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Differential Expression of Protein Kinase C Subtypes during Ginsenoside Rh2-Induced Apoptosis in SK-N-BE(2) and C6Bu-1 Cells

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We examined the modulation of protein kinase C (PKC) subtypes during apoptosis induced by ginsenoside Rh2 (G-Rh2) in human neuroblastoma SK-N-BE(2) and rat glioma C6Bu-1 cells. Apoptosis induced by G-Rh2 in both cell lines was confirmed, as indicated by DNA fragmentation and *in situ* strand breaks, and characteristic morphological changes. During apoptosis induced by G-Rh2 in SK-N-BE(2) cells, PKC subtypes α, β and γ were progressively increased with prolonged treatment, whereas PKC δ increased transiently at 3 and 6 h and PKC ε was gradually down-regulated after 6 h following the treatment. On the other hand, PKC subtype ζ markedly increased at 24 h when maximal apoptosis was achieved. In C6Bu-1 cells, no significant changes in PKC subtypes α, γ, δ, ε and ζ were observed during apoptosis induced by G-Rh2. These results suggest the evidence for a possible role of PKC subtype in apoptosis induced by G-Rh2 in SK-N-BE(2) cells but not in C6Bu-1 cells, and raise the possibility that G-Rh2 may induce apoptosis via different pathways interacting with or without PKC in different cell types.

Key words: Ginsenoside Rh2, Apoptosis, Protein kinase C, SK-N-BE(2) cells, C6Bu-1 cells

INTRODUCTION

Apoptosis is a form of active cell death, which is characterized by specific biochemical and morphological changes such as chromatin condensation, genomic DNA fragmentation, cell shrinkage, membrane blebbing and formation of membrane-bound apoptotic bodies that are eventually phagocytized by neighboring cells (Vaux *et al.*, 1994; Steller, 1995). 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 (Thompson, 1995).

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 or external stimuli; this response is mediated through a cascade of signal transductions (Thompson, 1995; Hoffman and Liebermann, 1994). Treatments that restore the ability to properly regulate apoptosis could be of considerable benefit in controlling some malignancies.

Protein kinase C (PKC) is a family of multiple isoenzymes and plays a crucial role in regulation of cellular proliferation and differentiation (Nishizuka, 1986). 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 (Nishizuka, 1992). The individual members of the PKC family show different tissue distribution, mode of activation, kinetic properties and substrate specificities, suggesting that individual or subgroups of PKC isoenzymes play discrete roles within the cells.

To elucidate a role of PKC in the regulation of apoptosis, many investigators modulated PKC activity using its activators, inhibitors or down-regulation in various model systems. However, the effect of these agents on apoptotic events in various cell types appears to vary with cell type and the individual apoptotic stimulus. Recently, it has been reported a role of PKC subtypes in various cell types. Furthermore, translocation of PKC to cellular sites and phosphorylation of the regulators of apoptosis or substrates of caspases,

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such as Bcl-2, p53 or lamins by PKC during apoptosis play important role in regulation of apoptosis. Therefore, different and conflicting effects of PKC on apoptosis in various model systems have been complicated to define the role of PKC in the regulation of apoptosis and the mechanism of the specific action of PKC or its subtypes.

It has been reported that G-Rh2, dammarane glycoside from *Panax ginseng* induced differentiations of mouse Bl6 melanoma cells (Odashima *et al.*, 1985; Ota *et al.*, 1987), F9 teratocarcinoma stem cells (Lee *et al.*, 1996), and HL-60 human promyelocytes (Kim *et al.*, 1998). In Bl6 melanoma, HeLa-S3, K562 and Meth-A cancer cell lines, G-Rh2 has been shown to arrest them at the G1 phase and/or to prolong the S phase (Fujikawa-Yamamoto *et al.*, 1987). In SK-HEP-1 cells, G-Rh2 arrested the cell cycle at the G1/S transition phase by selectively inducing the protein expression of $p27^{kipl}$ (Lee *et al.*, 1996) and induced apoptosis independently of Bcl-2 as well as in C6Bu-1 cells (Park *et al.*, 1997; Kim *et al.*, 1999).

In this study, we have demonstrated that the expression of PKC subtypes was modulated differently during apoptosis induce by G-Rh2 in human neuroblastoma SK-N-BE(2) and rat glioma C6Bu-1 cells.

MATERIALS AND METHODS

Materials

G-Rh2 from *Panax ginseng* was prepared as previously described (Kim *et al.*, 1998) and its chemical structure is shown in Fig. 1. Fetal bovine serum, trypsin and Dulbecco's Modified Eagles 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, γ and ζ subtypes were from GIBCO BLR (Grand Island, NY) and antibodies to PKC δ, ε and θ against subtype 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, 2 mM L-glutamine, 50 units/ml of penicillin and 50 μg/ml of streptomycin at 37°C in 5% CO_2 humidified atmosphere. The cells (2×10^5 cells/ml) were treated with G-Rh2 in a final concentration of 0.5% ethanol. This concentration of ethanol had no effect on cell growth and morphology.

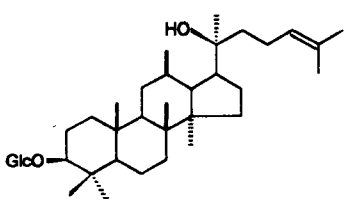

Fig. 1. Chemical structure of ginsenoside Rh2(G-Rh2).

Cell viability & morphology

The cells (2×10^5 cells/ml) were plated, maintained and treated in fresh medium with various concentrations of G-Rh2 for the indicated period. Cell viability was assayed with the sulforhodamine B (SRB) method (Skehan *et al.*, 1990). Morphology was observed under an inverted phase-contrast microscope with a 200× magnification.

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 analyze the DNA ladder by agarose gel electrophoresis, cells (2×10^6 cells/ml) were washed once with ice-cold PBS, resuspended in lysis buffer (0.5% Triton X-100, 5 mM Tris buffer, pH 7.4, and 20 mM EDTA) and incubated for 30 min at 4°C. Cell lysates were centrifuged for 15 min at 4°C. Soluble DNA was extracted with phenol-chloroform, precipitated in ethanol and treated with 1 mg/ml DNase-free RNAase (Promega) at 37°C for 1 h. Samples were electrophoresed on a 1.5% agarose gel containing 1 μg/ml of ethidium bromide. The soluble DNA was visualized by UV illumination and photographed using Polaroid 667 film.

Immunoblot analysis

The cells (2×10^6 cells/ml) were washed once with ice-cold PBS, sonicated (10 sec, 2 cycles) at 4°C in 200 μl of extraction buffer (10 mM Hepes, pH 7.4, 10 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 50 mM NaF, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin), and incubated on ice for 10 min. Cellular lysate was recovered after centrifugation at 10,000 × g for 10 min at 4°C. The protein was determined by a Bio-Rad protein assay kit with bovine serum albumin as the standard. Equal amounts of protein (10~20 μg) were subjected to 10% SDS-PAGE and transferred to nitrocellulose (Hybond-ECL, Amersham). Individual PKC subtype and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were immunoreacted with an appropriate dilution of primary antibodies to the individual PKC subtypes (α, β, γ, ζ, 1:500; δ, ε, θ; 1:200) and GAPDH (1:500) at room temperature for 1 h and incubated with a 1:1,600 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Sigma Chemical Co.) as a secondary antibody. Enhanced Chemiluminescence (ECL, Amersharm) was used to reveal the specific bands of PKC subtypes.

RESULTS

Apoptosis induced by G-Rh2

When SK-N-BE(2) and C6Bu-1 cells were treated with 10, 20, 30, 40, and 50 μM G-Rh2 for 3, 6, 12, 15 or 24 h, viability in both two cell lines was markedly decreased following treatment with 40 and 50 μM G-Rh2 (Fig. 2). In SK-N-BE(2) and C6Bu-1 cells treated with 50 μM G-Rh2 for various times, cell shrinkage was obvious, as shown for treatment at 16 h (Fig. 3C, D). However, simultaneous treatment with 10 μM cycloheximide and 50 μM G-Rh2 in SK-N-BE(2) and C6Bu-1 cells for 16 h prevented morphological characteristics of apoptosis induced by G-Rh_2 (Fig. 3E, F). Soluble DNA from cells exposed to 50 μM G-Rh2 for 0, 6, 8, 16, 24 or 30 h was subjected to agarose gel electrophoresis. As shown in Fig. 4, DNA fragmentation increased in SK-N-BE(2) and C6Bu-1 cells in a time-dependent manner. These results agreed with the decrease in cell viability and the induction of apoptotic morphological features shown above. The *in situ* TUNEL staining also indicated DNA strand breaks in apoptotic cells 3 h after exposure to 50 μM G-Rh2 (Fig. 5).

PKC subtypes during apoptosis induced by G-Rh2

To compare modulation of PKC subtypes during apo-

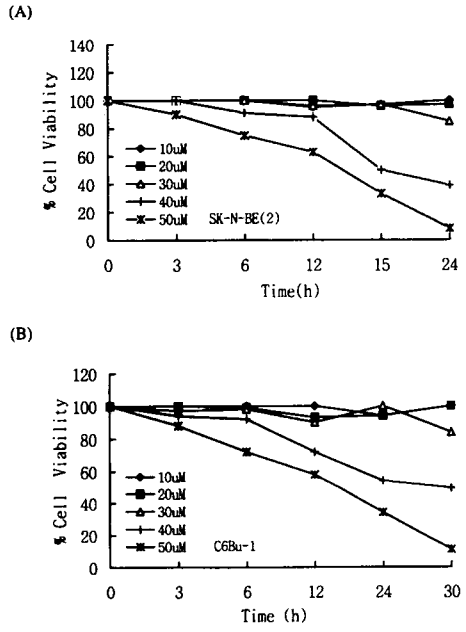

Fig. 2. The viability of SK-N-BE(2) (A) and C6Bu-1 cells (B) following treatment with ginsenoside Rh2. The cells (2×10^5/ml) were plated, maintained for 24 h and treated with ginsenoside Rh2 for 0, 3, 6, 12, 15, 24 or 30 h. The viability was determined with the SRB method. Data from three independent cultures (triplicated wells for each condition) are expressed as the mean.

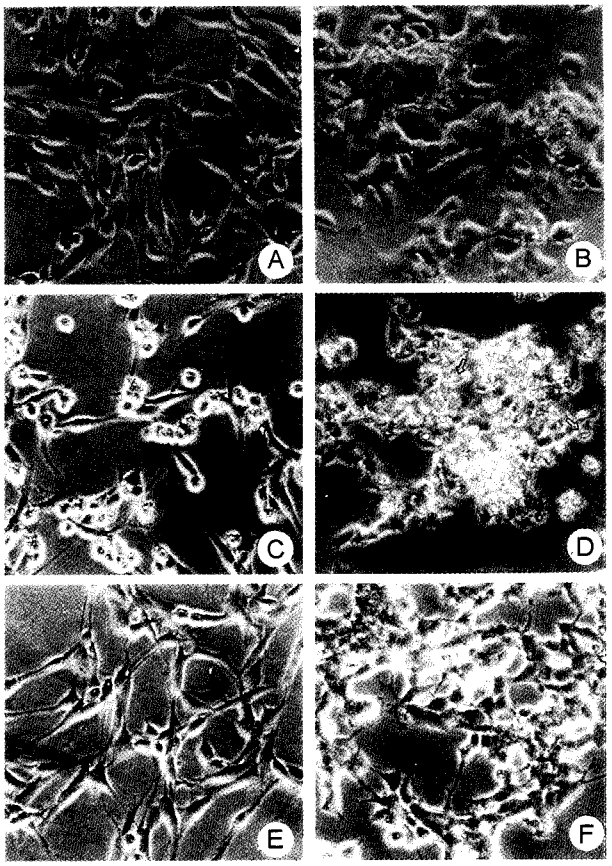

Fig. 3. Morphological changes induced by ginsenoside Rh2 in C6Bu-1 and SK-N-BE(2) cells. The cells (2×10^5/ml) were treated with or without 50 μM ginsenoside Rh2 with or without 10 μM cycloheximide for 16 h. A, C, E; C6Bu-1cells, B, D, F; SK-N-BE(2) cells, A, B; untreated control, C, D; treatment with ginsenoside Rh2 for 16 h, E, F; simultaneous treatment with cycloheximide and ginsenoside Rh2 for 16 h, arrows; apoptotic features (magnification × 200).

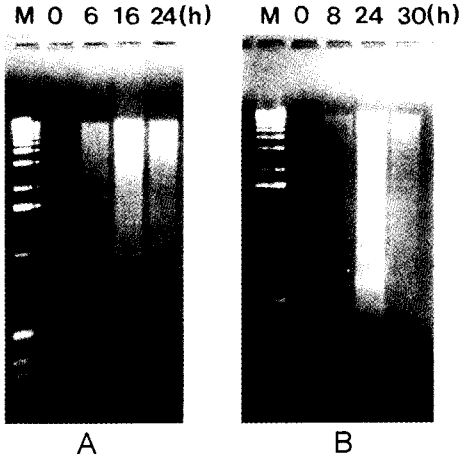

Fig. 4. DNA fragmentation in ginsenoside Rh2-treated SK-N-BE (2) (A) and C6Bu-1 cells (B). The cells were treated with 50 μM ginsenoside Rh2 for 0, 6, 8, 16, 24 or 30 h and then soluble DNA was analyzed on 1.5% agarose gel electrophoresis. M; DNA size markers.

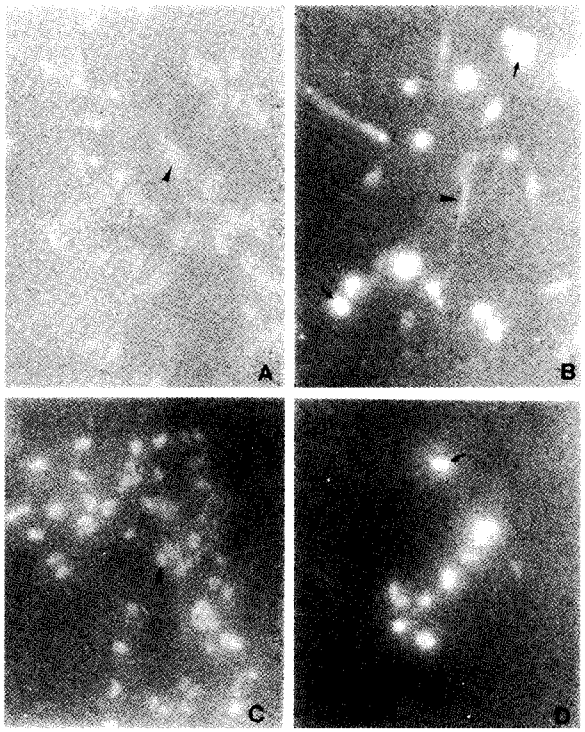

Fig. 5. TUNEL staining of ginsenoside Rh2-treated cells. The cells were treated with or without 50 μM ginsenoside Rh2 for 3 h. A, B; C6Bu-1 cells, C, D; SK-N-BE(2) cells, A, C; untreated control, B, D; ginsenoside Rh2-treated cells, arrow heads; normal, arrows; Fragmented and/or condensed nuclei.

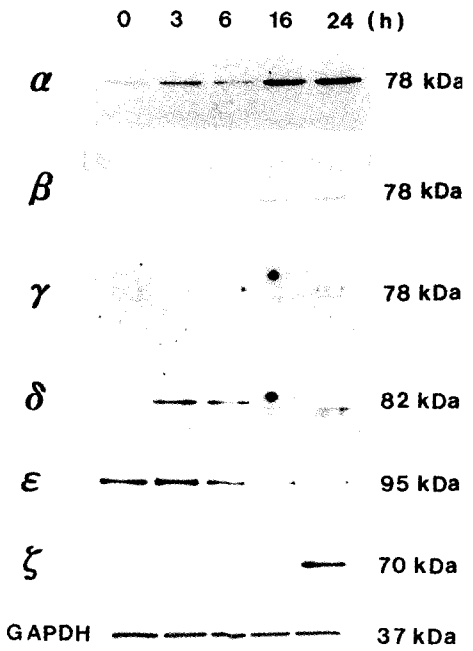

Fig. 6. Immunoblot analysis of PKC subtypes in ginsenoside Rh2- treated SK-N-BE(2). The cells were treated with 50 μM ginsenoside Rh2 for 0, 3, 6, 16 or 24 h. Equal amounts (10~20 μg) of protein were subjected to 10% SDS-PAGE electrophoresis. Immunodetection for each PKC subtype was performed as described in Materials and Methods.

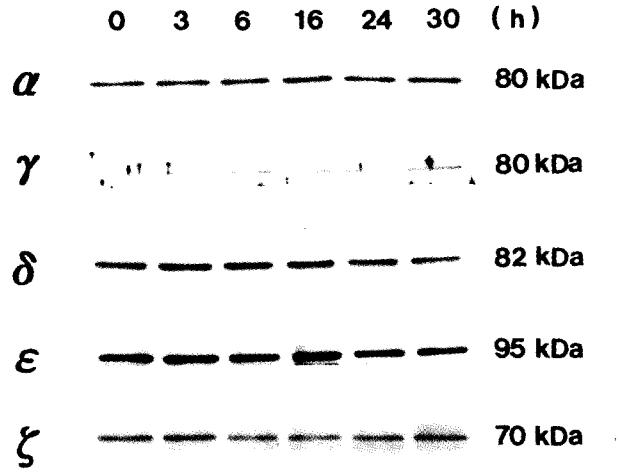

Fig. 7. Immunoblot analysis of PKC subtypes in ginsenoside Rh2- treated C6Bu-1 cells. The cells were treated with 50 μM ginsenoside Rh2 for 0, 3, 6, 16, 24 or 30 h. Equal amounts (10~20 μg) of protein were subjected to 10% SDS-PAGE electrophoresis. Immunodetection for each PKC subtype was performed as described in Materials and Methods

ptosis induced by G-Rh2 in SK-N-BE(2) and C6Bu-1 cells, the expression levels of PKC subtypes in SK-N-BE(2) and C6Bu-1 cells undergoing apoptosis by treatment with 50 μM G-Rh2 for 0, 3, 6, 16, 24 or 30 h were analyzed by immunoblot analysis.

As shown in Fig. 6, untreated SK-N-BE(2) cells expressed PKC α, δ and ε subtypes, while no PKC β, γ and ζ subtypes were detected. The level of PKC α, β and γ subtypes was progressively increased in the apoptosis-related manner. In contrast, the δ subtype was selectively increased at 3 and 6 h, early in the treatment, and e subtype gradually decreased at 6 h during the apoptotic process. Surprisingly, atypical PKC ζ subtype was detected with low level up to 16 h-treatment, however, its level increased markedly at 24 h when maximum apoptosis was achieved and cell viability was below 10%. During this process, no changes in the level of GAPDH were detected and PKC θ subtype was undetected in treated or untreated cells (data not shown). On the other hand, C6Bu-1 cells expressed PKC α, γ, δ, ε and ζ subtypes but not β subtype. In C6Bu-1 cells undergoing apoptosis induced by G-Rh2, no significant changes in the expression of α, γ, δ, ε and ζ subtypes were detected (Fig. 7). PKC β subtype was undetected during this process in C6Bu-1 cells (data not shown).

DISCUSSION

In this study, we found that G-Rh2 has a capacity to induce apoptosis in SK-N-BE(2) and C6Bu-1 cells. However, modulation of PKC subtypes by G-Rh2 in this process was distinct between the two cell lines.

The differential role of PKC subtypes in apoptosis has been demonstrated; PKC α and β expression was most

intense in human tonsil epithelial cells lacking the anti-apoptotic protein Bcl-2 and the cells were entering apoptosis (Knox *et al.*, 1993). 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 subtypes (Pongracz *et al.*, 1995). Other studies of HL-60 cells (Macfarlane and Manzel, 1994) and U937 cells (Pongracz *et al.*, 1996) using a selective activator of PKC β1 suggested that PKC β is a key regulator in myeloid cell apoptosis. Atypical PKC ζ subtype overexpressing U937cells were found to have an increased level of α and β subtypes (de Vente *et al.*, 1995). Treatment of PKC ζ overexpressing cells with TPA induced apoptotic cell death rather than differentiation, suggesting activation of a conventional subtype was necessary to induce apoptosis (de Vente *et al.*, 1995). It has been reported that the proteolytical activation of PKC δ and θ in apoptotic process (Emoto *et al.*, 1995: Emoto *et al.*, 1996; Denning *et al.*, 1998; Datta *et al.*, 1997), supporting a potential role for certain novel PKC subtypes in promoting apoptosis. Recent studies have demonstrated that the atypical PKC ζ and λ interact with Par-4 and abrogate the ability of Par-4 to induce apoptosis, suggesting that the atypical PKC subtypes exhibit anti-apoptosis (Diaz-Meco *et al.*, 1996). In addition, atypical PKC ι served to protect K562 cells against okadaic acid- and taxol-induced apoptosis, supporting a role for PKC ι in leukemia cell survival (Murray and Fields, 1997).

In addition, it has demonstrated that translocation of PKC δ and ε subtypes play important role in modulating apoptosis (Sawai *et al.*, 1997; Mayne and Murray, 1998). Evidences for a potential role of phosphorylation by PKC in regulating apoptosis have been reported; Interleukin-3, erythroprotein or bryostatin-1 suppressed apoptosis in growth factor-dependent myeloid cells by inducing the rapid hyperphosphorylation of Bcl-2 in a PKC-dependent mechanism (May *et al.*, 1994). Further study showed that Bcl-2 phosphorylation is required for anti-apoptosis function (Ito *et al.*, 1997). PKC βII phoshporylates mitotic lamin B, resulting in lamin disassembly (Goss *et al.*, 1994). Lamina disassembly is also required for apoptosis induced by different stimuli. Recently, it has found that lamin B phosphorylated by rapidly activated PKC α, and proteolyzed before DNA fragmentation during apoptosis in camptothecin-treated HL-60 cells, which is likely to affect nuclear and chromatin structure (Shimizu *et al.*, 1998). It has been demonstrated that p53 tumor suppressor, a positive regulator of apoptosis, is one of substrates for PKC and its tumor suppressor function is modulated by phosphorylation by PKC (Takenaka *et al.*, 1995).

Although the overall findings suggest that PKC subtype may be responsible for apoptosis induced by a variety of stimuli, current understanding of how specific PKC subtypes regulate certain cell function remains unclear. Alternation in distribution of PKC isoforms and phosphorylation of their substrates may play a crucial role in apoptosis.

In this study, the expression pattern of PKC subtypes revealed the presence of PKC α, δ and ε, while PKC β, θ and ζ were undetected in using SK-N-BE(2) cells. PKC ε is likely to be abundant in SK-N-BE(2) cells examined. Recently, Zeidman *et al.* (Zeidman *et al.*, 1999) have reported that 5 neuroblastomas cell lines including SK-N-BE(2) expressed PKC α, βII, δ and ε, while no PKC η or θ was detected, and PKC γ was found only in LAN 2 cells. PKC subtype expression pattern shown in our study consisted with that reported by Zeidman *et al.* (Zeidman *et al.*, 1999) except PKC β subtype. The expression of conventional PKC α, β and γ increased with accompanying apoptosis induced by G-Rh2. On the other hand, Gö6967, an inhibitor of classical subtypes only decreased cell number and caused apoptosis in SK-N-BE(2) cells (Zeidman *et al.*, 1999), implicating that the growth suppression in this cell line was likely due to be inhibition of conventional PKC α or βII subtype. Transient increase in PKC δ and down-regulation of PKC ε prior to progression of apoptosis markedly by G-Rh2 suggested that a certain role of novel PKC δ and ε may be required at initiation and/or progression of apoptosis. Interestingly, atypical PKC ζ was detected with low level 16 h after treatment with G-Rh2, markedly increased when maximum apoptosis was achieved, and cells almost died, suggesting PCK ζ may relate to terminate apoptosis. These results suggest that each PKC subtype may play a certain role at different stage during apoptosis induced by G-Rh2 in SK-N-BE(2) cells.

Untreated C6Bu-1 cells were found to express PKC α, γ, δ, ε, and ζ, but not β, in the present study. Although various changes in expression of PKC subtypes were observed in SK-N-BE(2) cells undergoing apoptosis induced by G-Rh2, no significant change was observed in PKC α, γ, δ, ε and ζ levels during apoptosis of C6Bu-1 cells.

Base on these observations, although G-Rh2 has the capacity to induce apoptosis in both SK-N-BE(2) and C6Bu-1 cell lines, it was suggested a possible role of PKC subtypes in apoptosis induced by G-Rh2 in SK-N-BE(2) cells and that G-Rh2 induced apoptosis via different pathways that might or might not be regulated by PKC in different cell types. To access a potential role of PKC subtypes, the effect of selective overexpression or antisense inhibition of expression of PKC subtype on G-Rh2-induced apoptosis is required in further study. Also, identification of substrates for PKC and alternation of phosphorylation of their substrates during apoptosis induced by G-Rh2 in SK-N-BE(2) cells will provide mechanism to modulate apoptosis by PKC subtype.

REFERENCES

Datta, R., Kojima, H., Yoshida, K. and Kufe, D., Caspase-3-mediated cleavage of protein kinase C θ in induction of apoptosis. *J. Biol. Chem.*, 272, 20317-20320 (1997).

Denning, M. F., Wang, Y., Nickoloff, B. J. and Wrone-Smith, T., Protein kinase C δ is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes. *J. Biol. Chem.*, 273, 29995-30002 (1998).

de Vente, J., Kiley, S., Garris, T., Bryant, W., Hooker, J., Posekany, K., Parker, P., Cook, P., Fletcher, D. and Ways, D. K., Phorbol ester treatment of U937 cells with altered protein kinase C content and distribution induces cell death rather than differentiation. *Cell Growth & Differ.*, 6, 371-382 (1995).

Diaz-Meco, M. T., Municio, M. M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L. and Moscat, J., The product of *par4*, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinese C. *Cell*, 86, 777-786 (1996).

Emoto, Y., Kisaki, H., Manome, Y., Kharbanda, S. and Kufe, D., Activation of protein kinase C δ in human myeloid leukemia cells treated with 1-β-D-arabinofuranosylcytosine. *Blood*, 87, 1990-1996 (1996).

Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W. W., Kamen, R., Weichselbaum, R. and Kufe, D., Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. *EMBO J.*, 14, 6148-6156 (1995).

Fujikawa-Yamamoto, K., Ota, T., Odashima, S., Abe, H. and Arichi, S., Different responses in the cell cycle of tumor cells to ginsenoside Rh_2. *Cancer J.*, 1, 349-352 (1987).

Goss, V. L., Hocevar, B. A., Thompson, L. J., Stratton, C. A., Burns, D. J. and Fields, A. P., Identification of nuclear β_{II} protein kinase C as a mitotic lamin kinase. *J. Biol. Chem.*, 269, 19074-19080 (1994).

Hoffman, B. and Liebermann, D. A., Molecular controls of apoptosis: differentiation/growth arrest primary response genes, proto-oncogenes, and tumor suppressor genes as positive & negative modulators. *Oncogene*, 9, 1807-1812 (1994).

Ito, T., Deng, X., Carr, B. and May, W.S., Bcl-2 phosphorylation required for anti-apoptosis function. *J. Biol. Chem.*, 272, 11671-11673 (1997).

Kim, Y. S., Jin, S. H., Lee, Y. H., Kim, S. I. and Park, J. D., Ginsenoside Rh2 induces apoptosis independently of Bcl-2, Bcl-x_L, or Bax in C6Bu-1 cells, *Arch. Pharm. Res.*, 22, 448-453 (1999).

Kim, Y. S., Kim, D. S. and Kim, S. I., Ginsenoside Rh_2 and Rh_3 induce differentiation of HL-60 cells into granulocytes: modulation of protein kinase C isoforms during differentiation by ginsenoside Rh_2. *Int. J. Biochem. Cell Biol.*, 30, 327-338 (1998).

Knox, K. A., Johnson, G. D. and Gordon, J., A study of protein kinase C isozyme distribution in relation to Bcl-2 expression during apoptosis of epithelial cells in vivo. *Exp. Cell. Res.*, 207, 68-73 (1993).

Lee, K. Y., Park, J. A., Chung, E., Lee, Y. H., Kim, S. I. and Lee, S. K., Ginsenoside-Rh2 blocks the cell cycle of SK-HEP-1 cells at the G1/S boundary by selectively inducing the protein expression of $p27^{kip1}$. *Cancer Lett.*, 110, 193-200 (1996).

Lee, Y. -N., Lee, H. -Y., Chang, H. Y., Kim, S. -I., Lee, S. -K., Park, B. -C. and Kim, K. -W., In vitro induction of differentiation by ginsenosides in F9 teratocarcinoma cells. *Eur. J. Cancer*, 32A, 1420-1428 (1996).

Macfarlane, D. E. and Manzel, L., Activation of β-isozyme of protein kinase C (PKCβ) is necessary and sufficient for phorbol ester-induced differentiation of HL-60 promyelocytes. *J. Biol. Chem.*, 269, 4327-4331 (1994).

May, W. S., Tyler, P. G., Ito, T., Armstrong, D. K., Qatsha, K. A. and Davidson, N. E., Interleukin-3 and bryostatin-1 mediate hyperphosphorylation of BCL2α in association with suppression of apoptosis. *J. Biol. Chem.*, 269, 26865-26870 (1994).

Mayne, G. C. and Murray, A. W., Evidence that protein kinase C ε mediates phorbol ester inhibition of calphostin C- and tumor necrosis factor-α-induced apoptosis in U937 histiocytic lymphoma cells. *J. Biol. Chem.*, 273, 24115-24121 (1998).

Murray, N. R. and Fields, A. P., Atypical protein kinase C ι protects human leukemia cells against drug-induced apoptosis. *J. Biol. Chem.*, 272, 27521-27524 (1997).

Nishizuka, Y., Studies and perspectives of protein kinase C. *Science*, 233, 305-312 (1986).

Nishizuka, Y., Intracelluar signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*, 258, 607-614 (1992).

Odashima, S., Ohta, T., Kohno, H., Matsuda, T., Kitakawa, I., Abe, H. and Arichi, S., Control of phenotypic expression of cultured B16 melanoma cells by plant glycosides. *Cancer Res.*, 45, 2781-2784 (1985).

Ota, T., Fujikawa-Yamamoto, K., Zong, Z. -P., Yamazaki, M., Odashima, S., Kitakawa, I., Abe, H. and Arichi, S., Plant-glycoside modulation of cell surface related to control of differentiation in cultured B16 melanoma cells. *Cancer Res.*, 47, 3863-3867 (1987).

Park, J. A., Lee, K. Y., Oh, Y. J., Kim, K. W. and Lee, S. K., Activation of caspase-3 protease via a Bcl-2-insensitive pathway during the process of ginsenoside Rh2-induced apoptosis. *Cancer Lett.*, 121, 73-81 (1997).

Pongracz, J., Deacon, E. M., Johnson, G. D., Burnett, D. and Lord, J. M., Doppa induces cell death but not differentiation of U937 cells: evidence for involvement of PKC-β1 in the regulation of apoptosis. *Leukemia Res.*, 20, 319-326 (1996).

Pongracz, J., Tuffley, W., Johnson, G. D., Deacon, E. M., Burnett, D., Stockley, R. A. and Lord, J. M., Changes in protein kinase C isoenzyme expression associated with apoptosis in U937 myelomonocytic cells. *Exp. Cell. Res.*, 218, 430-438 (1995).

Sawai, H., Okazaki, T., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Kishi, S., Umehara, H. and Domae, N., Ceramide induced translocation of protein kinase

C-δ and -ε to the cytosol. *J. Biol. Chem.*, 272, 2452-2458 (1997).

Shimizu, T., Cao, C. -X., Shao, R. -G. and Pommier, Y., Lamin B phosphorylation by protein kinase C α and proteolysis during apoptosis in human leukemia HL60 cells. *J. Biol. Chem.*, 273, 8669-8674 (1998).

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S. and Boyd, M. R., New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.*, 82, 1107-1112 (1990).

Steller, H., Mechanisms and genes of cellular suicide. *Science*, 267, 1445-1449 (1995).

Takenaka, I.., Morin, F., Seizinger, B. R. and Kley, N., Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases. *J. Biol. Chem.*, 270, 5405-5411 (1995).

Thompson, C. B., Apoptosis in the pathogenesis and treatment of disease. *Science*, 267, 1456-1462 (1995).

Vaux, D. L., Haecker, G. and Strasser, A., An evolutionary perspective on apoptosis. *Cell*, 76, 777-779 (1994).

Zeidman, R., Pettersson, L., Sailaja, P. R., Truedsson, E., Fagerström, S., Pahlman, S. and Larsson, C., Novel and classical protein kinase C isoforms have different functions in proliferation, survival and differentiation of neuroblastoma cells. *Int. J. Cancer*, 81, 494-501 (1999).