대한한의학회지 제29권 제1호(2008년 3월)
J Korean Oriental Med 2008;29(1):95-105

Original Article

Effect of *Polygonati Sibirici Rhizoma* on Cell Viability in Human Glioma Cells

Min-Soo Kim, Ji-Cheon Jeong

Dept. of Internal Medicine, College of Korean Medicine, Dongguk University

Objectives : Although herbal medicines containing flavonoids have been reported to exert anti-tumor activities, it has not been explored whether Hwang-Jeong (*Polygonati sibirici Rhizoma*, PsR) exerts anti-tumor activity in human glioma. The present study was therefore undertaken to examine the effect of PsR on cell viability and to determine its underlying mechanism in A172 human glioma cells.

Methods : Cell viability was estimated by MTT assay. Reactive oxygen species generation and mitochondrial membrane potential were measured by the fluorescence dyes. The phosphorylation of kinases was evaluated by western blot analysis and caspase activity was estimated using colorimetric assay kit.

Results : PsR resulted in loss of cell viability in a dose- and time-dependent manner. PsR did not increase reactive oxygen species (ROS) generation and the PsR-induced cell death was also not affected by antioxidants, suggesting that ROS generation is not involved in loss of cell viability. Western blot analysis showed that PsR treatment caused rapid reduction in phosphorylation of extracellular signal-regulated kinase (ERK) without changes in p38 and Jun-NH2-terminal kinase (JNK). U0126, an inhibitor of ERK, increased the PsR-induced cell death, but inhibitors of p38 and JNK did not affect the cell death. PsR induced depolarization of mitochondrial membrane potential. Caspase activity was not stimulated by PsR and caspase inhibitors did not prevent the PsR-induced cell death.

Conclusion : Taken together, these findings suggest that PsR results in human glioma cell death through caspase-independent mechanisms involving down-regulation of ERK.

Key Words : *Polygonati sibirici Rhizoma*, cell viability, ERK, mitochondrial membrane potential, human glioma cells.

Introduction

Glioblastoma multiforme (GBM) represents the most aggressive and invasive primary brain tumor in adults and acquires resistance against many therapeutic interventions[1]. Despite aggressive treatment including surgery, radiation, and chemotherapy, most patients die of the disease, with median survival of one year[2].

· received : 2008년 1월 29일
· accepted : 2008년 3월 7일
· Correspondence to : Ji-Cheon Jeong,
Dept. of Internal Medicine, Hospital of Korean Medicine, Dongguk University, Sukjang-Dong 1090-1, Gyeong-Ju City, Gyeong-Buk, Korea.
(Tel:+82-54-770-1265, Fax:+82-54-770-1500,
E-mail:jjcjh@paran.com)

Flavonoids are phenolic compounds widely distributed in almost every plants and act as pharmacologically active constituents in many herbal medicines. They have multiple biological, pharmacological and medicinal properties including anti-inflammatory, anti-allergic, antiviral, antithrombotic, antimutagenic, antineoplastic, and modulation of enzyme activities[3]. Therefore, there has been considerable interest in the anti-tumor activities of flavonoids[4-6]. Previous studies have shown that flavonoids induces growth inhibition and cell death in human glioma cells[7-9].

Oriental medicinal drugs that have flavonoids as their main constituents are generally nontoxic and may exert a diverse range of anti-tumor activities. Considering that many chemotherapeutic

agents have serious side effects and development of multidrug resistance further limits chemotherapy in cancer, oriental medicinal drugs may be a very promising group of compounds exerting the chemopreventive and chemotherapeutic effects.

Polygonati sibirici Rhizoma (PsR) has been known to enhance the force of heart and lung and to make strong bone and muscles. It also has been used as a therapeutic agent for diabetes[10]. PsR has been reported to improve cardiovascular diseases such as coronary artery disease, hypertension, and arteriosclerosis, reduce blood glucose levels, and exert antibiotic action[11]. *In vitro* studies have shown that PsR extraction affects nitrite level and nitric oxide synthase activity in rat's corpus cavernosum penis[12]. However, it has not explored whether PsR exerts any effect on the viability of glioma cells.

The present study was therefore undertaken to examine the effect of PsR on cell viability and to determine its molecular mechanism in A 172 human glioma cells.

Materials and Methods

1. Reagents

Trolox, *N*-acetylcysteine (NAC), ceramide, cisplatin, 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT), and propidium iodide were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Tween 20, U0126, SB203580, SP600125, z-DEVD-FMK, and DEVD-CHO were purchased from Calbiochem (San Diego, CA, USA). 3,3'-dihexyloxacarbocyamide [$DiOC_6(3)$] and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR, USA). Antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). All other chemicals were of the highest commercial grade available.

2. Extraction of Polygonati sibirici Rhizoma

The crushed Polygonati sibirici Rhizoma (300 g) was extracted 3 times, each time with 3 volumes of methyl alcohol at 60℃ for 24 h. The extract was filtered and evaporated under a reduced pressure using a rotary evaporator to yield 110.12g (yield 36.71%).

3. Cell culture

A172 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in 75-cm^2 culture flasks (Costar, Cambridge, MA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Invitrogen, Carsbad, CA, USA) containing 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA) at 37℃ in humidified 95% air / 5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on well tissue culture plates and used 1-2 days after plating when a confluent monolayer culture was achieved. Unless otherwise stated, cells were treated with PsR in serum-free medium.

4. Measurement of cell viability, proliferation and cell death

Cell viability was evaluated using a MTT assay[13]. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 hr at 37℃, the supernatant was removed and the formed formazan crystals in viable cells were

solubilized with 0.11 ml of dimethyl sulfoxide. A 0.1 ml aliquot of each sample was then translated to 96-well plates and the absorbance of each well was measured at 550 nm with ELISA Reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany). Data were expressed as a percentage of control measured in the absence of PsR. Unless otherwise stated, the cells were exposed to 50 μM PsR for 48 hr. Test reagents were added to the medium 30 min before PsR exposure.

Cell proliferation and cell death were estimated by counting the cell number and trypan blue exclusion assay, respectively. The cells were harvested using 0.025% trypsin and incubated with 4% trypan blue solution. The number of viable and nonviable cells was counted using a hemocytometer under light microscopy. Cells failing to exclude the dye were considered nonviable.

5. Measurement of reactive oxygen species

The intracellular generation of ROS was measured using DCFH-DA. The nonfluorescent ester penetrates into the cells and is hydrolyzed to DCFH by the cellular esterases. The probe (DCFH) is rapidly oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Cells cultured in 24-well plate were preincubated in the culture medium with 30 M DCFH-DAfor 1 hr at 37℃. After the preincubation, the cells were exposed to 50 μM PsR for various durations. Changes in DCF fluorescence was assayed using FACSort Becton Dickinson Flow Cytometer (Becton-Dickinson Bioscience, San Jose, CA, USA) and data were analyzed with CELLQuest Software.

6. Western blot analysis

Cells were harvest at various times after PsR treatment and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). Cell debris was removed by centrifugation at 10,000 g for 10 min at 4℃. The resulting supernatants were resolved on a 10% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with different primary antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. The signal was visualized using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

7. Measurement of mitochondrial membrane potential

The mitochondrial transmembrane potential was measured with DiOC6(3), a fluorochrome that is incorporated into cells depending upon the mitochondrial membrane potential[14]. Loss in DiOC6(3) staining indicates disruption of the mitochondrial inner transmembrane potential. Cells were stained with DiOC6(3) at a final concentration of 50 nM for 20 min at 37℃ in the dark. Cells were washed and resuspended in Hank's balanced salts solution containing Ca^{2+} and Mg^{2+}. The fluorescence intensity was analyzed with a FACScan flow cytometer using the fluorescence signal 1 channel.

8. Measurement of caspase-3 activity

Caspase-3 activity was measured by the caspase-3 colorimetric assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cells were collected by

centrifugation at 250g, and the supernatant was gently removed and discarded while the cell pellet was lysed by the addition of the cell lysis buffer at 4℃ for 10 min. Then the cell lysate was incubated with caspase-3 colorimetric substrate, DEVD-pNA at 37℃ for 1hr. The cleavage of the peptide was quantified spectrophotometrically at a wavelength of 405 nm.

9. Statistical analysis

The data are expressed as means SEM and the difference between two groups was evaluated using Student's t-test. Multiple group comparison was done using one-way analysis of variance followed by the Tukey post hoc test. A probability level of 0.05 was used to establish significance.

Results

1. Effect of PsR on cell viability

Cell viability was measured in cells exposed to 1, 5, and 10 mg/ml for various times. PsR resulted in loss of cell viability in dose- and time-dependent manner(Fig. 1). The cell viability was approximately 83, 56, and 32% of control after addition of 1, 5, and 10 mg/ml PsR for 24 hr, respectively. In subsequent experiments to measure loss of cell viability, cells were exposed to 5 mg/ml PsR for 24 hr.

To ascertain whether PsR affects cell proliferation, the cell number was counted in cells exposed to PsR. As shown in (Fig. 2), PsR decreased the cell number in a dose- and time-dependent manner. To determine if reduction in cell number was due to cell death, trypan blue exclusion assay was performed. As shown in (Fig. 2B), changes in cell death by PsR were similar to those estimated by counting cell number and MTT assay, suggesting that the reduction in cell number and cell viability by PsR was mainly due to induction of cell death.

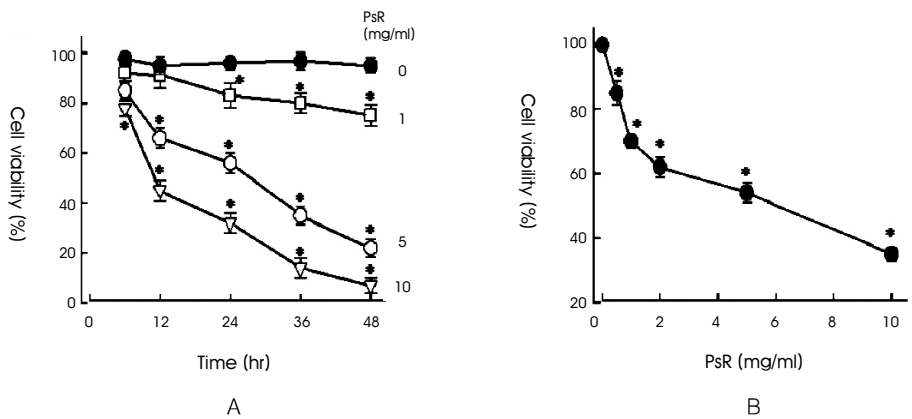


Fig. 1. Time-dependent effect of *Polygonati sibirici Rhizoma* (PsR) on cell viability. Cells were exposed to 1, 5, and 10 mg/ml PsR for various times (A). Cell viability was estimated by MTT reduction assay. Data are mean SEM of four independent experiments performed in duplicate. (B) Dose-dependent effect of PsR on cell viability. Cells were exposed to various concentrations of PsR for 24 hr. Cell death was estimated by MTT reduction assay. Data are mean SEM of four independent experiments performed in duplicate. *$p<0.05$ compared with control without PsR.

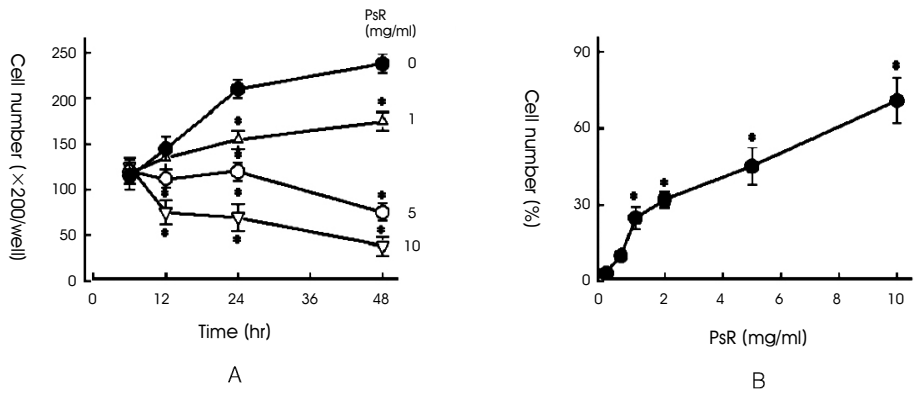


Fig. 2. Effect of *Polygonati sibirici Rhizoma* (PsR) on cell proliferation. Cells were exposed to 1, 5, and 10 mg/ml PsR for various times. Cell number was counted using a hemocytometer under light microscopy. Data are mean SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without PsR.

2. Role of ROS in PsR-induced cell death

To determine whether PsR induces ROS generation in A172 cells, the cells were exposed to PsR and changes in DCF fluorescence were measured using flow cytometry. As shown in (Fig. 3), Changes in ROS generation were not affected by PsR in cells exposed to 5 mg/ml PsR for 3-24 hr, whereas ceramide increased DCF fluorescence after 3 hr of treatment. Since ceramide has been reported to induce cell death via ROS generation in A172 cells[15], this agent was employed as a positive control.

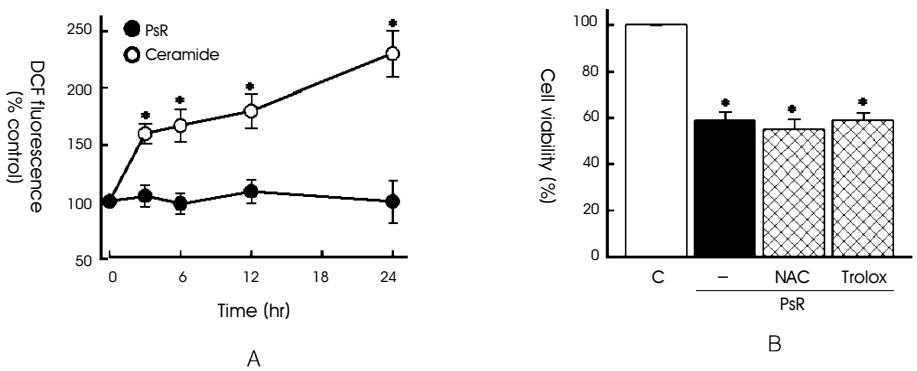


Fig. 3. Effects of *Polygonati sibirici Rhizoma* (PsR) on reactive oxygen species generation (A). Cells were exposed to 5 mg/ml PsR or 10 μM ceramide for various times and the DCF fluorescence intensity was measured by a flow cytometer. Ceramide was employed as a positive control. Data are mean SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without ceramide. (B) Effects of antioxidants on PsR-induced cell death. Cells were exposed to 5 mg/ml PsR for 24 hr in the presence or absence of 2 mM N-acetylcysteine (NAC) and 1 mM Trolox. Cell viability was estimated by MTT reduction assay. Data are mean SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without PsR.

To further determine whether ROS generation is not involved in the PsR-induced loss of cell viability, the effect of antioxidants NAC and Trolox on the cell viability was exmined. PsR-induced loss of cell viability was not affected by these antioxidants (Fig. 3B), indicating that PsR-induced cell death is not associated with ROS generation in A172 cells.

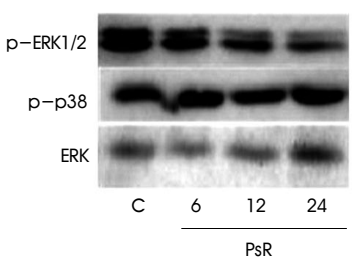
3. Roles of mitogen-activated protein kinase (MAPK) signaling in PsR-induced cell death

To investigate the role of MAPK activation in the PsR-induced cell death, MAPK activities were evaluated by detecting phosphorylation of MAPK subfamilies. Cells were exposed to 5 mg /ml PsRfor various times and activation of ERK, p38, and JNK was determined by Western blot analysis using antibodies specific to the respective phosphorylated form. PsR caused down-regulation of ERK in a time-dependent manner and activation of p38 was not affected by PsR (Fig. 4A). JNK phosphorylation was not detected (data not shown).

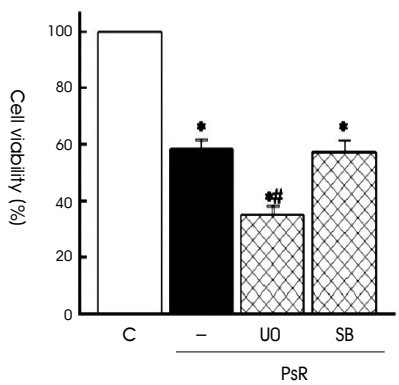
To evaluate whether these kinases are involved in the PsR-induced cell death, the effects of MAPK inhibitors on the cell death were examined. Cells were preincubated for 30 min with U0126 (ERK inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK inhibitor). Thereafter, the cells were exposed to 5 mg/ml PsR for 24 hr. Treatment of U0126 increased the cell death, whereas SB203580 did not affect the cell viability (Fig. 4B). SP60025 also was not effective in preventing the cell death (data not shown). These data suggest that down-regulation of ERK playsan important role in the PsR-induced cell death.

4. Role of mitochondria in PsR-induced cell death

To evaluate the role of mitochondria in PsR-induced cell death, changes in mitochon-



A



B

Fig. 4. Effects of *Polygonati sibirici Rhizoma* (PsR) on activation of MAPK. (A) Representative expression of phospho-ERK1/2 (p-ERK)and phospho-p38 (p-p38). Cells were exposed to 5 mg/ml PsR for various times. Total ERK was employed as a loading control. (B) Effects of MAPK inhibitors on PsR-induced cell death. Cells were exposed to 5 mg/ml PsR for 24 hrin the presence or absence of each 10 μM U0126 (U0) and SB203580 (SB). Cell viability was estimated by MTT assay. Data are mean SEM of three independent experiments performed in duplicate. *$p<0.05$ compared with control (C); #$p<0.05$ compared with PsR alone.

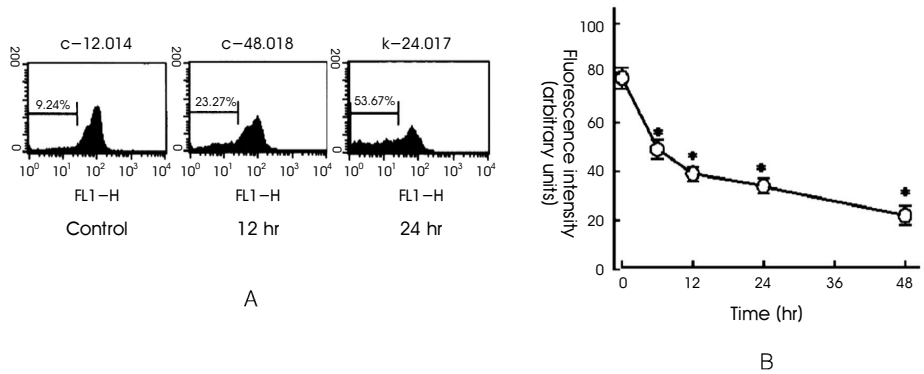


Fig. 5. Effect of *Polygonati sibirici Rhizoma* (PsR) on mitochondrial membrane potential. Cells were exposed to 5mg/ml PsR for 12 and 24 hr (A). Mitochondrial membrane potential was estimated by the uptake of a membrane potential-sensitive fluorescence dye DiCO6(3). The fluorescence intensity was analyzed with a flow cytometer. (B) Time-dependent effect of PsR on mitochondrial membrane potential. Cells were exposed to 5 mg/ml PsR for various times and mitochondrial membrane potential was estimated as described in (A). Data are mean SEM of three independent experiments performed in duplicate. . *p<0.05 compared with control without PsR.

drial membrane potential was examinedin cells exposed to PsR. Mitochondrial membrane potential was measured using the fluorescence dye in cells exposed to 5 mg/ml PsR for 12 and 24 hr. PsR caused disruption of mitochondrial membrane potential as evidenced by an increase in the proportion of cells with lower fluorescence intensity (Fig. 5A). Mitochondrial membrane

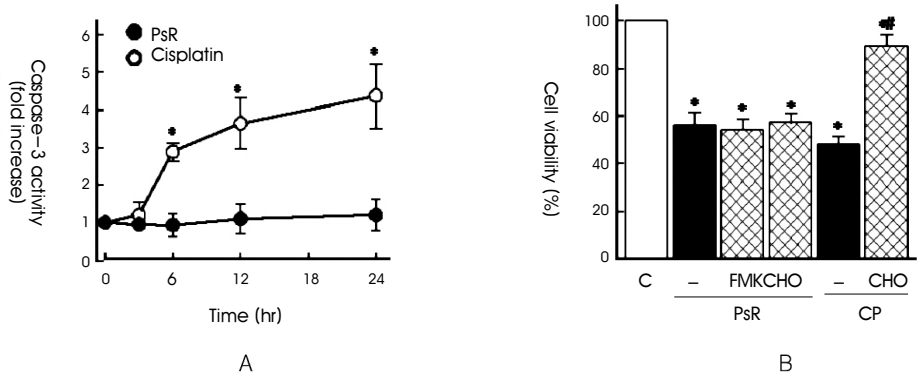


Fig. 6. (A) Effect of *Polygonati sibirici Rhizoma* (PsR) on caspase activation. Cells were exposed to 5 mg/ml PsR or 0.3 mM cisplatin (CP) for various imes. Caspase-3 activation was estimated by assay kit. Data are mean SEM of four independent experiments performed in duplicate. Cisplatin was employed as a positive control. *p<0.05 compared with control without cisplatin. (B) Effects of caspase inhibitors on cell death induced by PsR and CP. Cells were exposed to 5 mg/ml PsR or 0.3 mM CP for 24 hr in the presence or absence of each 20 μM of z-DEVD-FMK (FMK) and DEVD-CHO (CHO). Cell viability was estimated by MTT assay. Data are mean SEM of five independent experiments performed in duplicate. *p<0.05 compared with control (C) #p<0.05 compared with CP alone.

potential decreased after 6 hr of PsR treatment and the decrease was time- dependent up to 48 hr of treatment (Fig. 5B).

5. Role of caspase in PsR-induced cell death

Caspases play a key role during the execution phase in various forms of apoptosis[16]. To examine if caspase activation is involved in PsR-induced cell death, activity of caspase-3 was measured using assay kit in cells exposed to 5 mg/ml PsR for various times. As shown in (Fig. 6A), PsR did not affect the caspase activity, whereas cisplatin increased caspase-3 activity after 6 hr of treatment. Since cisplatin has been reported to induce caspase activation in A172 cells[17], cisplatin was employed as a positive control.

To evaluate ifcaspase is not involved in the PsR-induced cell death, the effect of caspase inhibitors on the cell viability was examined. Cells were exposed to PsR in the presence of the general caspase inhibitor z-DEVD-FMK and the caspase-3 inhibitor DEVD-CHO. The PsR-induced cell death was not affected by these inhibitors, the cisplatin-induced cell death was prevented by DEVD-CHO (Fig. 6B). These data indicate that PsR induces cell death through a caspase-independent mechanism.

Discussion

The present study demonstrated that PsR caused loss of cell viability in a dose-and time-dependent manner (Fig. 1). Cell viability was approximately 83, 56, and 32% of control after treatment of 1, 5, and 10 mg/ml PsR for 24 hr, respectively. PsR also inhibited cell proliferation as evidenced by reduction in the cell number (Fig. 2A). Similarly, PsR induced cell death in a dose-dependent fashion (Fig. 2B). Reduction in the cell number was similar to that of loss of cell viability by MTT assay and cell death by trypan blue exclusion assay. These results suggest that reduction in cell proliferation and loss of cell viability were attributed to cell death.

Besides the well-known antioxidant effect of flavonoids6, it may also behave as a pro-oxidant, generating ROS[18-19], which is responsible for cell death in some cancer cells[19-20]. In the present study, however, PsR did not increase ROS generation, whereas ceramide, used as a positive control, markedly increased ROS generation. The antioxidants NAC and Trolox also had no significant effect on the PsR- induced cell death (Fig. 3). Therefore, it seems unlikely that under our experimental conditions, the PsR -induced cell death is not attributed to ROS generation.

To explore the underlying mechanism of PsR-induced glioma cell death, the effect of PsR on activation of MAPKs was examined. MAPKs are highly conserved and act as second messengers in transducing the extracellular signals to the intracellular milieu for appropriate responses such as cell proliferation, differentiation, inflammation, malignant transformation, and apoptosis[21]. ERK1/2 are activated by a variety of extracellular signals including mitogens and contribute to the proliferative responses in cells, and are considered to be an essential common element of mitogenic signaling[22-23]. Their constitutive expression causes cell transformation and plays a putative role in the carcinogenesis process and drug resistance[24]. Several lines of evidence have describedan important role for ERK in glial survival signalingand glial tumorigenesis[25].

However, the effect of PsR on activation of MAPK is not explored. Activation of ERK

was inhibited by flavonoids in vascular smooth muscle cells[26], human epidermal carcinoma cells[27], and neuronal cells[28], whereas it was increased following flavonoid treatment in lung cancer cells[29]. These studies suggest that the effect of flavonoids on ERK activation may be dependent on cell types. In the present study, PsR caused a rapid inhibition of ERK phosphorylation without any effect on p38 and JNK (Fig. 4). To clarify the role of MAPK in the PsR-induced cell death, the effect of MAPK inhibitors was examined in cells exposed to PsR. The PsR-induced cell death was further increased by addition of the ERK inhibitor U0126, whereas inhibitors of p38 and JNK did not affect the cell death (Fig. 4). These data imply that down-regulation of ERK could be possible underlying molecular mechanism involved in the PsR-induced glioma cell death, a result consistent with findings in neuronal cells[28], human epidermal carcinoma cells[27], and human hepatoma cells[30].

A decrease in mitochondrial membrane potential plays an important role in cell death[31]. It has been reported that flavonoids such as quercetin induce loss of mitochondrial membrane potential in lungcancer cells[3], malignant HPB-ALL cells[32], and leukemia HL-60 cells[19]. However, the effect of PsRon mitochondrial function is not clear in glioma cells. In the present study, PsR caused a significant reduction in mitochondrial membrane potential (Fig. 5), indicating induction of depolarization of mitochondrial membrane potential.

Growth inhibition and cell death induced by flavonoids are associated with caspase activation in various cancer cells[3,20]. However, in the present study, we observed that PsR induced a caspase-independent cell death, as evidenced by failure of activation of caspase by PsR and no protection of PsR-induced cell death by caspase inhibitors (Fig. 6).

In conclusion, the present study demonstrated that PsR results in human glioma cell death through caspase-independent mechanisms involving down-regulation of ERK. Induction of cell death may be a promising therapeutic approach in cancer therapy. Our results suggest that PsR may be considered a potential candidate for both glioblastoma prevention and treatment. Further investigation is needed to validate the contribution of PsR to tumor therapy *in vivo*.

References

1. Ohgaki H, Kleihues P. Population-based studies on incidence, survival rates and genetic alterations in astrocytic and oligodendroglial gliomas. J Neuropathol Exp Neurol. 2005;64: 479-89.
2. DeAngelis LM. Brain tumors. N Engl J Med. 2001;344:114-23.
3. Yang JH, Hsia TC, Kuo HM, Chao PD, Chou CC, Wei YH, Chung JG. Inhibition of lung cancer cell growth by quercetin glucuronides via G2/M arrest and induction of apoptosis. Drug Metab Dispos. 2006;34:296-304.
4. Fresco P, Borges F, Diniz C, Marques MP. New insights on the anticancer properties of dietary polyphenols. Med Res Rev. 2006;26: 747-66.
5. Kanadaswami C, Lee LT, Lee PP, Hwang JJ, Ke FC, Huang YT, Lee MT. The antitumor activities of flavonoids. In Vivo. 2005;19:895 -909.
6. Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: promising anticancer agents. Med Res Rev. 2003;23:519-34.

7. Braganhol E, Zamin LL, Canedo AD, Horn F, Tamajusuku AS, Wink MR, Salbego C, Battastini AM. Antiproliferative effect of quercetin in the human U138MG glioma cell line. Anticancer Drugs. 2006;17:663-71.
8. Sharma V, Joseph C, Ghosh S, Agarwal A, Mishra MK, Sen E. Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. Mol Cancer Ther. 2007;6: 2544-53.
9. Shen SC, Lin CW, Lee HM, Chien LL, Chen YC. Lipopolysaccharide plus 12-o-tetradecanoylphorbol 13-acetate induction of migration and invasion of glioma cells *in vitro* and in vivo: Differential inhibitory effects of flavonoids. Neuroscience. 2006;140: 477-89.
10. Lee SI. Herbs. Seoul:Medical Herb Co. 1983: 128-9.
11. Wang YS. Pharmacological action and application of Chinese herbs. Beijing:People's Medical Publshing House. 1983:998-1000.
12. Lee SH, Shin HC, Jeong JC. Effects of Polygonati Sibirici Rhizoma extract on the nitrite level and nitric oxide synthase activity in rat's corpus cavernosum penis. J Oriental Chr Dis. 1999;5(1):163-75.
13. Denizot F, Lang R, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods. 1986;89:271-7.
14. Pastorino JG, Chen ST, Tafani M, Snyder JW, Farber JL. The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. J Biol Chem. 1998; 273:7770-5.
15. Kim WH, Choi CH, Kang SK, Kwon CH., Kim YK. Ceramide induces non-apoptotic cell death in human glioma cells. Neurochem Res. 2005;30:969-79.
16. Cohen GM. Caspases: the executioners of apoptosis. Biochem J. 1997;326:1-16.
17. Choi BK, Choi CH, Oh HL, Kim YK. Role of ERK activation in cisplatin-induced apoptosis in A172 human glioma cells. Neuro-Toxicology. 2004;25:915-24.
18. Miura YH, Tomita I, Watanabe T, Hirayama T, Fukui S. Active oxygens generation by flavonoids. Biol Pharm Bull. 1998;21:93-6.
19. Wang IK, Lin-Shiau SY, Lin JK. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL -60 cells. Eur J Cancer. 1999;35:1517-25.
20. Chen YC, Shen SC, Chow JM, Ko CH, Tseng SW. Flavone inhibition of tumor growth via apoptosis in vitro and in vivo. Int J Oncol. 2004;25:661-70.
21. Cobb MH. MAP kinase pathways. Prog Biophys Mol Biol. 1999;71:479-500.
22. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 2001;22:153-83.
23. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science. 1995;270:1326-31.
24. Brunet A, Pages G, Pouyssegur J. Constitutively active mutants of MAP kinase kinase (MEK1) induce growth factor-relaxation and oncogenicity when expressed in fibroblasts. Oncogene. 1994;9:3379-87.
25. Bhaskara VK, Sundaram C, Babu PP. pERK, pAkt and pBad: a possible role in cell proliferation and sustained cellular survival during

tumorigenesis and tumor progression in ENU induced transplacental glioma rat model. Neurochem Res. 2006;31:1163-70.

26. Moon SK, Cho GO, Jung SY, Gal SW, Kwon TK, Lee YC, Madamanchi NR, Kim CH. Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cell-cycle regulation, and matrix metalloproteinase-9. Biochem Biophys Res Commun. 2003;301:1069-78.
27. Singh RP, Tyagi AK, Zhao J, Agarwal R. Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein kinases and induction of apoptosis. Carcinogenesis. 2002;23: 499-510.
28. Spencer JP, Rice-Evans C, Williams RJ. Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability. J Biol Chem. 2003; 278:34783-93.
29. Nguyen TT, Tran E, Nguyen TH, Do PT, Huynh TH, Huynh H. The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. Carcinogenesis. 2004;25:647-59.
30. Granado-Serrano AB, Martin MA, Bravo L, Goya L, Ramos S. Quercetin Induces Apoptosis via Caspase Activation, Regulation of Bcl-2, and Inhibition of PI-3-Kinase/Akt and ERK Pathways in a Human Hepatoma Cell Line (HepG2). J Nutr. 2006;136:2715-21.
31. Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol. 1998;60:619-42.
32. Russo M, Palumbo R, Tedesco I, Mazzarella G, Russo P, Iacomino G, Russo GL. Quercetin and anti-CD95(Fas/Apo1) enhance apoptosis in HPB-ALL cell line. FEBS Lett. 1999; 462:322-8.