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Hepatocyte growth factor induces angiogenesis in injured lungs through mobilizing endothelial progenitor cells

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Abstract

Circulating endothelial progenitor cells (EPCs) play a pivotal role in angiogenesis. Hepatocyte growth factor (HGF) is known to induce proliferation and motility in endothelial cells, and to play a role in mitogenic and morphogenic actions. However, the role of HGF in EPC mobilization has not been clearly described yet. We investigated the effect of HGF on mobilizing EPCs and on angiogenesis in elastase-induced lung injury. HGF significantly increased the triple-positive (Sca-1$^+$, Flk-1$^+$, and c-kit$^+$) fraction in peripheral mononuclear cells in mice. The bone marrow-derived cells were recruited into the injured lungs, where they differentiated to capillary endothelial cells. HGF induced proliferation of both bone marrow-derived and resident endothelial cells in the alveolar wall. In conclusion, the present study suggests that HGF induces EPC mobilization from the bone marrow and enhances the proliferation of endothelial cells in vivo. These complex effects induced by HGF orchestrate pulmonary regeneration in emphysematous lung parenchyma.

Keywords: Hepatocyte growth factor; Angiogenesis; Endothelial progenitor cell; Bone marrow; Pulmonary emphysema

Hepatocyte growth factor (HGF) is a multifunctional cytokine that is a mitogen and a morphogen for many different cells and tissues [1,2]. HGF activates migration and proliferation of endothelial cells and induces angiogenesis. The angiogenic activity of HGF is mediated through its direct actions on endothelial cells [3,4], and its indirect actions that occur through an increase of endothelial cell mitogens [4,5]. Because of this angiogenic activity, HGF is known to improve coronary and peripheral artery diseases [6,7].

Circulating endothelial progenitor cells (EPCs) are present in peripheral blood, and they can differentiate into mature endothelial cells [8,9]. Released EPCs play a role in angiogenesis [8]. The release of EPCs from the bone marrow is regulated by a variety of growth factors and cytokines, such as vascular endothelial growth factor (VEGF) [10]. EPC is believed to originate directly from the hemangioblast (a common precursor of hematopoietic and endothelial cells [11]) or from hematopoietic stem cell [12,13]. HGF and c-met are expressed in hematopoietic environments, and play an important role in the growth and maintenance of hematopoietic progenitors [14]. In addition, human bone marrow stromal cells constitutively produce HGF, and this HGF promotes the growth of undifferentiated hematopoietic cells [15]. In spite of its angiogenic activity and its effect on hematopoietic cells, the

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role of HGF on the mobilization of EPCs has not been clearly described yet.

The aim of this study was to investigate the effect of HGF on EPC mobilization and angiogenesis. We used a murine model of elastase-induced lung injury, which is characterized by a loss of endothelial beds and mimics human pulmonary emphysema [16,17].

Materials and methods

Reconstitution of bone marrow. Mice transgenic for green fluorescent protein (GFP) on a C57BL/6 background were established at Osaka University, Japan [18]. Adoptive transfer of fetal liver cells was performed as previously described [17,19,20]. Briefly, recipient C57BL/6 male mice were irradiated using doses of 8 and 4 Gy, separated by 3 h. Bone marrow was reconstituted by injecting fetal liver cells (2×10^6 cells) intravenously. Three weeks after transplantation, $96.8 \pm 2.0\%$ of the circulating white blood cells were GFP-positive, indicating that the recipient C57BL/6 mice had been completely reconstituted with cells of GFP-mouse origin.

Induction of elastase-induced lung injury and treatment. After confirming GFP chimerism, lung injury and destruction of lung parenchyma were induced by intranasal instillation of porcine pancreas elastase (200 U/kg; Sigma, St. Louis, MO), as previously described [16,17]. Three weeks after elastase administration, the mice exhibited emphysema-like changes in the lungs. At this point, the mice were randomly divided into two groups (each group $n = 5$) and were administered either saline or recombinant human HGF [21,22] (1 mg/kg/day, i.p.) by daily injections for 12 days. At 13 days, mice were sacrificed by giving them an overdose of halothane.

Flow cytometry and histological analysis. A total of 0.5–1.0 ml of peripheral blood was obtained from each mouse. Peripheral blood mononuclear cells (PBMCs) were separated by a Ficoll–Hypaque density gradient (Lymphosepal; IBL, Gunma, Japan). Red blood cells were depleted by RBC lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, and 0.1 mM EDTA–2Na, pH 7.2). Sca-1^+/Flk-1^+/c-kit^+ cells, consistent with peripheral EPCs, were stained with FITC-labeled anti-murine Sca-1 antibody (BD Pharmingen, San Diego, CA), PE-labeled anti-murine fetal liver kinase 1/vascular endothelial growth factor receptor-2 (Flk-1/VEGF-R2) (BD Pharmingen), and APC-labeled anti-c-kit antibody (BD Pharmingen) and analyzed by a FACSCalibur (BD, San Jose, CA). For the histological evaluation, recipient lungs were fixed with 4% paraformaldehyde–PBS at a transpulmonary pressure of 20 cmH_2O. The number of thin and flat GFP-positive cells consistent with being endothelial and epithelial cells was counted in 200 separate alveoli from each mouse. Immunofluorescent staining using anti-CD34 (BD Pharmingen), anti-cytokeratin5&8 (Chemicon, Temecula, CA), and anti-CD45 antibodies (BD Pharmingen) was performed to identify the phenotype in the GFP-positive cells. Staining using anti-Ki67 antibody (DakoCytomation, Carpinteria, CA) was performed to identify proliferating cells. The extent of the emphysematous lesions was assessed by measuring the mean linear intercept (L_m) using the method of Ishizawa et al. and Thurlbeck [17,23]. Histological evaluations were performed blindly by three observers (K.I., T.S., and H.K.).

Table 1
Number of PBMCs and Sca-1^+/Flk-1^+/c-kit^+ cells in saline- and HGF-treated mice

Treatment	PBMCs	Sca-1^+/Flk-1^+/c-kit^+ cells
Saline	21.7 ± 1.7	1.1 ± 0.1
HGF	63.3 ± 8.8*	7.6 ± 1.1*

Values represent mean $\pm$ SEM $\times 10^5$ cells/mL.
* Significantly different from saline-treated mice ($P < 0.05$).

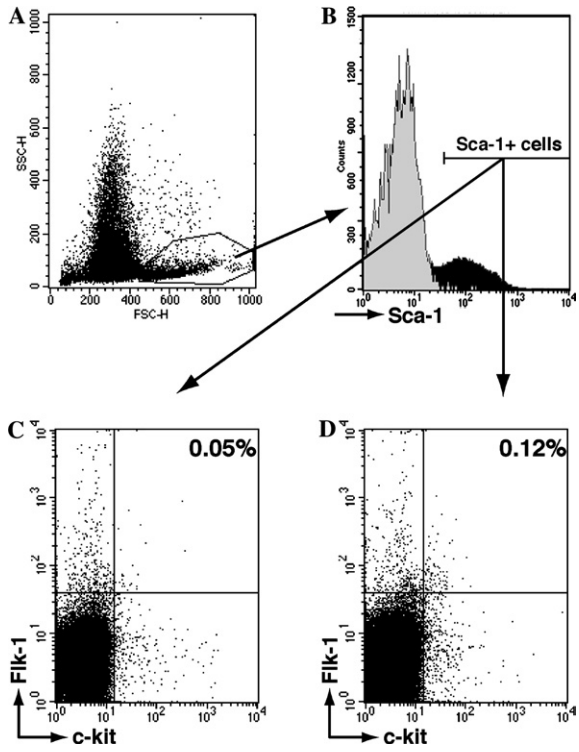

Fig. 1. Flow cytometric analysis of Sca-1, Flk-1, and c-kit expression in peripheral mononuclear cells. Peripheral blood was obtained from saline-treated and HGF-treated mice. Population of mononuclear cells in peripheral blood was gated (A), and then Sca-1^+ cells were selected (B, gray: negative control, black: Sca-1 staining). Sca-1^+/Flk-1^+/c-kit^+ triple-positive cells increased in HGF-treated mice (D) compared with saline-treated mice (C). Indicated percentages represent means ($n = 3$) of Sca-1^+/Flk-1^+/c-kit^+ population. SSC, side light scatter; FSC, forward light scatter.

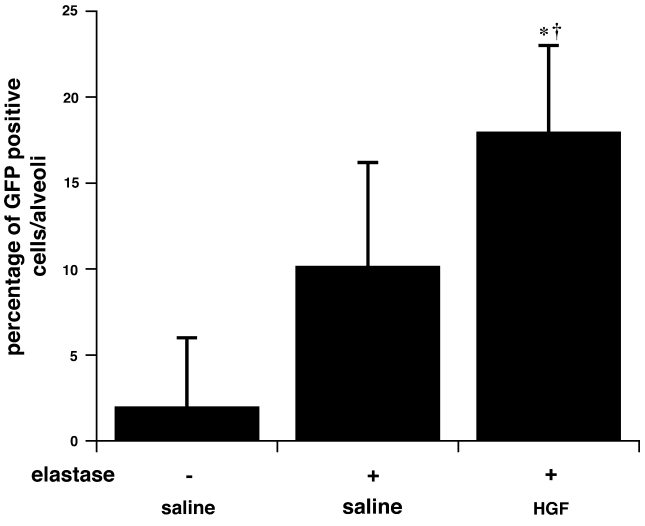

Fig. 2. Percentage of GFP-positive cells in alveoli. HGF increased bone marrow-derived, GFP-positive, cells in alveolar wall. *$P < 0.001$, versus saline without elastase, †$P < 0.001$ versus saline with elastase.

Serum VEGF concentration was measured using a mouse VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

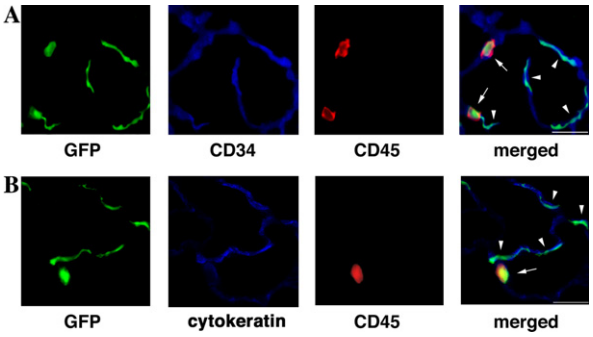

Fig. 3. Bon marrow-derived cells differentiated to capillary endothelial cells and alveolar epithelial cell. (A,B) Representative histological sections for HGF-treated lungs. Immunostaining for CD34 as a marker of endothelial cells or cytokeratin as a marker of epithelial cells, CD45 as a marker of hematopoietic cells, and GFP. Color staining: CD34, blue; CD45, red; and GFP, green. Note that $GFP^+/CD34^+/CD45^-$ cells and $GFP^+/cytokeratin^+/CD45^-$ were observed in the alveolar walls (light blue, arrowheads). $GFP^+/CD45^+$ cells, which were supposed to be hematopoietic cells in the capillary, were present (orange, arrows). Scale bars, 50 μm.

Statistical analysis. Data are expressed as means ± standard error of the mean. Comparisons were made by analyses of variance, and when overall differences were identified, multiple contrasts with a Bonferroni adjustment were used to identify which groups were significantly different. Statistical significance was defined as $P < 0.05$.

Results and discussion

The number of PBMCs, which contain an EPC population, was increased by HGF treatment compared with saline-treated mice (Table 1). Bone marrow-derived circulating EPCs express markers of both hematopoietic stem cells and hemangioblasts, such as Sca-1 and Flk-1/VEGF-R2 [20]. In addition, c-kit is known as another surface marker for EPCs [24]. Flow cytometry demonstrated that the $Flk\text{-}1^+/c\text{-}kit^+$ cells increased in peripheral Sca-1-positive population after HGF treatment (Fig. 1). The number of circulating $Sca\text{-}1^+/Flk\text{-}1^+/c\text{-}kit^+$ cells increased in HGF-treated mice (Table 1). These results suggested that HGF induced an increase in the number of peripheral $Sca\text{-}1^+/Flk\text{-}1^+/c\text{-}kit^+$ cells, consistent with EPC phenotype, from bone marrow into the

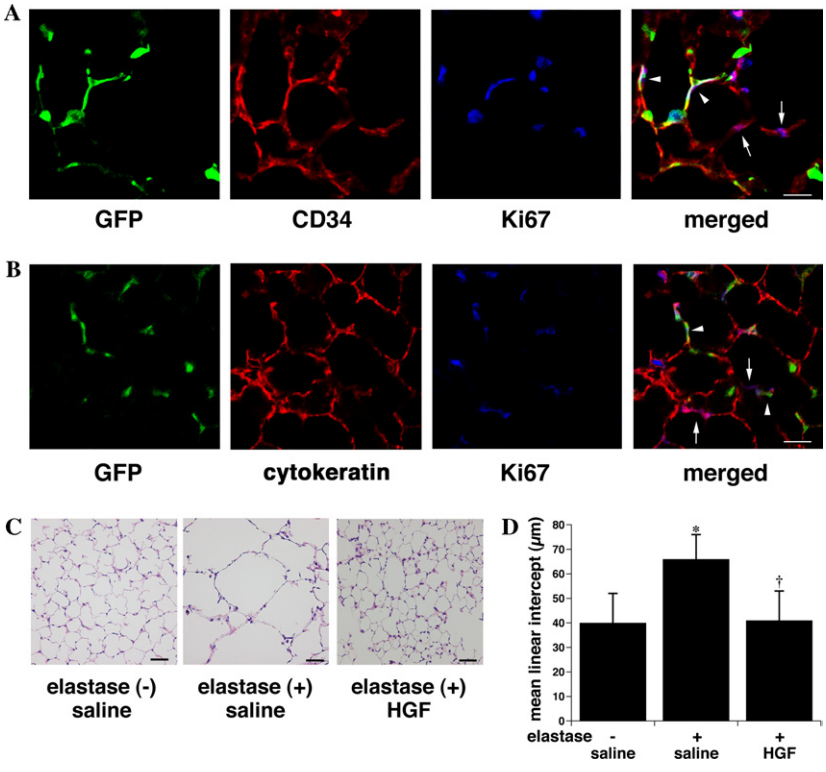

Fig. 4. HGF ameliorated elastase-induced emphysema-like changes. (A) Representative histological sections for HGF-treated lungs. Immunostaining for CD34 as a marker of endothelial cells, GFP, and Ki67 as a proliferating marker. Color staining: CD34, red; GFP, green; and Ki67, blue. *Note.* $GFP^+/CD34^+/Ki67^+$ (arrowheads) and $GFP^-/CD34^+/Ki67^+$ (arrows) cells were observed in the alveolar walls. Scale bars, 50 μm. (B) Representative histological sections for HGF-treated lungs. Immunostaining for cytokeratin as a marker of epithelial cells, GFP, and Ki67. Color staining: cytokeratin, red; GFP, green; and Ki67, blue. *Note.* $GFP^+/cytokeratin^+/Ki67^+$ (arrowheads) and $GFP^-/cytokeratin^+/Ki67^+$ (arrows) cells were observed in the alveolar walls. Scale bars, 50 μm. (C) Representative histological sections of the lungs from each group stained with hematoxylin and eosin. (D) The mean alveolar linear intercept (L_m) was used as a morphometric parameter of emphysema. Briefly, 20 fields at 400× magnification were randomly sampled on two slides from each mouse, and point counting was performed. The total distance divided by the number of alveolar intercepts gave the L_m. $^*P < 0.001$ versus saline without elastase, $^\dagger P < 0.001$ versus saline with elastase.

circulation, and that lung parenchyma could therefore receive more progenitor cells from the bone marrow.

Histological sections demonstrated that HGF induced a significant increase in the percentage of GFP^+ cells per alveolus (Fig. 2). In contrast, at baseline, only circulating white blood cells and alveolar macrophages were GFP-positive, and only a few GFP^+ cells were observed in the alveolar walls (Fig. 2). These results suggested that the regenerated alveoli were composed of bone marrow-derived cells (BMDCs). Thin flat GFP-positive cells that were morphologically consistent with capillary endothelial cells appeared in the alveolar wall of HGF-treated recipient lungs (Fig. 3A). To confirm the cell type of the GFP-positive cells in HGF-treated recipient mice, triple-color immunofluorescent staining was performed using CD45 to mark hematopoietic cells and CD34 to mark endothelial cells [25]. Flat $GFP^+/CD34^+/CD45^-$ cells were observed, suggesting differentiation toward endothelial cells (Fig. 3A). These results suggested that the EPCs mobilized by HGF were sequestered to injured lungs and that they participated in alveolar angiogenesis. GFP^+/cytokeratin$^+/CD45^-$ cells, suggestive of alveolar epithelial cells, were also present in the alveolar walls of HGF-treated recipient lungs (Fig. 3B). Therefore, HGF may also mobilize the progenitor cells with an epithelial phenotype.

Hepatocyte growth factor is known to stimulate not only migration but also proliferation in endothelial cells [4]. Immunostaining of CD34 and the proliferation marker Ki67 was performed to identify proliferative endothelial cells. $CD34^+/Ki67^+$ cells were found in both GFP-positive and GFP-negative populations after HGF treatment (Fig. 4A). These data confirmed that exogenous HGF strongly promotes cell proliferation in the alveolar wall. Interestingly, HGF induced endothelial proliferation not only in BMDCs but also in the resident endothelial cells. The same observation was seen in both cytokeratin and Ki67 staining (Fig. 4B), that is compatible with the previous report [26].

Vascular endothelial growth factor is known as another mitogen for endothelial cells. HGF induces VEGF from vascular smooth muscle cells in vivo [27]. However, in this model, HGF did not change VEGF concentration in peripheral blood compared with saline-treated mice (15.9 ± 3.7 vs. 18.0 ± 3.2 pg/mL, $P = 0.3$). It is known that HGF induces angiogenesis independently of VEGF [28], and acts in synergy with VEGF for the amplification of angiogenesis [29]. Our data suggest that direct effects of HGF on mobilization of EPCs and on endothelial proliferation are important for lung regeneration.

Histological analysis showed that HGF provided a significant reduction in emphysema (Fig. 4C). The L_m, used as a morphometric parameter of emphysema [23], showed a 90% reduction compared to saline-treated emphysematous lungs (Fig. 4D). HGF-induced mobilization of EPCs and endothelial proliferation in lung parenchyma are part of this lung regeneration process. In addition, HGF is known to be the principal mitogen for alveolar type II cells, and it contributes to alveolar epithelial repair [2]. Recently, Sakamaki et al. [30] reported that HGF was involved in compensatory lung growth after pneumonectomy in mice. Therefore, HGF is thought to have a role in both alveolar septation and angiogenesis resulting in lung regeneration.

In summary, we demonstrated that: (1) HGF induced mobilization of EPCs from bone marrow, (2) HGF induced alveolar angiogenesis in elastase-induced lung injury, (3) BMDCs, which were supposed to be of EPC origin, comprised part of the regenerated alveolar walls, and (4) this regeneration occurred not only through bone marrow stimulation but also as a proliferative effect induced by HGF. These complex effects induced by HGF orchestrate lung regeneration in emphysematous lung parenchyma. This is the first report to identify a ligand growth factor that helps to restore lung emphysema, an intractable respiratory disease. Our present findings may arouse further interest in the therapeutic possibilities of HGF in the amelioration of pulmonary emphysema.

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