

Hepatocyte growth factor (HGF) has an important role in many biological events such as angiogenesis and cell proliferation, as well as anti-fibrotic and anti-apoptotic effects. In addition, we found that HGF suppresses antigen-induced immune responses in the airway by suppressing dendritic cell functions, using a HGF-producing plasmid vector. In the present study, we examined whether delivery of the HGF protein in the lung attenuates allergic airway inflammation in a mouse model. Generally, HGF is rapidly cleared from organs. So, to achieve the efficient delivery of HGF, we prepared a slow-releasing form by mounting recombinant human (rh) HGF protein in biodegradable gelatin hydrogels. BALB/c mice were immunized and then challenged with ovalbumin (OVA) to induce eosinophilic airway inflammation. Intratracheal delivery of a very small amount of gelatin-coupled rhHGF (0.3 μ g) just once before the inhalation of OVA significantly suppressed eosinophilic airway inflammation. In addition, cytokine production in thoracic lymph nodes and the antigen-presenting capacity of lung CD11c⁺ cells

were reduced. In contrast, delivery of 1.0 μ g of rhHGF did not exhibit any significantly suppressive effect. These results suggest that the controlled release of rhHGF protein can suppress antigen-induced allergic immune responses in the lung. Therefore, HGF could be a novel therapeutic option for asthma.

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Introduction

Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes [1, 2] and as a scatter factor [3], targets various cell types [4]. HGF has many functions such as induction of angiogenesis, promotion of cell proliferation and migration [4], and inhibition of apoptosis [5, 6]. HGF exhibits these functions through its receptor, c-met [6]. It is also known to promote tumor progression [7, 8] and suppress the development of fibrosis after injury [9–11]. In addition, an immunosuppressive role of HGF has been recently highlighted. For example, in a mouse model of allogeneic heart

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transplantation, HGF reduced acute and chronic rejection of the allograft with the increased expression of TGF- β and IL-10, indicating that HGF might induce allograft tolerance [12].

Bronchial asthma is basically a Th2-type immune response in the airway, although a Th1-type response also plays some role. We reported that HGF markedly suppresses Ag-induced immune responses in the airway by inhibiting the functions of dendritic cells (DCs) in a mouse model of allergic airway inflammation [13]. In addition, Ito et al. [14] reported that HGF attenuates airway remodeling as well as suppresses airway inflammation. These studies strongly suggest HGF to be potential therapeutic option for chronic asthma with airway remodeling. However, this possibility has not been investigated further.

One problem with HGF is its rapid clearance. Generally, HGF vanishes rapidly from organs [15], so it is difficult to deliver enough HGF to the target tissue. Previously, we used a hydrodynamic-based transfer system [16] with a rat HGF expression plasmid. Although effective at delivering a sufficient amount of HGF to tissue, this is an artificial system and cannot be applied directly to patients. There are limited reports on the effect of administering HGF protein directly to target organs. We previously administered 100 μ g of a recombinant human (rh) HGF protein twice to bleomycin-treated mice and confirmed the beneficial effects against pulmonary fibrosis [11]. However, this dosage was too high for clinical application, so improvement of the delivery system was necessary.

The purpose of the present study is to elucidate whether intratracheal delivery of HGF protein has a suppressive effect on allergic airway inflammation. To achieve an efficient delivery of HGF protein, we applied a slow-releasing form of rhHGF mounted in biodegradable gelatin hydrogels [17, 18]. The gelatin-coupled HGF (0.3 μ g/body) reduced airway eosinophilia, cytokine production in the thoracic lymph nodes, and the antigen-presenting capacity of lung CD11c+ cells. These results suggested that HGF protein could be applicable to the treatment of asthma.

Animals and Methods

Mice

Male BALB/c mice, 6 weeks of age, were obtained from Charles River Breeding Laboratories Japan (Kanagawa, Japan). Male BALB/c TCR transgenic DO11.10 mice, aged 6–8 weeks, that express the peptide OVA_{323–339} were purchased from The Jackson

Laboratory (Bar Harbor, Me., USA). The mice were housed under conventional conditions in a specific pathogen-free setting. All of the animal experiments were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology (University of Tokyo, Tokyo, Japan).

Induction of Allergic Airway Inflammation

BALB/c mice were sensitized with 2 μ g of ovalbumin (OVA) in 2 mg of alum i.p. on days 0 and 11. Saline control mice received i.p. saline injections on days 0 and 11. The sensitized mice were then challenged with a 3% w/v OVA aerosol in PBS delivered by a nebulizer for 10 min every day on days 18–20. Saline control mice received PBS aerosol delivered by a nebulizer for 10 min on days 18–20.

Preparation of HGF-Containing Gelatin Hydrogels

A biodegradable hydrogel composed of acidic gelatin with an isoelectric point of 5.0 was prepared as a carrier of rhHGF, as reported [17, 18]. rhHGF was purchased from Toyobo (Osaka, Japan). It was diluted in a phosphate-buffered saline solution (PBS, pH 7.5) to 5 mg/ml and impregnated into freeze-dried hydrogels at 1% v/w. HGF-free, empty gelatin hydrogels were prepared in a similar way using PBS. Briefly, 100 μ g of rhHGF was diluted with 20 μ l of PBS (pH 7.5). The solution was dropped onto 2 mg of dried hydrogel (1% v/w), and left at 37°C for 4 h to obtain rhHGF-containing gelatin hydrogels. It was fully sorbed into the dried hydrogels during the swelling process because the volume was much less than that theoretically impregnated into each hydrogel. The gelatin or gelatin/rhHGF was dissolved in 100 μ l of PBS (HGF 100 μ g/PBS 100 μ l) and then diluted with an appropriate amount of PBS to be intratracheally injected into mice. Finally, 50 μ l of solution contained 0.3 μ g of rhHGF, and was used for intratracheal injection.

In vivo Treatment with HGF in the Effector Phase of Ag-Induced Immune Responses

Mice were sensitized twice and challenged with the OVA solution, as described above. Some of the mice received an intratracheal injection of gelatin-coupled rhHGF (0/0.3 μ g), or rhHGF protein alone (0, 0.1, 0.3, and 1.0 μ g, respectively) in PBS, or PBS alone just once on day 17. On day 21, the mice were subjected to analyses. Bronchoalveolar lavage fluid (BALF), thoracic lymph nodes and lung tissues were obtained as reported [19–22]. The cell count and cell differentials in BALF were calculated.

Conditions for Cell Culture

Throughout the study, complete DMEM was used for incubation as reported [19]. Cells were incubated in a 96-well flat-bottom microtiter assay plate in an incubator (37°C, 5% CO₂, 90% humidity) for specified periods. Some cells were treated with mitomycin C (10 μ g/ml; Sigma-Aldrich, St. Louis, Mo., USA) for 35 min in the incubator to inhibit their proliferation.

Preparation of Single Cell Suspensions from Thoracic Lymph Nodes and Lungs

Single cell suspensions for thoracic lymph nodes and lungs were prepared as reported [19]. Briefly, thoracic lymph nodes were collected and incubated at 37°C for 20 min after treatment with 0.1% (w/v) collagenase (Sigma-Aldrich)/complete DMEM solu-

tion. They were then minced, and single-cell suspensions were prepared by passing through the cell strainer. RBCs were removed by hypotonic lysis using RBC lysing buffer (Sigma-Aldrich). After two washes in HBSS, cells were cultured for experiments.

Cytokine Production of Thoracic Lymph Node Cells

Cell suspension was stimulated with OVA (10 µg/ml) for 3 days at 37°C, 5% CO₂, 90% humidity, as described above. Cytokine production in the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA).

ELISA and Cell Proliferation Assay

Concentrations of mouse IL-4, IL-5, IL-10, and IFN-γ were measured using the ELISA kit (BD Pharmingen, San Diego, Calif., USA), following the manufacturer's directions. Cell proliferation was measured based on the incorporation of BrdU using the BrdU cell proliferation ELISA kit (Roche, Mannheim, Germany). The data were analyzed with Microplate Manager III, Version 1.45 (Bio-Rad, Tokyo, Japan).

Positive Selection of Lung Antigen-Presenting Cells

Antigen-presenting cells were obtained with anti-mouse CD11c microbeads (Miltenyi Biotec, Auburn, Calif., USA) from the enriched cells obtained by the centrifugation. The purity of lung CD11c+ cells, confirmed by flow cytometry, was more than 85%. There was no significant difference in purity among each group of mice in any experiment. In the preliminary study, we applied Opti-Prep (Miltenyi Biotec) and then anti-mouse CD11c microbeads [19, 23–25], but the purity of the cells was not significantly different from that of the cells isolated without Opti-Prep.

Analysis of Antigen-Presenting Capacity in the Lung

To examine the effect of HGF on antigen presentation in the lung, antigen-presenting cells were obtained from the lung on day 21 using anti-mouse CD11c microbeads as previously described [13]. After mitomycin C treatment, the cells were cocultured (1×10^5 cells/ml) with CD4+ T cells (1×10^6 cells/ml) purified from the spleen of DO11.10 mice. After 3 days of coculture, cell proliferation was measured based on the incorporation of BrdU using BrdU cell proliferation ELISA kit (Roche). The data were analyzed with Microplate Manager III, Version 1.45 (Bio-Rad).

Statistical Analysis

Values are expressed as the mean $\pm$ SEM. Data were evaluated using a one-way ANOVA followed by the Student's t test for comparisons between two groups or the Turkey-Kramer test for comparisons among four groups. Values of $p < 0.05$ were considered to be significant.

Results

Treatment with HGF Alone Does Not Affect BALF Cells

We first examined the effect of the HGF protein during the effector phase of experimental allergic airway inflammation. Intratracheal treatment did not affect eo-

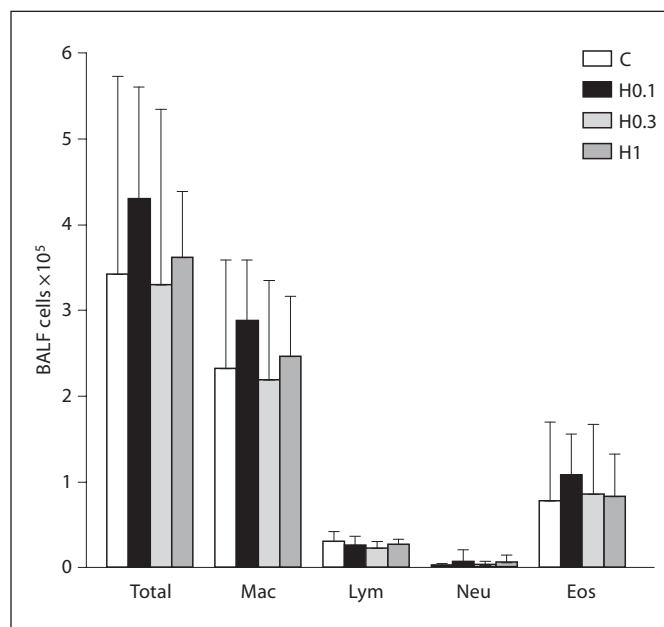


Fig. 1. Treatment with HGF alone does not affect BALF cells. Mice were sensitized and challenged with OVA as described in 'Materials and Methods'. On day 17, they received an intratracheal injection of 0 (C), 0.1 (H0.1), 0.3 (H0.3), or 1.0 (H1) µg of rhHGF, respectively. On day 22, BALF was collected, and inflammatory cells were counted. Data were obtained from 4–5 mice of each group.

sinophilic lung inflammation (fig. 1). Generally, exogenously administered HGF is removed from the organs within hours [15]. Then we tried using the biodegradable hydrogels.

Treatment with HGF-Containing Gelatin Hydrogels Significantly Suppresses the Development of Allergic Airway Inflammation

To deliver HGF effectively, we used a biodegradable hydrogel which incorporates HGF. Treatment of the mice with 0.3 µg of rhHGF-containing gelatin significantly suppressed eosinophilic airway inflammation in BALF (fig. 2a), as well as in lung tissue (fig. 2b–d).

Treatment with Gelatin Incorporating HGF Attenuated Cytokine Productions in Regional Lymph Nodes

We then examined the immunosuppressive effect of HGF by analyzing cytokine production in regional lymph nodes (fig. 3a–d). The amounts of IL-4, IL-5, IL-10, and IFN-γ were reduced in gelatin-HGF group compared to the control groups. The production of Th2 cytokines (IL-

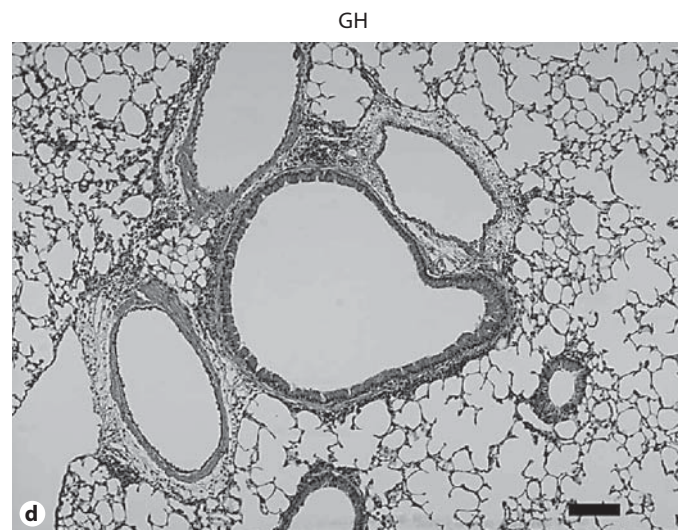
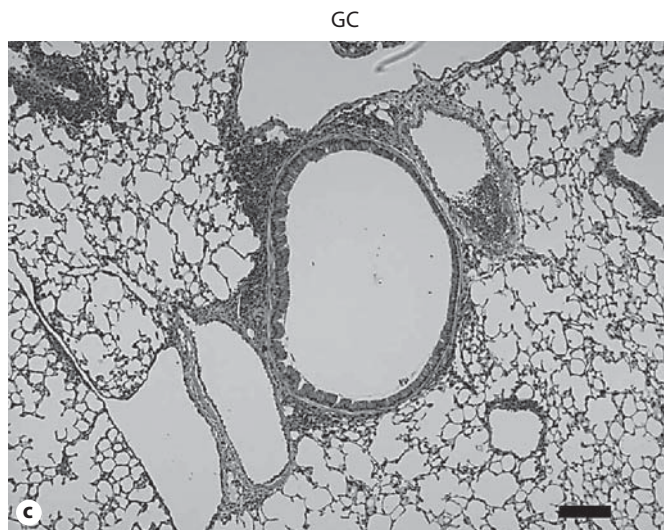
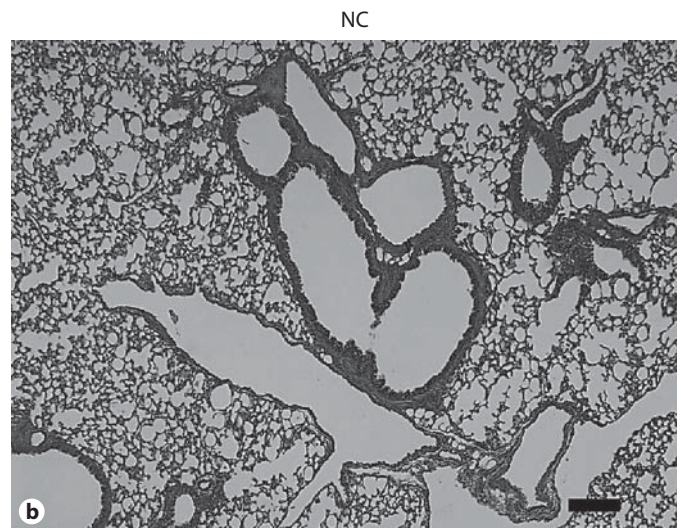
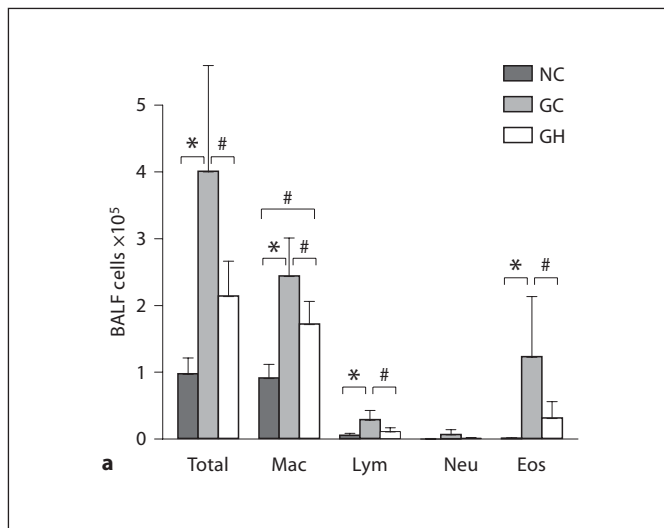


Fig. 2. Treatment with HGF-containing gelatin hydrogels significantly suppressed the development of allergic airway inflammation. Mice were sensitized and challenged with OVA, or injected with saline and challenged with PBS as described in 'Materials and Methods'. On day 17, mice received gelatin-coupled rhHGF (0.3 μ g; GH), control gelatin (GC), or the vehicle PBS (NC). On day 22, BALF and lung tissue were extracted. **a** Cell differentiation in BALF. **b–d** HE staining of lung tissue (**b** vehicle PBS; **c** control gelatin; **d** gelatin-coupled rhHGF). Scale bars: 100 μ m. * $p < 0.01$; # $p < 0.05$ (vs. control mice). Data were obtained from 5–6 mice of each group.

4, IL-5, IL-10) was predominantly suppressed, but that of Th1 cytokine (IFN- γ) was also suppressed. Cytokine production in the group given the HGF protein or gelatin alone was not suppressed.

Treatment with HGF in vivo Significantly Downregulates Antigen Presentation in the Lung

As shown in figure 3, treatment with HGF significantly suppressed the production of both Th1 and Th2

cytokines. As HGF suppresses inflammatory responses by altering DC functions [13], we then examined its effects on antigen presentation of CD11c⁺ cells by analyzing cell proliferation after incubation with CD4⁺ T cells from DO11.10 mice. As shown in figure 4, gelatin-HGF markedly reduced the antigen-presenting capacity of lung CD11c⁺ cells, although gelatin itself slightly decreased it.

Fig. 3. Treatment with gelatin incorporating HGF attenuated cytokine production in thoracic lymph nodes. Mice were sensitized and challenged with OVA as described in 'Materials and Methods'. On day 17, they received the PBS alone (C), gelatin in PBS solution (GC), 0.3 μ g of HGF in PBS solution (H), or 0.3 μ g of HGF coupled with gelatin in PBS solution (GH) by intratracheal injection, respectively. On day 21, thoracic lymph nodes were extracted. Levels of cytokine production for IFN- γ (a), IL-4 (b), IL-5 (c), and IL-10 (d) were measured after 3 days of incubation with OVA (10 μ g/ml). * $p < 0.01$; # $p < 0.05$ (vs. gelatin-coupled HGF-administered mice). Data were obtained from 3 mice of each group.

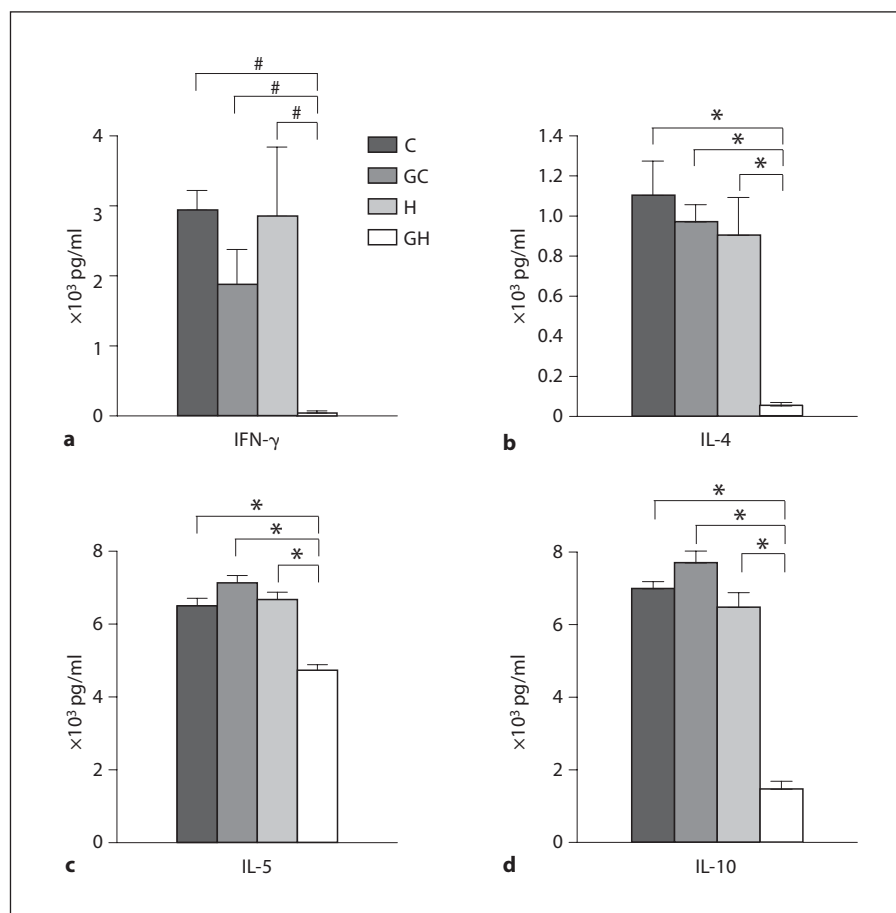
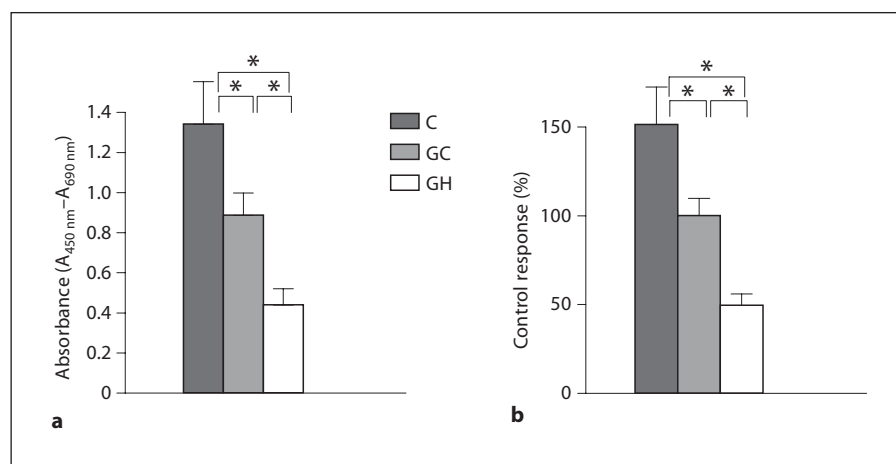


Fig. 4. Treatment with HGF in vivo significantly suppresses antigen presentation of thoracic lymph node cells. Mice were sensitized and challenged with OVA as described in 'Materials and Methods'. On day 17, mice received the vehicle PBS as a control (C), control gelatin (GC), or gelatin-coupled rhHGF (0.3 μ g: GH). On day 21, the antigen-presenting capacity was analyzed as described in 'Materials and Methods'. Data are expressed as the absorbance at 450 nm (reference wavelength: 690 nm) (a) and a percentage of the response of control (b). * $p < 0.01$. Data were obtained from 5 mice of each group.



Discussion

The results of the present study clearly showed that intratracheal delivery of the HGF protein suppresses allergic airway inflammation. A single dose of 0.3 μ g/body

of gelatin-coupled rhHGF delivered into lung tissue exerted an immunosuppressive effect (fig. 2), although 1.0 μ g/body of HGF protein had no effect (fig. 1). The gelatin-coupled HGF suppressed eosinophilic airway inflammation, cytokine production in the thoracic lymph nodes,

and furthermore, the antigen-presenting capacity of lung CD11c+ cells. As far as we know, this is the first report that clearly demonstrates the suppressive effect of the intratracheal delivery of HGF protein in any disease model of the lung.

HGF is rapidly removed from organs [15]. To achieve efficient delivery, several methods have been proposed [26, 27]. We previously used a hydrodynamic-based transfer system [16] with the rat HGF expression plasmid. With this method, the plasmid captured in the liver produces a sufficient amount of HGF, which is delivered to the lung via the bloodstream. Although these methods deliver a sufficient amount of HGF to tissue, they involve artificial systems and cannot be directly applied to patients.

As compared to gene delivery, the delivery of a recombinant protein itself is a simpler method with fewer potential side effects. So far, several studies have delivered the HGF protein itself by systemic injection. Yaekashiwa and co-workers [28] continuously delivered rhHGF by intraperitoneal injection using a minipump in bleomycin-induced model of pulmonary fibrosis. The total dosage of HGF delivered was 50 or 280 µg. In another study, Ito et al. [14] injected HGF (125, 250, 500 µg/kg/day) subcutaneously for 5–14 days [14], the total dosage ranging from 25 to 175 µg. Although these studies demonstrate a suppressive effect of systemic injection of rhHGF on lung fibrosis [28] or allergic airway inflammation [14], the amount of rhHGF was very large. On the other hand, we delivered rhHGF by intratracheal injection directly into the lung [11]. Although this method significantly suppressed the bleomycin-induced fibrosis, the total dosage of HGF was 200 µg.

Recently, we injected gelatin-coupled HGF subcutaneously and found that it suppressed collagen-induced arthritis [29]. In that study we confirmed that a biodegradable gelatin hydrogel worked effectively to release HGF

slowly. Here we applied this delivery system to allergic airway inflammation. The intratracheal injection of a much smaller amount of rhHGF (0.3 µg), as compared to previous studies, just once before the inhalation of OVA, significantly suppressed airway eosinophilia (fig. 2). In addition, it also reduced OVA-induced cytokine production in thoracic lymph nodes (fig. 3) and antigen-presenting capacity in the lung (fig. 4). The gelatin itself reduced the antigen-presenting capacity of lung CD11c+ cells (fig. 4). So, incorporation of gelatin may delay the function of antigen-presenting cells. In addition, gelatin-coupled HGF demonstrated a further reduction of antigen-presenting capacity. In contrast, the cytokine productions in thoracic lymph nodes were not significantly affected by gelatin treatment (fig. 3). Taken together, with a slow-releasing form of rhHGF, we obtained similar results to those obtained by the intravenous injection of a HGF plasmid [13]. These results clearly demonstrate that HGF was efficiently delivered into the lung with this system.

HGF has the potential to suppress experimental allergic airway inflammation by inhibiting functions of antigen-presenting cells [13] as well as airway remodeling [14]. Therefore, if delivered effectively, for example, using nebulizer, HGF could be a novel therapeutic option for asthmatic patients, and further studies should be conducted.

In summary, the intratracheal delivery of a small amount of rhHGF protein effectively suppressed antigen-induced allergic immune responses in the lung. HGF could be a novel therapeutic option for asthma.

Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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