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Ginsenoside Rh2 Induces Apoptosis Independently of Bcl-2, Bcl-x_L or Bax in C6Bu-1 Cells

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In ginsenoside Rh2-treated rat glioma C6Bu-1 cells, apoptotic morphological changes, such as cell shrinkage, chromatin condensation and pyknosis were confirmed by means of electron microscopy. To evaluate whether induction of apoptosis by ginsenoside Rh2 is mediated by the members of Bcl-2 family, we first established C6Bu-1 cells overexpressing Bcl-2. It was demonstrated that the expression of Bcl-2, Bcl-x_L and Bax was not altered in ginsenoside Rh2-treated C6Bu-1 cells. Bcl-2 overexpressing C6Bu-1 cells failed to prevent from ginsenoside Rh2-induced cell death. These results suggest the existence of other apoptotic pathway that requires induction of apoptosis by ginsenoside Rh2 rather than the pathway through Bcl-2, Bcl-x_L or Bax in C6Bu-1 cells.

Key words: Ginsenoside Rh2, Apoptosis, Bcl-2, Bcl-x_L, Bax, Glioma

INTRODUCTION

Apoptosis is recently implicated for the pathological mechanism for a variety of human diseases including cancer, autoimmune disease, viral infections, neurodegenerative disorders and AIDS (Thompson, 1995). Apoptosis is highly regulated process that involves activation of a cascade of molecular events, leading to cell death. It is characterized by plasma membrane blebbing, shrinkage, chromatin condensation, chromosomal DNA fragmentation, and formation of membrane-bound apoptotic bodies that are eventually phagocytized by neighboring cells (Steller, 1995). Apoptosis is induced by a variety of stimuli, such as Fas ligand, tumor necrosis factor (TNF), DNA-damaging drugs, and radiation (Reed, 1997). Despite of the diversity of apoptosis-inducing stimuli, activation of intracellular cysteine proteases, recently named caspases, plays a provital role in the initiation and execution of apoptosis. It has been identified that their substrates include poly (ADP-ribose) polymerase (PARP), lamins, protein kinase C δ, the 70kDa protein component of the U1 ribonucleoprotein and the catalytic subunit of the DNA-dependent protein kinase (Cohen, 1997).

The *bcl-2* gene was identified as a gene translocated in human follicular B-lymphoma that functions by inhibiting apoptosis (Tsujimoto *et al.*, 1985). Bcl-2 is the prototype of a family of proteins encoded by species from mammals to worm to virus. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Bcl-2 family members come in two functional categories; Bcl-2 subfamily including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1, NR-13, BHRF-1, LMW5-HL, ORF16, KS-Bcl-2, E1B-19K, and Ced-9 can inhibit apoptosis in the face of a wide variety of cytotoxic stimuli while Bax subfamily including Bax, Bak and Bok and BH3 subfamily including Bik, Blk, Hrk, BNIP3, Bim_L, Bad, Bid, and EGL-1 promote apoptosis (Adams and Cory, 1998). Bcl-2-related proteins either suppress or promote apoptosis by interacting with and fuctionally antagonizing each other. The proapoptotic proteins interact with antiapoptotic proteins via their BH3 domain in an antagonistic fashion to regulate cell survival which is determined by the ratio of the level of expression between proapoptotic and anti-apoptototic family (Zha *et al.*, 1996).

It has been shown that Bcl-2, or Bcl-x_L prevents various type of mammalian cells from apoptosis by preventing activation of caspases, indicating Bcl-2 family functions upstream of caspases (Monney *et al.*, 1996; Shimizu *et al.*, 1996; Smyth *et al.*, 1996). Recently, it has been proposed that Bcl-2, Bcl-x_L, and Bax might function as ion channel to regulate apoptosis (Antonsson *et al.*, 1997; Minn *et al.*, 1997; Vander Heiden *et al.*, 1997). In addition, it has been suggested that Bcl-2 have an antioxident activity (Hockenbery *et al.*, 1993).

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Ginsenoside Rh2 isolated from *Panax ginseng* belongs to protopanaxadiol dammarene glycosides. It has been reported that ginsenoside Rh2 induces functional and morphological differentiation of mouse Bl6 melanoma cells (Ota *et al.*, 1987), F9 teratocarcinoma stem cells (Lee *et al.*, 1996), and HL-60 human promyelocytes (Kim *et al.*, 1998). In several cancer cell lines, ginsenoside Rh2 has been shown to arrest cell cycle at the G1 phase and/or to prolong the S phase (Fujikawa-Yamamoto *et al.*, 1987; Lee *et al.*, 1996).

We have previously reported differential role of protein kinase C subtypes in ginsenoside Rh2-induced apoptosis in human neuroblastoma SK-N-BE(2) and rat glioma C6Bu-1 cells (Kim *et al.*, 1998). In the present study, we confirmed apoptotic features by transmission electron microscopy and examined whether ginsenoside Rh2-induced apoptosis is regulated by Bcl-2 family members, such as Bcl-2, Bcl-x_L and Bax in C6Bu-1 cells.

MATERIALS AND METHODS

Materials

Ginsenoside Rh2 from *Panax ginseng* was prepared as previously described (Kim *et al.*, 1998). Fetal bovine serum (FBS), trypsin and Dulbecco's Modified Eagle's Medium (DMEM) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Proteinase K was from USB (Cleveland, OH). Geneticin (G418) was from GIBCO BLR (Grand Island, NY) and monoclonal antibodies to Bcl-2 was purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibodies to Bcl-x_L and Bax were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell culture

Rat glioma C6Bu-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml of penicillin and 50 μg/ml of streptomycin at 37°C in 5% CO_2 humidified atmosphere. Cells (2×10^5/ml) were treated with ginsenoside Rh2 in a final concentration of 0.5% in ethanol. This concentration of ethanol had no effect on cell growth and morphology.

Cell viability

Cells (2×10^5) were plated, maintained and treated in serum-free medium or 3% FBS-containing medium with various concentrations of ginsenoside-Rh2 for the indicated time periods. Cell viability was assayed with the sulforhodamine B (SRB) method (Skehan *et al.*, 1990).

Eletron microscopy

Cells grown on Lab-Tek chambered slides were prefixed in cacodylate-buffered glutaraldehyde (2%), postfixed in 1% osmium tetraoxide, dehydrated in graded series of alcohol, and embedded in Epon (PolyBed 812). Sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss EM-900 electron microscope.

Preparation of Bcl-2 expressing cell lines

A full-length human Bcl-2 expression vector (CMV/Bcl-2) were obtained from Dr. Y. J. Oh at Yonsei University, Seoul. C6Bu-1 cells were trasfected with CMV/Bcl-2 or control expression vector (CMV/Neo) using by SuperFect™ transfection reagent (Qiagen) as recommended by the manufacturer. Subsequently, single neomycin-resistant colonies were selected and expanded in DMEM supplemented with FBS and 600 μg/ml G418. Stable C6Bu-1 cell lines expressing Bcl-2 were further selected and characterized by western blot analysis as described below. The resulting stable cell lines as well as cells expressing the CMV/Neo control vector were used for the present study.

Western blot analysis

Cells (2×10^6) were washed once, sonicated (10 sec, 2cycles) at 4°C in 200 μl of lysis buffer (10 mM Hepes, pH 7.4, 10 mM NaCl, 1 mM EDTA, 0.1 % Triton X-100, 50 mM NaF, 1 mM phenymethylsulphonyl fluoride, 10 μg/μl leupeptin, 20 μg/ml aprotinin) and incubated on ice for 10 min. Cellular lysates were recovered after centrifugation at $10{,}000 \times g$ for 10 min at 4°C. The protein contents were determined by a Bio-Rad protein assay kit with bovine serum albumin as a standard. Equal amounts of protein (10-30 μg) were subjected to 15% SDS-PAGE and transferred to nitrocellulose (Hybond-ECL, Amersham). Bcl-2, Bcl-x_L and Bax were immuno-reacted with an appropriate dilution of primary antibodies to each protein (Bcl-2, 1:500; Bcl-x_L, 1:200: Bax, 1:200) at room temperature for 1 h and incubated with horse-radish peroxidase conjugated anti-mouse IgG antibody (1:1,000 dilution, Amersharm) or anti-rabbit IgG antibody (1:4,000 dilution, Sigma Chemical Co.) as a secondary antibody. Enchanced Chemiluminescence (ECL, Amersharm) was used to reveal antibody binding.

RESULTS

Apoptotic morphology

To characterize morphorogical changes induced by ginsenoside Rh2, C6Bu-1 cells were examined by transmission electron microscopy following exposure with 50 μM ginsenoside Rh2 for 24 h. Fig. 1A showed characteristic features of untreated C6Bu-1 cells. In

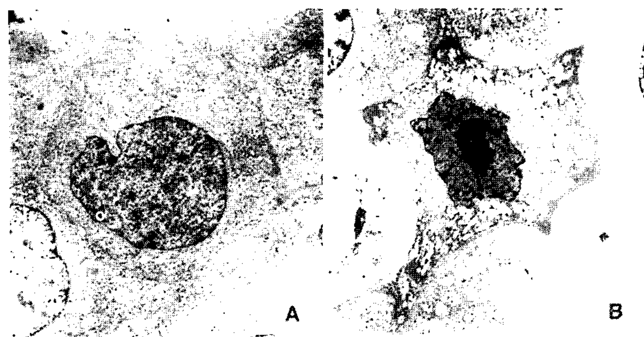


Fig. 1. Ginsenoside Rh2 largely induces apoptotic morphological changes in C6Bu-1 cells. The cells were treated with or without 50 μM ginsenoside Rh2 for 24 h. Samples were fixed and prepared for electron microscopy. A, untreated C6Bu-1 cell (×3,000); B, ginsenoside Rh2-treated C6Bu-1 cell (×4,000).

ginsenoside Rh2-treated cells, several apoptotic features including shrinkage of cellular and nuclear membrane, condenced chromatin around the nuclear periphery, nuclear pyknosis, and vacuoles in cytoplasm appeared (Fig. 1B). Interestingly, typical feature of necrosis such as irregularly scattered heterochromatin was accompanied during ginsenoside Rh2-induced cell death, suggesting that ginsenoside Rh2 induces mixed type of cell death.

Effects on Bcl-2, Bcl-x_L and Bax expression

In order to examine effects of ginsenoside Rh2 on the expression of endogenous Bcl-2, Bcl-x_L or Bax during apoptosis by ginsenoside Rh2, cells were treated with 50 μM ginsenoside Rh2 for 0, 3, 6, 16 and 24 h. As shown in Fig. 2, no significant changes were detected in the expression of Bcl-2, Bcl-x_L or Bax.

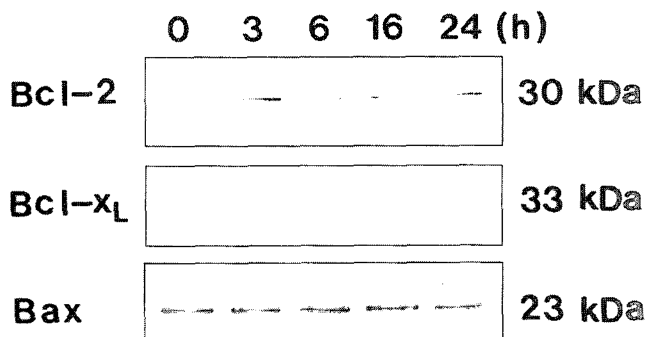


Fig. 2. The expression of Bcl-2, Bcl-x_L or Bax in ginsenoside Rh2-treated with C6Bu-1 cells. The cells were treated with 50 μM ginsenoside Rh2 for 0, 3, 6, 16 and 24 h. Cell lysates were prepared, and subjected to 15 % SDS-PAGE electrophoresis.

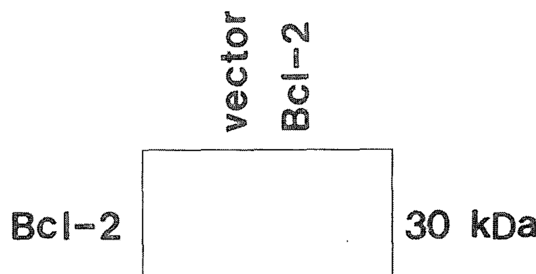
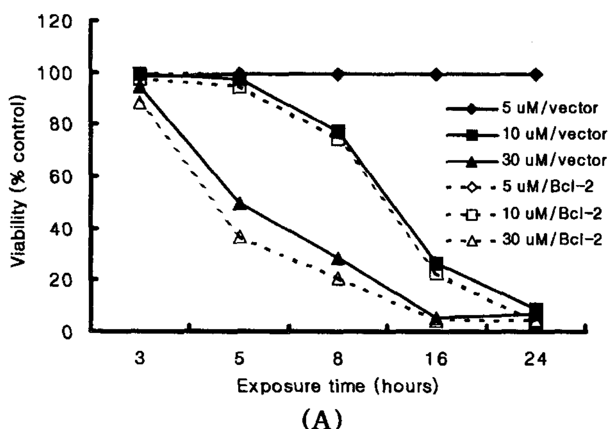


Fig. 3. Western blotting analysis of Bcl-2 expression in vector- or Bcl-2-transfected C6Bu-1 cells.



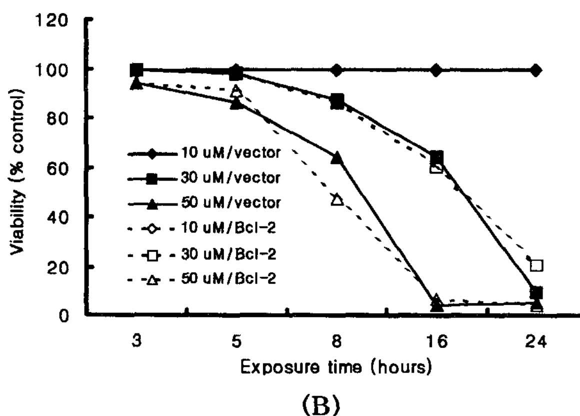


Fig. 4. Cell survival in responses to ginsenoside Rh2. 2×10^5 vector control or Bcl-2 overexpressing C6Bu-1 cells were treated with 5, 10, 30 and 50 μM ginsenoside Rh2 for 3, 5, 8, 16 and 24 h in serum-free media (A) or 3 % FBS-containing media (B). At the indicated times cell viability was assayed by SRB method.

Cell survival in Bcl-2 overexpressing C6Bu-1 cells

To examine whether Bcl-2 inhibits apoptotic cell death induced by ginsenoside Rh2, C6Bu-1 cells overexpressing human Bcl-2 were established. As demonstrated in Fig. 3, expression level in Bcl-2 transfectants was higher than

that in vector alone transfectants. When rate of cell survival was assayed following treatmentat with 5, 10, 30 and 50 μM ginsenoside Rh2 in serum-free or 3% FBS-containing medium, similar pattern of death kinetics were observed between control and Bcl-2 overexpressing cells under both culture conditions (Fig. 4A and B), indicating that Bcl-2 fails to protect the cells from ginsenoside Rh2-induced cell death.

DISCUSSION

The present study demonstated that i) ginsenoside Rh2 induces apoptosis without any alternation of protein level of Bcl-2, Bcl-x_L, and Bax and ii) Bcl-2 overexpression fails to inhibit apoptotic cell death induced by ginsenoside Rh2 in C6Bu-1 cells.

Anti-apoptotic proteins of Bcl-2 family such as Bcl-2 and Bcl-x_L play prominent roles in suppressing apoptosis and consequently enhancing cell survival in response to diverse apoptotic stimuli. On the other hand, Bax and Bak promotes apoptosis. It has been observed that cytochrome c is released from intermembrane space of mitochondria into the cytosol undergoing apoptosis induced by various stimuli including activation of cell surface death receptor such as Fas and TNF, growth factor deprivation, excessive DNA damage, and treatment with chemotherapeutical drugs (Reed, 1997). Both Bcl-2 and Bcl-x_L exert partly their protective roles against apoptosis through their ability to suppress the mitochondrial release of cytochrome c, wheras the pro-apoptotic Bax enhances cytochrome c release (Jürgensmeier *et al.*, 1998; Kharbanda *et al.*, 1997; Yang *et al.*, 1997). It was recently reported that Bax is localized in the cytoplasm and translocates to the mitochondria at the early stage of apoptosis (Zhang *et al.*, 1998; Wolter *et al.*, 1997). The up-regulation of Bax has been found in radiosensitive tissues after γ-irradiation, in lymphoid cells and epithelial cells of the small intestine, and other tissues for examples, in neurons following cerebral ischemia (Brady and Gil-Gōmez, 1998).

Although overexpression of Bcl-2 or Bcl-x_L has been found to inhibit the induction of apoptosis in malignant cells by a large number of agents including a wide variety of chemotherapeutic drugs, Bcl-2 overexpressing C6Bu-1 cells failed to inhibit ginsenoside Rh2-induced apoptosis in this study. Apoptotic cell death induced by a novel retinoid CD437 (6-3-(1-adamantyl)-4 hydroxyphenyl-2-naphtalene carboxylic acid) was shown to fail to prevent by overexpression of Bcl-2 or Bcl-x_L in HL-60 cells and was not accompanied by down-regulation of Bcl-2 or Bcl-x_L or up-regulation of Bax (Fontana *et al.*, 1998). These results were consistent with our data. In human hepatoma SK-HEP-1, cystein protease inhibitors, Ac-YVAD-CMK or Ac-DEVD-CHO, effectively prevented ginsenoside Rh2-induced apoptosis but Bcl-2 overexpression did not (Park *et al.*, 1997). Therefore, it is conceivable to assume that ginsenoside Rh2 induces apoptosis independent of Bcl-2.

The antiapoptotic activities of Bcl-2 and the related protein Bcl-x_L appeared to be regulated through their interaction with the structurally related proteins Bax and Bad (Oltvai *et al.*, 1993; Yang *et al.*, 1995). The ratio of each homodimeric and heterodimeric complex depends on the relative concentration of components. The expression of Bcl-2, Bcl-x_L or Bax was not altered by ginsenoside Rh2-induced apoptosis in C6Bu-1 cells, suggesting that ginsenoside Rh2 may induce apoptosis without any alternation in the heterodimerization between either Bcl-2 or Bcl-x_L complexes with Bax.

When tumor cells were induced to apoptotic cell death after intracellular acidification, Bcl-2 overexpression or interleukin 1β-converting enzyme inhibition did not protect against acid-induced cell killing, whereas acid-induced cell death depends on stress-activated protein kinases (SAPKs) signaling pathways (Zanke *et al.*, 1998), which are activated by cellular stress, such as UV irradiation, heat shock, IL-1, TNF-α, or chemotherapeutics (Kyriakis *et al.*, 1994). It is intriguing to examine whether SAPK activation may be necessary in signaling pathways that lead to cell death by ginsenoside Rh2 in C6Bu-1 cells in further study.

Currently, malignant gliomas are one of the most devastating cancers. The infiltrative growth pattern of these tumors precludes curative neurosurgery, and tumor cells fail to respond to irradiation, chemotherapy, or immunotherapy. Cancer cells have decreased ability to undergo apoptosis in response to some physiological stimuli. Although no currently available *in vivo* and *in vitro* brain tumor model exactly simulates human high grade brain tumor, various approches examining the anti-cancer activity of ginsenoside Rh2 and dissecting the molecular mechanisms associated with its proapoptotic action in brain tumor may be useful tool for developing new therapeutic strategies in the future.

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