Hypoxia-induced apoptosis in cultured glomerular endothelial cells: Involvement of mitochondrial pathways

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Background. Glomerular endothelial cells (GENs) play a key role in the preservation and reconstruction of the glomerular capillary network following injury, thus maintaining the tissue oxygenation. Accumulating evidence has shown that failure to maintain the microcirculation leads to irreversible glomerular injury and glomerular sclerosis. In this regard, the behavior of endothelial cells in a hypoxic milieu is of interest.

Methods. We exposed cultured GENs to hypoxia and observed apoptosis by annexin V assay. We examined mitochondrial signaling, focusing on Bcl2 and Bax by real-time polymerase chain reaction (PCR), immunocytochemistry, and immunoprecipitation. Furthermore, we examined the response to hypoxia in an overexpression model of Bcl2.

Results. Hypoxic treatment induced apoptosis in 12.8% ± 1.1% of GENs at 24 hours, and in 19.8% ± 0.9% at 24 hours followed by 8 hours of reoxygenation. The expression of Bcl2 mRNA decreased to 0.45 ± 0.15-fold at 24 hours, whereas that of Bax increased to 7.3 ± 1.2-fold 1 hour after reoxygenation, accompanied by translocation from the cytosol to mitochondria. These changes were associated with a decrease in mitochondrial membrane potentials and an increase in caspase-9 activity. Both overexpression of Bcl2 and inhibition of Bax protected GENs from hypoxic injury.

Conclusion. We conclude that changes of quantity and localization of Bcl2 and Bax contribute to hypoxia-mediated apoptosis of GENs in vitro. Further investigation into glomerular endothelial cell injury and intracellular signaling in a hypoxic milieu is required to better understand and ultimately prevent progression of chronic kidney disease.

Key words: hypoxia, apoptosis, glomerular endothelial cells, Bcl2, Bax, mitochondria.

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In this study, we exposed cultured GENs to a hypoxic milieu and observed apoptotic cell death, based on morphologic and biochemical criteria. Further insights into the death signaling have clarified an involvement of the mitochondrial pathways.

METHODS

Cell culture and hypoxia

GENs were isolated and cloned as reported previously [16]. In brief, glomeruli were isolated from male Sprague-Dawley rats by sieving and cultured in hepatoma G2-conditioned MDCB 107 medium and K1 medium at a 1:1 ratio. Outgrowth with the appearance of endothelial cells was isolated, cloned, and characterized by immunostaining with podocalyxin, nephrin, and ED-1 was all negative. Clones were further certified to be of endothelial origin from biochemical aspects, such as uptake of dil-acetyl low-density lipoprotein (LDL) and possession of angiotensin-converting enzyme (ACE) activity. Cells were cultured and maintained in RPMI 1640 (Nissui Seiyaku, Tokyo, Japan) containing 2000 mg/L glucose, supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA) and 10% NuSerum (BD Biosciences, Bedford, MA, USA) at 37°C under a humidified atmosphere of 5% CO2/95% air.

Hypoxic conditions were carried out using Anaerocult A.Mini (Merck, Darmstadt, Germany), which reduces the oxygen content to 0.2% in an hour. GENs were subjected to hypoxia upon reaching 80% confluence and were maintained in serum-free culture medium during the process. After hypoxic stimulation, all samples were processed as quickly as possible, usually within 6 minutes, to minimize the effect of reoxygenation.

Detection of apoptosis

Quantification of apoptotic cells was carried out by annexin V binding assays as described previously [18]. Briefly, hypoxia-treated cells in 60 mm culture plates (Falcon, NJ, USA) were collected in a 15 mL centrifuge tube (TPP, Transadingen, Switzerland). After a wash in phosphate-buffered saline (PBS), cells were double-stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide utilizing the annexin V-FITC Apoptosis Detection Kit (Medical and Biological Laboratories, Nagoya, Japan). Stained cells were later processed by flow cytometry analysis (FACScan and LYSIS II software; both from Beckton Dickinson, Franklin Lakes, NJ, USA). At least 10,000 cells were assayed per condition. The fraction of cells that were annexin V-positive and propidium iodide–negative were considered apoptotic.

To further confirm the apoptotic changes in GENs, propidium iodide–stained cells were visualized with a microscope equipped with fluorescence filters (Olympus, Tokyo, Japan) to observe nuclear changes such as blebbing or chromatin condensation. Furthermore, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining was performed in an attempt to detect internucleosomal DNA breakage. Control or hypoxia-treated cells were collected and fixed in 4% paraformaldehyde for 20 minutes at room temperature, and then attached to glass-slides. After treatment with protease K (10 μg/mL, 37°C, 15 min), samples were preincubated in TdT buffer (140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride, 30 mmol/L Tris-HCl, pH 7.2) for 5 minutes. TdT enzyme (Promega, Madison, WI, USA) and Fluorescein-12-dUTP (Roche, Mannheim, Germany) were added and incubated at 37°C for 1 hour. The reaction was stopped by rinsing the samples with 300 mmol/L NaCl containing 30 mmol/L sodium citrate.

Measurement of Bcl2 and Bax mRNA

To further characterize the expression and localization of Bcl2 and Bax inside the cells, we detected corresponding antigens with fluorescein-labeled antibodies. GENs were seeded on 4-well Lab-Tek chamber slides
Aliquots of protein samples were incubated with 1 l of 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P (NP) 40 lysis buffer (150 mmol/L NaCl, 1% Triton X-100, and 50 mmol/L Tris-HCl, pH 8.0) containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Aliquots of protein samples were incubated with 1 μg of anti-Bcl2 antibody or anti-Bax antibody at 4°C for 1 hour. Immunoprecipitates were captured with 25 μL of protein G sepharose beads (Amersham, Piscataway, NJ, USA) for another hour. The beads were spun down and washed three times with the lysis buffer. Immune complexes were eluted from the beads with 40 μL of sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 minutes, and resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE).

Immunoblot analysis was performed under reducing conditions. Separated samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Tokyo, Japan). Membranes were blocked with 2.5% skim milk and incubated with anti-Bcl2 antibody at 1/100 and alkaline phosphatase-conjugated antiamouse IgG (Promega) at 1/1000, or anti-Bax antibody at 1/200 and alkaline phosphatase-conjugated antirabbit IgG (Promega) at 1/1000. 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (Sigma Chemical Co., St. Louis, MO, USA) were used as a substrate. Coomassie Brilliant Blue (CBB) staining of the membranes confirmed equal loading and transfer.

Measurement of mitochondrial membrane potentials and caspase-9 activity

Changes in mitochondrial membrane potentials were measured semiquantitatively by flow cytometry. Cells treated as indicated were collected in 15 mL polystyrene centrifuge tubes and incubated with 10 μmol/L rhodamine-123 (Wako, Osaka, Japan) for 15 minutes. After a wash in PBS, cells were analyzed by fluorescence-activated cell sorter (FACS).

To measure the caspase-9 activity, a colorimetric assay kit (MBL) was used. In brief, aliquots of 120 μg protein samples were incubated in reaction buffer containing 10 mmol/L dithiothreitol (DTT) at 37°C for 1 hour. A 200 μmol/L ρ-nitroaniline (pNA)–conjugated Leu-Glu-His-Asp (LEHD-pNA) was used as a substrate. By measuring the optic absorbance at 405 nm with a microtiter plate reader, the caspase-9 activity was calculated.

Construction of the Bcl2 overexpression vector and stable transfectants

To ascertain the involvement and antiapoptotic effect of Bcl2 in hypoxia-induced GEN apoptosis, we constructed stable transfectants overexpressing rat Bcl2. An open reading frame coding for the rat Bcl2 was obtained from reverse transcription (RT)–PCR using total RNA of Lewis rat kidney. Primers used here were forward 5'-gcacagATGGCGCAAGCCGGGAGAAC-3' and reverse 5'-gtgtatgTCATTGTGCCCCAGGTATG-3', respectively. PCR reaction was performed at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds for 35 cycles, followed by final extension at 72°C for 7 minutes.

PCR fragments of 725nt were subcloned into pCR2.1 (Invitrogen, San Diego, CA, USA) and analyzed by dideoxy-sequencing. The sequence-confirmed fragment was digested with EcoR1 and ligated to the mammalian expression vector, pcDNA3.1(−) (Invitrogen). The vector plasmid (pcDNA-Bcl2) was stably transfected into GEN using Lipofectamine Reagent (Gibco Life Technologies, Rockville, MD, USA). Briefly, 2.0 × 10^5 GENs were seeded in a 60 mm culture dish. Eighteen to 24 hours later, the cells were washed with PBS and incubated in serum-free culture medium containing 4 μg of the vector plasmid and 8 μg of the cationic liposome for 9 hours. Colonies of cells transfected with pcDNA-Bcl2 and control plasmid (pcDNA-control) were selected in the culture medium containing 1200 μg/mL geneticin (Sigma Chemical Co.) and isolated utilizing cloning cylinders (Iwaki Glass, Chiba, Japan). The stably transfected clones were cultured and maintained in the culture medium containing 600 μg/mL geneticin. The expression level of Bcl2 was analyzed by Western blotting.

Antisense experiments

We investigated the role for Bax by introducing antisense oligonucleotides. GENs seeded on 96-well culture plates (TPP) were transfected with either antisense phosphorothioate oligonucleotide of Bax 5’-TGCTCCC CGGACCGTCCAT-3’ or corresponding sense
Fig. 1. Hypoxia induces apoptosis in glomerular endothelial cells (GENs). (A) When we exposed GENs to 0.2% O₂ for 24 hours (H24), 12.8% ± 1.1% of cells were categorized to apoptosis (P < 0.0001 vs. normoxic control, annexin V assay). In addition, 8 hours of reoxygenation following hypoxia (H24R8) induced a more prominent number of apoptotic cells (19.8% ± 0.9%, P < 0.0001 vs. control). Prolonged hypoxia for up to 32 hours (H32) failed to induce an increase in the number of apoptotic cells (N = 3, annexin V assay). (B) The cell viability was checked by promidium iodide staining. Exposure of GENs to 24 hours of hypoxia (H24) or hypoxia followed by 8 hours of reoxygenation (H24R8) resulted in 38.8% ± 8.3% and 47.5% ± 6.6% of surviving cells, respectively. Prolonged hypoxia up to 32 hours (H32) led to an equivalent number of viable cells (33.8% ± 2.3%) (N = 3). (C) The representative data of the annexin V assay. The cluster of cells in the right lower quadrant was determined apoptotic. FL1 denotes annexin V-fluorescein isothiocyanate (FITC); FL2 denotes promidium iodide (PI). *P < 0.05; **P < 0.01 vs. normoxic control.

Statistical analysis

Stat View version 5.0 (SAS Institute, Cary, NC, USA) was used for statistical analysis. The data obtained here were compared using unpaired Student t tests and expressed as means ± SEM. P values of <0.05 were considered statistically significant. For multiple comparisons, the data were adjusted with Bonferroni/Dunn method.
RESULTS

Hypoxia with or without reoxygenation induces apoptosis in cultured GENs

We tested the hypothesis that cultured GENs might proceed to apoptosis under hypoxic conditions. As seen in Figure 1A, 0.2% O2 for 24 hours (H24) resulted in 12.8% ± 1.1% of GENs becoming apoptotic (P < 0.0001 vs. normoxic control, annexin V assay). Of interest, 8 hours of reoxygenation following hypoxia (H24R8) induced an increase in the number of apoptotic cells (19.8% ± 0.9%, P < 0.0001 vs. control). Prolonged hypoxia for up to 32 hours (H32) failed to exaggerate induction of apoptosis. Representative data of the annexin V assays are shown in Figure 1C.

We also checked cell viability, which includes both apoptosis and necrosis, by propidium iodide staining. Exposure of GENs to 24 hours of hypoxia (H24) alone or followed by 8 hours of reoxygenation (H24R8) resulted in 38.8% ± 8.3% and 47.5% ± 6.6% of surviving cells, respectively. Prolongation of incubation under hypoxic conditions up to 32 hours (H32) did not affect the number of viable cells (33.8% ± 2.3%) compared with that after 24 hours of hypoxia (P = 0.56) (Fig. 1B).

Apoptotic changes were confirmed by nuclear staining and the TUNEL method. When we stained treated H24R8 cells with propidium iodide, a proportion of nuclei displayed nuclear blebbing and chromatin condensation, consistent with the pathologic changes of apoptosis (Fig. 2B), while in control cells, the above changes were absent (Fig. 2A). Furthermore, TUNEL staining showed the presence of nuclear breakage, a hallmark of apoptosis, in hypoxia-treated cells (Fig. 2D). In contrast, few positive stainings for TUNEL were detected in controls (Fig. 2C).

Changes in mRNA expression of mitochondrial pro- and antiapoptotic genes

In an attempt to gain some insights into death signaling, we checked the changes in mRNA expression of Bcl2 and Bax by real-time quantitative PCR. Figure 3A and B represents the temporal profiles of each gene. Twenty-four hours of hypoxia (H24) reduced Bcl2 expression to 0.45 ± 0.15 fold of the control level, although the difference did not reach statistical significance (P = 0.08). Subsequent reoxygenation of 1 to 4 hours did not cause significant changes in Bcl2 mRNA expression. Of interest, 8 hours of reoxygenation resulted in increased expression of Bcl2 mRNA (2.0 ± 0.3 fold, P = 0.005 vs. control, discussed below). In contrast to Bcl2, Bax expression reached its maximum 1 hour after reoxygenation (H24R1) (7.3 ± 1.2 fold, P < 0.0001), and then subsided gradually. With the above observations, we calculated the Bax/Bcl2 ratio, commonly used as an indicator of the death signal mediated by mitochondrial pathways. As shown in Figure 3C, the ratio was 2.6- and 6.7-fold at 24H and H24R1, respectively, returning to baseline 8 hours after reoxygenation.

A decrease in Bcl2 protein during hypoxia, an increase and translocation of Bax after reoxygenation

We further observed the expression of Bcl2 and Bax by immunocytochemistry. Bcl2 shows its signal in the...
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Fig. 3. Changes in Bcl2 and Bax mRNA expression. Changes in mRNA expression of Bcl2 and Bax were measured by real-time polymerase chain reaction (PCR). (A and B) The temporal profile of Bcl2 and Bax, respectively. Twenty-four hours of hypoxia (H24) reduced the Bcl2 expression to 0.45 ± 0.15 fold of the control level, \( P = 0.08 \), not significant. Subsequent reoxygenation of 8 hours (H24R8) resulted in increased expression of Bcl2 mRNA (2.0 ± 0.3 fold, \( P = 0.005 \) vs. control). In contrast to Bcl2, Bax expression reached its maximum 1 hour after reoxygenation (H24R1) (7.3 ± 1.2 fold, \( P < 0.0001 \)), which subsided gradually. (C) The Bax/Bcl2 ratio was 2.6- and 6.7-fold at H24, H24R1, respectively, which returned to the baseline 8 hours after reoxygenation. (N = 4, real-time PCR). **\( P < 0.01 \) vs. normoxic control.

perinuclear area, indicating that Bcl2 is a protein localized in mitochondria. The signal intensity decreased mildly following hypoxic treatment (Fig. 4A and B). Bax, on the other hand, was detected diffusely in the cytosol in normoxic controls, whereas it increased 2 hours after reoxygenation (H24R2). Importantly, the Bax protein changed its staining pattern from a diffuse to a perinuclear localization in some cells, indicating the translocation of Bax from the cytosol to mitochondria during apoptosis (Fig. 4C and D). In Figure 4C and D, double-staining was performed with MitoTracker to more clearly demonstrate the translocation of Bax, upon hypoxic stimulation. Negative controls included omission of the incubation step with the primary antibody, in which no positive staining was observed (not shown). In this study, the morphologic changes of necrotic cells, such as shrinkage and membrane disintegration, were not obvious in hypoxia-treated samples, probably reflecting the fact that dead GENs easily detach from the slides and are lost during staining. In Figure 4E, a representative figure of Western blotting is presented to show the relative decrease in Bcl2 and increase in Bax upon hypoxic stimulation.

To explore interactions between the translocated Bax and mitochondrial Bcl2, we performed immunoprecipitation to document the level of Bcl2-associated Bax.

Both in control and hypoxia-treated samples, anti-Bcl2 antibody coprecipitated Bax protein. However, anti-Bcl2 precipitated quantitatively similar amounts of Bax in each condition. Immunoprecipitation with anti-Bax antibody and subsequent immunoblotting with anti-Bcl2 antibody produced similar results (Fig. 5). Considering the increased expression of Bax, it seems reasonable to speculate that Bax does not form heterodimers with Bcl2 during hypoxia, upon translocation.

Mitochondrial membrane potentials and caspase-9 activity

Changes in mitochondrial membrane potentials were measured quantitatively by rhodamine-123 staining and flow cytometry analysis. Both hypoxia alone (H24) and hypoxia followed by reoxygenation (H24R2) caused a significant decrease in rhodamine-123 uptake, as compared to normoxic controls (Fig. 6A). In addition, we measured the relative caspase-9 activity, a cysteine protease activated upon stimulation of the mitochondria-mediated apoptotic death signal. As shown in Figure 6B, 24 hours of hypoxia (H24) caused a 440% ± 42% increase of caspase-9 activity, whereas hypoxia followed by 2 hours of reoxygenation (H24R2) resulted in a 543% ± 32% increase. Following reoxygenation, the elevated caspase-9 activity persisted throughout the observation period for up to 8 hours of reoxygenation. Prolonged reoxygenation period for up to 16 hours resulted in a more receded activity (220.5% ± 28.7%).

Bcl2 overexpression ameliorates hypoxia-associated GEN injury

The above findings have shown that hypoxia, alone or in combination with reoxygenation, causes GEN
apoptosis through the mitochondrial pathway. In this regard, we hypothesized that overexpression of Bcl2, an antiapoptotic gene, might ameliorate hypoxia-associated cell injury. Figure 7A shows the expression levels of Bcl2 and Bax in control and Bcl2-overexpressing clones in normoxia. The sample of the stable transfectants showed a significant increase in the amount of Bcl2 expression, which was detected as a strong band at 26 kD.

The relative amount of Bcl2 and Bax mRNA in control and Bcl2-overexpressing GENs was examined in normoxia (N), simple hypoxia (H24), and 1 hour following reoxygenation (H24R1). The amount of Bcl2 mRNA was apparently higher in overexpression clones than in control GENs at any condition tested. Interestingly, the expression levels of Bax seemed to be inversely suppressed in Bcl2-overexpressing clones as compared to
control GENs when they underwent hypoxic stimulation. [0.81 ± 0.09fold vs. 1.17 ± 0.35fold at H24 (P = 0.1436), 1.86 ± 0.43fold vs. 7.27 ± 1.25fold at H24R1 (P = 0.0175)].

With the overexpression model, we checked the anti-apoptotic effect of Bcl2 by annexin V assay. As shown in Figure 7C, stable clones with Bcl2 clearly demonstrated an antiapoptotic effect both in simple hypoxia (H24) (5.9% ± 1.7% vs. 12.8% ± 1.1% in control, P = 0.03) and hypoxia followed by reoxygenation (H24R8) (6.4% ± 3.5% vs. 19.8% ± 0.9%, P = 0.02). Figure 7D represents the cytoprotective role for Bcl2, as measured by propidium iodide staining. In both H24 and H24R8 groups, stable transfectants exhibited a cytoprotective effect over control clones. The proportion of surviving cells was 67.4% ± 5.9% as compared to 38.8% ± 8.3% in control (P = 0.049) at H24, and 88.7% ± 3.2% vs. 47.5% ± 6.6% (P = 0.005) at H24R8, respectively.

Figure 7E shows changes in caspase-9 activity. Relative caspase-9 activity was markedly reduced to 246% ± 36% at H24 (440% ± 42% in control, P = 0.0246) and 224% ± 29% at H24R2 (vs. 543% ± 32%, P = 0.002), respectively. Furthermore, the background difference of caspase-9 activity between control and Bcl2-overexpression clones was negligible in normoxia.

During the course of our experiments, we obtained five independent clones carrying the Bcl2-overexpression vector and analyzed two in detail, both of which behaved in the same manner against various degrees of hypoxic stimulation.

**Antisense treatment of Bax contributes to improvement in hypoxia-mediated GEN injury**

For the purpose of looking into the role Bax plays in hypoxia-induced GEN injury, we performed a loss-of-function study by using antisense oligonucleotide targeted against the mRNA sequence 1-20 of rat Bax [19]. Treatment of GENs with 1 μmol/L antisense oligonucleotide resulted in marked suppression of Bax mRNA at 48 hours after delivery (Fig. 8A). When we measured the cell damage by LDH assay, the percentage of LDH released outside the cells was significantly reduced in the antisense oligonucleotide group, both at H24 (14.8% ± 2.5% vs. 25.1% ± 1.7% in control, P = 0.0052) and H24R8 (10.5% ± 0.9% vs. 19.3% ± 2.1%, P < 0.0001) (Fig. 8B). These findings have led us to conclude that the inhibition of Bax is equally important in ameliorating hypoxia-induced GEN injury. It is also worth mentioning that the toxic effect of the sense (control) oligonucleotide was minimal at any time point we adopted. The apparent dissociation in the percentage of dead cells from the results by propidium iodide staining (Figs. 1B and 7C) most probably arises from the difference in the sensitivity of each methodology we used. We avoided using propidium iodide staining in antisense experiments since oligonucleotide delivery by cationic liposomes alone affected the membrane permeability of propidium iodide (not shown). Given these limitations, our findings clearly indicate the significance of Bax in hypoxia-mediated GEN injury.

**DISCUSSION**

In this study, we demonstrated that hypoxia, alone or in combination with reoxygenation, induces apoptotic cell death in cultured GENs. We have also shown that during the course of apoptosis, obvious changes in the expression of Bcl2 and Bax mRNA occur. A decrease in Bcl2 mRNA during simple hypoxia and an increase in Bax mRNA 1 hour after reoxygenation were the most notable quantitative changes during the observation period. The above changes were also demonstrated at the protein level by immunocytochemistry and Western blotting, in which Bcl2 staining turned faint during simple hypoxia, while that of Bax became stronger in some viable cells and translocated from the cytosol to mitochondria in others. Although they seemed to colocalize in mitochondria upon hypoxic stimulation, immunoprecipitation failed to demonstrate an increased amount of Bcl2-Bax heterodimers, similar to the results of Saikumar et al [20]. Furthermore, a decrease in mitochondrial membrane potentials and activation of caspase-9, a cysteine protease located just downstream, ensued, all of which are consistent with the current understanding of apoptotic cell death mediated by the mitochondrial pathway.
When we consider cell injury induced by hypoxia, the controversy arises not infrequently whether the low oxygen content per se induces cellular injury or reoxygenation following hypoxia contributes to the cell damage, and if so, to what degree each of the two distinct mechanisms are involved. In our study utilizing GENs, both of them led to changes in Bcl2 and Bax mRNA expression levels and contributed to apoptotic cell death. The rise in Bax mRNA 1 hour after reoxygenation was the most striking phenomenon during the course, which resulted in a marked increase in Bax/Bcl2 ratio. During the reoxygenation period, the Bax/Bcl2 ratio returned to the baseline 8 hours after reoxygenation. Although Bax mRNA still remained 1.5 ± 0.3 fold increased compared to the control level at that time, Bcl2, an antiapoptotic gene, was also proportionally increased (2.0 ± 0.3 fold vs. control), suggesting an adaptation to minimize the effects of hypoxia. A similar phenomenon was also reported by Misao et al [21], in which they studied autopsied specimens of hearts and detected enhanced Bcl2 protein in salvaged myocytes at the site of infarction. Although the downward caspase-9 activity still remained elevated at the end of the observation period, further examination at a later time course revealed its gradual
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N H24 H24R1

Treatment
9
8
7
6
5
4
3
2
1
0 Fold increase

P = 0.1436

# Control
# Bcl2

α Bcl2

26 kD

α Bax

23 kD

# Control
# Bcl2

Fig. 7. Bcl2 overexpression ameliorates hypoxia-associated cell injury. (A) The expression levels of Bcl2 and Bax in control and Bcl2-overexpressing clones. Stable transfectants exhibited an obviously stronger band at 26 kD (Bcl2) than control glomerular endothelial cells (GENs), while the expression level of Bax remained unaffected in normoxia. (B) The relative amount of Bcl2 and Bax mRNA in control and Bcl2-overexpressing GENs at various conditions. The amount of Bcl2 mRNA was significantly higher in overexpression clones than control GENs at any time point examined, while that of Bax seemed to be suppressed by Bcl2 overexpression under hypoxic conditions (0.81 ± 0.09 fold vs. 1.17 ± 0.35 fold at 24 hours (H24) (P = 0.1436), 1.86 ± 0.45 fold vs. 7.27 ± 1.25 fold at 1 hour of reoxygenation (H24R1) (P = 0.0175) [N = 3, real-time polymerase chain reaction (PCR)]. *P < 0.05; **P < 0.01 vs. control GENs. (C) With this model, we checked the antiapoptotic effect of Bcl2 by annexin V assay. Stable clones with Bcl2 clearly demonstrated an anti-apoptotic effect both in simple hypoxia (H24) (5.9% ± 1.7% vs. 12.8% ± 1.1% in control, P = 0.0271) and hypoxia followed by 8 hours of reoxygenation (H24R8) (6.4% ± 3.5% vs. 19.8% ± 0.9%, P = 0.02). (D) The cytoprotective role for Bcl2 is measured by propidium iodide staining. In both H24 and H24R8 groups, stable transfectants exhibited a consistent cytoprotective effect over control GENs. The proportion of surviving cells was 67.4% ± 5.9% (vs. 38.8% ± 8.3% in control GENs, P = 0.0485) at H24, 88.7% ± 3.2% (vs. 47.5% ± 6.6%, P = 0.0054) at H24R8, respectively (N = 3). *P < 0.05; **P < 0.01 vs. control GENs. (E) Changes in caspase-9 activity. Relative caspase-9 activity was markedly reduced to 246% ± 36% at H24 (vs. 440% ± 42% in control, P = 0.0246) and 224% ± 29% at H24R2 (vs. 543% ± 32%, P = 0.0018), respectively. There was no background difference between control and Bcl2 overexpression clones (N = 3).

decrease. Taken together, it seems a reasonable speculation that the normalized Bax/Bcl2 ratio was the initial termination process in the apoptotic death signaling. It is also important to point out the possibility that the persisting caspase-9 activity either reflects the slow inactivation of caspase-9 itself or suggests the importance of some antiapoptotic substance working downstream of caspase-9, such as inhibitors of apoptosis (IAPs) or heat shock proteins (HSPs), in hypoxia-induced apoptosis [22–24].

The significance of reoxygenation during the apoptotic process [25] suggests the possible involvement of reactive oxygen species (ROS). In support of this view, our preliminary experiments have shown that the stimulation of GENs with hydrogen peroxide for 1 hour (50 to 800 μmol) induces a dose-dependent increase in Bax mRNA, reaching 2.9 fold at 800 μmol.

In fact, mitochondria are the major source of superoxide anion production in cells [26] and the imbalance between production and inactivation of ROS may determine the cell fate under oxidative stress. Therefore, there is a possibility that administration of antioxidants, such as α-tocopherol (vitamin E) and allopurinol, may protect GENs against hypoxic injury, although a caution is required in its interpretation because of the potential problem in in vitro drug delivery [27].

Currently, it is generally accepted that hypoxia stabilizes p53 [28] and results in apoptosis through activation of Bax [29–31] or other mechanisms [32], although there are some controversies whether p53 is required in
the production of apoptosis in cardiac myocytes [33, 34]. There is also evidence of a direct association between p53 and hypoxia-inducible factor 1α (HIF-1α) [35] and that HIF-1α increases p53 levels [36]. In our experimental settings, however, we have not tested so far whether the increased Bax mRNA is associated with HIF-1α and p53.

Besides the mitochondrial pathway we have discussed here, there is another widely recognized apoptotic signaling pathway, the death receptor (Fas-FasL) pathway. Throughout the course of our experiments, we have mainly focused on mitochondria-centered intracellular signaling, which leaves the possibility of involvement of the death receptor pathways in hypoxia-induced endothelial cell death open.

**CONCLUSION**

We have shown that hypoxia, alone or in combination with reoxygenation, induces a significant proportion of apoptosis in cultured GENs. The intracellular signaling was characterized by a decrease in Bcl2 during hypoxia alone, and a marked increase and translocation of Bax immediately after reoxygenation followed by a decrease in mitochondrial membrane potentials and activation of
and observed involvement of mitochondrial pathways [Biochem Biophys Res Comm (in press)].

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