Differential Role of Protein Kinase C in Ginsenoside Rh_2 - induced Apoptosis in SK-N-BE(2) and C6Bu-1 Cells

Young Sook Kim, Sung-Ha Jin, You Hui Lee and Shin Il Kim

Korea Ginseng and Tobacco Research Institute, Taejon 305-345, Korea

ABSTRACT

Ginsenoside Rh_2 (G-Rh_2) from *Panax ginseng* induced morphological features of apoptosis and DNA fragmentation as a biochemical marker of apoptosis confirmed by TUNEL reaction and agarose gel electrophoresis in human neuroblastoma SK-N-BE(2) and rat glioma C6Bu-1 cells. During apoptosis by G-Rh_2, protein kinase C (PKC) isoforms were analysed by immunoblotting. In SK-N-BE(2) cells, the levels of α, β and γ subtypes were increased by undergoing apoptosis, while PKC ε isoform increased early in treatment (3 h and 6 h). In addition, PKC ε isoform gradually decreased during apoptosis by G-Rh_2 and PKC θ isoform was detected in neither untreated- nor G-Rh_2-treated SK-N-BE(2) cells (data not shown). However, no significant changes in the level of δ and ε isoforms were observed in C6Bu-1 cells undergoing apoptosis by G-Rh_2. These results suggest that PKC subtypes may play differential roles in apoptotic signal pathways and their roles can be cell type-specific in apoptosis induced by G-Rh_2.

Introduction

Cell death can be distingushed from apoptosis and necrosis. Apoptosis is a form of active cell death, which is characterized by specific biochemical and morphological features such as chromatin condensation, genomic DNA fragmentation, cell shrinkage, membrane blebbing and apoptotic body formation (1, 2). Recent evidences suggest that apoptotic cell death contributes to the pathogenesis of a variety of human diseases including cancer, autoimmune diseases, viral infections, neurodegenerative disorders, AIDS and osteoporosis (3).

In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death. Cancer cells, which fail to regulate cell proliferation and cell death, have a decreased ability to undergo apoptosis in response to some physiological stimuli or external stimuli; this response is mediated through a cascade of signal transductions (4, 5).

Protein kinase C (PKC) is a family of multiple isoenzymes and plays a crucial role in regulation of cellular proliferation and differentiation (6). The PKC family is divided into three subfamilies: conventional (α, βI, βII, γ), novel (ε, δ, η, θ, μ), and atypical (ζ, ι, λ) groups (7). The individual members of the PKC family show different tissue distribution, mode of activation, kinetic properties

and substrate specificities, suggesting that individual or subgroupings of PKC isoenzymes have specific discrete roles within the cells. In addition, many investigations indicate that PKC may modulate apoptosis in various cell types. However, conflicting results have been reported. Therefore, the role of PKC in the regulation of apoptosis and the mechanism of the specific action of PKC remain unclear.

Since it has been reported that G-Rh_2 from *Panax ginseng* causes morphologically and functionally the induction of differentiation in mouse Bl6 melanoma cells (8, 9), G-Rh_2 has been recently reported to differentiate F9 teratocarcinoma stem cells and HL-60 human promyelocytes into parietal endoderm-like cells and phenotypic granulocytes, respectively (10, 11). In Bl6 melanoma, HeLa-S3, K562 and Meth-A cancer cell lines, G-Rh_2 arrested the G1 phase and/or prolonged the S phase (12). In SK-HEP-1 cells, G-Rh_2 arrested the G1/S transition phase by selectively inducing the protein expression of $p27^{kip1}$ (13).

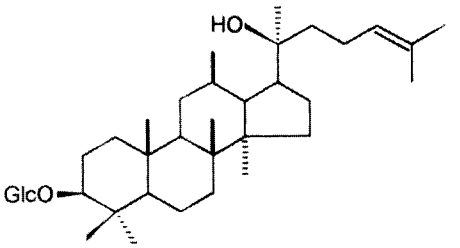

Fig. 1. Chemical structure of ginsenoside Rh_2.

In this study, we have demonstrated that G-Rh_2 induces apoptosis and modulates the expression of PKC isoform during apoptosis by G-Rh_2 in human neuroblastoma SK-N-BE(2) and rat glioma C6Bu-1 cells.

Materials and Methods

Materials

Ginsenoside Rh_2 (G-Rh_2) from *Panax ginseng* was prepared as previouly described (11) and its chemical structure is shown in Fig. 1. Fetal bovine serum, 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). PKC antibodies against peptides unique to α, β and γ isoforms were from GIBCO BLR (Grand Island, NY) and antibodies against δ, ε and θ, isoform specific peptides were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell culture

Human neuroblastoma SK-N-BE(2) and rat glioma C6Bu-1 cells were cultured in DMEM supplemented with 10 % fetal bovine serum, 2mM L-glutamine, 50units/ml of penicillin and 50 μg/ml of streptomycin at 37℃ in 5% CO_2 humidified atmosphere. The cells ($2x10^5$/ml) were treated with G-Rh_2 in the final concentration of 0.5% ethanol. This concentration of ethanol had no effect on cell growth and morphology.

Cell viability & morphology

The cells ($2x10^5$) were plated, maintained and treated in fresh medium with various concentrations of G-Rh_2 for the appropriate time. Cell viability was assayed with the sulforhodamine B (SRB) method (14). Morphology was observed under an inverted phase-contrast microscope with 200x.

Analysis of DNA fragmentation

In individual cells, DNA fragmentation was confirmed by TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick end labeling) reaction *in situ* using a Cell Death Detection kit (Boehringer Mannheim) under a fluorescence microscope (Nikon Microphot FXA). To analyse the DNA ladder by agarose gel electrophoresis, cells ($2x10^6$) were harvested, washed once with ice-cold PBS and resuspended in lysis buffer (10mM EDTA, 50mM Tris-HCl, pH 8.0 and 0.5% SDS containing 0.5mg/ml proteinase K) and incubated for 3hr at 50℃. DNA was extracted with 1 volume of phenol : chloroform (1:1) and then twice extracted with 1 volume of chloroform : isoamyl alcohol (24:1). The aqueous phase was adjusted to 0.5M NaCl and precipitated with 2 volumes of ethanol at -20℃ overnight. The DNA pellet was dissolved in TE buffer, treated with 0.1% RNAase (Promega) at 37℃ for 1hr and 5 μg DNA was electrophoresed on a 1.2% agarose gel containing 1 μg/ml of ethidium bromide. The DNA was visualized by UV illumination.

Immunoblotting anaylsis

The cells (2×10^6) were washed once, sonicated (10sec, 2cycles) at 4℃ in 200μl of extraction buffer (10mM Hepes, pH 7.4, 10mM NaCl, 1mM EDTA, 0.1%Triton X-100, 50mM NaF, 1mM phenylmethylsulphonyl fluoride, 10μg/ml leupeptin, 20μg/ml aprotinin) and incubated on ice for 10min. Cellular lysate was recovered after centrifugation at 12,000rpm for 10min at 4℃. The protein was determined by a Bio-Rad protein assay kit with bovine serum albumin as the standard. Equal amounts of protein (10~20μg) were subjected to 10% SDS-PAGE and transferred to nitrocellulose (Hybond-ECL, Amersham). Individual PKC isoform was immunoreacted with an appropriate dilution of primary antibodies to the individual PKC isoenzyme (α, β, γ, 1:500; δ, ε, θ; 1:200) at room temperature for 1h and incubated with a 1:1,600 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Sigma Chemical Co.) as a secondary antibody. Enchanced

Chemiluminescence (ECL, Amersharm) was used to reveal antibody binding.

Results

Apoptosis induced by G-Rh$_2$

When SK-N-BE(2) and C6Bu-1 cells were treated with 10, 20, 30, 40, and 50 μM G-Rh$_2$ for 3, 6, 12, 15, or 24 h, respectively, cell viability in two cell lines was markedly decreased following treatment with 40 and 50 μM G-Rh$_2$ (Fig. 2). In SK-N-BE(2) and C6Bu-1 cells treated with 50 μM G-Rh$_2$ for various times, morphological changes, cell shrinkage, membrane blebbing and apoptotic bodies were observed and Fig. 3 shows the data at 16 h treatment. However, simultaneous treatment with 10 μM cycloheximide and 50 μM G-Rh$_2$ in SK-N-BE(2) and C6Bu-1 cells for 16 h inhibited apoptotic

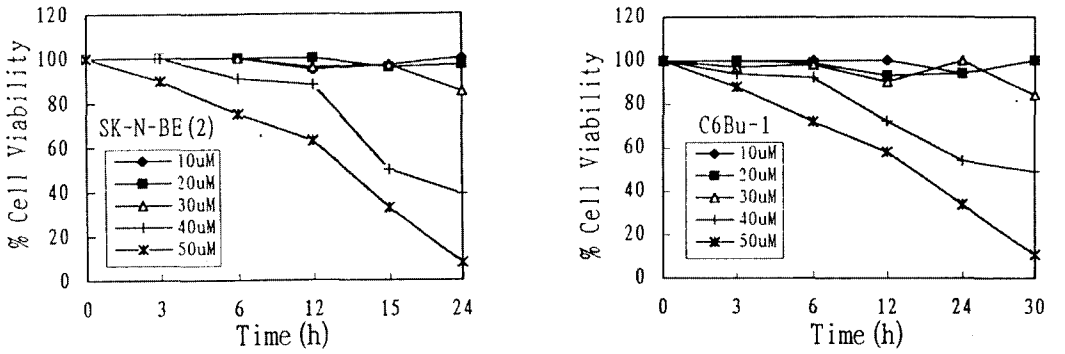

Fig. 2. The viability of SK-N-BE(2) and C6Bu-1 cells following treatment with ginsenoside Rh$_2$. The cells ($2x10^5$/ml) were plated, maintained for 24 h and treated with ginsenoside Rh$_2$ for 0, 3, 6, 12, 15, 24, or 30 h. The viability was determined with the SRB method. Data from three independent cultures (triplicated wells for each condition) are expressed as the mean.

morphological characteristics induced by G-Rh$_2$ (Fig. 3E, F). Genomic DNA from cells exposed to 50 μM G-Rh$_2$ for 0, 3, 6, 16, 24 or 30 h was subjected to agarose gel electrophoresis. As shown in Fig. 4, internucleosomal DNA fragmentation was induced by increasing the treated time in SK-N-BE(2) and C6Bu-1 cells treated with G-Rh$_2$. These results accompany the decrease in cell viability and the induction of apoptotic morphological features by G-Rh$_2$. Also, when DNA fragmentation was detected at the single cell level using *in situ* TUNEL staning , fluorescences in nuclei indicating DNA strand breaks in apoptotic cells were observed in two cancer cell lines 3 h after exposure to 50 μM G-Rh$_2$ (Fig. 5). These results reveal that G-Rh$_2$ induces apoptosis in SK-N-BE(2) and C6Bu-1 cells.

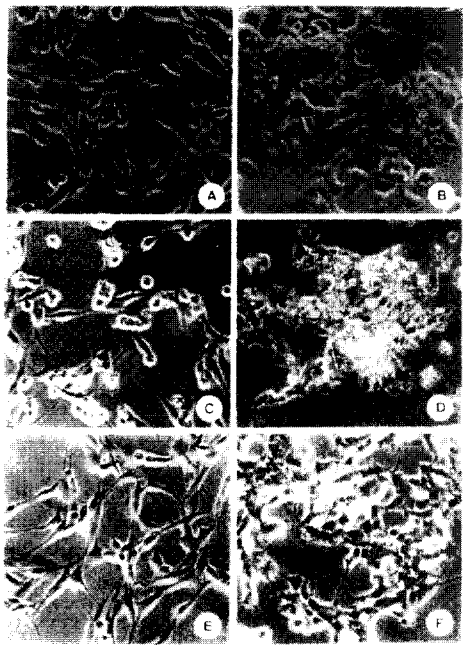

Fig. 3. Morphological changes induced by ginsenoside Rh_2 in C6Bu-1 and SK-N-BE(2) cells. The cells ($2x10^5$/ml) were treated with or without 50 μM ginsenoside Rh_2 or 10 μM cycloheximide for 16 h. A, C, E; C6Bu-1cells, B, D, F; SK-N-BE(2) cells, A, B; untreated control, C, D; treatment with ginsenoside Rh_2 for 16 h, E, F; simultaneous treatment with cycloheximide and ginsenoside Rh_2 for 16 h, arrows; apoptotic features.

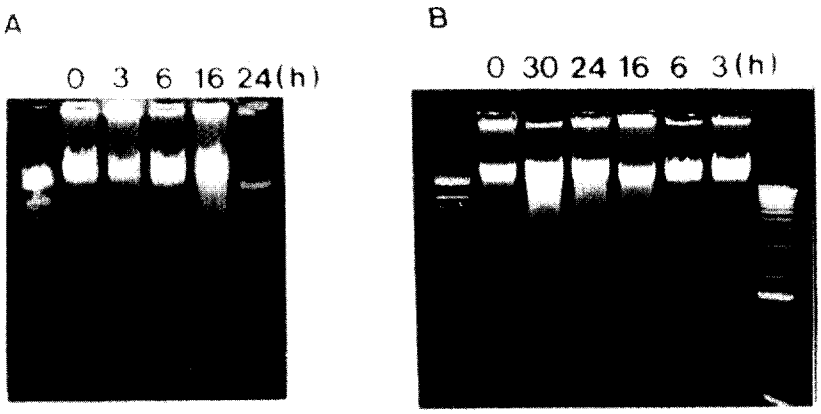

Fig. 4. DNA fragmentation in ginsenoside Rh_2-treated SK-N-BE(2) (A) and C6Bu-1 cells (B). The cells were treated with 50 μM ginsenoside Rh_2 for 0, 3, 6, 16, 24, or 30 h and then genomic DNA was prepared and analyzed on 1.2% agarose gel electrophoresis.

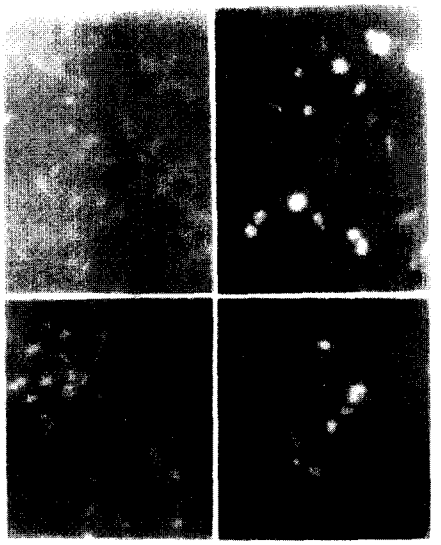

Fig. 5. TUNEL staining of ginsenoside Rh_2-treated cells. The cells were treated with or without 50 μM ginsenoside Rh_2 for 3 h. A, B; C6Bu-1 cells, C, D; SK-N-BE(2) cells, A, C; untreated control, B, D; ginsenoside Rh_2-treated cells, arrow heads; normal, arrows; DNA fragmentation.

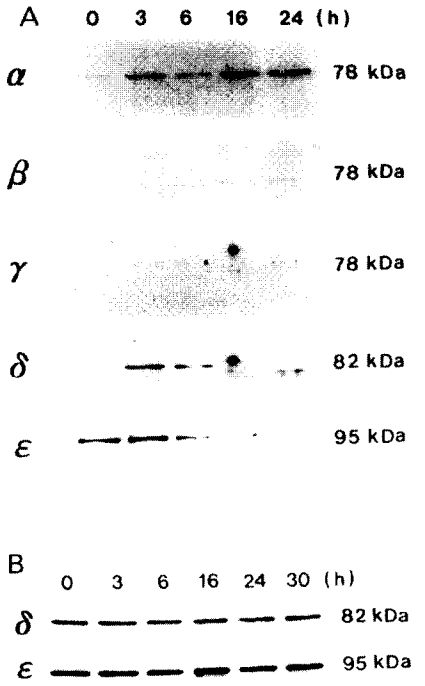

Fig. 6. Immunoblotting analysis of PKC isoforms in ginsenoside Rh_2- treated SK-N-BE(2) (A) and C6Bu-1 Cells (B). The cells were treated with 50 μM ginsenoside Rh_2 for 0, 3, 6, 16, 24, or 30 h, and then prepared cellular lysate. Equal amounts of protein were subjected to 10% SDS-PAGE electrophoresis. Immunodetection for each PKC isoform was performed.

To understand the role of PKC in apoptosis induced by G-Rh_2, the expression of PKC isoforms in SK-N-BE(2) and C6Bu-1 cells undergoing apoptosis by treatment with 50 μM G-Rh2 was analysed by immunoblotting. As shown in Fig. 6, the level of PKC α, β and γ isoforms was increased with concomitant apoptosis by G-Rh_2, while that of δ isoform was selectively increased early in the treatment, at 3 h and 6 h, and ϵ isoform gradually decreased during the apoptotic process in SK-N-BE(2) cells (Fig. 6A). However, in C6Bu-1 cells undergoing apoptotic cell death by G-Rh_2, the expression of δ and ϵ isoforms did not change (Fig. 6B). PKC θ isoform was detected in neither untreated- nor G-Rh_2-treated SK-N-BE(2) cells (data not shown).

Discussion

The differential role of PKC isoforms in apoptosis has been demonstrated; PKC α and β expession was most intense in human tonsil epitherial cells lacking bcl-2, which is a survival protein and the cells were entering apoptosis (15). Increased PKC β and reduced ζ expression were detected in spontaneous apoptotic U937 cells with changes in the intracellular location of PKC α and δ isoforms (16). Other studies of HL60 cells (17) and U937 cells (18) using a selective activator of PKC β I suggested PKC β is a key regulator in myeloid cell apoptosis. PKC ζ cells, which overexpressed the atypical ζ isoform, was found to have an increased level of α and β isoforms (19). TPA treatment of PKC ζ cells induced apoptotic cell death rather than differentiation, suggesting activation of a conventional isoform was necessary to induce apoptosis. It has been reported that PKC δ was proteolytically activated by ICE-like protease in ionizing radiation- and 1-β-D-arabinofuranosylcytosine (ara C) -induced apoptotic U937 cells (20, 21). The preferential downregulation of PKC ϵ was required for the suppression of chemotherapeutics-induced apoptosis in DU-145 human prostatic carcinoma (22) and sensitization of human HT58 B lymphoblastic cells to apoptosis (23). In addition, atypical PKC ι served to protect K562 cells against okadaic acid- and taxol-induced apoptosis, surpporting a role for PKC ι in leukemia cell servival (24). To learn the role of PKC in regulation of apoptosis PKC activators and inhibitors have been employed. However, the effect of these agents on apoptotic events in various cell types appears to vary with cell type and the individual apoptotic stimulus. These results may be due to the differential modulation of PKC isotypes with their separate and unique function in different cells. Although overall, these findings suggest that PKC isoform may be essentially responsible for apoptosis by a variety of stimuli, current understanding of how specific PKC isoforms regulate certain cell function is unclear. P53 (25) and Bcl-2 (26), regulators of apoptosis and nuclear lamim B (27) are substrates for PKC. Alternation in distribution of PKC isoforms in apoptotic cells and their substrates imply a possible role for PKC isoform.

We found in this study that G-Rh_2 has a capacity to induce apoptosis in SK-N-BE(2) and C6Bu-1 cells but distinctly modulates PKC isoforms in this process. In SK-N-BE(2) cells the expression of

conventional PKC α, β and γ increased when maximal apoptosis is achieved by G-Rh_2, while novel PKC ε isoform gradually decreased and novel PKC υ increased early in processed apoptosis. However, no significant change was observed in PKC υ and ε levels during apoptosis of C6Bu-1 cells. These results suggest that the role of each PKC isoform may be different in apoptosis by G-Rh_2 and involvement of PKC in the regulation of apoptosis may be dependent on cell type. In addition, G-Rh_2 can induce apoptosis via different signal pathways in different cell lines. Recently, it has been reported that inhibition of PKC β II by 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) enhanced ara-C-induced apoptosis through the reduction of bcl-2 level, suggesting the diacylglycerol formed in response to ara-C activates this signaling pathway (28). It is interesting whether modulation of PKC has an effect on the level of Bcl-2 and alternation in protein phosphorylation by PKC during apoptosis by G-Rh_2 in further study.

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