Contribution of Bcl-2, but Not Bcl-xL and Bax, to Antiapoptotic Actions of Hepatocyte Growth Factor in Hypoxia-Conditioned Human Endothelial Cells


Abstract—Angiogenic growth factors play important roles in angiogenic responses, such as vasculogenesis and angiogenesis in response to hypoxia. A novel angiogenic growth factor, hepatocyte growth factor (HGF), has been reported to inhibit endothelial cell death. However, its molecular mechanisms are largely unknown. Thus, we studied (1) the effects of HGF on hypoxia-induced endothelial apoptosis and (2) the molecular mechanisms of the antiapoptotic actions of HGF in endothelial cells. Severe hypoxia increased the cell death rate in human aortic endothelial cells, whereas HGF significantly attenuated cell death. In addition, hypoxic treatment resulted in a significant increase in apoptotic cells, whereas HGF could attenuate apoptosis, accompanied by attenuation of the increase in caspase-3–like activity ($P<0.01$). Of importance, HGF significantly increased Bcl-2, an inhibitor of apoptosis, in a dose-dependent manner under normoxic and hypoxic conditions ($P<0.01$), whereas hypoxic conditions resulted in a significant decrease in Bcl-2. In contrast, HGF failed to affect Bcl-xL, which is also well known as an inhibitor of apoptosis under both normoxic and hypoxic conditions, whereas Bcl-xL was significantly decreased in endothelial cells exposed to hypoxia ($P<0.01$). No significant change in Bax, a promoter of apoptosis, was also observed in endothelial cells under hypoxia, whereas HGF did not affect Bax. Overall, this study demonstrated that HGF prevented endothelial cell death induced by hypoxia through its antiapoptotic action. The antiapoptotic mechanisms of HGF in hypoxia-induced endothelial cell death largely depend on Bcl-2, but not Bcl-xL and Bax. (Hypertension. 2001;37:1341-1348.)

Key Words: hypoxia ■ blood vessels ■ vascular diseases ■ apoptosis ■ growth substances

The development of new blood vessels, or angiogenesis, begins with the activation of parent vessel endothelial cells. Growth factors that are shown to be mitogenic for endothelial cells in vitro and that also stimulate angiogenesis in vivo have been referred to as angiogenic growth factors. These include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). In addition, we and others have also identified hepatocyte growth factor (HGF) as being a member of the angiogenic growth factors. Moreover, we have found that HGF fulfills the characteristics of an endothelium-specific growth factor, similar to VEGF. VEGF and HGF are secreted by intact cells and are mitogenic exclusively for endothelial cells, whereas bFGF is neither secreted by cells nor is its mitogenic activity limited to endothelial cells only; it is also a potent mitogen for vascular smooth muscle cells. VEGF increases vascular permeability and stimulates monocyte migration through the endothelial layer, whereas HGF and bFGF do not seem to do so.

On the other hand, proliferation and cell death are considered 2 mechanically related phenomena. According to this view, cells are programmed to commit suicide by default and require specific extracellular factors to survive. Especially, the molecular events linking tyrosine kinase receptors to the antiapoptotic machinery of the cell have not been elucidated. However, a number of studies suggest that on activation, these receptors promote cell survival by (1) triggering specific signaling pathways, (2) modulating the activity of antiapoptotic molecules, and (3) inhibiting cell death effectors. Importantly, we reported that HGF linking to c-met, a tyrosine kinase receptor, can act as a protective factor against endothelial cell death induced by serum-free treatment, tumor necrosis factor-$\alpha$ treatment, and high-glucose conditions. Others have also reported antiapoptotic actions of bFGF under serum-free treatment and of VEGF under tumor necrosis factor-$\alpha$ treatment. However, the mechanisms by which endothelial cell death is prevented by these angiogenic growth factors are largely unknown. Because hypoxia is generally considered to represent a fundamental stimulus for angiogenesis, we especially investigated the effects of HGF.

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on apoptosis in human endothelial cells in response to hypoxia. Because the mechanisms responsible for their angiogenic activity remain enigmatic, we have addressed the following specific questions: (1) Does HGF have antiapoptotic actions on endothelial cell death induced by hypoxia? (2) How do endothelial cells undergo apoptosis in response to hypoxia? (3) How does HGF act as an antiapoptotic factor against endothelial cell death induced by hypoxia? Because neovascularization is a critical component in the development of collateral blood vessels that function to compensate for the compromised vascular function in response to hypoxia, it is extremely important to elucidate the molecular mechanisms of the antiapoptotic actions of angiogenic growth factors.

Methods

Cell Culture

Human aortic endothelial cells (passage 3) and coronary endothelial cells (passage 3) were obtained from Clonetics Corp and cultured in modified MCD1131 medium supplemented with 5% FCS, 50 µg/mL gentamicin sulfate, 50 µg/mL amphotericin B, 10 ng/mL epidermal growth factor, and 1 µg/mL hydrocortisone in the standard fashion.23 Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO2 with medium changes every 2 days. These cells showed the specific characteristics of endothelial cells by immunohistochemical examination and morphological observation. Briefly, human aortic endothelial cells tested positively for factor VIII antigen and for uptake of diacetylated LDL. All the cells were used within passages 4 to 7.

Hypoxic Treatment

Hypoxia was induced with an anaerobic device. Briefly, a hypoxic condition was achieved by using BBL GasPak (Becton Dickinson), which catalytically reduces O2 to undetectable levels by GasPak plus.23 In the preparation of experiments for determination of cell death, endothelial cells were grown to confluence. The medium was then changed to fresh defined serum-free (DSF) medium containing 10% fetal calf serum-containing medium with 5% CO2. At 4 days after transfection, lysing solution containing 100 µL of cell lysate supernatant were subjected to ELISA. The Western blot was performed for analysis of Bcl-2, Bcl-xL, Bax, and caspase-3 proteins. Endothelial cells were seeded onto 10-cm dishes previously described.14-25 Using membrane-permeable (H33342) dye in the assay allowed the determination of cell viability and plasma membrane integrity and the detection of any nonapoptotic toxic or necrotic death induced in the study groups.

To stain the cells for DNA, they were incubated with Hoechst 33342 (10 µg/mL in PBS) for 20 minutes at 37°C. The medium and a PBS rinse of the culture wells were collected before brief addition of trypsin/EDTA and decanting of cells. Culture wells were incubated with residual trypsin/EDTA for 3 minutes in humidified air at 37°C to achieve maximal cell detachment before the rinse with PBS and collection. Collected medium, the rinse, and trypsinized cells were pooled and collected by centrifugation at 1000 rpm for 5 minutes at 4°C. Cell pellets were resuspended in a small volume of serum-containing medium with 5 µg/mL Hoechst 33342. An aliquot was placed on a glass slide, covered with a glass coverslip, and viewed under fluorescence microscopy. Individual nuclei were visualized at ×400 to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells.

Although chromatin undergoes condensation during mitosis, these cells can be readily distinguished from apoptotic cells by their uniform and equatorial pattern of chromatin condensation compared with the randomly coalesced pattern typical of apoptotic cells. To quantify apoptosis, 400 nuclei from random microscopic fields were analyzed by 2 observers blinded to the treatment groups. The total number of apoptotic cells in each field was summed and expressed as a percentage of total cell number. At least 10 individual fields were evaluated per slide. Samples were coded so that the analysis was performed without knowledge of which treatment the cells had received. These observers were blinded to other data concerning the cells as well as to the results of the other observer. The reproducibility of the results has been reported.25 Briefly, intraobserver variability was 2.4±0.3%, and interobserver variability was 3.4±0.5% (mean±SD). Photographs were obtained with a fluorescence microscope (×200 and ×400, Olympus). The apoptotic index obtained is a cross-sectional and not a cumulative assessment at the time of harvest and may be an underestimate of the total apoptotic population, because apoptotic cells in the prenuclear condensation phase would be scored as normal and because late-stage apoptotic cells whose cellular membranes have disintegrated may not be detected.

Also, we used the measurement of cellular DNA fragmentation by use of a cellular DNA Fragmentation ELISA kit (Boehringer-Mannheim) to quantify apoptosis.26 Cultured endothelial cells were incubated with 10 µmol/L bromodeoxyuridine overnight at 37°C in 5% CO2. At 4 days after transfection, lysing solution containing BSA, EDTA, and Tween 20 was added to each well. DNA fragments in 100 µL of cell lysate supernatant were subjected to ELISA. The fluorescent signal was transferred to an anti-DNA–precoated microtiter plate and incubated for 60 minutes at 37°C. After they were washed, the samples were denatured and fixed by microwave irradiation for 5 minutes. After the microtiter plate was cooled for 10 minutes at −20°C, anti-bromodeoxyuridine peroxidase–conjugated solution was added, and the plate was incubated for 60 minutes at 37°C. Wells were again washed, trimethoxybenzoic acid substrate solution was added, and the plate was incubated for 30 minutes at room temperature. Stopping solution (25 µL at 1 mol/L H2SO4) was then added to each well. Absorbance was measured at 450 nm (reference wavelength 690 nm). We confirmed that the increase in apoptotic cells is associated with increased absorbance. Briefly, a concentration of 10 000 apoptotic cells per well reflects absorbance of 1.5 under the manufacturer’s recommended conditions. The sensitivity of the DNA fragmentation ELISA assay correlates well with the results obtained by the conventional [H]thymidine-based DNA fragmentation assay. In our experimental conditions, an increase in absorbance of 0.2 reflects an increase in cell number of 2000 apoptotic cells per well.

Western Blot

Western blot was performed for analysis of Bcl-2, Bcl-xL, Bax, and caspase-3 proteins. Endothelial cells were seeded onto 10-cm dishes.
(Corning). After treatment, total protein was extracted with RIPA buffer (50 mmol/L Tris chloride, 0.15 mol/L NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X, and 10 mmol/L NaF). Samples containing 10, 20, and 40 μg protein were run on 12.5% SDS-polyacrylamide gels. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane (Hybond ECL, Amersham), and incubated with a monoclonal antibody to Bcl-2 (anti-human mouse IgG, 1:25, Santa Cruz), Bcl-xL (anti-human rabbit IgG, 1:100, Santa Cruz), or Bax (anti-human rabbit IgG, 1:100, Santa Cruz) at 4°C overnight. Antibodies were diluted in 4% skimmed milk and 0.1% Tween 20 in PBS. The membranes were then washed and incubated with a 1:2000 dilution of mouse or rabbit immunoglobulin horseradish peroxidase–conjugated antibody (Amersham). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham) and Hyperfilm-MP (Amersham). It was confirmed that amounts of loaded proteins were equal by staining with Coomassie Brilliant Blue R (Sigma Chemical Co). Staining with Coomassie Brilliant Blue R revealed identical protein amounts in all samples of Western blotting (data not shown). Western blotting of tubulin with antitubulin antibody (anti-human mouse IgG, 1:25, Oncogene) was also performed to confirm equal protein amounts in all samples of Western blotting (data not shown).

Measurement of Caspase-3–Like Activity

Cells were lysed in buffer B containing 5 mmol/L EGTA, 5 mmol/L EDTA, 10 μmol/L digitonin, 2 mmol/L dithiothreitol, and 25 mmol/L HEPES, pH 7.4. The lysates were centrifuged at 900g for 3 minutes, and the supernatants were used for enzyme assay. Enzymatic reactions were carried out in buffer B containing 20 μg protein and 50 μmol/L acetyl-Asp-Glu-Val-Asp (DEVD)–aminotri fluoromethylcoumarin. The reaction mixtures were incubated at 37°C for 30 minutes, and fluorescent aminotri fluoromethylcoumarin formation was measured at 400-nm excitation and 505-nm emission by using a microplate fluorescence reader (Hitachi F-3000 and F-2000). One unit was defined as the amount of enzyme required to release 0.22 nmol of 7-amino-4-methylcoumarin per minute at 37°C.

Treatment of Caspase-3 Inhibitors

In the preparation of experiments for determination of cell death, endothelial cells were grown to confluence. The medium was then changed to fresh DSF medium containing a wide-spectrum inhibitor of caspase proteases (Z-DEVD.FMK, Enzyme Systems Products), or vehicle. The cells were then incubated. On day 2, an index of cell proliferation and cell damage was determined, as described above.

Materials

Human recombinant HGF was purified from the culture medium of Chinese hamster ovary cells or C-127 cells transfected with expression plasmid containing human HGF cDNA. VEGF and bFGF were obtained from Pepro Tech EC Ltd.

Statistical Analysis

All values are expressed as mean ± SEM. ANOVA with a subsequent Bonferroni test was used to determine the significance of differences in multiple comparisons. Values of P < 0.05 were considered statistically significant.

Results

Effects of HGF on Hypoxia-Induced Endothelial Cell Death and Apoptosis

First, we examined the effect of hypoxic treatment on endothelial cell growth. After 12 hours of hypoxia, some cells started to become round and eventually detached from the plate and floated in the medium, leaving many holes in the sheet of confluent cells (data not shown). The floating cells could be recovered with the medium and did not attach onto a new plate or proliferate. Consistent with this morphological observation, the number of living cells under hypoxia was significantly decreased compared with that under normoxia at 48 hours after treatment in a time-dependent manner (P < 0.01), as shown in Figure 1. In addition, hypoxic treatment significantly increased LDH release to the conditioned medium in a time-dependent manner (P < 0.01, Figure 1). Therefore, we examined the effects of HGF, VEGF, and bFGF, because these growth factors have been reported to act as survival factors against endothelial cell death. The addition of HGF resulted in partial, but significant, attenuation of cell death mediated by hypoxia in a dose-dependent manner, assessed by a WST cell count assay (Figure 2). Similarly, bFGF also abolished endothelial cell death induced by hypoxia at 10 and 100 ng/mL, whereas the inhibitory
effect of VEGF on cell death was observed only at 100 ng/mL, but not at 10 ng/mL. Similar results were obtained by using human coronary endothelial cells at 2 days after treatment (normoxia + vehicle 100%, hypoxia + vehicle 52±4% [P<0.01 versus vehicle], and 100 ng/mL HGF 78±4% [P<0.01 versus hypoxia + vehicle]). Values are expressed as percentage of cell number calculated from cell number treated with vehicle under normoxic conditions.

Therefore, we next examined the effects of HGF on hypoxia-induced apoptosis in human endothelial cells. Apoptotic cells were clearly observed in cells maintained under hypoxic conditions. In addition, consistent with previous reports, treatment of endothelial cells with hypoxia resulted in a significant increase in apoptotic cells, as assessed by cell morphology (Figure 3a, P<0.01). Of importance, the addition of HGF significantly attenuated hypoxia-induced endothelial apoptosis (Figure 3a, P<0.01). Although bFGF and VEGF also inhibited cell death induced by hypoxia, the degree of inhibitory effect of VEGF was significantly smaller than those of HGF and bFGF (P<0.05). A significant increase in apoptotic cells by hypoxia was also confirmed by DNA fragmentation ELISA (Figure 3b, P<0.01), whereas HGF significantly attenuated hypoxia-induced apoptotic changes in a dose-dependent manner (Figure 3b, P<0.01). The degree of inhibition of apoptosis by VEGF seems to be smaller than that of HGF and bFGF, whereas bFGF and VEGF also significantly decreased the DNA fragmentation rate. Moreover, we further evaluated apoptosis induced by hypoxia by the measurement of cysteine protease, caspase-3, because caspase-3 is essential for apoptotic death in mammalian cells and appears to be an attractive candidate. Caspase-3–like activity was markedly increased by hypoxia at 24 hours after the treatment, whereas HGF attenuated a significant increase in caspase-3–like activity (34.7±8.5 U/mL [normoxia] versus 65.9±6.9 U/mL [hypoxia], P<0.01). Of importance, the decrease in cell number was inhibited by Z-VAD, a wide spectrum inhibitor of caspases, but not Z-DEVD, a specific caspase-3 inhibitor, at 48 hours after treatment (Figure 4, P<0.01). In addition, the release of LDH into the conditioned medium induced by hypoxic treatment was also attenuated by Z-VAD rather than Z-DEVD at 48 hours after treatment (Figure 4, P<0.01). These results suggested that HGF prevented hypoxia-induced endothelial apoptosis in a caspase-3–like protease-dependent manner.

**Molecular Mechanisms of Antiapoptotic Actions of HGF in Hypoxia-Induced Endothelial Cell Death**

We next examined the molecular mechanisms of apoptosis induced by hypoxic treatment. Especially, we focused on the expression of Bcl-2 and Bax proteins. Bcl-2 and Bax are homologous proteins that have opposing effects on cell life and death, with Bcl-2 serving to prolong cell survival and Bax...
acting as an accelerator of apoptosis. The Bcl-2 and Bax proteins can form heterodimers in cells. Interestingly, hypoxic treatment significantly decreased Bcl-2 protein, as assessed by Western blotting, as shown in Figure 5 (P<0.01), whereas no significant change in Bax protein by hypoxic treatment was observed (Figure 6). Thus, the ratio of Bcl-2 to Bax was significantly decreased in cells maintained under hypoxia (data not shown, P<0.01).

To examine how HGF acts as an antiapoptotic factor in endothelial cell death induced by hypoxia, we also studied the molecular mechanisms of the antiapoptotic action of this angiogenic growth factor. Of importance, as shown in Figure 5, compared with vehicle, HGF partially, but significantly, attenuated the decrease in Bcl-2 protein by hypoxia (P<0.01). Similarly, bFGF and VEGF also attenuated a significant decrease in Bcl-2 protein by hypoxia. There was no significant difference in the stimulatory effect on Bcl-2 protein among HGF, bFGF, and VEGF. Moreover, HGF also significantly stimulated Bcl-2 protein even under normoxic conditions in a dose-dependent manner (Figure 5, P<0.01). The stimulatory effect of bFGF and VEGF was also observed at 10 and at 100 ng/mL under normoxic conditions. Unexpectedly, HGF as well as VEGF and bFGF failed to affect the Bax protein level (Figure 6). Thus, a significant increase in the ratio of Bcl-2 to Bax was observed in cells treated with HGF compared with vehicle (data not shown, P<0.01). Similarly, bFGF and VEGF also significantly increased the ratio of Bcl-2 to Bax. There were no significant differences among HGF, bFGF, and VEGF. Finally, we measured the protein level of another apoptosis-related gene, Bcl-xL, a Bcl-2–related gene that can function as a Bcl-2–independent regulator of apoptosis. Of importance, the protein level of Bcl-xL was markedly decreased in cells maintained under hypoxic conditions (P<0.01), whereas no apparent change was observed in cells treated with HGF, VEGF, or bFGF (Figure 7). In addition, none of them affected the Bcl-xL protein level under normoxic conditions (data not shown).
Angiogenesis constitutes a fundamental process underlying a variety of physiological and pathological situations. Insoluble extracellular matrix components and soluble endothelial cell mitogens in coordination modulate this complex multifaceted phenomenon. Moreover, neovascularization is an extremely important component in the development of collateral blood vessels, which function to compensate for compromised vascular function. Thus, angiogenic growth factors are important in the regulation of these processes. In the pathophysiology of the ischemic disease, when a major artery is obstructed, blood flow to the ischemic tissue is often dependent on collateral vessels. When spontaneous development of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, ischemia occurs. Preclinical studies have demonstrated that angiogenic growth factors (VEGF, bFGF, and HGF) can stimulate the development of collateral arteries in animal models of peripheral and myocardial ischemia.\(^{13-19}\) A concept called therapeutic angiogenesis. Most of the studies have used VEGF, also known as vascular permeability factor, because it is a secreted endothelial cell mitogen. More recently, the efficacy of therapeutic angiogenesis using VEGF gene transfer has been reported in human patients with critical limb ischemia.\(^{41,42}\) Thus, a strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia and myocardial infarction. However, the molecular mechanisms by which angiogenic growth factors can stimulate endothelial cell growth and maintain the endothelium are still unknown. This is of extreme importance, inasmuch as intact endothelium acts as a sensor and transducer of signals and also provides a nonthrombogenic surface at the blood–vascular wall interface. Hence, mechanisms that maintain the integrity of the endothelium are of interest in physiological and pathological states.

It has recently been postulated that regulation of cell death by apoptosis may be another determinant of vessel structure and lesion formation, in addition to classic paradigms defining the pathophysiology of vascular disease that have focused on the abnormal regulation of cell growth in response to growth factors.\(^{11}\) 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 ubiquitous phenomenon observed in a variety of cell types, including endothelial cells.\(^{31}\) Cell death due to hypoxia is a major concern in various clinical entities, such as ischemic diseases. Cell death by hypoxia has been generally believed to be manifested as necrosis.\(^{43}\) In contrast, recent biochemical observations have suggested the possibility of hypoxia-induced apoptosis.\(^{28,29}\) In the present study, we have demonstrated that hypoxic treatment induces endothelial cell death through the induction of apoptosis, consistent with previous findings.\(^{28,29}\) We have also demonstrated that hypoxia causes endothelial cell death through caspase-3–like protease-dependent apoptosis. Moreover, we explored upstream from the caspase pathway. Bcl-2 and Bax are homologous proteins that have opposing effects on cell life and death, with Bcl-2 serving to prolong cell survival and Bax acting as an accelerator of apoptosis.\(^{31,32}\) The Bcl-2 and Bax proteins can form heterodimers in cells.\(^{33}\) In addition, Bcl-xL, a Bcl-2–related gene that can function as a Bcl-2–independent regulator of programmed cell death (apoptosis), inhibits cell death on growth factor withdrawal at least as potently as Bcl-2.\(^{34,35}\) Bax also heterodimerizes with Bcl-xL in mammalian cells. Of importance, the present study revealed a significant decrease in Bcl-2 and Bcl-xL, antiapoptotic factors, and no change in Bax, a proapoptotic factor, by hypoxic treatment. Because our present data demonstrated a marked downregulation of Bcl-2 and Bcl-xL induced by hypoxia, the apoptosis induced by hypoxia may be due to an inappropriate decrease in antiapoptotic factors. However, the lack of change in Bax protein did not indicate that there was no contribution of Bax to endothelial apoptosis induced by hypoxia, because the translocation of Bax protein is dominant in the determination of apoptosis.\(^{44}\)

As mentioned earlier, angiogenic growth factors such as VEGF and bFGF act as antiapoptotic factors in endothelial cells.\(^{13-19}\) However, the actions of a novel member of the angiogenic growth factors, HGF, on endothelial cell death are largely unknown. Of importance, similar to bFGF and VEGF, HGF could rescue endothelial cells from death induced by hypoxia. The degree of the antideath effect of HGF seems to be equal to that of bFGF and VEGF. The inhibitory effect of HGF was also observed in coronary endothelial cells, suggesting that the phenomenon under hypoxia may be identical even in smaller vessels, such as the coronary artery. The present study has also demonstrated that HGF acts as an antiapoptotic factor in the apoptosis of endothelial cells induced by hypoxia. The prevention of hypoxia-induced endothelial apoptosis by HGF is supported by several lines of evidence: (1) the increase in morphological apoptotic changes induced by hypoxia was inhibited; (2) the increased DNA fragmentation by hypoxia was attenuated; and (3) upregulation of the activity of caspase-3, an interleukin 1–converting enzyme homologue that cleaves poly(ADP-ribose)polymerase.

**Discussion**

**Figure 7.** Left, Typical example of Western blot of Bcl-xL and tubulin proteins in endothelial cells treated with HGF, VEGF, and bFGF under hypoxic conditions at 48 hours. Right, Percent changes in protein level of Bcl-xL in endothelial cells treated with HGF, VEGF, and bFGF under hypoxic and normoxic conditions at 48 hours. There were \(n = 6\) per group calculated from 6 independent experiments. **\(P < 0.05\) and ***\(P < 0.01\) vs Untreat.
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early during apoptosis, by hypoxia\textsuperscript{30} was inhibited. Moreover, HGF significantly increased Bcl-2 protein without affecting Bax protein, thereby resulting in a significant increase in the ratio of Bcl-2 to Bax protein. Unfortunately, we failed to demonstrate the specific action of HGF on endothelial cell death, which was different from that of bFGF and VEGF. Unexpectedly, none of the angiogenic growth factors affected the decrease in Bcl-xL protein by hypoxia. This is consistent with a previous study reporting that only Bcl-2 is induced by FGF-2 in endothelial cells,\textsuperscript{18} although there is no report examining the effects of HGF and VEGF on Bcl-2 and Bcl-xL proteins in endothelial cells. Overall, HGFs should be classified as new members of the growth factors, with anti–cell-death actions in endothelial cells through the inhibition of apoptosis. HGF is known to stimulate phosphatidylinositol-37-kinase, protein tyrosine phosphatase 2, phospholipase C-\(C\), pp60\(^{c-src}\), grb2/hSos1, rho, and ras.\textsuperscript{45,46} In contrast, bFGF and VEGF also activated tyrosine kinase–linked signal transduction pathways. The mechanisms by which these growth factors prevented endothelial cell death mediated by the conditions in the present study are unclear. Recently, the bag-1 protein has been reported to interact with Bcl-2 protein and to cooperate with Bcl-2 protein to suppress apoptosis.\textsuperscript{47} Of importance, the bag-1 protein appears to inhibit cell death by binding to Bcl-2, the ras-1 protein kinase, and c-met (specific receptor of HGF),\textsuperscript{48} although the mechanism of inhibition remains enigmatic. The cooperative activation of these Bcl-2–related genes may also participate in the prevention of cell death by HGF. Interestingly, VEGF was upregulated and HGF was downregulated by hypoxic treatment.\textsuperscript{49,50} Some stimuli capable of inducing the development of neovascularization in vivo, specifically, certain cytokines and hypoxia, fail to stimulate endothelial cell proliferation in vitro, suggesting a role for additional mediators and/or cell types. Differential regulation among HGF, VEGF, and bFGF coupled with their ability to prevent endothelial cell death may explain the balance of endothelial cell growth and death, because it is assumed that specific angiogenic growth factors are either produced by cells recruited to the ischemic tissue or are locally regulated to mediate neovascularization. Overall, the present study demonstrated that hypoxic treatment resulted in significant endothelial cell death through the induction of caspase-dependent apoptosis by downregulation of Bcl-2 and Bcl-xL, whereas HGF prevented endothelial apoptosis through the upregulation of Bcl-2. Because we tested the mechanisms by using only an in vitro model in the present study, further studies using an in vivo model are necessary. Nevertheless, these findings may provide new perspectives for the development of therapeutic strategies to alter the progression of ischemic diseases, such as myocardial infarction and peripheral vascular disease.

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