Hepatocyte Growth Factor Prevents Endothelial Cell Death Through Inhibition of bax Translocation From Cytosol to Mitochondrial Membrane

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Injury of endothelial cells has been postulated to be an initial trigger of the progression of atherosclerosis in patients with diabetes. Previously, we demonstrated high d-glucose induced endothelial apoptosis through the bax-caspase pathway and the potential contribution of hepatocyte growth factor (HGF) to the pathogenesis of endothelial dysfunction. In this study, we analyzed the molecular mechanisms of the protective actions of HGF against endothelial cell death under high d-glucose conditions. High concentrations of d-glucose resulted in a significant increase in apoptosis and necrosis. In contrast, HGF attenuated high d-glucose–induced apoptosis and necrosis (P < 0.01). High d-glucose significantly increased bax protein, but not bcl-2, and activated caspase 3–like and 9, whereas HGF significantly increased bcl-2 expression without affecting bax level and attenuated the increase in caspase 3 and 9 activity. Interestingly, high d-glucose resulted in translocation of bax protein from cytosol to the mitochondrial membrane, whereas HGF inhibited the bax translocation. Importantly, this bax translocation was also completely blocked by overexpressed bcl-2. These findings suggest that HGF can activate bcl-2 expression and inhibit translocation of bax protein upstream of the mitochondria, thereby leading to the inhibition of caspase 3 and 9 activation. HGF may be an important factor in the maintenance of endothelial function. Diabetes 51:2604–2611, 2002

Dysfunction of endothelial cells is known to promote abnormal vascular growth such as that in atherosclerosis and arteriosclerosis (1). Diabetes is characterized by the premature development of microvascular and macrovascular diseases (2), and 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 (2–5). Loss of anti-proliferative substances from endothelial cells might be related to the development and progression of atherosclerosis arteriosclerosis in diabetic patients (4,5). From this viewpoint, we have focused on the role of hepatocyte growth factor (HGF) because it is a novel member of the angiogenic growth factors (6,7). Our previous studies demonstrated that local vascular HGF production was downregulated by high d-glucose through the activation of transforming growth factor-β (8) and that high d-glucose induced endothelial apoptosis through activation of the bax-caspase proteases pathway (9). In this study, we tried to find out the molecular mechanism of the protective actions of HGF in endothelial cells.

A variety of key events in apoptosis focus on mitochondrial function. The different signals that converge on mitochondria to trigger or inhibit these events and their downstream effects delineate several major pathways in physiological cell death. The effectors of apoptosis are now well known to be represented by a family of intracellular cysteine proteases known as caspases (10). A feature of apoptosis that impinges on caspases is altered mitochondrial function characterized by a reduction in the electrochemical gradient across the mitochondrial membrane and release of mitochondrial cytochrome c to cytoplasm (11), and it is inhibited by the presence of bcl-2 in these organelles (12,13). Cytochrome c is necessary for caspase 9 activation (13). Caspase 9 can function as an initiator caspase when mitochondrial dysfunction is the primary event in apoptosis, whereas it serves to amplify the apoptotic signaling of other initiator caspases under conditions in which disruption of mitochondria is a late event (14,15). In this study, we focused on the analysis of caspase activity and the bcl-2 family as the molecular mechanism of the apoptosis pathway. Because high d-glucose treatment induced endothelial apoptosis (9,16), we specifically investigated the effects of HGF on apoptosis induced by high d-glucose in human endothelial cells. Here, we address the following questions: 1) Does HGF have anti-apoptotic actions on endothelial cell death induced by high d-glucose? 2) How does HGF act as an anti-apoptotic factor against endothelial cell death?

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HGF, hepatocyte growth factor; HM, heavy membrane; LM, light membrane; PI, propidium iodide; rHGF, recombinant HGF; VEGF, vascular endothelial growth factor.
RESEARCH DESIGN AND METHODS

Cell culture. Human aortic endothelial cells (passage 3) were obtained from Clonetech (San Diego, CA) and cultured in modified MCD131 medium in the standard fashion (9).

Cell death assay. The extent of cell death was assessed using a commercially available kit (Wako, Osaka, Japan) to measure released lactate dehydrogenase activity from dead cells because loss of cell membrane integrity was observed in both necrotic and apoptotic cells (9,17). After subconfluence, the cells were then incubated overnight with serum-free medium. On day 1, the medium was changed to serum-free medium containing HGF or vehicle, both with D-glucose (5–25 mmol/l). Cells were also stained with Hoechst 33258 (bisbenzimide, 10 μmol/l; Wako) and propidium iodide (PI) (10 μmol/l; Molecular Probes, Eugene, OR) (18). Because Hoechst 33258 stains all nuclei and PI stains nuclei of cells with a disrupted plasma membrane, nuclei of viable, necrotic, and apoptotic cells were observed as blue intact nuclei, red round nuclei, and fragmented (or condensed) nuclei, respectively, under fluorescence microscopy (Olympus BX60). Caspase activity was assayed by using fluorogenic tetrapeptide substrates as previously described (9).

Analysis of protein levels by Western blotting. Western blotting was performed for analysis of bax and bcl-2 proteins (Santa Cruz Biotechnology, Santa Cruz, CA) (9). Briefly, endothelial cells were resuspended in hypotonic buffer (10 mmol/l Tris, pH 7.5, 5 mmol/l MgCl2, 1 mmol/l EGTA, and 1 mmol/l dithiothreitol) and then homogenized. Nuclei and unbroken cells were separated at 120g for 5 min as the low-speed pellet (P1). This supernatant was centrifuged at 10,000g for 10 min to collect the heavy membrane (HM) pellet. This supernatant was centrifuged at 100,000g for 30 min to yield the light membrane (LM) pellet and final soluble fraction. The HM and LM fraction were resuspended in radioimmunoprecipitation assay buffer, and samples were run on 12.5% SDS-PAGE. Another fractionation method was performed using digitonin as described previously (19). Western blotting of tubulin using anti-tubulin antibody (anti-human mouse IgG, 1:100; Oncogene) was also using digitonin as described previously (19). Western blotting of tubulin using anti-tubulin antibody (anti-human mouse IgG, 1:100; Oncogene) was also using digitonin as described previously (19). Western blotting of tubulin using anti-tubulin antibody (anti-human mouse IgG, 1:100; Oncogene) was also using digitonin as described previously (19).

RESULTS

HGF inhibits cell death induced by high D-glucose in human endothelial cells. Initially, we examined the effect of high D-glucose on human endothelial cells. Consistent with our previous reports (8,9), high D-glucose treatment resulted in a significant increase in LDH release in a time-dependent manner, whereas recombinant (r)HGF significantly attenuated high D-glucose–induced endothelial cell death (Fig. 1A, P < 0.01). Cytotoxicity was assayed by the morphological features of the cells using double staining with Hoechst 33258 (blue) and PI (red) (18). Nuclei of viable, necrotic, and apoptotic cells were observed as blue intact nuclei, red intact nuclei, and fragmented (or condensed) nuclei, respectively (Fig. 1B). Endothelial cells showed decreased viability, with increased apoptosis and necrosis after exposure to 25 mmol/l high D-glucose, whereas endothelial cells treated with rHGF (100 ng/ml) showed fewer apoptotic and necrotic cells than high D-glucose–treated cells (Fig. 1C, P < 0.01).

Activity of caspase 3–like protease and caspase 9 protease. To determine the effect of HGF on the increase in caspase activity induced by high D-glucose, the proteolytic activity of caspase 3(like) and 9 was measured. As shown in Fig. 2A, the relative activity of caspase 3(like) proteases was increased and continued to be elevated after treatment with 25 mmol/l D-glucose compared with 5 mmol/l D-glucose in endothelial cells. Moreover, the activity of caspase 9 located upstream of caspase 3 was also significantly increased at 8 or 16 h after stimulation (Fig. 2B). Thus, we examined the effects of a specific caspase 3 inhibitor, z-DEVD, and a wide-spectrum caspase inhibitor, z-VAD, on cell death induced by high D-glucose. Addition of z-VAD as well as z-DEVD significantly attenuated LDH release from cell death induced by high D-glucose (Table 1, P < 0.01). These data suggest that addition of rHGF also markedly inhibited activation of caspase 3(like) and 9 proteases during high D-glucose treatment.

Analysis of the apoptosis-related proteins bax and bcl-2. To further examine the molecular mechanisms of the apoptosis pathway, we focused on the expression of bcl-2 and bax proteins. High D-glucose treatment significantly increased bax protein, as assessed by Western blotting (Fig. 3A, P < 0.01), whereas no significant change in bcl-2 protein was observed. Interestingly, rHGF significantly increased bcl-2 protein under both control and high D-glucose conditions (Fig. 3B, P < 0.01) but failed to affect the decreased bax protein level. We further examined the effect of overexpressed bcl-2 in endothelial cells with respect to LDH release and caspase 3(like) activity. Although treatment of endothelial cells with 25 mmol/l D-glucose resulted in a significant increase of LDH release compared with 5 mmol/l D-glucose at 3 days after treatment, overexpression of the bcl-2 gene in endothelial cells attenuated the increase in LDH release induced by 25 mmol/l D-glucose (Fig. 4A, P < 0.01). Similarly, overexpression of the bcl-2 gene resulted in significant attenuation of the increase of D-glucose–induced caspase 3(like) activity (Fig. 4B, P < 0.01). These results support that HGF prevented endothelial apoptosis induced by high D-glucose through bcl-2 upregulation.

HGF inhibits translocation of bax protein. Recently, several studies demonstrated that translocation of bax protein from the cytoplasm to the mitochondrial membrane is critical in apoptosis (23,24). Therefore, we also focused on the translocation of this pro-apoptotic protein. A substantial portion of bax protein was found consistently in the cytosol fraction (soluble) as well as in the mitochondria-enriched HM fraction, as documented by the mitochondrial markers (Porin/VDAC, a protein of the outer mitochondrial membrane) (Fig. 5A). After D-glucose stimulation, bax protein moves from the cytosol to the HM fraction (Fig. 5A), consistent with our recent report (9). Of importance, rHGF attenuated the translocation of bax protein induced by D-glucose, and overexpression of bcl-2 gene completely blocked the translocation of bax protein. This bax translocation during apoptosis was also assessed using another fractionation method (19). As shown in Fig. 5B, although organellar bax protein was increased and cytosolic bax protein was decreased during high D-glucose conditions in endothelial cells, treatment of rHGF attenuated these changes in both organellar and cytosolic fractions. More importantly, this bax translocation was also completely blocked by bcl-2 overexpression. These results suggest that increased bcl-2 protein induced by rHGF...
Anti-Apoptotic Actions of HGF in Endothelial Cells

FIG. 1. A: LDH release in endothelial cells treated with high D-glucose with or without rHGF. Values are expressed as the percentage of LDH release compared with that under D-glucose (5 mmol/l). *P < 0.01 vs. 5 mmol/l D-glucose.

B: Morphological changes of endothelial cells maintained under high glucose conditions at 72 h after treatment, assessed by Hoechst 33258 and PI staining. Because Hoechst 33258 stains all nuclei and PI stains nuclei of cells with a disrupted plasma membrane, nuclei of viable, necrotic, and apoptotic cells were observed as blue intact nuclei, red round nuclei, and fragmented (or condensed) nuclei, respectively. 5 mmol/l D-glucose = maintained under normal glucose conditions (5 mmol/l); 25 mmol/l D-glucose = maintained under high D-glucose conditions (25 mmol/l); 25 mmol/l D-glucose + 100 ng/ml HGF = treated with high D-glucose (25 mmol/l) and human rHGF (100 ng/ml).

C: Percent increase in apoptotic and necrotic endothelial cells (total count 500) under high D-glucose conditions with or without rHGF at 48 h, assessed by Hoechst and PI staining. *P < 0.05 vs. 5 mmol/l D-glucose without rHGF and HGF without rHGF. §P < 0.05 vs. D-glucose without rHGF and HGF without rHGF.
would block the translocation of bax protein from the cytosol to the mitochondria.

**DISCUSSION**

Regulation of cell death by apoptosis may be another determinant of vessel structure and lesion formation (1). In response to a variety of stimuli and circumstances, cells have an intrinsic capacity to activate a gene-directed program that commits the cell to a suicidal death, described as apoptosis. It has become increasingly clear that the process of cell death by apoptosis is a relatively

**TABLE 1**

Effect of caspase inhibitors on LDH release induced by high v-glucose

<table>
<thead>
<tr>
<th>LDH release (% increase)</th>
<th>P</th>
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<tbody>
<tr>
<td>5 mmol/l v-glucose</td>
<td>21.16 ± 1.44</td>
</tr>
<tr>
<td>25 mmol/l v-glucose</td>
<td>39.22 ± 2.24</td>
</tr>
<tr>
<td>25 mmol/l v-glucose + 100 μmol/l z-DEVD</td>
<td>24.37 ± 1.52</td>
</tr>
<tr>
<td>25 mmol/l v-glucose + 100 μmol/l z-VAD</td>
<td>20.61 ± 1.02</td>
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Data are means ± SE. n = 8 per group. 5 mmol/l v-glucose = treated with serum-free medium and v-glucose (5 mmol/l); 25 mmol/l v-glucose = treated with serum-free medium and v-glucose (25 mmol/l), + 100 μmol/l z-DEVD = pretreatment with 100 μmol/l z-DEVD 1 h before v-glucose treatment; + 100 μmol/l z-VAD = pretreatment with 100 μmol/l z-VAD 1 h before v-glucose treatment. *vs. 5 mmol/l v-glucose; † vs. 25 mmol/l v-glucose.
ubiquitous phenomenon in a variety of cell types, including endothelial cells (25). However, apoptosis in vivo in endothelial cells due to hyperglycemia has not been proven. Using the current technology, it is extremely difficult to find apoptosis in vivo for evaluation in endothelial cells. It is speculated that early apoptotic cells in endothelium might be detached due to mechanical stress of the vessel in vivo because the increase in apoptosis may lead to the acceleration of the disappearance of capillary endothelium and weakening of intracellular junctions. Indeed, induction of apoptosis has been reported to lead to an alteration of the endothelial barrier function, resulting from the distortion of monolayer architecture as a consequence of the reduced size and altered shape of the apoptotic endothelial cells (26). Nevertheless, an increase in apoptosis during hyperglycemia was reported (27–29). Interestingly, the blastcysts from bax-deficient mice were protected from glucose-induced apoptosis (27), and in streptozotocin-induced hyperglycemic rats, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining in aortic sections showed a sixfold
increase of positive cells in the media of diabetic aorta, whereas electron microscopy demonstrated the typical apoptotic cells and bodies in the media of aorta from diabetic, but not control, rats (28). Moreover, we and others demonstrated (in vitro and in vivo study) that high glucose increased apoptosis in cultured endothelial cells (9,16), and programmed cell death of retinal microvascular cells occurs in situ in human and experimental diabetic retinopathy (29). From these data, the apoptosis in endothelial cells seems to occur in vivo. In this study, we demonstrated that HGF attenuated high D-glucose–induced endothelial cell death through upregulated bcl-2.

Caspases appear to be important for the progression of apoptotic cell death. Interestingly, the present studies reveal a significant increase in caspase 3 and 9 activities and bax, a pro-apoptotic factor, from high D-glucose. Recent studies have also documented that the subcellular localization of these molecules determines the fate of cells, in addition to classic diagrams defining the quantity. Bcl-2 and bax contain a hydrophobic segment at their COOH-terminal end (23,30), which is believed to serve as a membrane anchor. Subcellularly, bcl-2 protein is localized in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrane (31). Recently, analysis using fluorescence microscopy indicated that bax protein is mainly localized within the cytosol of healthy cells (32,33). However, after delivery of death signals to cells in culture, bax protein moves to the mitochondria and other membrane sites and triggers a catastrophic change of mitochondrial function (23,24). We demonstrated that after high D-glucose treatment, bax protein inserts into the mitochondrial membranes, while it is located in the cytosol and in association with intracellular membranes, including mitochondria under normal glucose conditions. Translocation of bax protein into the mitochondrial membrane was accompanied by a significant increase in caspase 3(-like) and 9 activity. These results demonstrate that bax translocation from the cytosol to the mitochondrial membrane occurred under physiological conditions, thereby leading to pathological tissue destruction.

Importantly, the present studies also demonstrated that rHGF significantly increased bcl-2 protein without affecting bax protein and attenuated the high glucose–induced caspase 3(-like) and 9 activation. Anti-apoptotic action of HGF through bcl-2 induction may be effective against not only high glucose conditions, but also other stimulation involved in the activation of the mitochondrial-mediated apoptotic pathway, because HGF attenuated the caspase 3 activation induced by tumor necrosis factor-α due to phosphatidylinositol 3-kinase pathway involved in Akt activation (7,34). In this study, we do not conclude that these anti-apoptotic actions of HGF are unique as vascular endothelial growth factor (VEGF) and fibroblast growth factor. In addition, the expression of VEGF and its receptor were also decreased in the myocardium of diabetic rats (35), similar to HGF (36). However, the potential unique mechanism of HGF is the ability of direct association between Bcl-2 and c-met (specific receptor of HGF) via bag-1 protein. The bag-1 protein has been reported to interact with the bcl-2 protein and to cooperate with the bcl-2 protein to suppress apoptosis (37). Of importance, the bag-1 protein appears to inhibit cell death by binding to bcl-2, the raf-1 protein kinase, and c-met (38). The cooperative activation of these bcl-2–related genes may also participate in the prevention of cell death by HGF, although further studies are necessary. Bcl-2 has been suggested to exert anti-apoptotic activity by two mechanisms: sequestration of the proforms of two major caspases—pro-caspase 9 and pro-caspase 8—and inhibition of apoptogenic mitochondrial changes, including cytochrome c release and Δψ loss, resulting in apoptosis inducible factor release in isolated mitochondria (39,40).

To further study these findings, we investigated the effect of overexpression of the bcl-2 gene. Overexpressed bcl-2 attenuated the LDH release and caspase 3(-like) activation induced by high D-glucose. More importantly, overexpressed bcl-2 completely inhibited the bax translocation from the cytosol to the mitochondria. Although bax induced apoptosis by release of cytochrome c from mitochondria and bcl-2 blocked apoptosis by retaining cytochrome c in the mitochondria, the present study also suggested a third action of bcl-2 in preventing apoptosis.

**FIG. 6.** Potential mechanisms of anti-apoptotic action of HGF. High D-glucose treatment increased bax protein without affecting bcl-2 protein and also induced bax translocation from cytosol to the mitochondrial membrane. On the other hand, because HGF significantly increases bcl-2 protein without affecting bax protein, it may also block the translocation of bax. These changes in bax protein released cytochrome c from the mitochondria, resulting in activation of the caspase cascade. Therefore, upregulation of bcl-2 induced by HGF blocks the release of cytochrome c through both a direct action on the mitochondria and a blockade of bax translocation.
because bcl-2 inhibited the translocation of bax upstream of mitochondria and blocked the release of cytochrome c from mitochondria, thereby inhibiting caspase 3-like and 9 activation in human endothelial cells. Recent reports have also documented the action of overexpressed bcl-2 on bax translocation (19). Thus, bcl-2 can potentially block three distinct steps in the apoptotic pathway. It has also been reported that HGF can protect cell death through the phosphorylation of bad via phosphatidylinositol 3-kinase and increase bcl-XL (41). Of importance, because bax itself could not be phosphorylated, the target of Akt via phosphatidylinositol 3-kinase was still unclear in this model, which may be different from the regulation of bad. Interestingly, other reports demonstrated that bax translocation was regulated by the conformational change resulting in the exposure of its BH3 domain, and phosphatidylinositol 3-kinase prevents apoptosis through the inhibition of conformation of the bax BH3 epitope (42). Therefore, another possible pathway in the kinase-regulated anti-apoptotic action of HGF should be examined in the near future.

Overall, we demonstrated that high D-glucose results in the induction of bax protein and translocation of bax from the cytosol to the mitochondria that lead to caspase 3 and 9 activation, whereas HGF exhibited an anti-apoptotic action through the induction of bcl-2 and inhibition of translocation of bax (Fig. 6). HGF may have a potential therapeutic role in endothelial function, especially from the viewpoint of cell death.

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