Increase in Peripheral Blood Flow by Intravenous Administration of Prostaglandin E1 in Patients with Peripheral Arterial Disease, Accompanied by Up-Regulation of Hepatocyte Growth Factor

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Since endothelial damage is a trigger for the progression of atherosclerosis, we evaluated the clinical utility of prostaglandin E1 (PGE1) in relation to peripheral blood flow and regulation of hepatocyte growth factor (HGF), an angiogenic growth factor, in patients with peripheral arterial disease (PAD). Fourteen male patients with PAD who showed the characteristic symptoms of arteriosclerosis obliterans (Fontaine I: n = 2; Fontaine II: n = 4; Fontaine III: n = 2; Fontaine IV: n = 6), confirmed by angiography, were enrolled in this study. Patients were administered synthetic PGE1 at a dose of 120 μg per day for 14 consecutive days. Measurement of peripheral blood flow and serum HGF concentration was performed before PGE1 treatment and after 14 days of administration. Interestingly, intravenous administration of PGE1 for 2 weeks significantly increased the blood flow as assessed by a laser Doppler imager (p < 0.01). In patients with Fontaine III and IV, serum HGF concentration was significantly higher than that in patients with Fontaine I or II and normal subjects. Of importance, administration of PGE1 further increased serum HGF concentration as compared to that before treatment (p < 0.01). The increase in circulating HGF might work as a compensatory mechanism to decrease local HGF expression in patients with PAD, since HGF acts as an angiogenic growth factor with anti-apoptotic actions on endothelial cells. Moreover, to confirm the stimulatory effect of PGE1 on HGF in vessels, we employed an in vitro culture system. PGE1 increased HGF production and the growth of human cultured vascular endothelial cells. The stimulatory effect of PGE1 on HGF production might be due to an increase in cAMP, since forskolin and 8-bromo-cAMP induced HGF production. In conclusion, we demonstrated that administration of PGE1 stimulated peripheral blood flow, accompanied by an increase in systemic HGF concentration. Also, our in vitro data suggested that PGE1 augmented not only the systemic HGF level, but also local HGF production, probably through cAMP accumulation, resulting in improvement of endothelial function and blood flow. (Hypertens Res 2004; 27: 85–91)

Key Words: peripheral arterial disease, hepatocyte growth factor, endothelial cell, cAMP, prostaglandin E1
Introduction

Endothelial dysfunction is one of the triggers for the development of atherosclerosis followed by vascular remodeling, since endothelial cells have an important role as a biological barrier in the suppression of growth of vascular smooth muscle cells (VSMC), maintenance of vascular tone and protection from monocyte and platelet adhesion, etc. (1–5). Severe damage to endothelial cells induced by a variety of cytokines and adhesion molecules has been assumed to be responsible for cardiovascular diseases such as myocardial infarction and arteriosclerosis. Therefore, for the treatment of vascular diseases, it would be worthwhile to develop a treatment to protect the function of endothelial cells. Indeed, re-endothelialization using endothelium-specific growth factor is becoming a useful application as gene therapy for atherosclerosis and restenosis after angioplasty (6–8).

Prostaglandin E1 (PGE1), a potent vasodilator and platelet aggregation inhibitor, is well known as a useful drug for peripheral arterial disease (PAD). PGE1 has been reported to relax the contraction of VSMC (9) and increase blood flow in peripheral arteries (10), followed by improvement of endothelial function (11). In this study, we focused on the relationship between PGE1 and hepatocyte growth factor (HGF), a potent angiogenic growth factor with anti-apoptotic actions on endothelial cells. In the present study we examined 1) the effect of PGE1 on the circulating HGF system in patients with PAD and 2) the effect of PGE1 on the regulation of HGF in human cultured endothelial cells.

Methods

Clinical Study

Subjects and Study Design

The subjects were 14 male patients with PAD aged 61 to 78 years (68 ± 4 years). All patients showed characteristic symptoms of arteriosclerosis obliterans (ASO) (Fontaine I: n = 2; Fontaine II: n = 4; Fontaine III: n = 2; Fontaine IV: n = 6) as confirmed by angiography. Patients were administered synthetic PGE1 at a dose of 120 μg per day for 14 consecutive days. All patients took an oral prostaglandin I2 analogue and phosphodiesterase 3 (PDE3) inhibitor (cilostazol) continuously throughout the study. Measurement of peripheral blood flow and serum HGF concentration was performed before PGE1 treatment and 14 days after administration. Also, to estimate the normal level of serum HGF, serum HGF concentration was measured in 10 age- and sex-matched volunteers (63 ± 3 years) with no risk factors for atherosclerosis.

Laser Doppler Imaging

The measurement of peripheral blood flow with a laser Doppler imager was performed as described previously (12, 13). Peripheral blood supply was measured using a laser Doppler blood flowmeter (Laser Doppler Image; Moor Instruments, Devon, UK). The laser Doppler imager used a 12-mW helium-neon laser beam that sequentially scans a 15 mm² 15 cm² surface area at extremely high speed in order to measure the peripheral blood flow. Using this method, the blood flow at 1 mm under the surface can be measured. While scanning, blood cells which are in motion shift with the frequency of projected light according to the Doppler principle. The perfusion signal is subdivided into 14 different intervals, and each interval is displayed in a separate color. Low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as white. The stored perfusion values behind the color-coded pixels remain available for data analysis. The mean voltage and maximum voltage in the thumb were calculated.

Blood Sampling and HGF Measurement

Antecubital venous blood was taken during the morning, at 0700–0900, after an overnight fast. Serum was immediately separated by centrifugation at 4°C, and stored at -20°C until assay. Serum HGF concentration was assayed using a recently developed enzyme-immunoassay (EIA) for use in humans (14). Briefly, rabbit anti-human HGF immunoglobulin G (IgG) was coated on a 96-well plate (Corning, New York, USA) and incubated at 4°C for 15 h. After blocking with 3% bovine serum albumin in phosphate-buffered saline (PBS), the sample was added to each well, and the preparation was incubated for 2 h at 25°C. Wells were washed three times with PBS containing 0.025% Tween 20 (PBS-Tween), then biotinylated rabbit anti-human HGF IgG was added and the preparation was incubated for 2 h at 25°C. After washing with PBS-Tween, wells were incubated with hors eradish peroxidase-conjugated streptavidin-biotin complex in PBS-Tween. The enzyme reaction was initiated by adding substrate solution composed of 2.5 mg/ml o-phenylenediamine, 100 mmol/l sodium phosphate, 50 mmol/l citric acid, and 0.015% H2O2. The enzyme reaction was halted by adding 1 mol/l H2SO4, and absorbance at 490 nm was measured. The antibody against human HGF reacts with only human HGF, and not with rat HGF.

Measurement of Tissue HGF Concentration

Vascular specimens were obtained from patients with PAD at the time of open surgical repair. The harvested specimens were immediately frozen in liquid nitrogen and stored at -70°C until measurement of HGF concentration. The tissue was thawed at 4°C, weighed, and homogenized by polytron in an assay solution. Each specimen was centrifuged at 20,000 x g for 30 min at 4°C to remove the lysates. The concentration of tissue HGF was determined by EIA with an anti-human HGF antibody as described previously (14).
In Vitro Study

Cell Culture of Human VSMC and Measurement of HGF in Conditioned Medium

Human VSMC were seeded on 6-well plates at a density of \(5 \times 10^4\) cells/cm\(^2\) and cultured to confluence. After replacing the medium with fresh defined serum-free medium (DSF) with or without platelet derived growth factor (PDGF) (10 ng/ml; Biosource, Camarillo, USA) and following culture with or without cAMP agents (forskolin or 8-bromo-cAMP) for 24 h and 48 h, the concentration of HGF in the medium was determined by EIA using anti-human HGF antibodies, as described previously (14). DSF medium was supplemented with insulin (5 \(\times 10^{-7}\) mol/l), transferrin (5 mg/ml) and ascorbate (0.2 mmol/l), as previously described (15).

Measurement of Intracellular cAMP Concentration

Intracellular cAMP was measured using an EIA kit from Amersham (Buckinghamshire, UK). In brief, culture medium was removed at the indicated times, and cells were washed twice in PBS and a third time in the same buffer containing 3-isobutyl-1-methylxanthine. Cells were then lysed by the addition of ice-cold trichloroacetic acid (5%). The trichloroacetic acid-soluble supernatant was removed from the wells, extracted three times with 10 ml ether, dried, and resuspended in 0.4 ml sodium acetate buffer (pH 6.2) per sample. Then EIA was performed.

Cell Culture of Human Aortic Endothelial Cells

Human aortic endothelial cells (passage 5) were obtained from Clonetics Corp. (San Diego, USA) and cultured in modified MCDB131 medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor (bFGF) and 1 \(\mu\)mol/l dexamethasone in the standard fashion (16, 17). After replacing the medium and following culture with or without PGE1 (10 \(^{-6}\) mol/l) for 24 h, the concentration of HGF in the medium was determined by EIA using anti-human HGF antibodies as described previously (14), and an index of cell proliferation was determined by using a water-soluble tetrazolium cell counting kit (WST; Wako Pure Chem. Ind., Osaka, Japan), because this compound produces a highly water-soluble formazan dye, which makes the assay procedure easier to perform (18). These cells showed the specific characteristics of endothelial cells by immunohistochemical examination and morphological observation. All the cells were used within passages 5–6.

Fig. 1. (a) Typical image of peripheral blood flow analyzed by a laser Doppler imager. These panels show color-coded images representing blood flow distribution. Low or no perfusion is displayed as dark blue, whereas the highest perfusion is displayed as white. Pre., before treatment with PGE1; Post., after 14 consecutive days of PGE1 administration. (b) Quantitative analysis of peripheral blood flow. Values are expressed as voltage. Pre, pre-treatment; 1W, after 1 week of treatment, 2W, after 2 weeks of treatment.

Materials

PGE1 was obtained from Ono Pharmaceutical Co. (Osaka, Japan).

Statistical Analysis

All values are expressed as the mean \(\pm\) SEM. All experiments were performed at least three times. Analysis of variance with subsequent Duncan’s test was used to determine the significance of differences in multiple comparisons. Differences with values of \(p < 0.05\) were considered statistically significant.

Results

Clinical Study

Intravenous administration of PGE1 did not affect blood pressure or heart rate in the patients with PAD (data not shown). Laser Doppler imaging demonstrated that treatment with PGE1 resulted in a significant increase in peripheral blood supply (\(p < 0.05\); Fig. 1). In the patients with Fontaine III and IV, serum HGF concentration was significantly higher than that in patients with Fontaine I or II or normal subjects (\(p < 0.05\); Fig. 2). On the other hand, tissue HGF level in the vascular wall of patients with PAD was significantly
decreased as compared to that in normal segments of the vessels (Fig. 3). The decrease in tissue HGF concentration in the atherosclerotic lesions was consistent with previous reports that tissue HGF concentration in ischaemic or atherosclerotic lesions was down-regulated in experimental animals (19–21). Therefore, the increase in circulating HGF level in the patients with severe symptoms of PAD suggests the existence of compensatory mechanisms in response to the decrease in vascular HGF level. This is also consistent with our previous reports showing that serum concentration of HGF is significantly elevated dependent on the severity of hypertension (22, 23). Although details of the origin of circulating HGF are still unclear, it is speculated that HGF production might increase in organs such as the liver, kidney and lung, which are known as the representative HGF-producing organs. Of importance, administration of PGE1 further increased serum HGF concentration as compared to that before treatment (p<0.05; Fig. 4). The increase in circulating HGF might have protective actions on endothelial cells, since HGF functions as an angiogenic growth factor with anti-apoptotic actions on endothelial cells. However, the relationship between the degree of HGF induction and improvement of clinical symptoms was negative (data not shown) because the number of subjects in this study was not large.

Effect of PGE1 on Local HGF Production in Culture System

To clarify the mechanisms of the stimulatory effect of PGE1 on HGF expression, we employed an in vitro culture system. Since the promoter region of the HGF gene contains a cAMP responsive element (24), we speculated that PGE1 directly activates HGF production in local vessels through cAMP accumulation. Indeed, incubation with forskolin (30 µmol/l) as well as 8-bromo-cAMP (1 mmol/l) stimulated local HGF production in human vascular smooth muscle cells in a time-dependent manner (Fig. 5a), accompanied by cAMP accumulation (Fig. 5b). The increase in local HGF production was at least in part due to the accumulation of cAMP, since (R)-p-adenosine-3’,5’-cyclic phosphorothioate (an inhibitor of the cAMP-dependent protein kinase; Rp-cAMP) significantly attenuated the increase in local HGF production by 8-bromo-cAMP (Fig. 5c). Moreover, treatment with either PGE1 or cilostazol (CSZ), a PDE3 inhibitor that induces cAMP, stimulated local HGF production in human endothelial cells, as shown in Fig. 6a. Interestingly, addition of either of these drugs to cultured human endothelial cells induced endothelial cell growth (Fig. 6b). These data suggested that administration of PGE1 induces local HGF production both in vascular smooth muscle cells and endothelial cells, resulting in improvement of endothelial dysfunction and induction of endothelium-dependent vasorelaxation.

**Fig. 2.** Serum HGF concentration in patients with or without PAD. Normal, subjects with no risk factor for atherosclerosis (n = 10); I & II, patients with PAD classified as Fontaine I or II (n = 6); III & IV, patients with PAD classified as Fontaine III or IV (n = 8). * p<0.05 vs. III & IV.

**Fig. 3.** Tissue HGF concentration in vascular specimens harvested from patients with ASO or aneurysm.

**Fig. 4.** Serum HGF concentration before treatment and after 1 or 2 weeks of treatment. Pre, pre-treatment; 1W, after 1 week of treatment; 2W, after 2 weeks of treatment. * p<0.05 vs. Pre.
The pathophysiological role of endothelial cells is becoming important, since locally synthesized compounds from endothelial cells and VSMC have been postulated to control local vascular function (25–27). From this viewpoint, the maintenance of endothelial cells is very important, given their antiproliferative and vasodilating actions. Indeed, disruption or dysfunction of endothelial cells results in loss of multiple endothelium-derived substances (PGI2, NO, CNP), which in turn results in a shift of balance of VSMC growth to abnormal growth such as that seen in atherosclerosis. Because some clinical trials have suggested the association of endothelial dysfunction with vascular events (28–30), therapy focused on endothelial function has recently been proposed in the clinical field of vascular disease. Based on these findings, we focused on the effect of PGE1 on HGF in the present study, since HGF is a unique growth factor stimulating angiogenesis and preventing apoptosis of endothelial cells.

HGF is a mesenchyme-derived pleiotropic factor which regulates cell growth, cell motility, and morphogenesis of
various types of cells, and is thus considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis (31–33). Moreover, tissue HGF appears to play an important role in the pathogenesis of atherosclerosis, especially in endothelial function. Indeed, our previous reports demonstrated that the area covered by endothelial cells was significantly increased in balloon-injured vessels by in vivo transfer of the HGF gene, and induction of NO content in balloon-injured vessels transfected with the HGF gene was observed in accordance with the recovery of endothelial vasodilator properties in response to acetylcholine (34). The present study demonstrated that the increase in peripheral blood flow by intravenous administration of PGE1 was accompanied by a significant increase in serum HGF concentration. Because HGF has been shown to improve endothelial function and stimulate angiogenesis in several animal models as well as in human gene therapy (19, 20), increased serum HGF might have contributed to the improvement of endothelial function and the increase in peripheral blood flow induced by PGE1. Systemic HGF may act in tissue regeneration as a humoral mediator, in addition to autocrine-paracrine local HGF production. However, since peripheral blood flow assessed by laser Doppler imaging reflects not only endothelial function but also relaxation of vascular smooth muscle cells, more detailed studies (e.g., of vasodilator response to reactive hyperemia using plethysmography, flow-mediated vasodilatation (FMD), etc.) will be needed to show improvement of endothelial function by PGE1-induced HGF production. This is a limitation of our study.

Interestingly, PGE1 stimulated HGF production in cultured human endothelial cells. Our in vitro findings are supported by several lines of evidence: 1) The promoter region of the HGF gene contains a cAMP responsive element (24); 2) The increase in cAMP concentration by forskolin as well as 8-bromo-cAMP and PGE1 was confirmed; and 3) The increase in HGF concentration was attenuated by Rp-cAMP (an inhibitor of cAMP-dependent protein kinase). Moreover, we demonstrated that incubation of PGE1 with endothelial cells resulted in a significant increase in the number of endothelial cells, as compared to vehicle, through induction of HGF. Thus the PGE1-induced increase in local HGF production may have therapeutic value through its improvement of endothelial function, in addition to its blockade of VSMC growth. Since our clinical study demonstrated that administration of PGE1 to patients with PAD induced the production of serum HGF, the beneficial effect of PGE1 is considered to be mediated by induction of both systemic and local HGF production.

Here, we demonstrated that administration of PGE1 stimulated peripheral blood flow, accompanied by an increase in systemic HGF concentration. Also, our in vitro data suggested that PGE1 increased not only the systemic HGF level, but also local HGF production, probably through cAMP accumulation, resulting in improvement of endothelial function and blood flow.

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References

16. Wertheimer SJ, Myers CL, Wallace RW, Parks TP: Inter-


