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FULL PAPER

Hepatocyte growth factor transduces different intracellular signals in aortic and umbilical venous endothelial cells

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Abstract

Endothelial cells are important for maintenance of vascular integrity by producing a variety of bioactive molecules such as nitric oxide (NO). Recent evidence has suggested that there are some differences in characteristics between endothelial cells from different origins. Here we examined responses of two typical endothelial cells to hepatocyte growth factor (HGF), which induces endothelium-dependent relaxation of microvessels. Stimulation of human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) with HGF increased endothelial NO synthase activity, accompanied with an increase of activity-related site-specific phosphorylation of protein kinase B/Akt. However, HGF stimulated phosphorylation of p38 mitogen-activated protein kinase (MAPK) only in HUVEC, but not in BAEC, while it induced phosphorylation of p44 /p42 MAPK in both cells. These results suggest that HGF transduces different intracellular signals between aortic and umbilical venous endothelial cells, and that the differences might represent divergent endothelial responses to growth factors, especially those that activate receptor-tyrosine kinases.

Key words : Akt, endothelial cell, HGF, MAPK, nitric oxide synthase.

Introduction

Vascular endothelial cells play a crucial role in many physiological functions such as the transport of substances between blood and tissues, the modulation of the vascular tone, the activation and migration of white blood cells, the control of blood coagulation and fibrinolysis[3]. All endothelial cells are derived from an identical embryonic origin, but

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recent evidence has demonstrated that endothelial cells show definite morphological and molecular differences not only among organs but also in vascular segments (arterial/capillary/venous) in the same organ[4,6,17,18]. For example, endothelial cells from lung, heart and brain express different types of lectin-binding proteins on their plasma membranes[4], and those from lung, kidney and brain bind selectively to structurally different peptides[17]. In addition, Wang et al. have reported differences in cell-adhesion-mediated activation of intracellular signals between lung microvascular and arterial endothelial cells[20], implying divergent endothelial responses to extracellular stimuli including angiogenic factors.

Hepatocyte growth factor (HGF), also known as Scatter factor, is a mesenchyme-derived multifunctional cytokine with a plethora of biological effects including mitogenesis, motogenesis, morphogenesis, and organogenesis[5], and possibly involved in tumor invasion and metastasis[13]. HGF also stimulates endothelial cell motility, proliferation and organization into capillary-like tubes[19]. In addition to these angiogenic effects, HGF acts as a vasorelaxation factor of microvessels, possibly through nitric oxide (NO) production[14]. Moreover, we lately demonstrated HGF stimulation of endothelial NO synthase (eNOS) activity by phosphoinositide 3-kinase (PI 3 K) / Akt-dependent phosphorylation in a Ca^{2+}-sensitive manner[12].

Bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) are most commonly used for various experiments as typical endothelial cells. However, it remains to be elucidated whether there are differences in the responses to angiogenic growth factors, possibly reflecting the characteristics attributed to the origins and whether HGF transduces divergent signals. To test these hypotheses, in the present study, we compared HGF-induced intracellular signals between the two types of endothelial cells.

Materials and Methods

Materials

Human recombinant HGF was a generous gift of Mitsubishi Pharma Co. (Tokyo, Japan). Antibodies against phospho-specific p44/p42 MAPK (Thr-202/Tyr-204), p44/p42 MAPK, phospho-specific p38 MAPK (Thr-180/Tyr-182), p38 MAPK, phospho-specific Akt (Thr-308), and Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-eNOS antibody and calmodulin were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Wako Pure Chemicals Co. (Osaka, Japan), respectively.

Cell culture and treatment

BAEC from Cell Systems (Kirkland, WA, USA) were maintained in CS-C Complete Medium Kit (Cell Systems) on Type I-collagen-coated plates (Asahi Techno Glass Co., Tokyo, Japan) at 37℃ and 5 % CO_2 under humidified conditions. HUVEC obtained from ATCC (Manassas VA, USA) were cultured on the collagen-coated plates in MCDB-104 medium (Morinaga Institute of Biological Science, Yokohama, Japan) containing 10% bovine calf serum and 12.5 mM HEPES, supplemented with brain-extracted growth factors (Morinaga Institute of Biological Science). After these cells were grown to confluence, they were serum-starved in phenol red-free Medium 199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine and 0.2% bovine serum albumin (BSA) overnight prior usage. All the experimental treatments were carried out using fresh serum-starvation medium.

Measurement of NOS activity

The eNOS activity was quantified as the conversion of L-[U-^{14}C] arginine to L-[U-^{14}C] citrulline as previously described[7] with mi-

nor modifications. Briefly, confluent cells were serum-starved and the medium replaced before the treatments. Following the experimental treatments, cells were harvested in ice-cold homogenization buffer containing 50mM Tris-HCl, pH 7.4, 250 mM sucrose, 0.1 mM EGTA, 0.1mM EDTA, 1 mM dithiothreitol, and a protease inhibitor cocktail, Complete (1 tablet / 50 mL, Roche Diagnostics, GmbH, Mannheim, Germany). For each sample 10μL of homogenate was incubated in duplicate at 37℃ for 10min in a100μL of reaction mixture containing 50 mM HEPES, pH 7.9, 1 mM dithiothreitol, 1mM $CaCl_2$, 0.1mM tetrahydro-L-biopterin (BH_4), 1 mM NADPH, 10 μM FAD, 10 μg/mL calmodulin and 1.43μM L-[U-^{14}C] arginine (Amersham Pharmacia Biotech, Buckinghamshire, UK). The reaction was terminated by addition of 200μL of a stop solution containing 100 mM HEPES and 10 mM EDTA, pH 5.2. The reaction mixture was then applied to 0.5mL neutralized AG 50 W-X 4 resin (Na^+ form 200-400 mesh, Bio-Rad Labs, Hercules, CA, USA) column to separate L-[U-^{14}C] citrulline. The flow through was analyzed by liquid scintillation counting, and enzyme activity expressed as fmol of L-[U-^{14}C] citrulline produced/mg protein of homogenate/10min. Protein concentration was determined by the Lowry method[10] using BSA as a standard.

Western blot analysis

Following the experimental treatments, cells were washed with ice-cold PBS and scraped in ice-cold lysis buffer (50mM HEPES, pH 7.5, 150mM NaCl, 5 mM EDTA, 20mM sodium fluoride, 10mM sodium pyrophosphate, 2 mM sodium vanadate, 1 %NP 40 and a protease inhibitor cocktail, Complete). Harvested cells were incubated on ice for 30min followed by centrifugation at 12,000x *g* for 20min at 4 ℃ to obtain cell lysate. Aliquots of the cell lysate (30μg of each sample) were resolved on SDS-PAGE under reducing conditions and protein electroblotted onto polyvinylidene difluoride membrane (Immobilon™; Millipore, MA, USA). The membrane was blocked in 5 % skim milk overnight at 4 ℃ followed by incubation with a primary antibody overnight at 4 ℃, and then exposure to a horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Zymed Lab. Inc., San Francisco, CA, USA) for 1 h at room temperature. Visualization was performed using the enhanced chemiluminescence ECL (Amersham) detection system according to the instructions. Intensities of immunoreactive bands in the Western blots were densitometrically analyzed on a Macintosh computer using the public domain NIH Image program (U.S. National Institutes of Health; available on the Internet at http : // rsb.info.nih.gov/nih-image/).

Statistical Analysis

Results are expressed as means ± S.E. of 3 - 4 independent experiments. Statistical analysis was done using analysis of variance (ANOVA) and Fischer's test at $p<0.05$.

Results

We first tested the expression of eNOS protein in HUVEC and BAEC, and compared

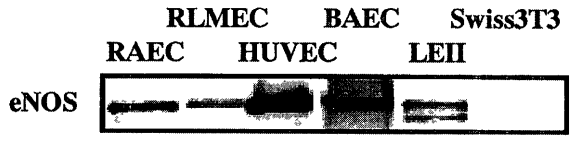

Figure 1. Western blot analysis of eNOS protein in various types of endothelial cell.
Cell lysates were prepared from HUVEC, BAEC, rat aortic and lung microvascular endothelialcells (RAEC and RLMEC), mouse lung microvascular endothelial cell line (LEII) and Swiss 3T3 fibroblast as negative control, and 40 μg of each cell lysate was separated by SDS-PAGE and examined for eNOS protein expression by Western blot. Shown are representative blots of three independent experiments.

Table 1. HGF increases eNOS activity in HUVEC and BAEC

Cell line	Basal activity	HGF stimulation	Fold
HUVEC	1063.0±164.1	2336.0±171.9*	2.4±0.5
BAEC	3554.7±779.7	6708.0±964.1*	2.1±0.3

Confluent HUVEC and BAEC were serum-starved overnight and stimulated with HGF (40ng/ml) for 20min. The eNOS activity in the cell homogenate was determined as the conversion of L-[U-^{14}C] arginine to L-[U-^{14}C] citrulline, and expressed as fmol of citrulline produced/mg protein of homogenate/ 10min and the mean±S.E. of four independent experiments. *, $p<0.05$ vs. basal activity.

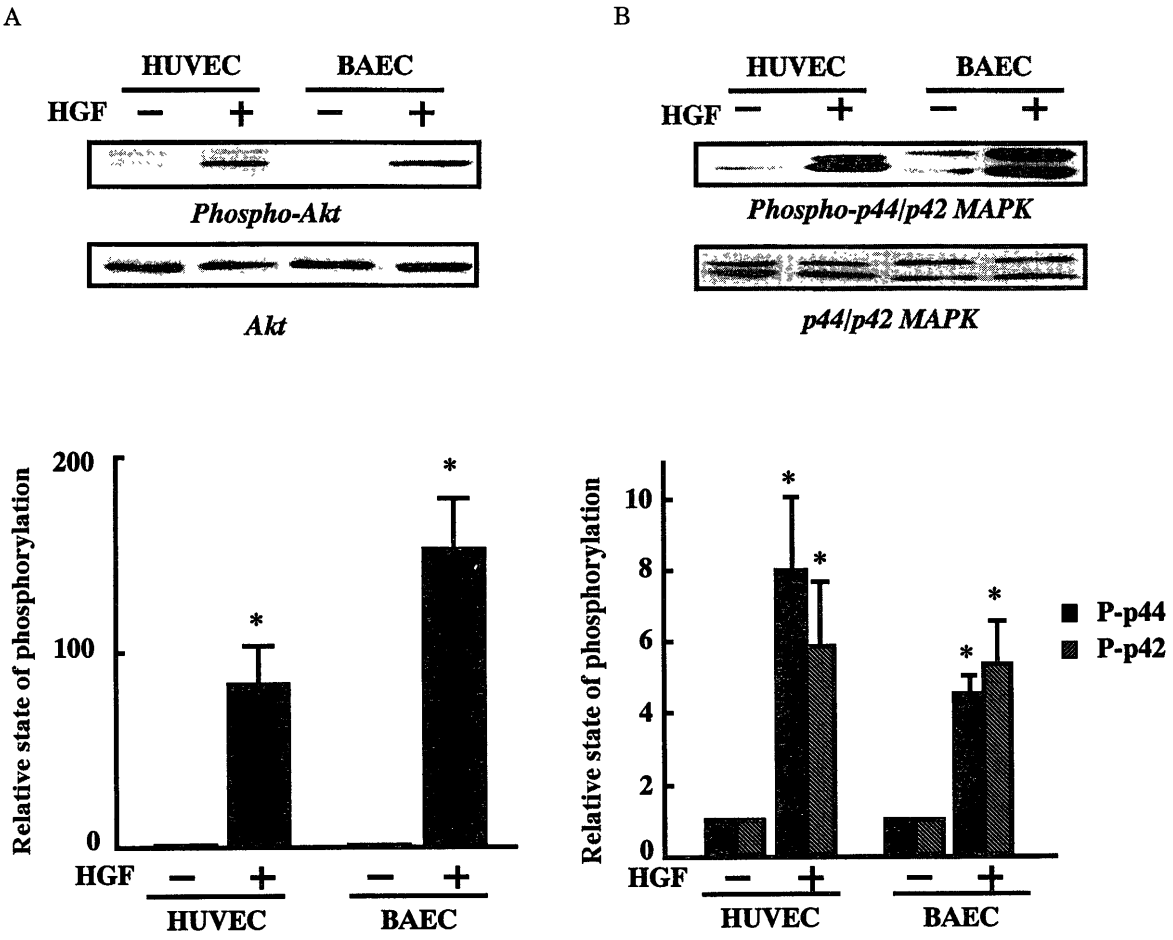

Figure 2. HGF induces Akt and p44/p42MAPK phosphorylation in HUVEC and BAEC.
Serum-starved HUVEC and BAEC were stimulated with HGF (40ng/ml) for10min. The cell lysate (30μg/lane) was resolved on 10% SDS-PAGE, and examined for activation of p44/p42 MAPK and Akt with anti-phospho-p44/p42 MAPK (Thr 204/Tyr 202) and anti-phospho-Akt (Thr-308) antibodies and for their protein content (total) with respective antibodies. Shown are representative blots of three independent experiments for Akt (A) and p44/p42MAPK (B), respectively. Densitometric analyses as fold increase relative to controls (untreated). Results represent the mean±S.E. of three independent experiments. *, $p<0.05$ vs. without HGF addition.

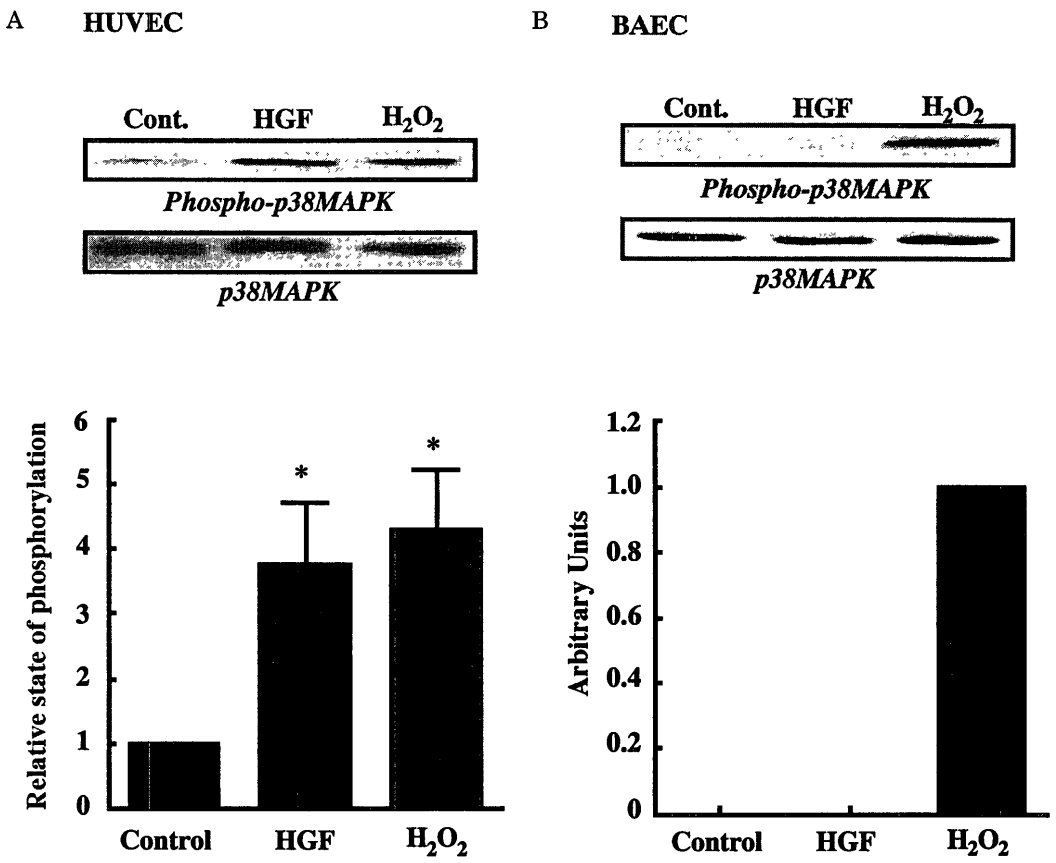

Figure 3. HGF induces p38 MAPK phosphorylation in HUVEC, but not in BAEC.
Serum-starved HUVEC (A) and BAEC (B) were stimulated with either HGF (40ng/ml) or H_2O_2 (1 mM) for 10 min. The cell lysate (30μg/lane) was resolved on 10% SDS-PAGE, and examined for activation of p38 MAPK with anti-phospho-p38 MAPK (Thr-180/Tyr-182) antibodies and for p38 MAPK protein content (total). Shown are representative blots of three independent experiments. Densitometric analyses are expressed as fold increase relative to controls (A). As no signal for phospho-p38 MAPK was detected in control and HGF -stimulated BAEC, densitometric value for H_2O_2-stimulated cells is shown as arbitrary units (B). Results represent the mean ± S.E. of three independent experiments. *, $p < 0.05$ vs. without HGF addition.

with those in other endothelial cells, primary cultured rat aortic and lung microvascular endothelial cells (RAEC and RLMEC)[11] and mouse lung microvascular endothelial cell line (LEII)[1] and Swiss 3T3 fibroblast as negative control. As shown in Fig. 1, all of the endothelial cells tested, but not Swiss 3T3 cells, expressed eNOS protein, but the levels of the protein in HUVEC and BAEC were much higher than those of primary cultured endothelial cells and LEII cells. We also examined eNOS activity in HUVEC and BAEC as the conversion of arginine to citrulline. Basal eNOS activity in HUVEC was relatively lower than that in BAEC, and the stimulation of the cells with HGF for 20 min significantly increased eNOS activity by almost the same magnitude (Table 1).

HGF acts on endothelial cells through tyrosine kinase receptor, c-Met, and subsequent activation of both PI3K and p44/p42MAPK[2,16], and Akt which lies down-stream of PI 3 K di-

rectly regulates eNOS activity by phosphorylation[12]. We next examined the effects of HGF stimulation on Akt and p44/p42MAPK phosphorylation, using the antibodies to detect activity-related site-specific phosphorylation. As shown in Figs. 2 A and 2 B, HGF potently stimulated phosphorylation of Akt (Thr 308) and p44/p42MAPK (Thr-202/Tyr204) without affecting their total protein contents, indicating activation of both protein kinases. There was no apparent difference in these responses to HGF between HUVEC and BAEC.

Recently, differences in p38 MAPK activation have been reported between lung microvascular and arterial endothelial cells treated with anti-intercellular adhesion molecule-1 (ICAM-1) antibody for its cross-linking[20]. To test whether HGF activates p38 MAPK, we examined activity-related site-specific p38 MAPK phosphorylation. Stimulation of HUVEC with HGF induced p38 MAPK phosphorylation (Thr-180/Tyr-182), while it did not cause any noticeable changes in total p38 MAPK (Fig. 3 A). In contrast, stimulation of BAEC with HGF failed to induce p38 MAPK phosphorylation, although significant amount of p38MAPK was detected (Fig. 3B). Moreover, hydrogen peroxide (H_2O_2) induced phosphorylation similarly in HUVEC and BAEC (Fig. 3).

Discussion

We here demonstrated that HGF/c-Met signaling stimulated pathways leading to eNOS activation, Akt and p44/p42 MAPK phosphorylation in the two types of endothelial cells, HUVEC and BAEC, but p38 MAPK activation only in HUVEC. Our findings are in accordance with HGF activation of Akt and p44/p42MAPK pathways in human aortic endothelial cells, necessary for HGF-induced mitogenic and antiapoptotic actions[16].

Recently we have demonstrated that HGF stimulates eNOS activity through PI 3 K / Akt-dependent eNOS phosphorylation in BAEC[12] : that is, HGF initially activates PI3K to produce 3 -phosphoinositide, leading to Akt phosphorylation at Thr308 by 3 -phosphoinositide-dependent protein kinase-1 (PDK-1), and consequently to Akt-mediated eNOS phosphorylation and activation. Stimulation of HUVEC with HGF similarly increased eNOS activity as in BAEC, possibly through Akt-mediated pathway, as the activation of eNOS was accompanied with an increase of Akt phosphorylation. The eNOS activation obviously indicates enhancement of NO production, and might explain the mechanism of HGF-induced vasorelaxation[14] and some other HGF-induced changes including angiogenesis[19], limiting of neointimal proliferation[21] and tissue regeneration after myocardial ischemic damage[15]. Furthermore, our results suggest conserved eNOS activation, Akt and p44/p42 MAPK pathways by HGF in, at least HUVEC and BAEC, vascular endothelial cells of divergent origins.

As mentioned above, although HGF activated Akt, p44/p42MAPK and eNOS in both HUVEC and BAEC, it stimulated activity-related site-specific p38 MAPK phosphorylation only in HUVEC. In BAEC, HGF failed to induce p38 MAPK phosphorylation even though p38 MAPK protein is present and H_2O_2 did induce its phosphorylation. These findings suggest that some machinery leading to p38 MAPK activation through c-Met, a tyrosine receptor kinase, are lacking in BAEC. Similarly, even in the same species, treatment of arterial endothelial cells with anti-ICAM-1 antibody[20] or proinsulin C-peptide (Kitamura, T. et al. manuscript in submission) fails to activate the p38 MAPK pathway, while treatment of lung microvascular endothelial cells does. Collectively, the deficiency of the machinery to activate p38 MAPK seems to be due

to the origin of endothelial cells, especially those derived from major arteries.

The activation of p38 MAPK has been shown to be elicited by a series of cytokines, growth factors, and autonomic neurotransmitters and also stress factors such as UV irradiation and H_2O_2, leading to phosphorylation of some transcription factors[9]. Actually, we have recently shown that stimulation of LEII mouse lung capillary endothelial cells with proinsulin C-peptide increased p38 MAPK activity, thereby leading to phosphorylation of cyclic AMP responsive element (CRE) binding protein (CREB) /activating transcription factor 1 (ATF 1) and consequent binding of these transcription factors to CRE[8]. Therefore, it is likely that HUVEC, a venous type of endothelial cell, is controlled differently from BAEC at transcription levels by HGF and possibly by other growth factors that activate receptor-tyrosine kinases.

In summary, we have demonstrated that HGF transduces different intracellular signals between aortic and umbilical venous endothelial cells, and the difference, at least in terms of p38 MAPK activation, might be worthy consideration prior to experimental usage.

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