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Role of hepatocyte growth factor in endothelial regulation: prevention of high D-glucose-induced endothelial cell death by prostaglandins and phosphodiesterase type 3 inhibitor

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Summary Injury of endothelial cells (EC) has been postulated as the initial trigger of the progression of atherosclerosis in patients with diabetes mellitus. We previously reported that decrease in a novel endothelium-specific growth factor, hepatocyte growth factor (HGF), by high D-glucose might be a trigger of endothelial injury. However, the physiological role of the local vascular HGF system has not yet been clarified. To investigate the role of HGF in endothelial injury, we initially examined the effects of HGF on endothelial injury induced by serum deprivation. Decrease in EC number by serum deprivation was significantly attenuated by addition of HGF as well as recombinant basic fibroblast growth factor, whereas vascular endothelial growth factor showed no effect. Apoptotic changes in EC induced by serum deprivation were also significantly attenuated by addition of HGF ($p < 0.01$). Given the protective action of HGF, we next studied the physiological role of local HGF production in endothelial regulation. We focused on the protective actions of prostaglandin (PG) I_2, PGE and a phosphodiesterase type 3 inhibitor (cilostazol) on endothelial injury by high glucose, since these agents are widely used in the treatment of peripheral arterial disease which is frequently observed in diabetic patients. Treatment of human aortic EC with PGE_1, PGE_2, and a PGI_2 analogue (beraprost sodium) as well as cilostazol stimulated EC growth. HGF concentration in conditioned medium from EC treated with PGE_1, PGE_2 or PGI_2 analogue as well as cilostazol was significantly higher than that with vehicle ($p < 0.01$). Interestingly, treatment with PGI_2 analogue or cilostazol attenuated high D-glucose-induced EC death, which was abolished by neutralizing anti-HGF antibody. Moreover, decreased local HGF production by high D-glucose was also significantly attenuated by PGI_2 analogue or cilostazol. Finally, we tested the effects of PGE, PGI_2 analogue and cilostazol on local HGF production in human aortic vascular smooth muscle cells (VSMC). Although high D-glucose treatment resulted in a significant increase in VSMC number, PGI_2 analogue and/or cilostazol treatment had no effects on VSMC growth. However, the decrease in local HGF production by high D-glucose was significantly attenuated by addition of PGI_2 analogue or cilostazol.
Overall, this study demonstrated that treatment with PGE, PGI_2 analogue or cilostazol prevented aortic EC death induced by high D-glucose, probably through the activation of local HGF production. Increased local vascular HGF production by prostaglandins and cilostazol may prevent endothelial injury, potentially resulting in the improvement of peripheral arterial disease. [Diabetologia (1997) 40: 1053–1061]

Keywords Diabetes mellitus, atherosclerosis, vascular HGF system, vascular remodelling, apoptosis.

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Abbreviations: WST-1, 4-[4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazdio]-1, 3-benzene disulfonate; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; VSMC, vascular smooth muscle cells; PG, prostaglandins; PDE, phosphodiesterase; FGF, fibroblast growth factor; MTT, 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide.

Endothelial cells are known to secrete various vasoactive substances. Recently, it has been hypothesized that endothelial cells may also modulate vascular growth, because many anti-proliferative factors such as nitric oxide and vascular natriuretic peptides are secreted by endothelial cells [1–3]. Therefore, it is apparent that dysfunction of endothelial cells may promote abnormal vascular growth such as in atherosclerosis and arteriosclerosis [4, 5]. Our previous studies demonstrated that hepatocyte growth factor (HGF) is a novel member of endothelium-specific growth factors, whose mitogenic activity is the most potent [6, 7]. Moreover, the presence of a local HGF system (HGF and its receptor, c-met) in endothelial cells and vascular smooth muscle cells (VSMC) was also demonstrated *in vitro* as well as *in vivo* [8]. Thus, it is important to know the physiological role of HGF in endothelial regulation, as the loss of anti-proliferative substances from endothelial cells might be related to the development and progression of atherosclerosis/arteriosclerosis in diabetes mellitus (DM) and hypertension. In this study, we initially examined whether HGF has a protective role in endothelial regulation using human cultured aortic endothelial cells.

On the other hand, prostaglandin (PG) I_2 and PGE have been reported to have a cytoprotective action on endothelial cells and to inhibit VSMC growth [9–11]. PGI_2 and PGE are well known to stimulate cyclic AMP (cAMP) which plays a pivotal role in the regulation of glucose metabolism, platelet aggregation and VSMC relaxation. Cyclic AMP is synthesized from ATP by adenylate cyclase, and converted to 5'-AMP by phosphodiesterase (PDE) [12]. PGI_2 and PGE directly activate adenylate cyclase, resulting in the accumulation of cAMP. One of the rate-limiting steps in the cascade of the cAMP pathway is PDE. Type 3 PDE, which is known as cAMP-PDE and degrades cAMP and is inhibited by cGMP, mainly exists in platelets, VSMC, cardiac myocytes and adipose tissue [12]. Therefore, PDE_3 inhibitors inhibit platelet aggregation and VSMC relaxation through the activation of cAMP [13–15]. Indeed, these agents, which cause accumulation of cAMP, are widely used for the treatment of peripheral arterial disease observed in diabetes [16–18]. Diabetes is characterized by the premature development of microvascular and macrovascular disease [19–21], and hyperglycaemia is an independent risk factor for the development of cardiovascular disease [22]. Interestingly, high glucose treatment caused endothelial cell death through a decrease in local HGF production [23]. As addition of recombinant HGF prevented the endothelial cell death induced by high glucose [23], HGF seems to play a pivotal role in the regulation of endothelial cells. Therefore, in this study, we examined the effects of PGE, a stable oral PGI_2 analogue (beraprost sodium) and a PDE_3 inhibitor (cilostazol) on local HGF production in vascular cells, to clarify the potential role of HGF in endothelial injury induced by high D-glucose.

Materials and methods

Cell culture. Human aortic endothelial cells (passage 5) and human aortic VSMC (passage 5) were obtained from Clonetics Corp. (San Diego, Calif., USA), and cultured in modified MCDB131 medium supplemented with 5% fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 10 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor (FGF) and 1 μmol/l dexamethasone in the standard fashion [24, 25]. Cells were incubated at 37 °C in a humidified atmosphere of 95% air-5% CO_2 with medium changes every 2 days. These cells showed the specific characteristics of endothelial cells and VSMC, on immunohistochemical examination and morphological observation. Briefly, human aortic endothelial cells tested positive for factor VIII antigen and for uptake of di-acetylated LDL. In contrast, human aortic VSMC also tested positive for α-actin, and negative for expression of factor VIII antigen. All the cells were used between passage 5–6.

Counting of cell number. An index of cell proliferation was determined using the WST-cell counting kit which is similar to MTT assay (Wako, Osaka, Japan) [23, 26–28]. Tetrazolium salt has been used to develop a quantitative colorimetric assay for cell growth. The assay detects living, but not dead cells, and the signal generated is dependent on the degree of activation of the cells. For this purpose, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is widely used [29]. In this study, we used an alternative to MTT, sulfonated tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1), since this compound produces a highly water-soluble formazan dye, which makes the assay procedure easier to perform [28]. Briefly, 16.3 mg WST-1 and 0.2 mmol/l 1-methoxy-5-methyl-phenazinium methylsulfate were dissolved in 20 mmol/l HEPES buffer (pH 7.4). Then, 10 μl of the reaction solution was immediately added to 100 μl culture medium per well and the cells were then incubated for an additional 2 h. The plate was read on a Bio-Rad Model 3550 Microplate reader, using a test wavelength of 450 nm, and a reference wavelength of 650 nm. We confirmed that serum-stimulated increase in cell number is associated with increased absorbance at 450 nm (data not shown).

Treatment by serum deprivation. In the preparation of experiments for determination of cell death, endothelial cells were grown to confluence. After reaching confluence, the medium was changed to fresh defined serum-free medium containing HGF, basic FGF, vascular endothelial cell growth factor (VEGF), or vehicle. Defined serum-free medium was supplemented with insulin (5×10^{-7} mol/l), transferrin (5 mg/ml), and ascorbate (0.2 mmol/l), as previously described [30]. The cells were then incubated. Every 2 days, the medium was changed to fresh defined serum-free medium containing HGF, basic FGF, VEGF, or vehicle. On days 2 and 4, an index of cell proliferation was determined, as described above.

Apoptotic cells induced by serum deprivation were also counted at 4 days. Hoechst 33342 (Sigma, St. Louis, Mo., USA) was used to observe nuclear morphology by fluorescence microscopy under ultraviolet light. It was prepared extemporaneously in distilled water at 1 mg/ml and added to the culture medium at a final concentration of 10 μg/ml [31, 32].

After 2 h of incubation at 37 °C, cells were washed twice in phosphate buffered saline and resuspended at a concentration of 10^6 cells/ml in phosphate buffered saline containing 1 % (w/v) paraformaldehyde. Cell deposits of approximately 40 000 cells were applied to glass slides. After staining with Hoechst 33342, the morphological features of cell nuclei were observed with an epifluorescence microscope. For each sample, 300 cells were examined. The number of apoptotic cells was counted under microscopy (magnification, × 100) in a blinded manner. The total number of apoptotic cells in each section was summed and expressed as a percentage of total cell number. At least ten individual sections were evaluated per slide. Samples were coded so that the analysis was performed without knowledge of which treatment each cells had received. The reproducibility of the results was assessed. Intraobserver variability was determined from triplicate measurements performed by one observer for all sections. The mean ± SD difference among measurements made by the same observer was 2.4 ± 0.3 %. Interobserver variability was determined from measurements of ten randomly selected sections performed by a second observer in addition to the first observer. The difference between measurements made by the two observers was 3.4 ± 0.5 %. These observers were blinded to other data concerning the cells, as well as to the results of the other observer.

Treatment with prostaglandins and cilostazol. Endothelial cells and VSMC were seeded onto uncoated 96-well tissue culture plates (Corning, N.Y., USA). In the preparation of experiments for determination of cell count, the cells were grown to sub-confluence. After cells reached 80 % confluence, the medium was changed to fresh defined serum-free medium for VSMC or endothelial cells, containing PGE, PGI_2, cilostazol or vehicle, both with and without high glucose (5 to 25 mmol/l). The cells were then incubated overnight. On day 1, the medium was again changed to fresh medium with normal or high D-glucose. On day 4, an index of cell proliferation was determined using a WST-cell counting kit, as described above.

Measurement of HGF in conditioned medium. Human endothelial cells and VSMC were seeded on 6-well plates (Corning) at a density of 5×10^4 cells/cm^2 and cultured for 48 h. After replacing the medium with fresh defined serum-free medium and following culture for 48 h, the concentration of HGF in the medium was determined by enzyme-immunoassay using anti-human HGF antibodies, as described previously [33]. Rabbit anti-human HGF IgG was coated on a 96-well plate (Corning) at 4 °C for 15. After blocking with 3 % bovine serum albumin in phosphate-buffered saline phosphate buffered saline, conditioned medium 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 horseradish peroxidase-conjugated streptoavidin-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 % H_2O_2. The enzyme reaction was halted by adding 1 mol/l H_2SO_4, and absorbance at 490 nm was measured. This ELISA specifically detects human HGF, but not rat HGF [33].

Effect of neutralizing anti-HGF antibody. The effect of endogenously produced HGF in human endothelial cells and VSMC was examined by a neutralization procedure, using rabbit anti-human HGF antibody [34]. For the antibody, the IgG fraction (purified with protein A-agarose) was able to neutralize a biological activity of 10 ng/ml HGF, at a concentration of 10 μg/ml. Normal rabbit serum IgG fraction (10 μg/ml) was employed as a control.

Protocol: Group 1: Incubation of endothelial cells with anti-HGF antibody (final 10 μg/ml) for 48 h; group 2: Incubation of endothelial cells with normal IgG (final 10 μg/ml) for 48 h group 3: Incubation of endothelial cells without normal IgG or anti-HGF antibody for 48 h.

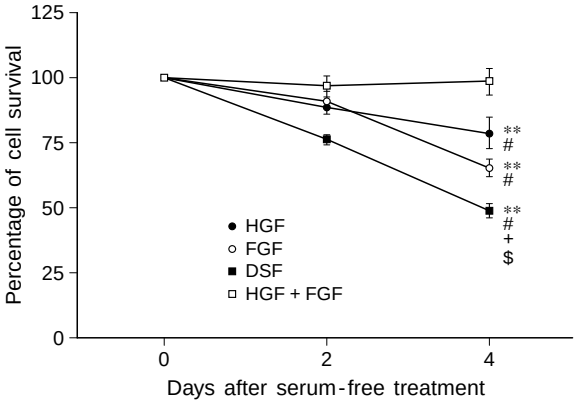

Fig. 1. Effect of exogenously added HGF and FGF on human aortic endothelial cell number under serum deprivation. Values as percentage of cell survival compared to serum (10 % fetal calf serum) treatment. $n = 8$ per group. DSF = vehicle, HGF = human recombinant HGF (10 ng/ml) added to endothelial cells maintained in DSF, FGF = human recombinant FGF (10 ng/ml) added to endothelial cells maintained in DSF, HGF = human recombinant HGF (10 ng/ml) added to endothelial cells maintained in DSF, HGF + FGF = human recombinant HGF (10 ng/ml) and human recombinant FGF (10 ng/ml) added to endothelial cells maintained in DSF. $^{**}p < 0.01$ vs serum (10 % fetal calf serum) treatment; $\#p < 0.01$ vs HGF + FGF; $+ p < 0.01$ vs HGF; $\$p < 0.01$ vs FGF

Materials. Human recombinant HGF was purified from the culture medium of Chinese hamster ovary cells or C-127 cells, and transfected with expression plasmid containing human HGF cDNA [35, 36]. PGE_1 and PGE_2 were obtained from Sigma. PGI_2 analogue (beraprost sodium) was donated by Kaken Pharmaceutical Company (Tokyo, Japan). Cilostazol was donated by Otsuka Pharmaceutical Company (Osaka, Japan).

Statistical analysis. All values are expressed as mean ± SEM. Analysis of variance with subsequent Bonferroni's/Dunnet test was employed to determine the significance of differences in multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of HGF on endothelial cell death induced by serum deprivation. Initially, we examined the effect of HGF treatment on cell death induced by serum deprivation. Serum deprivation caused endothelial cell death. Some cells started to become round and eventually detached from the plate and floated in the medium 4 h after serum deprivation, leaving many holes in the sheet of confluent cells. The floating cells could

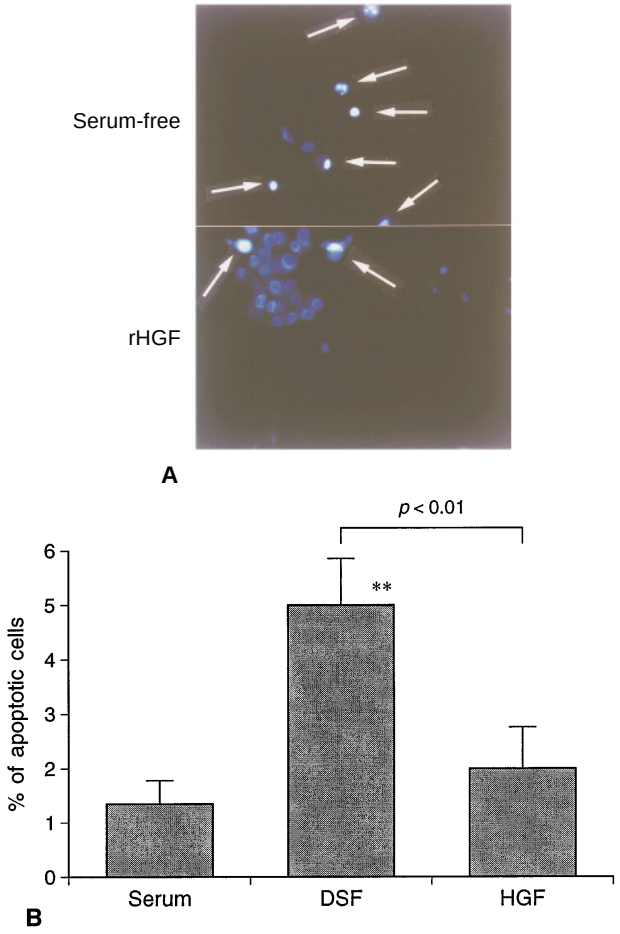

Fig. 2. (A) Morphological changes in nuclei of endothelial cells treated with serum deprivation. **(B)** Effect of exogenously added HGF on apoptosis of human aortic endothelial cell in serum deprivation. Values are percentage of apoptotic cells. $n = 8$ per group. Serum = 10 % fetal calf serum added to endothelial cells, DSF = vehicle added to endothelial cells maintained in DSF, HGF = human recombinant HGF (10 ng/ml) added to endothelial cells maintained in DSF. $^{**}p < 0.01$ vs serum (10 % fetal calf serum) treatment

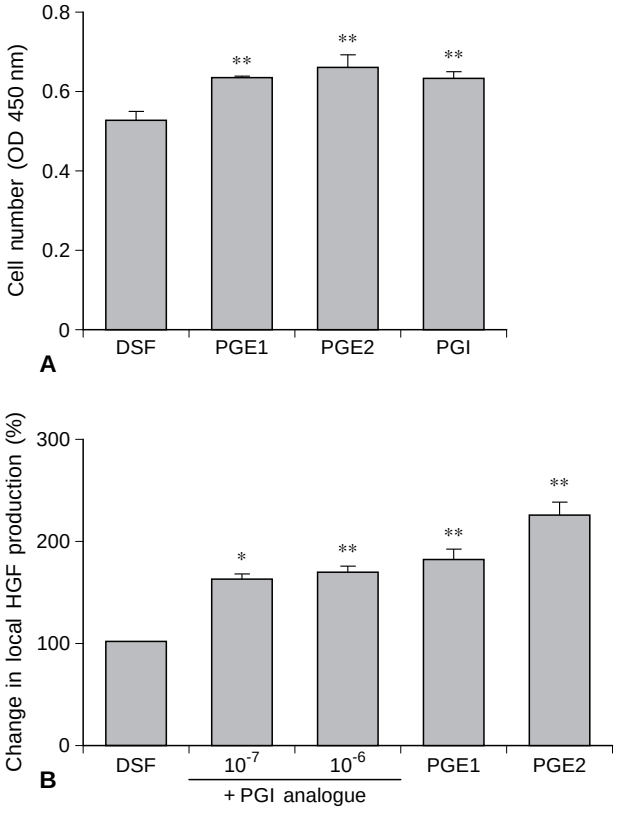

Fig. 3. (A) Effects of PGE_1, PGE_2 and PGI_2 analogue on number of endothelial cells. $n = 8$ per group. DSF = cells treated with 0.5 % FCS medium, PGE1 = cells treated with 0.5 % FCS medium and PGE_1 (10^{-6} mol/l), PGE2 = cells treated with 0.5 % FCS medium and PGE_2 (10^{-6} mol/l), PGI = cells treated with 0.5 % FCS medium and PGI_2 analogue (10^{-6} mol/l). $^{**}p < 0.01$ vs DSF. **(B)** Effects of PGE_1, PGE_2 and PGI_2 analogue on HGF concentration in conditioned medium of human aortic endothelial cells. $n = 8$ per group. DSF = cells treated with 0.5 % FCS medium, + PGI analogue = cells treated with 0.5 % FCS medium and PGI_2 analogue, PGE1 = cells treated with 0.5 % FCS medium and PGE_1 (10^{-6} mol/l), PGE2 = cells treated with 0.5 % FCS medium and PGE_2 (10^{-6} mol/l). Values are expressed as HGF concentration adjusted for cell number. $^{**}p < 0.01$ vs DSF

be recovered with the medium, and neither attached onto a new plate nor proliferated (data not shown). Consistent with this morphological observation, cell death rate after serum deprivation was significantly increased in a time-dependent manner, as shown in Figure 1. Addition of HGF (10 ng/ml) or FGF (10 ng/ml) resulted in partial attenuation of cell death mediated by serum deprivation. Moreover, co-incubation with HGF and bFGF completely abolished endothelial cell death, similar to serum (10 % fetal calf serum) treatment. Moreover, addition of VEGF (10 ng/ml) to HGF and bFGF had no additive effect on survival rate of endothelial cells (data not shown). Serum deprivation resulted in a significant increase in apoptotic endothelial cells assessed by morphology, as shown in Figure 2 a. Serum deprivation significantly increased the apoptotic cells, which was abolished by the addition of serum ($p < 0.01$). Importantly, addition of recombinant HGF also prevented apoptosis of endothelial cells induced by serum deprivation (Fig. 2 b). These results were also confirmed by nuclear staining using propidium iodide (data not shown).

Effect of PGE_1, PGE_2, PGI_2 analogue and cilostazol on human endothelial cells. Initially, we examined the effect of prostaglandins on number of human endothelial cells. Treatment of endothelial cells with PGE_1, or PGE_2 as well as PGI_2 analogue stimulated an increase in the number of human aortic endothelial cells (Fig. 3 a). DNA synthesis of endothelial cells was also increased by PGI_2 analogue (data not shown). As mentioned earlier, HGF is a novel endothelium-specific growth factor. Therefore, we studied the role of local HGF production in prostaglandin-treated endothelial cells. Treatment with PGE_1, or

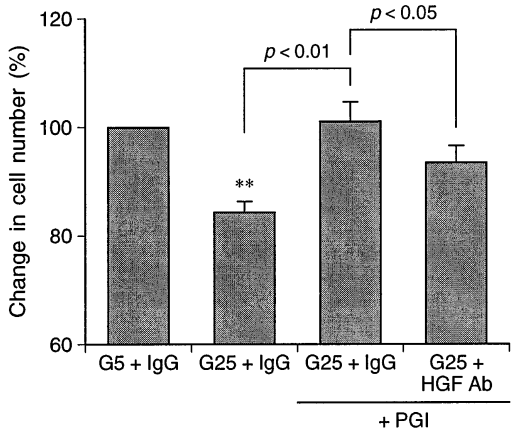

Fig. 4. Inhibition of endothelial cell death induced by high D-glucose by addition of PGI_2 analogue. $n = 8$ per group. G5 = cells treated with 0.5% FCS medium and D-glucose (5 mmol/l), G25 = cells treated with 0.5% FCS medium and D-glucose (25 mmol/l), + IgG = cells treated with IgG control, + HGF-Ab = cells treated with anti-HGF antibody, + PGI = cells treated with 0.5% FCS medium and PGI_2 analogue (10^{-6} mol/l). Values are expressed as percentage of G5 + IgG after adjusting for cell number. $^{**}p < 0.01$ vs cells treated with 0.5% FCS medium and D-glucose (5 mmol/l)

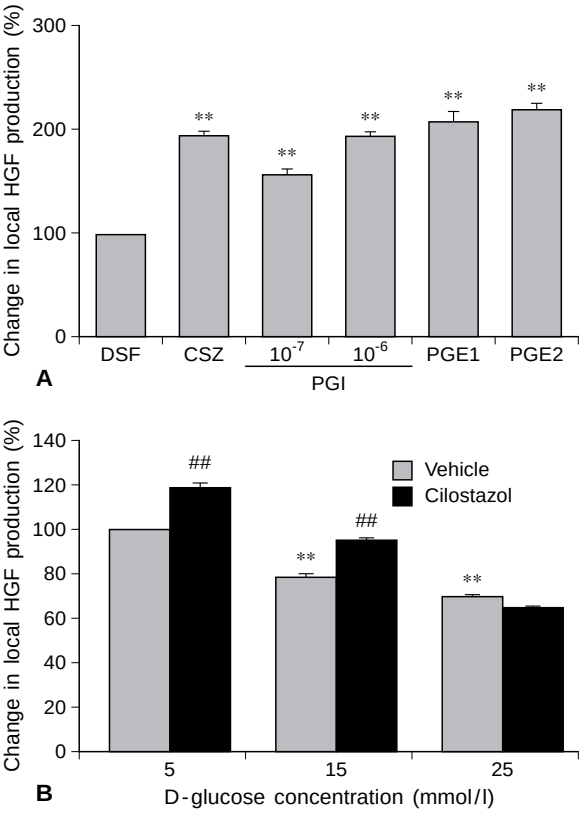

Fig. 5. (A) HGF concentration in medium of human aortic endothelial cells treated with cilostazol and PGI_2 analogue. $n = 8$ per group. DSF = cells treated with 0.5% FCS medium, CSZ = cells treated with 0.5% FCS medium and cilostazol, PGI analogue = cells treated with 0.5% FCS medium and PGI_2 analogue. Values are HGF concentration adjusted for cell number. $^{**}p < 0.01$ vs cells treated with DSF. **(B)** Percent change in local HGF production in medium of human aortic endothelial cells treated with cilostazol under normal and high D-glucose conditions. $n = 8$ per group. 5 = cells treated with 0.5% FCS medium and D-glucose (5 mmol/l), 15 = cells treated with 0.5% FCS medium and D-glucose (15 mmol/l), 25 = cells treated with 0.5% FCS medium and D-glucose (25 mmol/l), Vehicle = cells treated with vehicle, CSZ = cells treated with cilostazol (10^{-6} mol/l). Values are expressed as percent change in HGF concentration adjusted for cell number. $^{**}p < 0.01$ vs cells treated with 5 mmol/l D-glucose, $^{\#\#}p < 0.01$ vs cells treated with vehicle

PGE_2 as well as PGI_2 analogue significantly stimulated local HGF production in human aortic endothelial cells (Fig. 3b). Therefore, we hypothesized that PGE and PGI_2 analogue improved endothelial dysfunction through stimulation of local HGF production. To test this hypothesis, we studied the effect of prostaglandins on endothelial cell death induced by high D-glucose. As shown in Figure 4, treatment with high D-glucose (25 mmol/l) resulted in a significant decrease in endothelial cell number, whereas addition of PGI_2 analogue attenuated endothelial cell death induced by high D-glucose. Of importance, the prevention of endothelial cell death by PGI_2 analogue was significantly abolished by addition of neutralizing anti-HGF antibody. As previously reported, no significant changes were observed in cell number of endothelial cells treated with high mannitol or L-glucose [23].

Local HGF secretion of endothelial cells treated with cilostazol was also examined, since cilostazol is also used in the treatment of peripheral arterial disease. As shown in Figure 5a, treatment with cilostazol significantly stimulated local HGF production in human aortic endothelial cells under basal conditions. The stimulatory action of PGE, a PGI_2 analogue and cilostazol on local HGF production may be due to the accumulation of cAMP, as treatment with forskolin (30 μmmol/l) as well as 8-bromo cAMP (1 mmol/l) stimulated local HGF production (vehicle; 1.54 ± 0.19, forskolin; 12.19 ± 0.60, 8-bromo cAMP; 12.54 ± 0.38, cilostazol; 5.2 ± 0.49 ng/10^6 cells, $p < 0.01$ vs vehicle). Of importance, cilostazol significantly attenuated the decrease in local HGF production induced by high-D-glucose, whereas high D-glucose treatment resulted in a significant decrease in local HGF production (Fig. 5b). However, cilostazol treatment failed to show attenuation of local HGF production with high D-glucose treatment at 25 mmol/l. Therefore, we also studied the effect of cilostazol on growth of endothelial cells. Cilostazol stimulated growth of endothelial cells under basal conditions (Fig. 6a). Consistent with the previous report [23, 37], high D-glucose treatment induced endothelial cell death. Cilostazol significantly attenuated the decrease in number of endothelial cells at a high D-glucose concentration of 15 mmol/l, but not 25 mmol/l (Fig. 6a), in parallel with stimulation of local HGF production (Fig. 5b). Of importance, the prevention

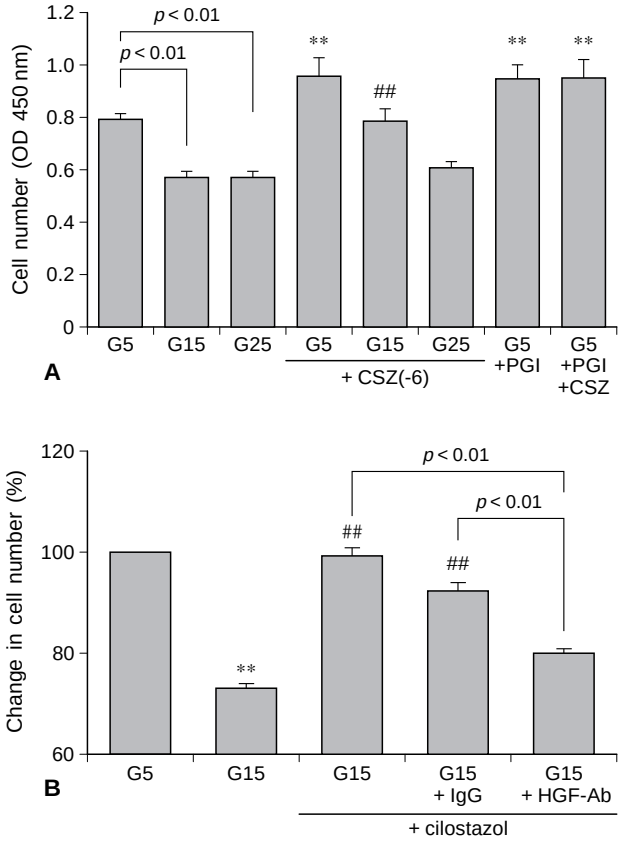

Fig. 6. (A) Inhibition of endothelial cell death induced by high D-glucose by addition of cilostazol and PGI_2 analogue. $n = 8$ per group. G5 = cells treated with 0.5% FCS medium and D-glucose (5 mmol/l), G15 = cells treated with 0.5% FCS medium and D-glucose (15 mmol/l), G25 = cells treated with 0.5% FCS medium and D-glucose (25 mmol/l), + CSZ(-6) = cells treated with cilostazol (10^{-6} mol/l), + PGI = cells treated with PGI_2 analogue (10^{-6} mol/l). $^{**}p < 0.01$ vs cells treated with 0.5% FCS medium and D-glucose (5 mmol/l), $^{\#\#}p < 0.01$ vs cells treated with 0.5% FCS medium and D-glucose (15 mmol/l). **(B)** Inhibition of prevention of high D-glucose-induced endothelial cell death by cilostazol by addition of neutralizing anti-HGF antibody. $n = 8$ per group. G5 = cells treated with 0.5% FCS medium and D-glucose (5 mmol/l), G15 = cells treated with 0.5% FCS medium and D-glucose (15 mmol/l), + IgG = cells treated with IgG control, + HGF-Ab = cells treated with anti-HGF antibody, + cilostazol = cells treated with 0.5% FCS medium and cilostazol (10^{-6} mol/l). Values are percentage of G5. $^{**}p < 0.01$ vs cells treated with 0.5% FCS medium and D-glucose (5 mmol/l), $^{\#\#}p < 0.01$ vs cells treated with 0.5% FCS medium and D-glucose (15 mmol/l)

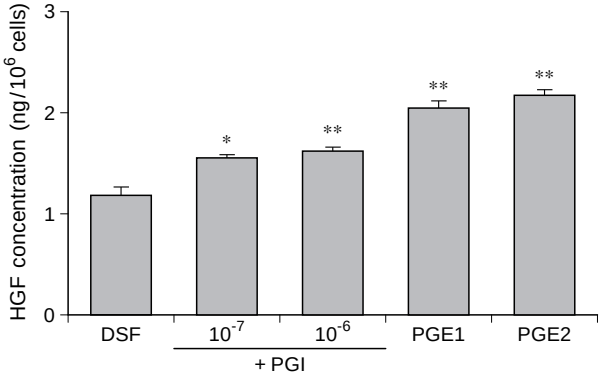

Fig. 7. Effects of PGE_1, PGE_2 and PGI_2 analogue on HGF concentration in the conditioned medium of human aortic VSMC. $n = 8$ per group. DSF = cells treated with DSF, PGI = cells treated with DSF and PGI_2 analogue (10^{-6} and 10^{-7} mol/l), PGE1 = cells treated with DSF and PGE_1 (10^{-6} mol/l), PGE2 = cells treated with DSF and PGE_2 (10^{-6} mol/l). $^{*}p < 0.05$, $^{**}p < 0.01$ vs DSF. Values are HGF concentration adjusted for cell number

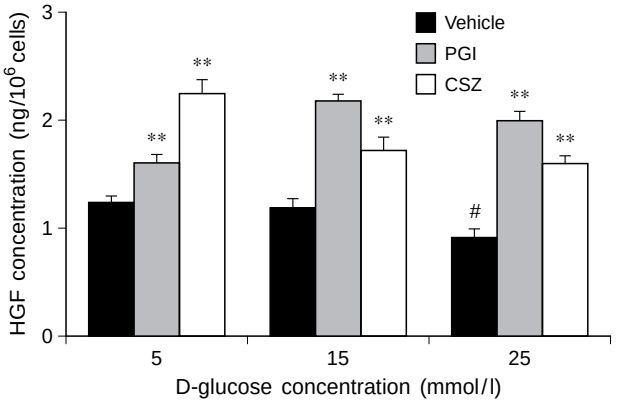

Fig. 8. Inhibition of decrease in local HGF concentration induced by high D-glucose by addition of PGI_2 analogue and cilostazol. $n = 8$ per group. 5 = cells treated with DSF and D-glucose (5 mmol/l), 15 = cells treated with DSF and D-glucose (15 mmol/l), 25 = cells treated with 0.5% FCS medium and D-glucose (25 mmol/l), vehicle = cells treated with vehicle, PGI = cells treated with DSF and PGI_2 analogue (10^{-6} mol/l), CSZ = cells treated with cilostazol (10^{-6} mol/l), CSZ + PGI = cells treated with cilostazol (10^{-6} mol/l) and PGI_2 analogue (10^{-6} mol/l). Values are HGF concentration adjusted for cell number. $^{**}p < 0.01$; $^{+}p < 0.05$ vs cells treated with 0.5% FCS medium and D-glucose (5 mmol/l)

of endothelial cell death by cilostazol was significantly abolished by addition of neutralizing anti-HGF antibody (Fig. 6b).

Effects of PGE_1, PGE_2, PGI_2 analogue and PDE_3 inhibitor on human VSMC. Since the presence of a local HGF system has also been detected in human aortic VSMC [8, 23], we next examined the effects of prostaglandins and cilostazol on local HGF secretion from human aortic VSMC. Similar to endothelial cells, treatment with PGE_1 and PGE_2 as well as PGI_2 analogue resulted in a significant increase in local HGF secretion from VSMC in a dose-dependent manner, as shown in Figure 7. Addition of PGI_2 analogue significantly attenuated the decrease in local HGF production induced by high D-glucose treatment. Cilostazol treatment also increased local HGF production in human VSMC (Fig. 8). Of importance, addition of cilostazol significantly attenuated the decrease in local HGF production induced by high D-glucose treatment. Finally, the direct effect of PGI_2

Table 1. Effects of cilostazol and PGI_2 analogue on number of human aortic VSMC at various glucose concentrations

mmol/l	Vehicle	PGI	Cilostazol	PGI + cilostazol
5	0.406 ± 0.018	0.409 ± 0.020	0.437 ± 0.015	0.420 ± 0.025
15	0.463 ± 0.032[a]	0.441 ± 0.026[a]	0.450 ± 0.023[a]	0.455 ± 0.036[a]
25	0.532 ± 0.031[a]	0.509 ± 0.030[a]	0.500 ± 0.019[a]	0.525 ± 0.018[a]

Values are expressed as absorbance at OD 450 nm. $n = 8$ per group. 5 mmol/l = cells treated with DSF and D-glucose (5 mmol/l), 15 mmol/l = cells treated with DSF and D-glucose (15 mmol/l), 25 mmol/l = cells treated with 0.5% FCS medium and D-glucose (25 mmol/l), PGI = cells treated with DSF and PGI_2 analogue (10^{-6} mol/l), cilostazol = cells treated with DSF and cilostazol (10^{-6} mol/l), PGI + cilostazol = cells treated with DSF, cilostazol (10^{-6} mol/l) and PGI_2 analogue (10^{-6} mol/l). [a] $p < 0.01$ vs cells treated with DSF and D-glucose (5 mmol/l)
Values of VSMC number treated with vehicle was previously published [23]

analogue and cilostazol on VSMC growth was examined under normal and high D-glucose treatment. High D-glucose treatment stimulated VSMC growth. Addition of PGI_2 analogue as well as cilostazol failed to alter VSMC growth with and without high D-glucose treatment (Table 1).

Discussion

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 [38–40]. Moreover, we found that HGF fulfills the characteristics of an endothelium-specific growth factor [6, 7]. Therefore, we hypothesized that HGF may also have an important role in the regulation of endothelial cells. Indeed, we have previously reported that endothelial cell death induced by high D-glucose is due to decreased local HGF production in vascular cells through transforming growth factor-β (TGF-b) activation [23]. This phenomenon should be important, since dysfunction of endothelial cells causing loss of multiple endothelium-derived substances (PGI_2, nitric oxide, C-type natriurectic peptide) would result in the progression of arteriosclerotic vascular changes in diabetes [19–21].

Therefore, we initially examined the protective action of HGF on endothelial cell injury induced by serum deprivation, which is known as a mediator of apoptosis in endothelial cells [41, 42]. Of interest, HGF could abrogate the cell death of endothelial cells mediated by serum deprivation, through the inhibition of apoptosis (Figs. 1 and 2). Although, currently, bFGF is only known to prevent apoptosis of endothelial cells mediated by serum deprivation [41], HGF also has the ability to prevent apoptosis of human aortic endothelial cells induced by serum deprivation. HGF should be classed as a new member of the growth factors with anti-cell death actions in endothelial cells through the inhibition of apoptosis. The mechanisms by which HGF prevented endothelial cell death mediated by these conditions in this study are unclear. HGF is known to stimulate phosphatidylinositol-37-kinase (PI3 K), protein tyrosine phosphatase 2, phospholipase C-r, $pp60^{c\text{-}src}$, grb2/hSos1, the rho- and ras [43–48]. The activation of these signal transduction pathways suggests that HGF will act to prevent cell death.

In this study, we next focused on the effects of PGE, PGI_2 analogue and cilostazol, which are well known to improve peripheral arterial disease in patients with diabetes [16–18]. The present study demonstrated that PGE and PGI_2 analogue as well as cilostazol treatment attenuated endothelial cell death induced by high D-glucose. Our present study cannot determine whether these agents inhibit apoptosis, resulting in an increase in cell number, or whether these agents may have no effect on apoptosis, but work primarily as a mitogen. Although the overall change in cell population may reflect a combination of the rate of cell replication and the rate of cell death, it is important to increase the overall change induced by these agents, to maintain endothelial function. More importantly, the prevention of high D-glucose-induced endothelial cell death by these drugs was abolished by addition of neutralizing antibody against HGF (Figs. 4 and 6b), suggesting that the protective action of PGI_2 and cilostazol on endothelial cell death is in part due to the activation of local HGF production. These results are consistent with our previous data that addition of recombinant HGF protected endothelial cells from death induced by high D-glucose [23]. Given that high D-glucose initiates apoptosis of endothelial cells [37], increased HGF production by these agents might attenuate apoptosis of endothelial cells. This increase in local HGF production by PGE, PGI_2 analogue and cilostazol may be due to increase in cAMP. Since the promoter region of HGF gene contains a cAMP responsive element [49], accumulation of cAMP may stimulate local HGF production probably through a cAMP responsive element. Indeed, treatment with forskolin as well as an 8-bromo cAMP analogue stimulated local HGF production.

Finally, we studied the effects of a PGI_2 analogue and cilostazol on local HGF production in VSMC. Since multiple VSMC-derived substances have

profound influences on the maintenance of endothelium [4, 5], secretion of endothelial protectants from VSMC is very important in the control of endothelial function. As local HGF production in VSMC was decreased in association with the increased D-glucose concentration [23], decrease in local HGF production in VSMC by high D-glucose may affect endothelial cell growth in a paracrine manner. Of importance, decreased local HGF production by high D-glucose was significantly attenuated by PGI_2 analogue and cilostazol. As mentioned earlier, endothelial cells secreted anti-proliferative substances, resulting in the inhibition of VSMC growth, while VSMC promoted endothelial cell growth by production of local HGF. Therefore, loss of local HGF secretion from endothelial cells and VSMC by high D-glucose treatment may promote endothelial dysfunction. Increased local HGF production from VSMC by cilostazol and PGI_2 analogue may improve endothelial dysfunction induced by high D-glucose. Increased vascular HGF production by these agents may contribute to the usefulness of PGI_2 analogue and cilostazol in the treatment of peripheral arterial disease such as arteriosclerosis obliterans, observed in diabetes, although further studies are needed.

Overall, we demonstrated that treatment with PGI_2 analogue or cilostazol prevented aortic endothelial cell death induced by high D-glucose, probably through the activation of local HGF production. Increased local vascular HGF production by prostaglandins and cilostazol may prevent endothelial injury, potentially resulting in the improvement of peripheral arterial disease.

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References

1. Tare M, Parkington HC, Coleman HA, Neild TO, Dusting GJ (1990) Hyperpolarization and relaxation of atrial smooth muscle caused by nitric oxide derived from the endothelium. Nature 346: 69–71
2. Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, Imura H (1992) Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β. Possible existence of "vascular natriuretic peptide system". J Clin Invest 90: 1145–1149
3. Itoh H, Pratt RE, Dzau VJ (1990) Atrial natriuretic polypeptide inhibits hypertrophy of vascular smooth muscle cells. J Clin Invest 86: 1690–1697
4. Dzau VJ, Gibbons GH (1991) Endothelium and growth factors in vascular remodeling of hypertension. Hypertension 18 [Suppl III]:III–115–III–121
5. Dzau VJ (1993) The role of mechanical and humoral factors in growth regulation of vascular smooth muscle cells and cardiac myocytes. Curr Opin Nephrol Hypertens 2: 27–32
6. Nakamura Y, Morishita R, Nakamura S et al. (1996) A vascular modulator, hepatocyte growth factor, is associated with systolic blood pressure. Hypertension 28: 409–413
7. Nakamura Y, Morishita R, Higaki J et al. (1996) Hepatocyte growth factor (HGF) is a novel member of endothelium-specific growth factors: additive stimulatory effect of HGF with basic fibroblast growth factor, but not vascular endothelial growth factor. J Hypertens 14: 1067–1072
8. Nakamura Y, Morishita R, Higaki J et al. (1995) Expression of local hepatocyte growth factor system in vascular tissues. Biochem Biophys Res Commun 215: 483–488
9. Kainoh M, Nishio S, Nakadate T (1992) Cytoprotective action of beraprost sodium against peroxide-induced damage in vascular endothelial cells. Pharmacology 45: 61–70
10. Koh E, Morimoto S, Jiang B et al. (1993) Effects of beraprost sodium, a stable analogue of prostacyclin, on hyperplasia, hypertrophy and glycosaminoglycan synthesis of rat aortic smooth muscle cells. Artery 20: 242–252
11. Nilsson J, Olsson AG (1984) Prostaglandin E_1 inhibits DNA synthesis in arterial smooth muscle cells stimulated with platelet-derived growth factor. Atherosclerosis 53: 77–82
12. Beavo JA, Conti M, Heaslip RJ (1994) Multiple cyclic nucleotide phosphodiesterases. Mol Pharmacol 46: 399–405
13. Tani T, Sakurai K, Kimura Y, Ishikawa T, Hidaka H (1992) Pharmacological manipulation of tissue cyclic AMP by inhibitors. Effects of phosphodiesterase inhibitors on the functions of platelets and vascular endothelial cells. Adv Second Messenger Phosphoprotein Res 25: 215–227
14. Tanaka T, Ishikawa T, Hagiwara M, Onoda K, Itoh H, Hidaka H (1988) Effects of cilostazol, a selective cAMP phosphodiesterase inhibitor on the contraction of vascular smooth muscle. Pharmacology 36: 313–320
15. Souness JE, Hassall GA, Parrott DP (1992) Inhibition of pig aortic smooth muscle cell DNA synthesis by selective type III and type IV cyclic AMP phosphodiesterase inhibitors. Biochem Pharmacol 44: 857–866
16. Fujitani K, Kambayashi J, Murata K et al. (1995) Clinical evaluation of combined administration of oral prostacyclin analogue beraprost and phosphodiesterase inhibitor cilostazol. Pharmacol Res 31: 121–125
17. Numano F, Kishi Y, Ashikaga T (1992) Clinical studies of phosphodiesterase inhibitors for cardiovascular disease. Adv Second Messenger Phosphoprotein Res 25: 395–407
18. Uchikawa T, Murakami T, Furukawa H (1992) Effects of the anti-platelet agent cilostazol on peripheral vascular disease in patients with diabetes mellitus. Arzneimittelforschung 42: 322–324
19. Kannel WB, McGee DL (1979) Diabetes and glucose tolerance as risk factors for cardiovascular disease: the Framingham study. Diabetes Care 241: 2035–2038
20. Graier WF, Wascher TC, Lackner L, Toplak H, Krejs GJ, Kukovetz WR (1993) Exposure to elevated D-glucose concentrations modulates vascular endothelial cell vasodilatory response. Diabetes 42: 1497–1505
21. Hsueh WA, Anderson PW (1993) Systemic hypertension and the renin-angiotensin system in diabetic vascular complications. Am J Cardiol 72: 14H-21H
22. Henry RR, Genuth S (1996) Forum one: current recommendations about intensification of metabolic control in non-insulin-dependent diabetes mellitus. Ann Intern Med 124: 175–177
23. Morishita R, Nakamura S, Nakamura Y et al. (1997) Potential role of endothelium-specific growth factor, hepatocyte

growth factor, in endothelial damage in diabetes mellitus. Diabetes 46: 138–142
24. Wertheimer SJ, Myers CL, Wallace RW, Parks TP (1992) Intercellular adhesion molecule-1 gene expression in human endothelial cells. J Biol Chem 267: 12030–12035
25. Bonin PD, Leadley RJ, Erickson LA (1993) Growth factor-induced modulation of endothelial-1 binding to human smooth muscle cells. J Cardiovasc Pharmacol 22:S125–S127
26. Iwaki T, Iwaki A, Fukumaki Y, Tateishi J (1995) αB-crystallin in C6 glioma cells supports their survival in elevated extracellular K^+: the implication of a protective role of αB-crystallin accumulation in reactive glia. Brain Res 673: 47–52
27. Shirahata S, Watanabe J, Teruya K et al. (1995) E1A and ras oncogenes synergistically enhance recombinant protein production under control of the cytomegalovirus promoter in BHK-21 cells. Biosci Biotech Biochem 59: 345–347
28. Ishiyama M, Shiga M, Sasamoto K, Mizoguchi M, He P (1993) A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chem Pharm Bull 41: 1118–1122
29. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63
30. Libby P, O'Brien KV (1983) Culture of quiescent arterial smooth muscle cells in a defined serum-free medium. J Cell Physiol 115: 217–223
31. Lizard G, Deckert V, Duprez L, Moisant M, Gambert P, Lagrost L (1996) Induction of apoptosis in endothelial cells treated with cholesterol oxides. Am J Pathol 148: 1625–1638
32. Haas-Kogan DA, Kogan SC, Levi D, Dazin P, T'ang A, Fung YKT, Israel MA (1995) Inhibition of apoptosis by the retinoblastoma gene product. Embo J 14: 461–472
33. Yamada A, Matsumoto K, Iwanari H et al. (1995) Rapid and sensitive enzyme-linked immunosorbent assay for measurement of HGF in rat and human tissues. Biomed Res 16: 105–114
34. Tsuda H, Iwase T, Matsumoto K et al. (1992) Immunohistochemical localization of hepatocyte growth factor protein in pancreas islet A-cells of man and rats. Jpn J Cancer Res 83: 1262–1266
35. Nakamura T, Hagiya M, Nishizawa T et al. (1989) Molecular cloning and expression of human hepatocyte growth factor. Nature 342: 440–443
36. Seki T, Ihara I, Sugiyama M et al. (1990) Isolation and expression of cDNA for different forms of hepatocyte growth factor from human leukocytes. Biochem Biophys Res Commun 172: 321–327
37. Baumgartner-Parzer SM, Wagner L, Pettermann M, Grillari J, Gessi A, Waldhausl W (1995) High-glucose-triggered apoptosis in cultured endothelial cells. Diabetes 44: 1323–1327
38. Matsumoto K, Nakamura T (1991) Roles of HGF as a pleiotropic factor in organ regeneration. In: Goldberg ID, Rosen EM (eds) Hepatocyte growth factor-scatter factor (HGF-SF) and C-met receptor. Birkhäuser, Basel, pp 225–249
39. Nakamura T (1991) Structure and function of hepatocyte growth factor. Prog Growth Factor Res 3: 67–86
40. Boros P, Miller CM (1995) Hepatocyte growth factor: a multifunctional cytokine. Lancet 345: 293–295
41. Araki S, Shimada Y, Kaji K, Hayashi H (1990) Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. Biochem Biophys Res Commun 168: 1194–1200
42. Lee SW, Tsou AP, Chan H, Thomas J, Petrie K, Eugui EM, Allison G (1988) Glucocorticoids selectively inhibit the transcription of the interleukin 1β gene and decrease the stability of interleukin 1β mRNA. Proc Natl Acad Sci USA 85: 1204–1208
43. Matsumoto K, Nakamura T (1993) Roles of HGF as a pleiotropic factor in organ regeneration. In: Goldberg ID, Rosen EM (eds) Hepatocyte growth factor-scatter factor (HGF-SF) and C-met receptor. Birkhauser, Basel. 225–249
44. Ponzetto C, Bardelli A, Zhen Z et al. (1994) A multifunctional docking site mediates signaling and transformation by the HGF/SF receptor family. Cell 77: 261–271
45. Rosen EM, Nigam SK, Goldberg ID (1994) Scatter factor and the c-met receptor: a paradigm for mesenchymal/epithelial interaction. J Cell Biol 127: 1783–1787
46. Canley LG, Cantley LC (1995) Signal transduction by the hepatocyte growth factor receptor, c-met. Activation of the phosphatidylinositol 3-kinase. J Am Soc Nephrol 5: 1872–1881
47. Ridley AJ, Comoglio PM, Hall A (1995) Regulation of scatter factor/hepatocyte growth factor responses by ras, rac and rho in MDCK cells. Mol Cell Biol 15: 1111–1122
48. Takaishi K, Sasaki T, Kato M et al. (1994) Involvement of Rho p21 small GTP-binding protein and its regulation in HGF-induced cell motility. Oncogene 9: 273–279
49. Okajima A, Miyazawa K, Kitamura N (1993) Characterization of the promoter region of the rat hepatocyte-growth-factor/scatter-factor gene. Eur J Biochem 213: 113–119