Glycine Prevents Apoptosis of Rat Sinusoidal Endothelial Cells Caused by Deprivation of Vascular Endothelial Growth Factor

YAN-JUN ZHANG, KENICHI IKEJIMA, HAJIME HONDA, TSUNEO KITAMURA, YOSHIYUKI TAKEI, AND NOBUHIRO SATO

Apoptosis of sinusoidal endothelial cells (SECs) is one of the initial events in the development of ischemia-reperfusion injury of the liver. Glycine has been shown to diminish ischemia-reperfusion injury in the liver and improve graft survival in the rat liver transplantation model. Here, we investigated the effect of glycine on apoptosis of primary cultured rat SECs induced by vascular endothelial growth factor (VEGF) deprivation. Isolated rat SECs were cultured in EBM-2 medium supplemented with 10% fetal bovine serum (FBS) and growth factors including 20 ng/mL VEGF for 3 days. SECs at 3 days of culture showed spindle-like shapes; however, cells started shrinking and detaching from dishes by VEGF deprivation. Apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated d-uridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining in these conditions. Control SECs contained only a few percent of TUNEL-positive cells; however, they started increasing 4 hours after VEGF deprivation, and the percentage of TUNEL-positive cells reached about 50% at 8 hours and almost 100% at 16 hours after VEGF deprivation. Interestingly, this increase in TUNEL-positive cells after VEGF deprivation was prevented significantly when glycine (1-10 mmol/L) was added to the medium, the levels being around 60% of VEGF deprivation without glycine. Furthermore, strychnine (1 μmol/L), a glycine receptor antagonist, inhibited this effect of glycine, suggesting the possible involvement of the glycine receptor/chloride channel in the mechanism. Moreover, Bcl-2 protein levels in SECs were decreased 8 hours after VEGF deprivation, which was prevented almost completely by glycine. It is concluded that glycine prevents apoptosis of primary cultured SECs under VEGF deprivation. (HEPATOLOGY 2000;32:542-546.)

Liver sinusoidal endothelial cells (SECs) have been demonstrated to play an important role in the development of various types of liver injury. In particular, the cell death of SECs is one of the initial events in ischemia-reperfusion injury in the liver,[1] and the degrees of injury in SECs have inversely correlated well with the graft survival in liver transplantation. Recently, several reports demonstrated that apoptosis occurs primarily in SECs after cold storage–reperfusion of the liver,[2-4] indicating that the apoptosis of SECs most likely plays a pivotal role in primary graft nonfunction. In this aspect, prevention of apoptosis in SECs appears to be important for successful liver transplantation. On the other hand, vascular endothelial growth factor (VEGF) is one of the key factors for the survival and proliferation of endothelial cells.[5] Indeed, the starvation of serum or growth factor has been used for the induction of apoptosis in various types of endothelial cells in culture. It was also reported that VEGF promotes extended survival of SECs in the primary culture.[6]

Glycine, a nonessential amino acid, is known to possess various physiological functions besides being a source of protein synthesis and metabolism. In the central nervous system, glycine works as a neurotransmitter, and a glycine-receptor is identified in nerve cells.[7] Furthermore, cyto- and organ-protective effects of glycine has been reported in renal tubules,[8-10] isolated hepatocytes,[11-13] and an ischemia-reoxygenation injury model using perfused liver *ex vivo*.[14] It has also been demonstrated that a diet containing glycine is preventive against endotoxin-induced mortality and liver injury[15] and early alcohol-induced liver injury in the rat.[16] Moreover, the organ rinse solution containing glycine increased graft survival in the rat liver transplantation model,[17] and intravenous administration of glycine to the recipient also improved survival in rat liver transplantation.[18] The effect of glycine rinse on cold ischemia-reperfusion injury in the liver was further confirmed in human liver transplantation.[19] More recently, Nishimura et al. reported that hypoxia-reoxygenation injury in isolated SECs was reduced by glycine.[20] These observations support the hypothesis that glycine is cytoprotective in various types of cells including SECs; however, it is not clear whether glycine can prevent apoptotic cell death in SECs. Therefore, the purpose of this study was to investigate the effect of glycine on VEGF deprivation–induced apoptosis in primary cultured SECs. Furthermore, we investigated the mechanism by which glycine prevents apoptosis in SECs. The preliminary account of this work has been reported elsewhere in abstract form.[21]

MATERIALS AND METHODS

Isolation and Primary Culture of Liver Sinusoidal Endothelial Cells. SECs were isolated from male Wistar rats weighing 350 to 400 g (Charles River Japan Inc., Saitama, Japan) by collagenase perfusion and differential centrifugation using Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the method described by Braet et al., with some modifications.[22] All animals used in this study received humane care, and the protocol was approved by the commit-

Abbreviations: SEC, sinusoidal endothelial cell; VEGF, vascular endothelial growth factor; GBSS, Gey's balanced salt solution; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TdT, terminal deoxynucleotidyl transferase; dUTP, d-uridine triphosphate; TUNEL, TdT-mediated dUTP-biotin nick end labeling; TBS, Tris-buffered saline.

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tee of laboratory animals according to institutional guidelines. Briefly, the liver was perfused *in situ* through the portal vein with Gey's balanced salt solution (GBSS) without calcium at 37°C for 10 minutes at a flow rate of 20 mL/min, followed by GBSS with calcium containing 0.05% collagenase A (Roche Diagnostics Co., Mannheim, Germany) at 37°C for 10 minutes at a flow rate of 10 mL/min. Subsequently, the liver was excised and further digested by shaking in 100 mL fresh GBSS with calcium containing 0.05% collagenase A, 0.001% DNase (Roche Diagnostics Co.), and 5.3 mL heat-inactivated fetal bovine serum (FBS) for 5 minutes at 37°C. The cell suspension was then filtered through nylon gauze and centrifuged at 100*g* for 4 minutes. The supernatant enriched in SECs was centrifuged for 6 minutes at 350*g*, and then the pellet was layered on the double layer of Percoll gradients (25% and 50%) and centrifuged immediately at 900*g* for 20 minutes. The fraction of SECs was collected and washed in phosphate-buffered saline (PBS). The viability of isolated SECs judged by the Trypan blue exclusion test was above 95% in all isolations. The purity of SECs examined by a phase-contrast microscope was 89.5% ± 3.5%. Furthermore, the purity of cultured SECs was confirmed by the uptake of acetylated low-density lipoprotein and ovalbumin (data not shown). Cells were seeded onto collagen-coated polyethylene dishes (Sumilon, Sumitomo Bakelite Co., Tokyo, Japan) and cultured in EBM-2 medium (Bio Whittaker Co., Walkersville, MD), supplemented with the EGM-2 bullet kit (Bio Whittaker Co.; containing long R insulin-like growth factor-1, human epidermal growth factor, and human fibroblast growth factor-B) and 20 ng/mL human recombinant VEGF (Wako Junyaku Co., Osaka, Japan) in humidified air with 5% CO_2 at 37°C for 3 days before experiments.

Induction of Cell Death in Primary Cultured SECs and Evaluation of Apoptosis. At day 3 of culture, cell death of primary cultured SECs was induced by deprivation of VEGF by exchanging the culture media with VEGF-free EBM-2. Cells were then further cultured in a CO_2 incubator for up to 24 hours. Morphological changes of SECs were observed and photographed under a phase-contrast microscope (F-601 AF Quartz Data, Nikon Optics, Tokyo, Japan).

Apoptosis of SECs was detected by a terminal deoxynucleotidyl transferase (TdT)-mediated d-uridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) technique using a fluorescein apoptosis detection system kit (Apoptosis Detection System, Fluorescein, Promega Corp., Madison, WI) according to the manufacturer's instructions. Briefly, cells were fixed with 4% formalin, permeabilized with 0.2% Triton X-100 in PBS on ice, and incubated in an equilibration buffer containing 5 μmol/L fluorescein-12-dUTP and 25 U TdT for 1 hour at 37°C to stain fragmented DNA. Subsequently, 0.2 μg/mL propidium iodide in PBS was applied for 15 minutes to stain both apoptotic and nonapoptotic cells. Thus, propidium iodide stains both apoptotic and nonapoptotic cells red throughout the cytoplasm, whereas fluorescein-12-dUTP is incorporated at the 3′-OH ends of fragmented DNA, resulting in localized green fluorescence within the nucleus of apoptotic cells. The stained cells were observed under a fluorescence microscope (Axiophot ZVS 3C75DE, Carl Zeiss Inc., Goleta, CA), and photographs were taken. To calculate the percentage of apoptotic SECs, TUNEL-positive cells were counted in a middle-power field (200×). Four random fields were examined in a blinded manner in each preparation.

Western Blotting for Bcl-2. Cultured SECs were washed with ice-cold PBS and harvested by scraping, followed by a centrifugation at 1,500 rpm for 5 minutes. The cell pellet was homogenized in a buffer containing 66 mmol/L Tris (pH 8.0), 5 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, and protease/phosphatase inhibitors (40 μg/mL bestatin [Roche Diagnostics], 20 mmol/L β-glycerophosphate [Sigma Chemical Co., St. Louis, MO], 10 mmol/L *p*-nitrophenyl phosphate [Roche Diagnostics], 500 μmol/L Pefabloc [Roche Diagnostics], 700 ng/mL pepstatin A [Roche Diagnostics], 2 μg/mL aprotinin [Roche Diagnostics], 500 μmol/L Na_3VO_4 [Sigma Chemical], and 500 ng/mL leupeptin [Roche Diagnostics]). After centrifugation at 15,000 rpm for 15 minutes, the supernatant was collected and protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The whole-cell protein extracts (20 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA) using a semidry transfer apparatus. Membranes were blocked with 20% FBS in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for nonspecific bindings of antibodies, and then hybridized with a polyclonal rabbit anti–Bcl-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in 20% FBS in TBS-T for 2 hours at room temperature. Membranes were washed 3 times with TBS for 5 minutes and incubated with a horseradish peroxidase–conjugated anti-rabbit IgG (Santa Cruz Biotechnology) in 20% FBS in TBS-T for 1 hour at room temperature. Following 3× washes with TBS for 5 minutes, an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech, Denver, CO) was applied for the detection of specific bands according to the manufacturer's instructions.

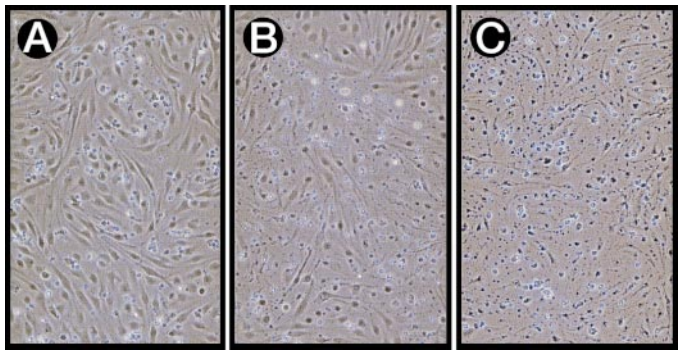

FIG. 1. Morphological changes in primary cultured rat SECs under VEGF deprivation. SECs were isolated from rat livers by collagenase digestion and cultured for 3 days before the experiment, as described in Materials and Methods. Cells were then cultured in fresh media without VEGF (VEGF deprivation) for 24 hours. Representative phase-contrast views of SECs from 5 individual experiments are shown. (A) Control SECs 3 days after inoculation. (B) SECs 12 hours after VEGF deprivation. (C) SECs 24 hours after VEGF. (Original magnification ×200.)

Statistical Analysis. Data were expressed as mean ± SD, and ANOVA and the Tukey's *post-hoc* test were used for the multiple comparisons of the differences in mean values. $P < .05$ was selected before the analyses to determine the significance.

RESULTS

VEGF Deprivation Causes Apoptosis in Primary Cultured Rat SECs. Under a phase-contrast microscope, the primary cultured rat SECs showed spindle-like shapes and were nearly confluent at 3 days of culture. However, cells tended to shrink during the time course of VEGF deprivation, and finally, cells started detaching from dishes after 24 hours of VEGF deprivation (Fig. 1), indicating that VEGF deprivation caused cell death in SECs. To clarify whether the cell death of SECs as a result of VEGF deprivation is apoptosis, TUNEL staining was performed (Fig. 2). As expected, most cells 3 days after culture in EBM-2 medium containing VEGF were negative for TUNEL staining, and TUNEL-positive cells were only 2.0% in this condition. However, the TUNEL-positive cells start to increase in number 4 hours after VEGF deprivation, and almost all cells were TUNEL-positive 16 hours after VEGF deprivation (Fig. 3). The nuclei of TUNEL-positive cells showed condensation and fragmentation under a high-power view, representing typical features of apoptotic cells (data not shown).

Effects of Glycine on VEGF Deprivation–Induced Apoptosis of SECs. To evaluate the protective effect of amino acids against the apoptosis of SECs, we used this VEGF deprivation–treated

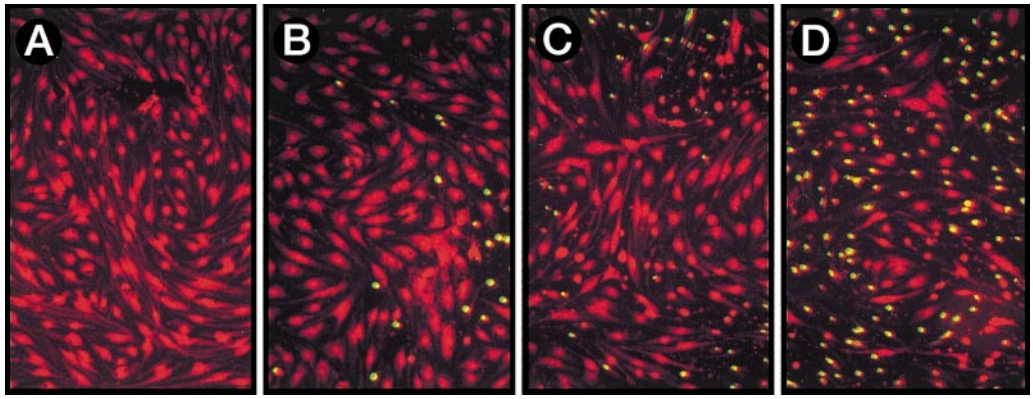

FIG. 2. Apoptosis of primary cultured rat SECs under VEGF deprivation. Experimental design as in Fig. 1. Apoptotic cells were detected by TUNEL staining as described in detail in Materials and Methods. Photographs were taken under fluorescent microscopy. (A) Control SECs 3 days after inoculation. (B) SECs 4 hours after VEGF deprivation. (C) SECs 8 hours after VEGF deprivation. (D) SECs 12 hours after VEGF deprivation. Representative photographs from 5 individual experiments are shown. (Original magnification ×200.)

apoptosis in primary cultured SECs. Interestingly, the increase in TUNEL-positive cells as a result of VEGF deprivation was significantly prevented by the addition of glycine (1-10 mmol/L) in the medium in a dose dependent manner (Fig. 4). The percentages of apoptotic cells in the presence of glycine were almost half 4 hours and 8 hours after VEGF deprivation (Fig. 5). To investigate whether this antiapoptotic effect on SECs is glycine-specific, the effects of other amino acids were examined. The percentages of apoptotic cells 8 hours after VEGF deprivation were 59.8% ± 13.4%, 66.0% ± 10.4%, 60.5% ± 8.0%, 62.1% ± 5.0%, and 71.3% ± 15.2% in the presence of 10 mmol/L alanine, valine, glutamine, methionine, and serine, respectively, which had no significant difference as compared with VEGF-deprived controls. These observations indicated that the amino acids other than glycine tested here had no protective effect on the apoptosis in primary cultured SECs, and the effect of glycine was unique.

Strychnine Antagonizes the Effect of Glycine on the Apoptosis in SECs. To test the hypothesis that this antiapoptotic effect of glycine in SECs is mediated through glycine receptors on the plasma membrane, we tried strychnine, a putative glycine receptor antagonist.[23] Figure 6 shows the percentage of TUNEL-positive cells 8 hours after VEGF deprivation in the presence of glycine (10 mmol/L) and strychnine (1 μmol/L). The percentage of TUNEL-positive cells was 40.3% ± 2.0% when strychnine was added in combination with glycine, which was significantly higher than that of glycine alone ($P < .05$). In contrast, the percentage of TUNEL-positive cells 8 hours after VEGF deprivation in the presence of strychnine alone (1 μmol/L) was 50.8% ± 9.3%, which was not significantly different from VEGF deprivation alone. These findings indicated that a low dose of strychnine inhibited, at least in part, the antiapoptotic effect of glycine in primary cultured SECs.

Bcl-2 Expression in SECs Under VEGF Deprivation. The expression of Bcl-2, an antiapoptotic protein, in SECs was detected by Western blotting (Fig. 7). As expected, the 25- to 26-kd specific band for Bcl-2 was detected clearly in the control SECs 3 days after inoculation. However, the expression of Bcl-2 was decreased dramatically in the SECs 8 hours after VEGF deprivation. Surprisingly, the decrease of Bcl-2 as a

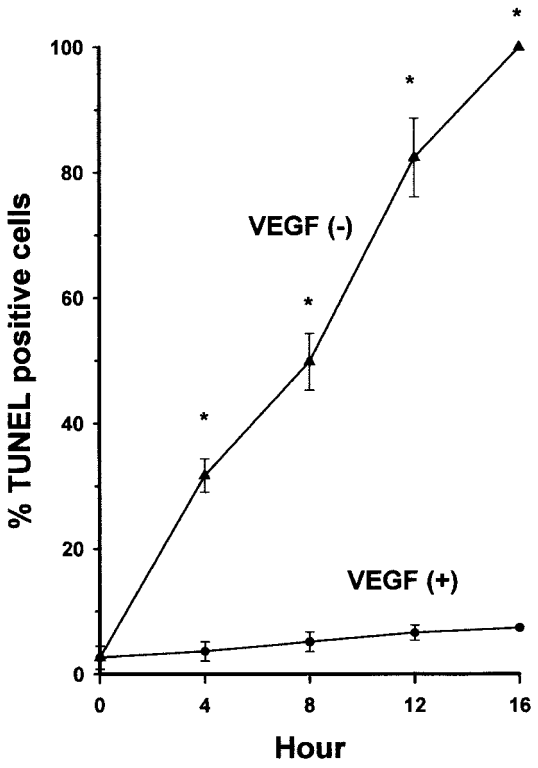

FIG. 3. Time course of VEGF deprivation–induced apoptosis in rat SECs. Experimental design as in Fig. 2. TUNEL-positive cells were counted in a middle-power field (×200) in each condition, and the percentages of TUNEL-positive cells were plotted. Data represent mean ± SD of 5 individual experiments (*$P < .05$ by ANOVA and Tukey's *post-hoc* test as compared with controls).

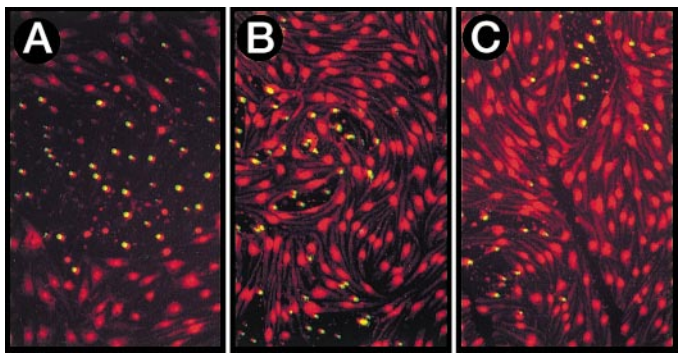

FIG. 4. Effect of glycine on VEGF deprivation–induced apoptosis in rat SECs. Experimental design as in Fig. 2. Glycine (1-10 mmol/L) was added in the medium without VEGF. (A) SECs 8 hours after VEGF deprivation (control). (B) SECs 8 hours after VEGF deprivation in the presence of 1 mmol/L glycine. (C) SECs 8 hours after VEGF deprivation in the presence of 10 mmol/L glycine. Representative photographs from 5 individual experiments are shown. (Original magnification ×200.)

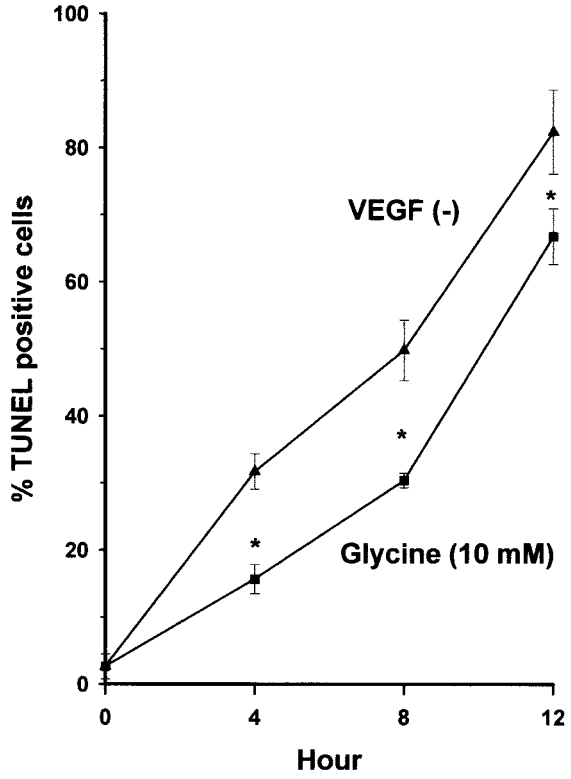

FIG. 5. Effect of glycine on VEGF deprivation–induced apoptosis in rat SECs. Experimental design as in Fig. 4. Percentages of TUNEL-positive cells were plotted. Data represent mean ± SD of 5 individual experiments (*$P < .05$ by ANOVA and Tukey's *post-hoc* test as compared with VEGF-deprivation groups).

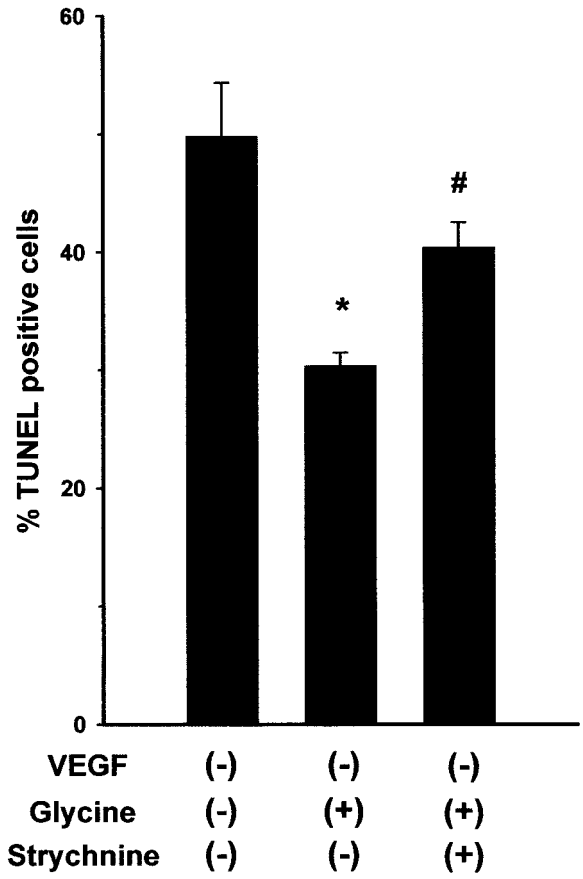

FIG. 6. Strychnine antagonizes the antiapoptotic effect of glycine in rat SECs. Experimental design as in Fig. 4. Strychnine (1 μmol/L), a glycine receptor antagonist, was added to the media without VEGF supplemented with glycine (10 mmol/L), and cells were incubated for 8 hours before TUNEL staining. Percentages of TUNEL-positive cells were plotted. Data represent mean ± SD from 5 individual experiments (*$P < .05$ as compared with controls; #$P < .05$ as compared with glycine alone by ANOVA and Tukey's *post-hoc* test).

result of VEGF deprivation was prevented almost completely in the presence of 10 mmol/L glycine.

DISCUSSION

Deprivation of growth factors is well known to induce apoptosis in various types of cells. In this study, we demonstrated that VEGF deprivation caused apoptotic cell death in primary cultured SECs (Figs. 1 and 2). TUNEL-positive SECs increased dramatically during the time course of VEGF deprivation (Fig. 3), and the TUNEL-positive nuclei showed condensation and fragmentation in shape, which are characteristic morphological features of apoptosis. It was reported that SECs express VEGF receptors (Flt-1 and KDR/Flk-1) abundantly, and VEGF maintains isolated SECs for an extended time course in culture, indicating that VEGF is one of the essential survival factors for SECs.[6] More recently, Moriga et al. reported that VEGF is preventive against sinusoidal endothelial damage and apoptosis induced by cold ischemia-reperfusion.[24] These findings support the hypothesis that VEGF plays a crucial role in the damage of SEC during cold ischemia-reperfusion, including liver transplantation. Therefore, we evaluated the effect of glycine on apoptotic cell death in SECs induced by VEGF deprivation. Interestingly, glycine prevented this VEGF deprivation–induced apoptosis in SECs in a dose-dependent manner (Fig. 4). Furthermore, the other amino acids we tested (alanine, valine, glutamine, methionine, and serine) had no effect in this model, which indicated that this antiapoptotic effect of glycine is a unique property rather than a nonspecific effect of amino acids. It is concluded, therefore, that glycine has an antiapoptotic effect in SECs.

It is well known that glycine acts as a neurotransmitter in the central nervous system, and a glycine receptor has been identified and well characterized. The glycine receptor in the

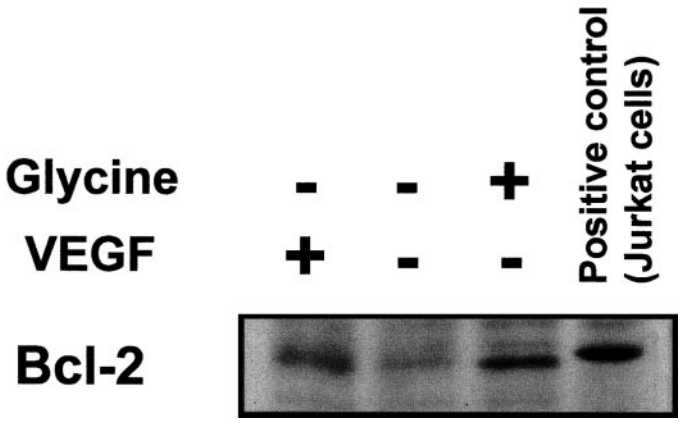

FIG. 7. Effect of glycine on Bcl-2 expression in rat SECs under VEGF deprivation. Bcl-2 in rat SECs was detected by Western blotting using a polyclonal antibody for Bcl-2 as described in Materials and Methods. Specific bands at 25 to 26 kd are shown. *First lane*: SECs 3 days after inoculation (control); *second lane*: SECs were incubated in the media without VEGF for 8 hours; *third lane*: SECs were incubated in the media without VEGF in the presence of glycine for 8 hours; *fourth lane*: whole-cell extracts from Jurkat cells (positive control).

central nervous system constitutes a chloride channel on the plasma membrane, and glycine causes the influx of chloride ion, leading to hyperpolarization of the plasma membrane, thereby counteracting depolarization stimuli.[7] Recently, Kupffer cells, resident macrophages in the liver, have been demonstrated to possess a similar type of receptors, by which glycine prevents the production of inflammatory cytokine production.[25] These observations suggest a possibility that SECs contain a similar type of glycine receptor on the plasma membrane. To clarify whether the antiapoptotic effect of glycine observed here is through this possible glycine receptors, strychnine, a putative glycine receptor antagonist, was tested (Fig. 6). Interestingly, the antiapoptotic effect of glycine was antagonized in part by a low dose of strychnine (1 μmol/L). It is postulated, therefore, that SECs most likely contain a glycine receptor, through which glycine elicits antiapoptotic properties. It is not clear how the glycine receptor modulates apoptotic cell death, but one possibility is that this glycine receptor/chloride channel increased the chloride influx, which may alter the osmolarity of cytoplasm, thereby preventing the shrinkage of cells during the apoptotic process.

On the other hand, Bcl-2, an antiapoptotic protein locating on the mitochondrial membrane,[26] is recognized as one of the key factors in growth factor deprivation–induced apoptosis in endothelial cells. Indeed, it has been reported that VEGF increases the expression of Bcl-2 in human umbilical vein endothelial cells, and the overexpression of Bcl-2 prevents the apoptotic cell death caused by growth factor deprivation in human umbilical vein endothelial cells.[27] Furthermore, the adenoviral gene transfer of Bcl-2 has been shown to reduce ischemia-reperfusion injury in the liver.[28] In the present study, it was demonstrated that Bcl-2 protein levels decreased dramatically after 8 hours of VEGF deprivation in isolated SECs (Fig. 7), which may be one of the key regulating factors of apoptosis under this condition. Surprisingly, glycine prevented this decrease of Bcl-2 protein levels almost completely. Although the precise mechanisms by which glycine prevented the decrease of Bcl-2 is unclear, these findings support the hypothesis that glycine prevents apoptosis of SECs by maintaining Bcl-2 protein levels.

In conclusion, glycine is protective against apoptotic cell death in SECs. Glycine most likely acts through glycine receptor on SECs, and it also prevents the decrease of Bcl-2 under VEGF deprivation. This antiapoptotic effect of glycine in SECs might be involved, at least in part, in the mechanism by which glycine prevents various types of liver injury in which apoptosis of SECs plays an important role.

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