

Overexpression of GD3 synthase induces apoptosis of vascular endothelial ECV304 cells through downregulation of Bcl-2

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Received 25 February 2004; revised 13 April 2004; accepted 13 May 2004

Available online 25 May 2004

Edited by Vladimir Skulachev

Abstract The disialoganglioside GD3 plays a major role in proliferation, differentiation, and apoptosis. It has been reported that ganglioside GD3 can induce apoptosis through bcl-2 mediated mitochondrial pathway. However, the relationship between ganglioside GD3 and B-cell/CLL lymphoma 2 (Bcl-2) is not fully understood. In this study, we have demonstrated that the downregulation of Bcl-2 by overexpression of CMP-NeuAc:GM3 α -2,8-sialyltransferase (GD3 synthase) results in an accelerated apoptosis in vascular endothelial cells (ECV304), as evidenced by DNA fragmentation and caspase-3 activation. In addition, phosphorylation of AKT and cyclic-AMP responsive element binding protein (CREB) was reduced by GD3 synthase overexpression. Moreover, the activation of CREB as a transcriptional factor was also inhibited, as evidenced by electrophoretic mobility shift assay. Therefore, we conclude that GD3 synthase has an apoptotic effect on ECV304 cells through downregulation of Bcl-2 expression via dephosphorylation of AKT and CREB.

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Keywords: GD3 synthase; ECV304 cell; Bcl-2; Apoptosis

1. Introduction

Endothelial cells (ECs) form the lining of blood vessels and regulate vascular function and homeostasis [1]. A number of studies have shown that apoptosis of EC is important in the pathogenesis of cardiovascular diseases, including diabetic microangiopathy, angiogenesis and atherosclerosis [2–5]. Two major apoptosis pathways have been defined in mammalian cells, the death receptor pathway and the mitochondria pathway [5]. The B-cell/CLL lymphoma 2 (Bcl-2) family proteins consist of both anti-apoptotic and pro-apoptotic members that regulate apoptosis, mainly by controlling the release of cytochrome *c* from mitochondria [6]. These two groups of proteins can form both homo- and heterodimers and neutralized each

other's function. Consequently, they can function either independently or in concert to regulate apoptosis [6]. Upregulation of anti-apoptotic protein Bcl-2 expression has been identified as a critical mechanism by which growth factors promote cell survival in vascular EC [7,8]. On the other hand, many apoptotic agents such as hypoxia, cytokines and oxidized low density lipoprotein (LDL) were reported that they lower the expression of Bcl-2 [9–11].

Gangliosides are complex glycosphingolipids containing sialic acid residues and the changes in composition of gangliosides have been observed during cell proliferation, cell cycle phase, brain development and differentiation [12]. Among these gangliosides, the disialoganglioside GD3, which is weakly expressed in most normal tissues, but highly expressed during development and in pathological conditions such as cancer, neurodegenerative disorders and atherosclerosis, is responsible for diverse events such as proliferation, differentiation, and apoptosis [13]. Recently, it was reported that high concentrations of GD3 induce apoptosis in aortic smooth muscle cells through recruiting reactive oxygen species [14]. In addition, ganglioside GD3 can induce apoptosis through mitochondrial pathway [15,16] and the forced expression of Bcl-2 significantly prevents GD3-induced apoptosis [16,17]. But the molecular mechanism of GD3-induced apoptosis is not fully elucidated.

On the basis of these findings reported, we hypothesized that the endogeneously synthesized ganglioside GD3 might act as a pro-apoptotic agent to vascular EC. In this study, therefore, we have demonstrated that the downregulation of Bcl-2 by CMP-NeuAc:GM3 α -2,8-sialyltransferase (GD3 synthase) results in an accelerated apoptosis in vascular endothelial ECV304 cells. In addition, AKT and cyclic-AMP responsive element binding protein (CREB) signaling pathway was inactivated by GD3 synthase overexpression, as evidenced by Western blot analysis. Moreover, the activation of CREB as a transcription factor was also inhibited by GD3 synthase, as evidenced by electrophoretic mobility shift assays (EMSA). This constitutes the first report to demonstrate that GD3 synthase has an effect on the expression of an anti-apoptotic protein Bcl-2.

2. Materials and methods

2.1. Cell culture

ECV304 cells, an immortalized human vascular EC line [18], were obtained from American Type Culture Collection (Rockville, MD). ECV304 cells were cultured in Dulbecco's modified Eagle's medium

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Abbreviations: GD3 synthase, CMP-NeuAc:GM3 α -2,8-sialyltransferase; CREB, cyclic-AMP responsive element binding protein; Bcl-2, B-cell/CLL lymphoma 2; Bax, Bcl-2-associated X protein; TLC, thin layer chromatography; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assays

(DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Gaithersburg, MD) and 1% penicillin/streptomycin (Life Technologies, Inc.), and maintained at 37 °C in an atmosphere of 5% CO₂. For DNA fragmentation assay, the cells were grown in DMEM containing 10% FBS to subconfluent monolayers, washed twice with phosphate-buffered NaCl solution (PBS), and incubated with serum-free DMEM for 48 h.

2.2. Expression plasmids

To construct the GD3 synthase expressing plasmid, a 1.1 kb DNA fragment including the human GD3 synthase coding region was amplified by PCR using primer oligonucleotides (sense 5'-CTAAGCTTATGAGCCCCTGCGGGCGCCGCGACAA-3' and antisense 5'-ATCTCGAGTCTAGGAAGTGGGCTGGAGT-GAGGTATCTTC-3') and human fetal brain cDNA as a template. The sense and antisense primers contain *Hind*III and *Xho*I restriction sites (underlined), respectively. The fragment was purified from 1% agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and digested with the appropriate restriction enzyme, and ligated using T4 ligase (Takara Bio Inc., Shiga, Japan) into pcDNA3 vector, to generate pcDNA-GD3. To identify the constructed vector, restriction mapping and DNA sequencing were carried out.

2.3. Cell transfection and generation of stably transfected cell clones

Transfection of ECV304 cells was performed by electroporation, as previously described [19]. The pcDNA3 and pcDNA-GD3 plasmids were dissolved in PBS at a concentration of 300 µg/ml. A 400-µl aliquot of the cell suspension (1×10^7 cells/ml) was then mixed with 100 µl of the DNA solution in a Gene Pulser Cuvette (0.4 cm² electrode gap; Bio-Rad, Richmond, CA) on ice. Electroporation was carried out with a Bio-Rad Gene-pulser apparatus set to field strength of 300 V/cm (capacitance 500 µF, resistance 300 Ω). The pulse-treated cells were kept on ice for 10 min and transferred to 60-mm Petri dishes containing 37 °C DMEM with 10% FBS and cultured for 48 h. After incubation, the transfected cells were cultured in the presence of 1000 µg/ml G418 (Life Technologies, Inc.). After 21 days in the selective medium, individual G418-resistant colonies were isolated. Three positive clones, which are highly expressing GD3 synthase as determined by reverse transcription-polymerase chain reaction (RT-PCR), were used in further study.

2.4. Assessment of intracellular GD3 levels

Assessment of intracellular GD3 levels was performed as described previously [20]. Cultured cells were washed twice with ice-cold PBS and cells were scraped from the dishes and homogenized. Gangliosides were extracted according to the method of Svennerholm and Fredman [21] and analyzed by high-performance thin layer chromatography (HPTLC) using analytical precoated Silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). All plates were first activated by heating to 100 °C for 30 min. Samples were spotted onto plates with a Hamilton syringe in chloroform:methanol:0.25% KCl (5:4:1, v/v/v). Authentic GD3 (Wako Chemical Co., Osaka, Japan) was used as standard. GD3 was immunodetected by using the 4F6 anti-GD3 monoclonal antibody (1:100; Covalab, Lyon, France). The plates were incubated for 1 h at room temperature with the primary antibody, washed twice with PBS-Tween 20, and then incubated for 45 min at room temperature with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:200; Sigma, St. Louis, MO). The GD3 bands were identified by enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). The bands were scanned and quantified by densitometric analysis using TotalLab software (BioSystematica, Devon, UK) of Frog Gel Image Analysis System (CorebioSystem, Seoul, Korea). The results shown are means of three independent experiments.

2.5. DNA fragmentation assay

Total DNA was extracted according to the method of Lee and Shacter [22]. Briefly, the cultured cells were harvested, washed with PBS, and lysed in buffer containing 10 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% Triton X-100, 200 µg/ml RNase, and 200 µg/ml proteinase K. After DNA was precipitated with isopropanol, it was then resuspended in a Tris-EDTA solution. Samples were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining and UV transillumination. A 1-kb DNA ladder served as a marker for estimating DNA fragment size.

2.6. RT-PCR analysis

Total RNA was extracted from parent, pcDNA3-transfected and pcDNA-GD3-transfected ECV304 cells, using an TRIzol reagent (Invitrogen). Then, 1 µg of RNA from each sample was reverse-transcribed into cDNA, using a Superscript™ pre-amplification system (Invitrogen). The cDNA obtained was further amplified by PCR with the following primers: GD3 synthase (460 bp), 5'-TGTGGT-CCAGAAAGACATTTGTGGA-3' (sense) and 5'-TGGAGTGAGG-TATCTTCACATGGGT-3' (antisense); Bcl-2 (369 bp), 5'-GGAG-GATTGTGGCCTTCTTTGAG-3' (sense) and 5'-TATGCACCCAG-AGTGATGCAGGC-3' (antisense); Bcl-2-associated X protein (Bax) (260 bp), 5'-TGAAGTGGACAACAACATGGAGC-3' (sense) and 5'-GGTCTTGGATCCAGACAAACAGC-3' (antisense); β-actin (275 bp), 5'-CAAGAGATGGCCACGGCTGCT-3' (sense) and 5'-TCCTTCTGCATCCTGTCGGCA-3' (antisense). The use of equal amounts of mRNA in the RT-PCR assays was confirmed by analyzing the expression levels of β-actin. The PCR product of Bcl-2 was quantitated by densitometric analysis.

2.7. Western blot analysis

The cultured cells were homogenized in a sample buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% NaN₃, 100 µg/ml PMSF, 1 µg/ml aprotinin, and 1% Triton X-100. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad). Twenty µg samples of total cell lysates were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes using the Hoefer electrotransfer system (Amersham Biosciences). To detect Bcl-2, Bax, caspase-3, p-AKT, AKT, p-ERK, ERK, p-p38, p38, p-CREB, CREB and GAPDH proteins, the membranes were incubated with the Bcl-2, Bax (Zymed, San Francisco, CA), caspase-3, p-AKT, AKT, p-p38, p38 (Santa Cruz, Santa Cruz, CA), p-ERK, ERK (New England Biolabs, Beverly, MA), p-CREB, CREB (Upstate Biotechnology, Lake Placid, NY) and GAPDH antibodies (Chemicon, Temecula, CA). Detection was performed using a secondary horseradish peroxidase-linked anti-mouse and rabbit antibody, and an enhanced chemiluminescence system (Amersham Biosciences).

2.8. Electrophoretic mobility shift assays

Nuclear extracts from parent, pcDNA3-transfected and pcDNA-GD3-transfected ECV304 cells were prepared as described previously [28]. EMSA was performed using a gel shift assay system kit (Promega) according to the manufacturer's instructions. Briefly, double-stranded oligonucleotides containing the consensus sequence for CREB (5'-ACCGTGTGACGTTACGC-3') were end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase and used as probes for EMSA. Nuclear extract proteins (2 µg) were preincubated with the gel shift binding buffer (4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)) for 10 min and then incubated with the labeled probe for 20 min at room temperature. Each sample was electrophoresed in a 4% non-denaturing polyacrylamide gel in 0.5 × TBE buffer at 250 V for 20 min. The gel was dried and subjected to autoradiography.

3. Results

3.1. Expression of GD3 synthase in ECV304 cells

To investigate the role of GD3 synthase on human vascular EC, pcDNA-GD3 expression vector harboring the human GD3 synthase cDNA and pcDNA3 empty vector were transfected into the ECV304 cells by electroporation. After selection of the G418-resistant colonies, the expression of GD3 synthase mRNA was highly expressed in pcDNA-GD3-transfected cells compared with that in the parent and pcDNA3-transfected cells (Fig. 1A). In addition, in pcDNA-GD3-transfected cells, the overexpression of ganglioside GD3 was also identified by TLC immunostaining with anti-GD3 antibodies (Fig. 1B). These results show that the enzymatically active GD3 synthase is synthesized in the pcDNA-GD3-transfected cells.

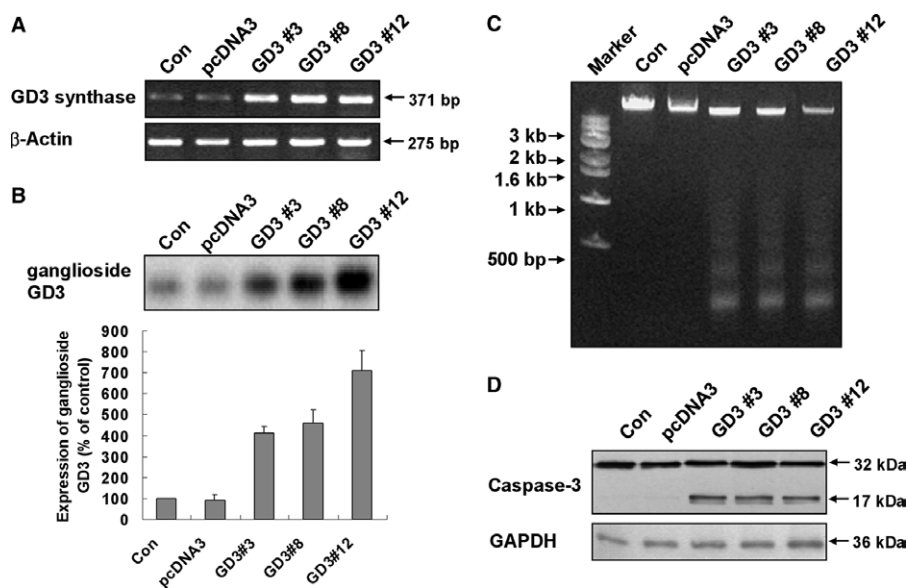


Fig. 1. Apoptosis of ECV304 cells induced by overexpression of GD3 synthase. (A) ECV304 cells were transfected with the pcDNA-GD3 expression vector harboring the human GD3 synthase cDNA. After selection of the G418-resistant colonies, three transfectants (GD3#3, GD3#8 and GD3#12) were compared to parent ECV304 cells (Con) and pcDNA3-transfected cells (pcDNA3) for GD3 synthase expression by RT-PCR analysis, using β -actin as an internal control. (B) A representative TLC immunostained with anti-GD3 antibodies and densitometric analysis of three independent experiments. Values are expressed as percentages of control and present means \pm S.E. (C) After deprivation of serum for 48 h, nucleosomal DNA was isolated and separated by electrophoresis on 1.5% agarose gels. The DNA was stained with ethidium bromide and visualized under UV light. Similar patterns of gel electrophoresis were obtained in three separate experiments. (D) After deprivation of serum for 48 h, the activation of caspase-3 was determined by Western blot analysis. Similar data were obtained in three independent experiments.

3.2. Induction of apoptosis by GD3 synthase overexpression

The DNAs from parent, pcDNA3-transfected and pcDNA-GD3-transfected cells, which were cultured in serum-free condition for 48 h, were resolved by electrophoresis. As shown in Fig. 1C, the agarose gel electrophoresis of DNA isolated from the GD3 synthase overexpressing cells showed a ladder of DNA fragment. In addition, overexpression of GD3 synthase results in caspase-3 activation, as revealed by the detection of the caspase-3 p17 fragment (Fig. 1D). This indicates that GD3 synthase induces the apoptosis of ECV304 cells.

We next examined the molecular mechanisms of apoptosis induced by GD3 synthase overexpression. Especially, we have focused on the Bcl-2 and Bax proteins, which are main regulators in mitochondrial pathway of apoptosis [6]. Interestingly, in pcDNA-GD3-transfected cells, Bcl-2 mRNA and protein levels were significantly decreased, as assayed by RT-PCR and Western blot analysis, whereas no significant change was observed in Bax (Fig. 2). Thus, the ratio of Bcl-2 to Bax was significantly decreased in GD3 synthase transfected cells.

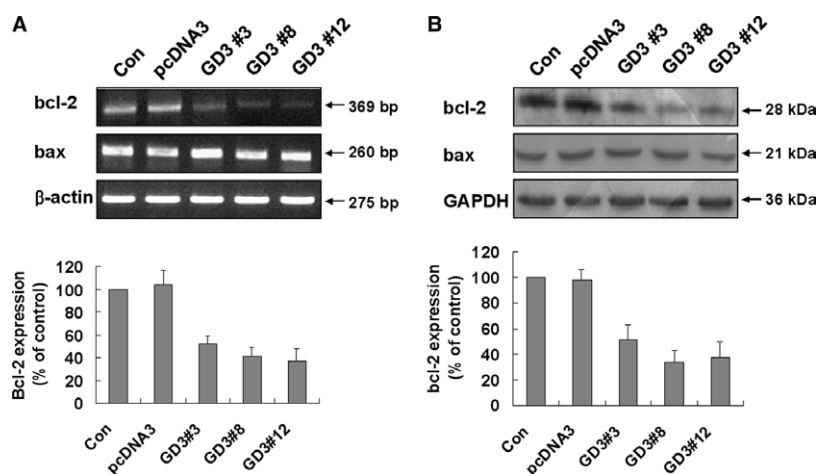


Fig. 2. Overexpression of GD3 synthase downregulated the expression of Bcl-2. Parent (Con), pcDNA3-transfected (pcDNA3) and pcDNA-GD3-transfected (GD3#3, GD3#8 and GD3#12) cells (5×10^5 cells/ml) were cultured for 24 h and washed twice with ice-cold PBS. (A) The mRNA levels of Bcl-2 and Bax were measured by RT-PCR with the total RNA isolated from each cell, using β -actin as an internal control. Densitometric intensities of Bcl-2 bands in three independent experiments were analyzed using the TotalLab software. Values are expressed as percentages of control and present means \pm S.E. (B) Each of the cell lysates with equal protein content (20 μ g) were electrophoresed and immunoblotted for Bcl-2 and Bax using GAPDH as an internal control. The blots were scanned, and the intensities of Bcl-2 bands in three independent experiments were quantitated and expressed using the same methods as above.

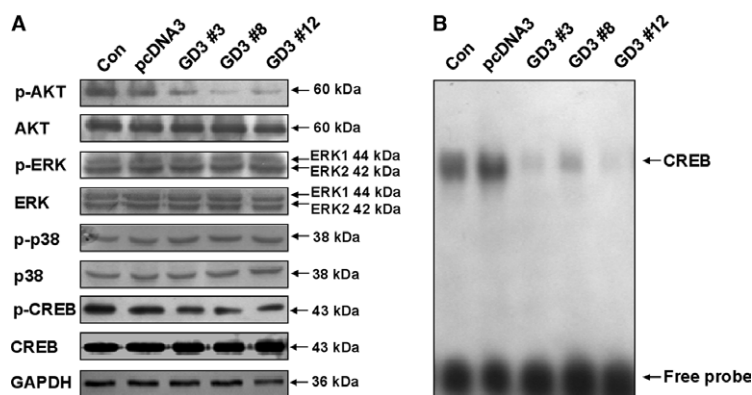


Fig. 3. Overexpression of GD3 synthase inhibited the phosphorylation of AKT and CREB, and translocation of CREB into the nucleus. Parent (Con), pcDNA3-transfected (pcDNA3) and pcDNA-GD3 transfected (GD3#3, GD3#8 and GD3#12) cells (5×10^5 cells/ml) were cultured for 24 h and washed twice with ice-cold PBS. (A) The cell lysates containing equal protein content (20 μ g) were separated by SDS-PAGE and analyzed by Western blotting. (B) The nuclear extracts were incubated with ³²P labeled probe, containing the consensus sequence for CREB from the region of Bcl-2 promoter. The DNA-protein complexes were analyzed on a 4% non-denaturing polyacrylamide gel.

3.3. Inhibition of the activation of AKT and CREB by GD3 synthase overexpression

To investigate molecular mechanism of Bcl-2 downregulation, we have examined the activation of AKT, ERK, p38 and CREB. As shown in Fig. 3A, the expression of AKT and CREB in parent, pcDNA3-transfected and pcDNA-GD3-transfected ECV304 cells was similar, but the phosphorylated AKT and CREB were dramatically decreased in pcDNA-GD3-transfected cells, as evidenced by Western blot analysis. But the expression and activation of ERK and p38 have no significant changes.

To additionally confirm that GD3 synthase overexpression is involved in the CREB-mediated transcriptional activation of Bcl-2, we examined the binding of CREB to oligonucleotides that contain the sequence for the CRE binding site from the Bcl-2 promoter by EMSA. As shown in Fig. 3B, the intensity level of the shifted band in the nuclear lysates from pcDNA-GD3-transfected cells was lower than those for the nuclear lysates from parent and pcDNA3-transfected cells (Fig. 3B). These results suggest that overexpression of GD3 synthase regulates AKT/CREB-mediated transcription of *Bcl-2* gene.

4. Discussion

The disialoganglioside GD3 is synthesized by α -2,8-sialyl-transferase, called the GD3 synthase (ST8Sia I or sialyltransferase II), which is a type II transmembrane protein of approximately 40 kDa, through catalyzing the addition of a second sialic acid residue to one of its immediate precursor GM3 [24,25]. To examine the function of endogenously synthesized GD3 synthase in EC, we constructed GD3 synthase expressing vector, pcDNA-GD3, and transfected the vector into immortalized human umbilical vein EC line, ECV304 cells [18]. The transfected cells highly expressed GD3 synthase and consequently ganglioside GD3 (Fig. 1A and B).

It has been reported that the ganglioside GD3 is weakly expressed in normal vascular tissues and vascular ECs [26,27]. But the expression of ganglioside GD3 is important in the pathologic conditions, including angiogenesis, diabetic microangiopathy and atherosclerosis [28–30]. Moreover, apoptosis

of EC plays a key role in such diseases [2–4,31]. Recently, it was reported that the ganglioside GD3 induces mitochondrial damage and apoptosis [15,16]. Thus, we examined the apoptotic effect of endogenously expressed ganglioside GD3 synthase in ECV304 cells. The agarose gel electrophoresis of DNA isolated from the cells overexpressing GD3 synthase showed a ladder of DNA fragments (Fig. 1C). In addition, the activation of caspase-3 was determined in the cells overexpressing GD3 synthase (Fig. 1D). This indicates that GD3 synthase induces the apoptosis of ECV304 cells.

Three different mechanisms that were involved in GD3-induced apoptosis have been reported, i.e., direct targeting to mitochondria in a Bcl-2-controlled manner [16], suppressing the nuclear factor- κ B-dependent survival pathway [32] and recruiting reactive oxygen species [14]. In the regulatory mechanism of EC apoptosis, Bcl-2 is a well-known inhibitor of apoptosis by forming a dimer with the pro-apoptotic protein, Bax [33]. In addition, several studies have reported that expression of Bcl-2 is suppressed in apoptosis of human vascular ECs [9–11]. Moreover, it was reported that GD3-induced apoptosis was reversed by forced expression of Bcl-2 [15,16]. Therefore, we assumed that the downregulation of Bcl-2 expression might be related with the apoptosis of pcDNA-GD3-transfected cells. As shown in Fig. 2, levels of Bcl-2 mRNA and protein were significantly decreased in pcDNA-GD3-transfected cells compared with those of parent and pcDNA3-transfected cells, whereas no significant change was observed in Bax. Thus, the ratio of Bcl-2 to Bax was significantly decreased in GD3 synthase-transfected cells.

The *Bcl-2* gene is transcriptionally regulated by many transcription factors including CREB, p53, *Wilms' tumor-1* gene and estrogen receptor [34–37]. However, the basal activity of Bcl-2 mediated primarily by CREB [34,38] and CREB mediates the induction of Bcl-2 by a number of growth factors through a signaling pathway mediated by ERK, p38 and AKT [23,39,40]. Therefore, we examined the AKT, ERK and p38 signal pathway. The results showed that the activation of AKT and CREB was dramatically decreased in pcDNA-GD3-transfected cells, whereas the activation of ERK and p38 has no significant change (Fig. 3A). In addition, binding activity of CREB to the CRE site of Bcl-2 promoter was significantly

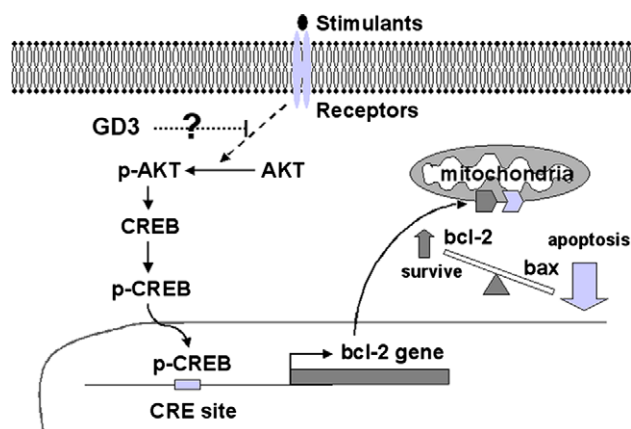


Fig. 4. Schematic representation of ganglioside GD3-induced apoptosis in ECV304 cells. Ganglioside GD3, which is produced by over-expressed GD3 synthase, may downregulate the expression of anti-apoptotic protein, Bcl-2, through inhibition of AKT/CREB signal pathway. The downregulation of Bcl-2, and consequent imbalance between Bcl-2 and Bax can cause apoptosis in ECV304 cells.

reduced in pcDNA-GD3-transfected cells, as evidenced by EMSA (Fig. 3B). These results suggest that endogeneously expressed ganglioside GD3 downregulates AKT/CREB-mediated transcription of *Bcl-2* gene.

In conclusion, the overexpression of GD3 synthase downregulates the expression of anti-apoptotic protein, Bcl-2, through inhibition of AKT and CREB signal pathway. The downregulation of Bcl-2, and consequent imbalance between Bcl-2 and Bax can cause apoptosis in ECV304 cells (Fig. 4). Therefore, this is, to our best knowledge, the first report to demonstrate that endogeneously expressed ganglioside GD3 has an apoptotic effect on ECV304 cells through downregulation of Bcl-2 expression.

Acknowledgements: This work was supported by National Research Laboratory Program, KISTEP, MOST, Korea (No. M10203000024-02J0000-01300 to C.-H. Kim).

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