

Hepatocyte Growth Factor Suppresses Production of Reactive Oxygen Species and Release of Eosinophil-Derived Neurotoxin from Human Eosinophils

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Key Words

Hepatocyte growth factor • Asthma • Eosinophil • Reactive oxygen species • Degranulation

Abstract

Background: Reactive oxygen species (ROS) and eosinophilic granule proteins such as eosinophil-derived neurotoxin (EDN) are known to damage bronchial tissue and cause airway hyperresponsiveness (AHR) in asthma. Hepatocyte growth factor (HGF) regulates various biological activities and is known to be a multifunctional factor. In our previous study, we found that HGF suppressed allergic airway inflammation and AHR in a murine model of asthma. However, there have been few reports regarding the detailed mechanism of the anti-allergic effect of HGF in asthma. In this study, we investigated the potential of recombinant HGF to regulate the production of ROS and the release of EDN from human eosinophils. **Methods:** Eosinophils were isolated from subjects with mild eosinophilia by modified CD16-negative selection. We investigated the expression of CD69, an activation marker of eosinophils, on eosinophils, using flow cytometry. Further, ROS production from eosinophils was analyzed using luminol-dependent chemiluminescence, and

EDN release was measured by ELISA. **Results:** Treatment with HGF suppressed interleukin-5-induced upregulation of CD69 expression, ROS production and EDN release from human eosinophils. **Conclusion:** Taken together, these data suggest that in asthma, HGF attenuates allergic airway inflammation and AHR through at least the suppression of ROS production and EDN release from eosinophils.

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Introduction

Asthma is a complex chronic airway disorder characterized by allergen-induced airway inflammation, elevated serum IgE and airway hyperresponsiveness (AHR). Eosinophils play an important role in the mechanism of allergic airway inflammation [1]. The activation of eosinophils, in particular, is a critical event in allergic inflammatory response [1]. Eosinophils activated by various cytokines such as interleukin-5 (IL-5) produce reactive oxygen species (ROS) [1, 2]. Previous studies have reported that ROS such as hydrogen peroxide (H₂O₂) is elevated in exhaled breath of asthmatic patients [3, 4]. Moreover, ROS secreted from eosinophils results in tis-

sue damage at inflamed sites, vasodilation and AHR [5, 6]. Thus, ROS is recognized as one of the most important events in the pathogenesis of allergic inflammation. On the other hand, activated eosinophils also release cytotoxic granular proteins including eosinophil-derived neurotoxin (EDN) [1, 7, 8], which plays an important role in allergic inflammation [1], and causes expression of CD69, which is an activation marker of eosinophils [9, 10]. Therefore, some studies have previously reported that the degree of activation markers such as EDN release and CD69 expression is strongly correlated with the severity of allergic disease including asthma and allergic rhinitis [1, 11].

Hepatocyte growth factor (HGF) was originally identified and cloned as a potent mitogen for mature hepatocytes and has a variety of cellular functions [12–14]. There is now evidence that HGF plays an essential part in parenchymal repair and protection in various organs. Thus, HGF is widely recognized as a multifunctional cytokine and a humoral mediator [13]. For example, in lungs, HGF prevented the onset and progression of lung fibrosis in vivo [15] and induced the apoptosis of myofibroblasts in lung fibrosis in vitro [16]. Previously, we found that in a murine model of asthma, HGF attenuated AHR and allergen-induced airway remodeling and inflammation, involving eosinophils and lymphocytes [17]. Moreover, we reported that HGF directly inhibited the factor-induced chemotaxis of human eosinophils in the absence of Th2 cytokines or granulocyte macrophage colony-stimulating factor [18]. However, as far as we know, the functional role of HGF in the activation of eosinophils, including the production of ROS and the release of granule proteins from eosinophils, has not been studied previously.

In this study, to define the direct effect of HGF in regulating the activation of eosinophils, we examined whether HGF modulates CD69 expression on human eosinophils, and further, whether it has an effect on the production of ROS and the release of EDN from human eosinophils.

Methods

Eosinophil Purification

Peripheral blood was obtained from subjects with mild eosinophilia including nonatopic asthma, allergic rhinitis and healthy donors. All subjects gave informed consent. Eosinophils were isolated by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll (Pharmacia, Uppsala, Sweden) density gradients, modified from the method of Hansel et al. [19, 20].

The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Eosinophils (>99% purity) were then suspended in Hank's balanced salt solution (Life Technologies, Grand Island, N.Y., USA) with 1% fetal calf serum.

CD69 Surface Expression

After incubation of purified eosinophils (1×10^6 cells) with human recombinant HGF (hrHGF, 2.0, 20, 200 ng/ml; R&D Systems, Minneapolis, Minn., USA) or medium in RPMI-1640 for 1 h, the eosinophils were further cultured with or without 10 ng/ml IL-5 (R&D Systems) in 24-well tissue culture plates for 24 h in 37°C humidified air with 5% CO₂. After washing once, the cells were incubated with CD69/FITC (mouse IgG1; Dako, Glostrup, Denmark). An FITC-conjugated IgG1 isotype-matched control monoclonal antibody (mAb; Dako) was applied to assess the degree of nonspecificity. The percentage of eosinophils expressing a specific marker was determined relative to the expression level on the isotype-matched control, and mean fluorescent intensities were analyzed as an index for the expression of CD69 using a FACScan flow cytometer (Becton-Dickinson, San Jose, Calif., USA) according to a previous study [21].

Luminol-Dependent Chemiluminescence

ROS production from eosinophils was examined by luminol-dependent chemiluminescence according to previous reports [22, 23]. Purified eosinophils (1×10^6 cells/ml) cultured in RPMI-1640 (Gibco, Grand Island, N.Y., USA) were incubated with or without hrHGF (2.0, 20, 200 ng/ml) for 1 h at 37°C. Then, ROS production was evoked by adding 50 µl of calcium ionophore A23187 (Sigma, St. Louis, Mo., USA; final concentration 1×10^{-5} M) to 100 µl of eosinophil suspension (5×10^4 cells) containing 0.25 mM luminol (Futaba Medical, Tokyo, Japan). Maximal and integral intensity chemiluminescence was determined for 60 min using an Argus-50/2D luminometer (Hamamatsu Photonics, Hamamatsu, Japan).

EDN Release

EDN release from eosinophils was examined by EDN ELISA kit (MBL Co., Ltd., Nagoya, Japan) according to previous reports [21, 24, 25]. Purified eosinophils (1×10^6 cells) cultured in RPMI-1640 with 0.1% HSA were preincubated with or without hrHGF (2.0, 20, 200 ng/ml) for 1 h at 37°C. These cells were further cultured with or without 10 ng/ml of IL-5 for 4 h under the same conditions. The supernatants were separated by centrifugation, and the concentration of EDN was measured by ELISA.

Adhesion Molecule Surface Expression

After incubation of purified eosinophils (1×10^6 cells) with 200 ng/ml of hrHGF or medium in RPMI-1640, the eosinophils were further cultured with or without 10 ng/ml of IL-5 in 24-well tissue culture plates for 16 h in 37°C humidified air with 5% CO₂ according to a previous report [26]. After washing once, the eosinophils were reacted with an mAb against CD11b (Nichirei, Tokyo, Japan), CD11a (Dako), CD18 (Dako) and CD49d (Immunotech, Marseille, France) for 30 min at 4°C and stained by FITC-conjugated secondary antibodies (Biosource International, Camarillo, Mass., USA) under the same condition for 30 min. After washing the cells, the stained cells were analyzed using a FACScan flow cytometer [27].

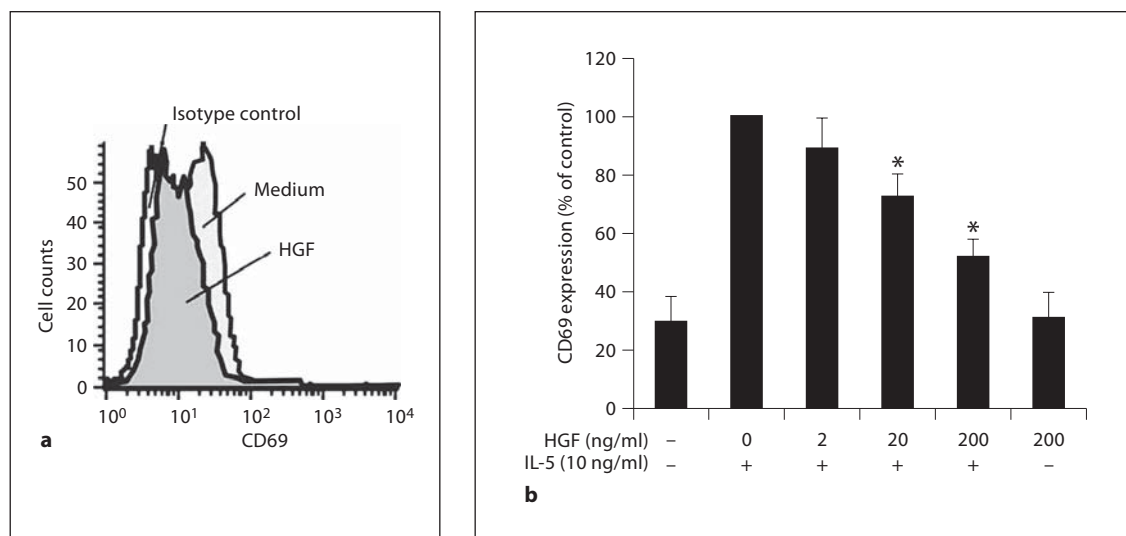


Fig. 1. Effect of HGF on IL-5-induced CD69 expression of eosinophils. Purified eosinophils with or without HGF. The assay of CD69 expression was performed using fluorescence-activated cell-sorting analysis (FACS). Cells stained with isotype-matched mouse IgG were used for negative control staining. **a** Representative histogram. **b** Bar graph data show the mean of 4 experiments. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$, versus without HGF.

Western Blot Analysis

Purified eosinophils (1×10^6 cells) were treated with or without hrHGF (200 ng/ml) for 1 h at 37°C followed by stimulation with or without 10 ng/ml of human IL-5 for 5 min. The reaction was terminated by the addition of 15 volumes of ice-cold Hank's balanced salt solution containing 1 mM Na_3VO_4 . The cells were pelleted by centrifugation and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4 , 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 10% glycerol, and 1 $\mu\text{g/ml}$ aprotinin, leupeptin and pepstatin). After 20 min on ice, detergent-insoluble materials were removed by centrifugation at 4°C at 15,000 g. The supernatants were mixed with SDS sample buffer and boiled for 4 min. SDS-polyacrylamide (10%) gels (Ready Gel J) were obtained from Bio-Rad (Tokyo, Japan). The electrophoresed gel was blotted onto Hybond ECL membranes (Amersham, Arlington Heights, Ill., USA).

Blots were incubated in blocking buffer containing 5% bovine serum albumin in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl and 0.05% Tween-20, pH 7.6) for 30 min followed by incubation in the primary 0.1 $\mu\text{g/ml}$ of Ab [mouse mAb against phospho-p38 from Cell Signaling Technology, Beverly, Mass., USA, and mouse mAb against phospho-extracellular signal-regulated kinases (ERKs) from Santa Cruz Biotechnology, Santa Cruz, Calif., USA] for the phosphorylation state of signaling proteins with gentle agitation overnight at 4°C. After washing 3 times in TBS-T buffer, blots were incubated for 30 min with horseradish peroxidase-conjugated secondary 0.04 $\mu\text{g/ml}$ of Ab directed against the primary Ab. The blots were developed with the ECL substrate according to the manufacturer's instructions. Blots were subsequently re-probed with another Ab (rabbit polyclonal anti-p38 and anti-ERK2; Santa Cruz Biotechnology) for the non-phosphorylation state of signaling proteins after stripping in a buffer of 62.5 mM

Tris-HCl (pH 6.7), 100 mM 2-ME and 2% SDS at 56°C for 30 min. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit Abs were obtained from Santa Cruz Biotechnology.

Statistical Analysis

Two groups of data were compared using Student's t test. The p value for significance was set at $p < 0.05$. All results were expressed as the mean $\pm$ SEM.

Results

Treatment with HGF Suppresses IL-5-Induced Upregulation of Eosinophil CD69 Expression

The effect of HGF on CD69 surface expression of eosinophils was investigated. Compared with eosinophils incubated with only the vehicle, IL-5 increased CD69 expression of purified human eosinophils (control; fig. 1). hrHGF significantly inhibited the IL-5-induced upregulation of CD69 expression, which is an activation marker of eosinophils (fig. 1).

Treatment with HGF Suppresses the Production of ROS from Eosinophils

To determine the effects of HGF on the production of ROS from eosinophils, purified human eosinophils were treated with hrHGF, and ROS evoked with calcium ionophore (A23187) was measured using a luminometer.

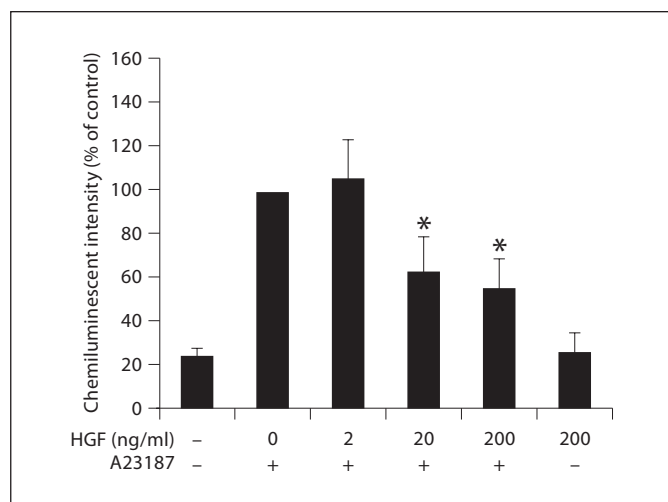


Fig. 2. Effect of HGF on ROS production from eosinophils stimulated by calcium ionophore A23187. Purified eosinophils were preincubated with increasing concentrations of HGF for 1 h. The assay was performed using a luminometer. Data are expressed as the mean of 4 experiments $\pm$ SEM. * $p < 0.05$, versus without HGF.

Compared with eosinophils incubated with only the vehicle, A23187 increased ROS production of purified human eosinophils (control; fig. 2). Preincubation of eosinophils with hrHGF significantly prevented the production of ROS in a dose-dependent manner (fig. 2). On the other hand, HGF itself did not induce ROS from eosinophils.

Treatment with HGF Suppresses Eosinophil EDN Release Stimulated by IL-5

Next, we investigated whether HGF affected IL-5-induced EDN release from eosinophils. EDN release from purified human eosinophils was induced by IL-5. Preincubation of eosinophils with hrHGF significantly prevented the release of EDN (fig. 3).

Treatment with HGF Has No Effect on Surface Adhesion Molecule Expression on Eosinophils

The signal from adhesion molecules plays a critical role in activation, degranulation and ROS production from eosinophils [28, 29]. Therefore, we also examined whether HGF modulates the surface expression of CD11a, CD11b, CD18 and CD49d on eosinophils. In agreement with a foregoing report [26], IL-5 induced CD11b and CD18 expression. However, a flow-cytometric study revealed that HGF had no effect on the surface expression of adhesion molecules (data not shown). Thus, these data may suggest that the inhibitory effects of HGF on degran-

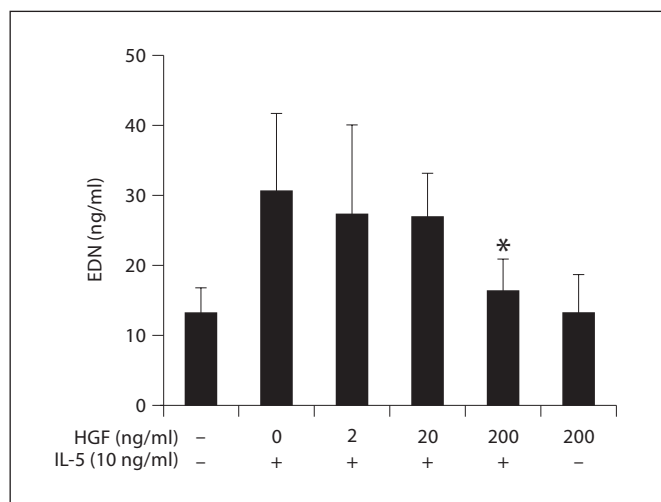


Fig. 3. Effect of HGF on EDN release from eosinophils stimulated by IL-5. Purified eosinophils were preincubated with increasing concentrations of HGF for 1 h. The assay was performed using ELISA. Data are expressed as the mean of 4 experiments $\pm$ SEM. * $p < 0.05$, versus without HGF.

ulation and ROS production were caused independent of adhesion molecules.

Treatment with HGF Inhibits IL-5-Induced Activation of Mitogen-Activated Protein Kinase Signaling Pathway in Eosinophils

Both p38 and ERK1/2 mitogen-activated protein (MAP) kinase also play an important role in eosinophil activation [24, 25]. Thus, the MAP kinase signaling pathway was studied to further explore the inhibition mechanisms of HGF. To investigate the MAP kinase pathway activity, eosinophils were stimulated with IL-5 followed by electrophoresis and Western blotting with anti-p-ERK or anti-p-p38 Ab. Treatment with 200 ng/ml of HGF partially inhibited eotaxin-induced phosphorylation of ERK1/2 as well as p38 (fig. 4).

Discussion

The eosinophil is believed to be an important effector cell in allergic diseases such as asthma, and the production of ROS and the release of cytotoxic granular proteins from activated eosinophils are essential for tissue damage in allergic inflammation [1–8]. In the present study, we investigated the functional role of HGF in ROS production, EDN release and activation of human eosinophils and found that HGF suppressed eosinophil activation in-

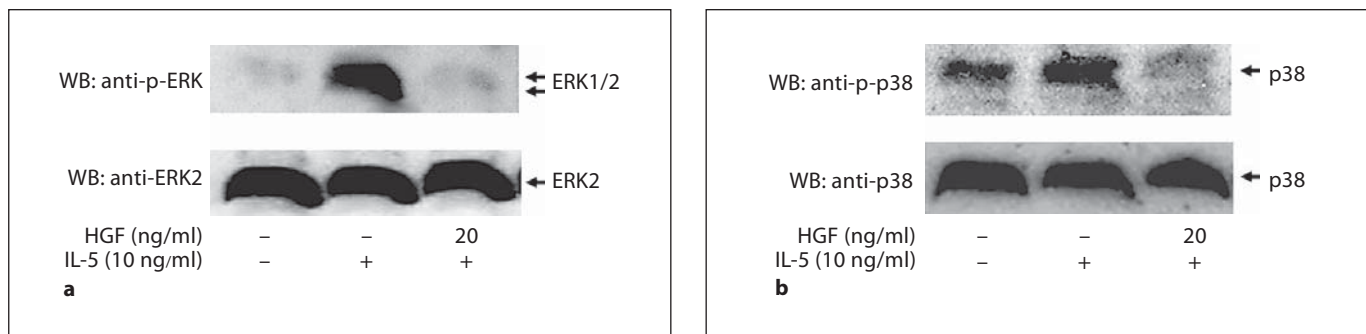


Fig. 4. Examination of the effect of the intracellular signaling pathway on the inhibitory effect of HGF. Purified eosinophils were incubated with or without 200 ng/ml of HGF for 1 h. After incubation, eosinophils were stimulated with IL-5 (10 ng/ml) for 5 min. Phosphorylated ERK1/2 (p-ERK) and total ERK2 (ERK2), and phosphorylated p38 MAPK (p-p38) and total p38 MAPK (p38) were determined by Western blot (WB) analysis. HGF partially abrogated the eotaxin-induced phosphorylation of ERK (a) and p38 (b). The membranes were reprobed with anti-ERK2 or anti-p38 Ab incubated with the same amounts of proteins loaded on the gels. Experiments were repeated at least 3 times with a different cell preparation.

duced by IL-5 and inhibited secretion of ROS and EDN. This is the first report to show the important role of HGF in the production of ROS and granule proteins from eosinophils.

ROS, which is released by activated eosinophils at the site of allergic inflammation, injures epithelial cells and causes vasodilation and AHR in asthma [5, 6]. Thus, ROS may play an important role in the pathogenesis of asthma. Ichiki and coworkers [30] reported that thioredoxin, which has a protective function against the cellular damage caused by ROS, suppressed AHR and allergic airway inflammation in a murine model of asthma. On the other hand, HGF is secreted by several cell types such as fibroblasts and macrophages in the lung and stimulates morphogenesis and organogenesis in a wide range of tissues and organs [13, 31]. Regarding oxidative stress, recent studies have shown that HGF could suppress oxidative stress-induced action as an antioxidant factor in various kinds of cells such as hepatocytes and airway epithelial cells [32, 33]. Moreover, Li and coworkers [34] reported that HGF protected rat mesangial cells from high-glucose-mediated oxidative stress by suppressing ROS production. Consistent with this study, in our present study, we showed that HGF suppressed the production of ROS from eosinophils activated by calcium ionophore (fig. 2). Previously, we also found that HGF administration inhibited AHR in lung tissue of asthmatic mice [17]. These findings may suggest that HGF attenuates AHR in asthma through the inhibition of ROS production from eosinophils.

EDN, an eosinophil granule protein and a member of the eosinophil-associated ribonuclease family, has antiviral activities against the respiratory syncytial virus [35], and EDN-derived peptide induces apoptosis of sarcoma cells [36]. On the other hand, eosinophil degranulation is also a very important reaction in the pathophysiology of asthma. Granule proteins including EDN secreted from eosinophils have toxicity against respiratory epithelium, which is manifested by epithelial cell desquamation, damage to epithelial cells and alternations in ciliary beating, and they induce AHR [1]. In this study, HGF treatment suppressed the EDN release from eosinophils activated by IL-5 (fig. 3) and inhibited the activation of human eosinophils, which is essential for degranulation as well as ROS production from eosinophils (fig. 1). IL-5 is an important effector for degranulation and ROS production of eosinophils [1]. Moreover, a recent study showed that transforming growth factor- β (TGF- β) induced ROS production [37]. On the other hand, HGF is a strong suppressor of TGF- β [38], and further, in our previous study, treatment with HGF inhibited the elevation of TGF- β as well as of IL-5 levels in bronchoalveolar lavage fluid of asthmatic mice [17]. Therefore, these data may indicate that in asthma, HGF may not only attenuate the production of Th2 cytokines and growth factors such as TGF- β but also suppress ROS production and degranulation through the direct inhibition against eosinophil activation by remaining cytokines and growth factors. Recently, we reported that HGF negatively regulated allergic airway inflammation by eosinophils not only through the suppression of Th2 cytokines but also

through direct action against eosinophil migration [18]. Taken together, HGF may strongly modulate allergic inflammation and AHR by the double suppression including both indirect and direct actions in vivo.

Adhesion molecules have been considered to play an important role in allergic diseases. Previous studies have reported that an increase in ROS production from eosinophils can be mediated by signaling through adhesion molecules and their ligands [27, 39]. In this study, HGF had no effect on the expression of adhesion molecules such as β_1 and β_2 integrins on human eosinophils. On the other hand, Min and coworkers [40] reported that HGF suppressed vascular endothelial growth factor-induced expression of adhesion molecules on human vein endothelial cells. This conflict between findings may also be related to different cell types or stimulation conditions. Thus, further studies are necessary to clarify the physiologic role of HGF. In another study, ROS production required calcium influx [41, 42]. We have shown that HGF inhibited ROS production from eosinophils stimulated by calcium ionophore. In our previous study, HGF suppressed Ca^{2+} influx induced by eotaxin in human eosinophils. Therefore, the suppressive effect of HGF on ROS production may be caused by inhibition of Ca^{2+} influx. Regarding EDN release, eosinophils activated by IL-5 or eotaxin release granule proteins such as EDN through activation of MAP kinase [24, 25]. We previously found that in human eosinophils, HGF treatment suppressed activation of the MAP kinase pathway by eotaxin [17]. Furthermore, in this study, HGF treatment inhibited the ERK1/2 and p38 MAP kinase pathways activated by IL-5

in human eosinophils (fig. 4). Therefore, these findings may suggest that HGF suppresses eosinophil degranulation through the inhibition of factor-induced MAP kinase activation.

Although no study has reported the HGF levels in serum or bronchoalveolar lavage fluid of asthmatics, in general, HGF levels in humans are altered by various diseases. For example, serum HGF levels in fulminant hepatitis patients are significantly higher than those in healthy subjects (16.40 ± 14.67 vs. 0.27 ± 0.08 ng/ml) [43]. Further, serum HGF concentrations in patients with bacterial pneumonia also increased to 0.96 ± 0.27 ng/ml compared with healthy subjects (0.30 ± 0.02 ng/ml) [44]. In this study, the higher level of hrHGF was used according to previous reports [45, 46], because a functional role of HGF in eosinophil activation was more clear.

In summary, we have defined an inhibitory function of HGF in eosinophil activation including ROS production and degranulation of eosinophils, which play a pivotal role in allergic airway inflammation and AHR of asthma. However, the detailed mechanism of these negative actions of HGF remains unclear. Although HGF is also recognized as a homeostatic mediator that restores abnormal conditions in organisms, the biological potential of HGF is extremely obscure in allergic responses in particular. Therefore, it is necessary to clarify what kind of signal pathway plays an important role in the action of HGF against allergic response. Finally, this result may help to elucidate the mechanism of allergic diseases and suggests that HGF has potential in the treatment of allergic disorders.

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