Potential Role of an Endothelium-Specific Growth Factor, Hepatocyte Growth Factor, on Endothelial Damage in Diabetes

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Endothelial cells are known to secrete various antiproliferative and vasodilating factors. Although injury of endothelial cells has been postulated as an initial trigger of the progression of atherosclerosis in patients with diabetes, the mechanisms of endothelial injury in diabetes are not yet clarified. Therefore, it is important to know the effects of high glucose on the factors that may influence endothelial cell growth. A novel member of endothelium-specific growth factors, hepatocyte growth factor (HGF), is produced in vascular cells. To investigate the effects of high glucose on vascular cells, we examined *1*) the effects of high glucose on endothelial cell and vascular smooth muscle cell (VSMC) growth and *2*) the effects of high glucose on local HGF production in endothelial cell and VSMC. Treatment of human aortic endothelial cell with a high concentration of D-glucose, but not mannitol and L-glucose, resulted in a significant decrease in cell number. Interestingly, addition of recombinant HGF attenuated high D-glucose–induced endothelial cell death. Therefore, we measured local HGF secretion of endothelial cell. Importantly, local HGF production was significantly decreased by high D-glucose treatment. In contrast, high D-glucose treatment resulted in a significant increase in the number of human aortic VSMCs, whereas local HGF production was significantly decreased in accordance with increase in D-glucose concentration. No significant changes in numbers were observed in VSMC treated with high mannitol and L-glucose. We also studied the mechanisms of local HGF suppression by high D-glucose. High D-glucose treatment stimulated transforming growth factor-β (TGF-β) concentration in endothelial cell and VSMC. Decreased local vascular HGF production was abolished by addition of anti-TGF-β antibody. As TGF-β inhibited local HGF production in endothelial cell and VSMC, increased TGF-β induced by high D-glucose may suppress local HGF production. This study demonstrated that high D-glucose induced endothelial cell death, stimulated VSMC growth, and decreased local HGF production through the stimulation of TGF-β production both in endothelial cell and VSMC. Overall, decrease in a local endothelial stimulant, HGF, by high D-glucose may be a trigger of endothelial injury in diabetes, potentially resulting in the progression of atherosclerosis. *Diabetes* 46:138–142, 1997

Endothelial cells are known to secrete various vasoactive substances. Recently, it has been hypothesized that endothelial cells may also modulate vascular growth, because many antiproliferative factors, such as nitric oxide (NO) 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. Diabetes is characterized by the premature development of microvascular and macrovascular disease (4). Hyperglycemia is an independent risk factor for the development of cardiovascular disease. The fact that glucose uptake by vascular cells is largely insulin-independent renders vascular cells vulnerable to glucose-induced injury when the extracellular glucose concentration is elevated (4–7). The loss of antiproliferative substances from endothelial cells might be related to the development and progression of atherosclerosis in diabetic patients (6,7). On the other hand, the growth of vascular smooth muscle cells (VSMC) is controlled by a balance of growth inhibitors and growth promoters, and in the normal adult vessel, this balance results in a very low rate of growth of smooth muscle. However, with endothelial dysfunction, this balance is shifted such that proliferation of smooth muscle cells occurs (8,9). Given the importance of endothelial cells, we sought an endothelium-specific growth factor. Our previous studies demonstrated that hepatocyte growth factor (HGF) is a member of endothelium-specific growth factors, whose mitogenic activity is the most potent among basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor, interleukin-1 and -6 (10,11). Moreover, the presence of a local HGF system (HGF and its receptor, c-met) in endothelial cells and VSMC was also demonstrated in vitro as well as in vivo (12). Interestingly, its regulation is controlled by the balance of transforming growth factor-β (TGF-β), angiotensin II, and HGF itself, since TGF-β and angiotensin II are strong suppressors and HGF itself is a positive regulator of local HGF production (13–17). There-

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bFGF, basic fibroblast growth factor; DSF, defined serum free medium; ELISA, enzyme-linked immunosorbent assay; HGF, hepatocyte growth factor; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β; VSMC, vascular smooth muscle cell.

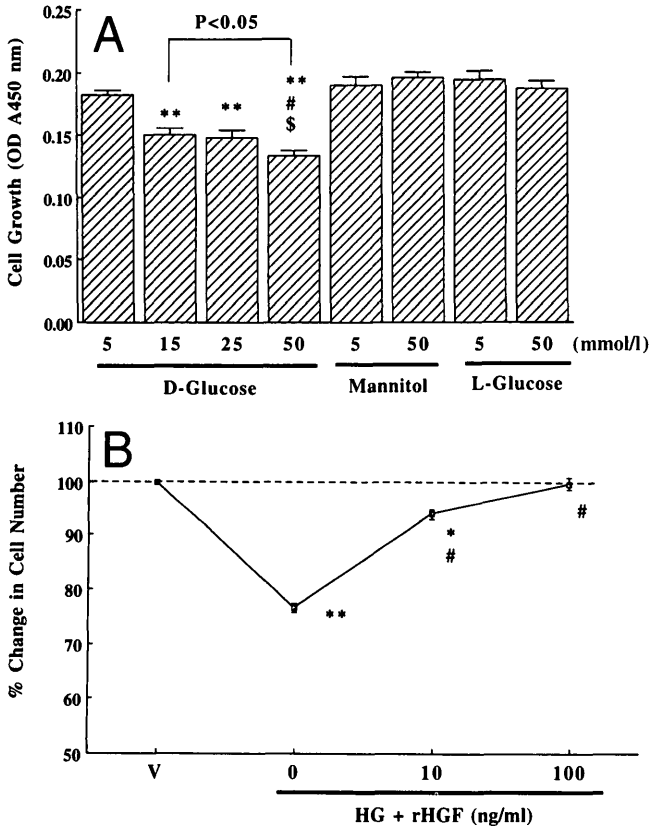

FIG. 1. *A*: **effects of D-glucose, mannitol, and L-glucose on human aortic endothelial cell number. n = 8 per group. D-Glucose, cells treated with 0.5% FCS medium and D-glucose (5–50 mmol/l); mannitol, cells treated with 0.5% FCS medium and mannitol (5 and 50 mmol/l); L-glucose, cells treated with 0.5% FCS medium and L-glucose (5 and 50 mmol/l). **P < 0.01 vs. D-glucose (5 mmol/l), ##P < 0.01 vs. mannitol (50 mmol/l), \$\$$P$ < 0.01 vs. L-glucose (50 mmol/l). *B*: stimulatory effect of recombinant HGF on human aortic endothelial cell injured by high D-glucose. n = 8 per group. V, cells treated with 0.5% FCS medium and D-glucose (5 mmol/l); HG ± HGF, human recombinant HGF (0–100 ng/ml) added to endothelial cells treated with 0.5% FCS medium and D-glucose (25 mmol/l). **P < 0.01 vs. V, #P < 0.01 vs. cells treated with 0.5% FCS medium and D-glucose (25 mmol/l) without HGF.**

fore, in this study, we examined effects of high glucose on local HGF production in vascular cells to clarify the potential role of HGF on endothelial injury in diabetic patients.

RESEARCH DESIGN AND METHODS

Cell culture. Human aortic endothelial cells (passage 5) and human aortic VSMC (passage 5) were obtained from Clonetics Corp. (San Diego, CA) and cultured in modified MCDB131 medium supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, 10 ng/ml epidermal growth factor, 2 ng/ml bFGF, and 1 μmol/l dexamethasone in the standard fashion (18,19). Cells were incubated at 37°C in a humidified atmosphere of 95% air–5% CO_2 with media changes every 2 days. These cells showed the specific characteristics of endothelial cells and VSMC by immunohistochemical examination and morphological observation. Briefly, human aortic endothelial cells were tested by positive results for factor VIII antigen and for uptake of di-acetylated LDL. In contrast, human aortic VSMC were also tested by positive results for α-actin and negative results for expression of factor VIII antigen. All the cells were used within passage 5–6.

Counting of cell number. Endothelial cells and VSMC were seeded onto uncoated 96-well tissue culture plates (Corning, NY). In the preparation of experiments for determination of cell count, the cells were grown to subconfluence. After 80% confluence, the medium was changed to fresh defined serum free medium (DSF) containing HGF (10 ng/ml) or vehicle, both with or without high glucose (5 to 105 mmol/l). The cells were then incubated overnight. On day 1, the medium was again changed to fresh DSF with normal or high D-glucose, mannitol, or L-glucose. On day 4, an index of cell proliferation was determined using WST-cell counting kit that is similar to MTT assay (Wako, Osaka, Japan) (10,11,20–22). 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 (23). In this study, we used an alternative of MTT, i.e., 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 (22). Briefly, 16.3 mg of WST-1 and 0.2 mmol/l 1-methoxy-5-methyl-phenazinium methylsulfate were dissolved in 20 mmol/l HEPES buffer (pH 7.4). Ten microliters of the reaction solution was immediately added to 100 μl of a culture medium per well, and the cells were then incubated for an additional 2 h. The plates were 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).

Measurement of HGF and TGF-β in conditioned medium. Human endothelial cells and VSMC were seeded on six-well plates (Corning) at a density of 5×10^4 cells/cm^2 and cultured for 48 h. After replacing the medium with fresh DSF 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 (24). Rabbit anti-human HGF IgG was coated on a 96-well plate (Corning) at 4°C for 15 h. After blocking with 3% bovine serum albumin in phosphate-buffered saline (PBS), 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. Similarly, TGF-β concentrations in the medium were measured by enzyme-linked immunosorbent assay (ELISA) (TGF-β ELISA kit, Amersham, Amersham, U.K.). TGF-β concentration in cultured medium from endothelial cells was directly measured by ELISA, while concentration in cultured medium from VSMC was measured after conversion from inactive form to active form of TGF-β.

Effect of neutralizing anti-TGF antibody. Augmentation of decreased local HGF production in human endothelial cells and VSMC was characterized as TGF-β–specific by a neutralization procedure, using rabbit anti-human TGF-β (R&D Systems). The IgG fraction (purified with Protein A-agarose) was able to neutralize the biological activity of TGF-β. Normal rabbit serum IgG fraction was employed as a control.

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 (25,26).

Statistical analysis. All values are expressed as means ± SE. All experiments were repeated at least three times. Analysis of variance with subsequent Bonferroni's/Dunnet test was used to determine the significance of differences in multiple comparisons. Multiple regression analyses were used to assess the relations between blood pressure and other parameters. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of high glucose on human endothelial cells. Initially, we examined the effect of high glucose on number of human endothelial cells. Treatment of endothelial cells with a high concentration of D-glucose resulted in a significant decrease in cell number in a dose-dependent manner, as shown in Fig. 1*A*. No significant changes were observed in the cell number of endothelial cells treated with high mannitol or L-glucose (Fig. 1*A*). Therefore, endothelial cell death was due to the cytotoxicity of high D-glucose, but not to high osmolarity. Interestingly, addition of recombinant HGF significantly attenuated high D-glucose–induced endothelial cell death in a dose-dependent manner, as shown in Fig. 1*B*. Given the protective action of HGF on D-glucose–induced

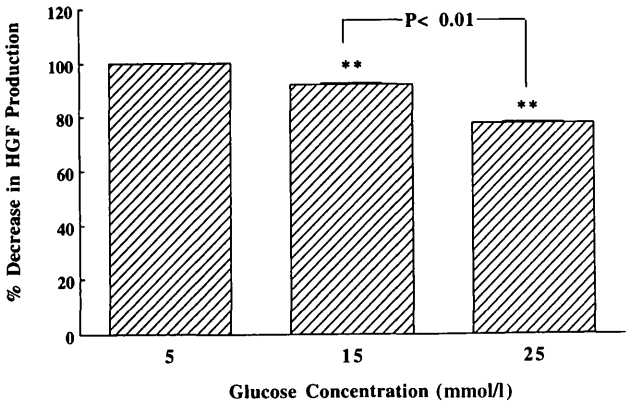

FIG. 2. HGF concentration in medium of human aortic endothelial cells treated with D-glucose at various concentrations. n = 8 per group. 5, Cells treated with 0.5% FCS medium and D-glucose (5 mmol/l); 15, cells treated with 0.5% FCS medium and D-glucose (15 mmol/l); 25, cells treated with 0.5% FCS medium and D-glucose (25 mmol/l). Values are expressed as HGF concentration adjusted for cell number. **P < 0.01 vs. cells treated with 0.5% FCS medium and D-glucose (5 mmol/l).

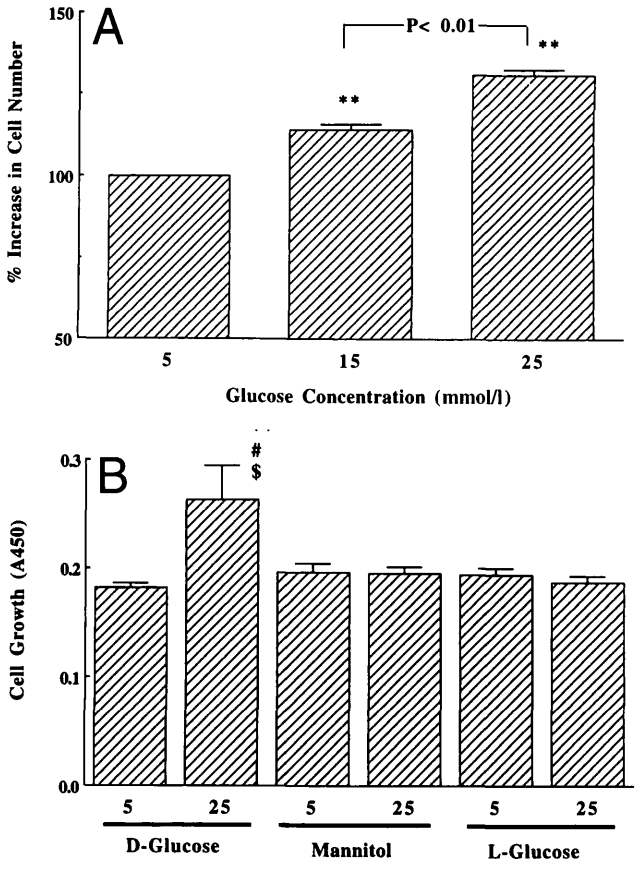

FIG. 3. *A*: stimulatory effects of D-glucose on human aortic VSMC number. n = 8 per group. 5, Cells treated with 0.5% FCS medium and D-glucose (5 mmol/l); 15, cells treated with 0.5% FCS medium and D-glucose (15 mmol/l); 25, cells treated with 0.5% FCS medium and D-glucose (25 mmol/l). **P < 0.01 vs. D-glucose (5 mmol/l). *B*: effect of high D-glucose, mannitol, and L-glucose on human aortic VSMC number. n = 8 per group. 5, Cells treated with 0.5% FCS medium and D-glucose, mannitol, or L-glucose (5 mmol/l); 25, cells treated with 0.5% FCS medium and D-glucose, mannitol, or L-glucose (25 mmol/l). **P < 0.01 vs. D-glucose (5 mmol/l); #P < 0.01 vs. mannitol (25 mmol/l); $$P$ < 0.01 vs. L-glucose (25 mmol/l).

endothelial cell death, we hypothesized that glucose might affect local HGF production as an endogenous endothelial protectant by endothelial cells. Therefore, we measured local HGF secretion of endothelial cells with and without high-glucose treatment. Importantly, local HGF production was significantly decreased by high D-glucose treatment in a dose-dependent manner (Fig. 2). Therefore, we also studied the mechanisms of local HGF suppression by high D-glucose. As previously reported, addition of TGF-β suppressed local HGF production by endothelial cells and VSMC (16). Therefore, we evaluated the effect of D-glucose on TGF-β concentration in the medium of endothelial cells. High D-glucose treatment stimulated release of active TGF-β by endothelial cells assessed by ELISA (5 mmol/l; 1.15 ± 0.24 ng/ml, 25 mmol/l; 1.67 ± 0.07 ng/ml, P < 0.05). Indeed, addition of neutralizing antibody against TGF-β attenuated the decrease in HGF concentration induced by high D-glucose (D-glucose 5 mmol/l, 0.118 ± 0.002 ng/ml; D-glucose 25 mmol/l ± IgG control, 0.055 ± 0.001 ng/ml; D-glucose 25 mmol/l ± anti-TGF antibody, 0.071 ± 0.002 ng/ml; P < 0.01).

Effect of high glucose on human VSMC. Next, the effect of high glucose on growth of human aortic VSMC was also examined. In contrast to the results in endothelial cells, high D-glucose treatment resulted in a significant increase in the number of VSMCs in a dose-dependent manner, as shown in Fig. 3*A*. No significant changes were observed in the cell number of VSMCs treated with high mannitol or L-glucose (Fig. 3*B*). Addition of recombinant HGF did not alter the growth of VSMC (Table 1), which is consistent with the previous findings that HGF did not stimulate VSMC growth (9,11). Similar to endothelial cells, local HGF production was significantly decreased in accordance with increase in D-glucose concentration (5 mmol/l, 0.42 ± 0.02 ng/ml; 25 mmol/l, 0.31 ± 0.03 ng/ml; P < 0.01). High D-glucose treatment stimulated release of TGF-β by VSMC assessed by ELISA (5 mmol/l, 1.36 ± 0.01 ng/ml; 25 mmol/l, 1.68 ± 0.01 ng/ml; P < 0.01). Moreover, the addition of a neutralizing antibody against TGF-β completely attenuated the decrease in HGF concentration induced by high D-glucose (Fig. 4).

DISCUSSION

Locally synthesized compounds from endothelial cells and VSMC have been postulated to control local vascular function (1–3,8,9). Therefore, disruption or dysfunction of endothelial cells causing loss of multiple endothelium-derived substances (PGI_2, NO, CNP) would result in a shift of balance of VSMC growth to abnormal growth, such as that in atherosclerosis. Numerous papers have reported the loss of vasodilating properties of resistance vessels in diabetic patients (6,7). These changes in vascular tone may be due to the decrease in NO content and increase in vascular hypertrophy and/or growth induced by diabetes. In this study, we demonstrated that high D-glucose treatment induced endothelial cell death in a dose-dependent manner, whereas mannitol and L-glucose, as controls for osmolarity, did not. These results revealed the cytotoxicity of high D-glucose, consistent with previous findings (6,7,27). We focused on the action of HGF, since HGF fulfills the characteristics of an endothelium-specific growth factor. HGF is a mesenchyme-derived pleiotropic factor that regulates cell growth, cell motility, and morphogenesis of various types of cells and is thus considered a humoral mediator of epithe-

TABLE 1
No stimulatory effects of recombinant HGF on human aortic VSMC number

	Cell number (absorbance at 450 nm)
D-glucose	
5 mmol/l + vehicle	0.406 ± 0.018
25 mmol/l + vehicle	0.532 ± 0.031*
5 mmol/l + rHGF 100 ng/ml	0.420 ± 0.025
25 mmol/l + rHGF 100 ng/ml	0.525 ± 0.018*

n = 8 per group. D-glucose 5 mmol/l = cells treated with 0.5% FCS medium and D-glucose (5 mmol/l), D-glucose 25 mmol/l = cells treated with 0.5% FCS medium and D-glucose (25 mmol/l), + rHGF = cells treated with 0.5% FCS medium and recombinant HGF 100 ng/ml. *P < 0.01 vs. D-glucose (5 mmol/l).

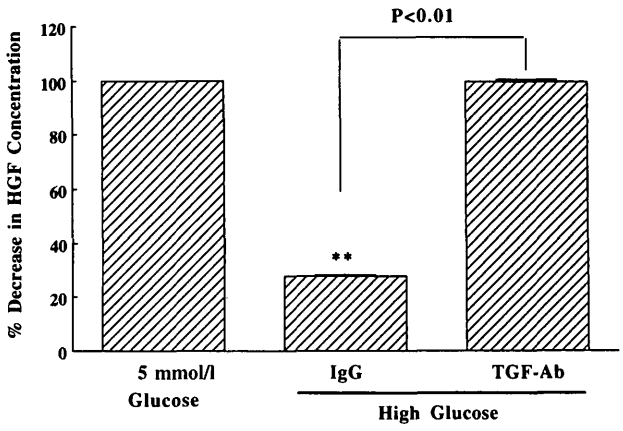

FIG. 4. Inhibition of decrease in local HGF concentration induced by high D-glucose by addition of neutralizing antibody against TGF-β. n = 8 per group. 5 mmol/l, Cells treated with 0.5% FCS medium and D-glucose (5 mmol/l); High Glucose, cells treated with 0.5% FCS medium and D-glucose (25 mmol/l); IgG, cells treated with IgG control; TGF-Ab, cells treated with anti-TGF-β antibody. Values are expressed as HGF concentration adjusted for cell number. **P < 0.01 vs. cells treated with 0.5% FCS medium and D-glucose (5 mmol/l).

lial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis (28–30). Of importance, addition of recombinant HGF rescued endothelial cells from death induced by high D-glucose (Fig. 1*B*), suggesting the potential role of endogenous HGF secretion in vascular cells. Given that high D-glucose initiates apoptosis of endothelial cells (31), HGF might attenuate apoptosis of endothelial cells.

Interestingly, a local HGF system (HGF and its receptor, c-met) has been identified in endothelial cells and VSMC in vitro and in vascular tissues in vivo (12). Therefore, we measured local HGF production under high D-glucose treatment to investigate whether high D-glucose affects local HGF production. Our present data showed that local HGF production was significantly suppressed by high D-glucose treatment. As discussed earlier, our preliminary data demonstrated that TGF-β and angiotensin II strongly inhibited local HGF production, and HGF itself stimulated HGF production in both endothelial cells and VSMC (17). To clarify the role of TGF-β in the local HGF system, we measured TGF-β concentration in the medium of endothelial cells treated with normal and high D-glucose. Our present data revealed a significant increase in active TGF-β release from endothelial cells treated with high D-glucose as compared with those with normal glucose. This result is consistent with the previous reports that high D-glucose increased local TGF-β production (32–34). In atherosclerotic lesions, TGF-β is upregulated, as assessed by in situ hybridization and immunohistochemical technique (35,36). In experimental diabetic models, activation of the vascular renin angiotensin system and TGF-β was also reported in the vasculature (37–39). Taken together, the activation of local TGF-β and the vascular renin angiotensin system in diabetes may negatively regulate local HGF production in vascular tissues, although the present study did not show in vivo results.

In contrast to endothelial cells, the number of VSMCs was markedly increased by treatment with high D-glucose. Numerous papers have reported that high D-glucose stimulated VSMC growth, probably due to increased activation of protein kinase C (6,40–42), consistent with our present findings. Alternatively, it is also reported that high D-glucose–initiated release of free reactive species is involved in D-glucose–mediated change in endothelial cells and smooth muscle cells (43). Moreover, local HGF production in VSMC was decreased in association with the increased D-glucose concentration. Similar to endothelial cells, local TGF-β production was significantly increased by high D-glucose treatment. Of importance, the decrease in local HGF production by high D-glucose was completely abolished by addition of neutralizing antibody against TGF-β (Fig. 4). As previously reported (1–3), endothelial cells secreted antiproliferative 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 in this study may promote endothelial dysfunction. Interestingly, our clinical data indicate that serum HGF concentration was negatively correlated with HbA_{1c}, suggesting loss of this endothelial protectant in accordance with the severity of diabetes (unpublished observation). Decreased serum HGF concentration in diabetes may promote the progression of arteriosclerotic vascular changes, although further studies are needed.

Overall, we demonstrated that high D-glucose, but not mannitol and L-glucose, induced aortic endothelial cell death (which was attenuated by addition of recombinant HGF) and stimulated VSMC growth. Local HGF production in endothelial cells and VSMC was markedly suppressed by high D-glucose, probably due to increased TGF-β concentration. These results suggest that decreased local HGF production may promote the progression of arteriosclerotic vascular changes in diabetes.

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