Effect of *Fructus ligustri Lucidi* Extract on Cell Viability in Human Glioma Cells

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It is unclear whether *Fructus ligustri Lucidi* (FLL) extract anti-proliferative effect in human glioma cells. The present study was therefore undertaken to examine the effect of FLL on cell viability and to determine the underlying mechanism in A172 human glioma cells. Cell viability and cell death were estimated by MTT assay and trypan blue exclusion assay, respectively. Apoptosis was measured by Annexin-V binding assay and cell cycle analysis. Activation of kinases and caspase-3 was estimated by Western blot analysis. FLL resulted in apoptotic cell death in a dose- and time-dependent manner. FLL-induced cell death was not associated with reactive oxygen species generation. Western blot analysis showed that FLL treatment caused down-regulation of PI3K/Akt pathway, but not ERK. The PI3K/Akt inhibitor LY984002 sensitized the FLL-induced cell death and overexpression of Akt prevented the cell death. FLL induced caspase-3 activation and the FLL-induced cell death was prevented by caspase inhibitors. These findings indicate that FLL results in a caspase-dependent cell death through a PI3K/Akt pathway in human glioma cells. These data suggest that FLL may serve as a potential therapeutic agent for malignant human gliomas.

Key words : f*ructus ligustri Lucidi*, cell viability, apoptosis, PI3K/Akt, caspase activation, human glioma cells

Introduction

Natural products derived from plants have recently received much attention as potential chemopreventive and chemotherapeutic agents. Among them great attention has been given to natural products with polyphenols. These substances were described to play as chemopreventive agents[1,2]. Since conventional chemotherapeutic agents have serious side effects and development of multidrug resistance further limits chemotherapy in cancer, the development of chemopreventive strategies is an urgent priority in public health. These polyphenols compounds may inhibit various stages in the carcinogenesis process by affecting molecular events in the initiation, promotion, and progression stages. They may also increase the expression of proapoptotic genes and thereby prevent or delay tumor development[3].

Fructus Ligustri Licidi (FLL) has been used in traditional Korean herb medicine for long times mainly to treat dizziness, tinnitus, rheumatic pains, backache, and blurred vision[4,5]. Although the active components of FLL are not defined, it has been reported that FLL has anti-viral effect[6] and improves Ca^{2+} balance in aged female rats by increasing serum 1,25 (OH)2D3 levels and vitamin D-dependent CaBPs expression[7]. However, little information is available regarding the effect of FLL on glioma cell viability.

The present study was undertaken to investigate whether FLL affects cell viability and to characterize its molecular mechanism in A 172 human glioma cells.

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Materials and Methods

1. Reagents

N-acetylcysteine (NAC), Trolox, rapamycin, ciglitazone, 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, LY984002, PD98059, VAD-FMK, and DEVD-CHO were purchased from Calbiochem (SanDiego, CA, USA). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was 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 FLL

The crushed *Fructus ligustri Lucidi* (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 84.97 g (yield 28.32%).

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 FLL in serum-free medium.

4. Measurement of cell viability and cell death

Cell viability was evaluated using a MTT assay[8]. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 h 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 FLL. Test reagents were added to the medium 30 min before FLL exposure.

Cell death was estimated by 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 apoptosis

1) Annexin-V staining

Phosphotidylserine exposure on the outer layer of the cell membrane was measured using the binding of annexin V - fluorescein isothiocyanate (FITC). Cells were harvested and washed with cold PBS, incubated for 15 min with annexin V - FITC and propidium iodide and analyzed by flow cytometry (Becton Dickinson, FranklinLakes, NJ, USA).

2) Cell cycle analysis

Cells were grown in 6-well plates and were treated as indicated. Then, attached and floating cells were pooled, pelleted by centrifugation, washed in PBS, and fixed with cold 70% ethanol containing 0.5% Tween 20 at 4℃ overnight. Cells were washed and resuspended in 1.0 ml of propidium iodide solution containing 100 mg of RNase A/ml and 50 mg propidium iodide/ml and incubated for 30 min at 37℃. Apoptotic cells were assayed using FACSort Becton Dickinson Flow Cytometer at 488 nm and data were analyzed with CELLQuest Software. Cells with sub-G1 propidium iodide incorporation were considered as apoptotic. The percentage of apoptotic cells was calculated as the ratio of events on sub-G1 to events from the whole population.

6. Measurement of reactive oxygen species (ROS)

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 mM DCFH-DA for 1 h at 37℃. After the preincubation, the cells were exposed to 5 mg/ml FLL for various durations. Changes in DCF fluorescence was assayed using FACSort Becton Dickinson Flow Cytometer (Becton-Dickinson Bioscience, SanJose, CA, USA) and data were analyzed with CELLQuest Software.

7. Western blot analysis

Cells were harvest at various times after FLL 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 12% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fatdried milk at room temperature for 30 min and incubated with primary antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody. The signal was visualized using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

8. Transfection

To modulate the activity of Akt and extracellular

signal-regulated kinase (ERK), a transient transfection of constitutively active forms of Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase1 (MEK1), an upstream kinase of ERK, was performed. Cells were seeded in six well plates and grown to 70% confluence. A 2 μg cDNA each was transiently transfected using Lipofectamine (Invitrogen, Carsbad, California, USA) according to manufacturer's guidelines. After 4 hr incubation at 37℃, cells were maintained in normal culture media for 24 h.

9. Measurement of caspase-3 activity

Caspase-3 activity was measured by Western blot analysis using the procaspase-3 specific antibody as described above.

10. 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 FLL on cell viability and cell death

Cell viability was measured in cells exposed to 1-30 mg/ml for 24 and 48 h. FLL resulted in loss of cell viability in a dose-dependent manner (Fig. 1A). To evaluate the time-dependent effect of FLL on loss of cell viability, cells were exposed to 5 mg/ml FLL for various times. As shown in Fig. 1B, the cell viability was significantly decreased after 24 h of FLL treatment, although stimulation of cell proliferation was present at 3 h of treatment. To determine if loss of cell viability was attributed to cell death, trypan blue exclusion assay was performed. FLL induced cell death with patterns similar to those estimated by MTT assay (Fig. 2), suggesting that the reduction in cell viability by FLL was mainly due to induction of cell death. To determine whether FLL-induced cell death was due to apoptosis, cells were exposed to 5 mg/ml FLL for 24 h and apoptosis was estimated by annexin-V staining assay and cell cycle analysis. Cytometric analyses of annexin V-positive cells demonstrated a 46.68% increase in cells treated with FLL as shown in right lower and upper quadrants (Fig. 3A). We further confirmed the onset of apoptotic cell death by the DNA content analysis by flow cytometry. The results showed that the proportion of the cells in the sub-G1 phase (apoptotic cells) was increased 5.11% of control to 39.29 after FLL treatment (Fig. 3B) and the increase in apoptosis was significant after 24 h (Fig. 3C). These results indicate that FLL induces loss of cell viability largely through apoptosis.

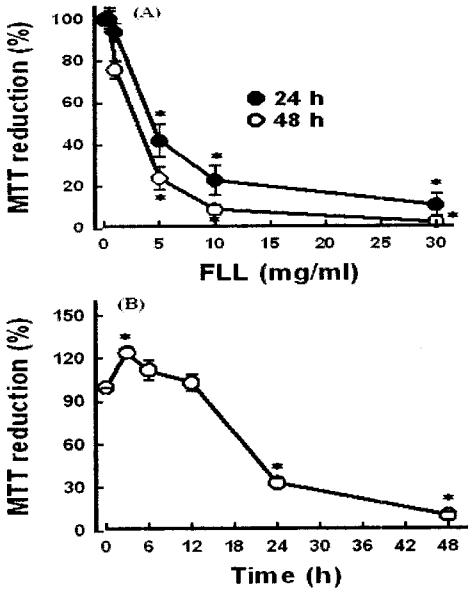

Fig. 1. Effect of *Fructus ligustri Lucidi* (FLL) on cell viability. Cells were exposed to various concentrations of FLL for 24 and 48 h (A). Cell viability was estimated by MTT reduction assay. Data are mean± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without FLL. (B) Time dependency of FLL-induced loss of cell viability. Cells were exposed to 5mg/ml FLL for various times. 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 FLL.

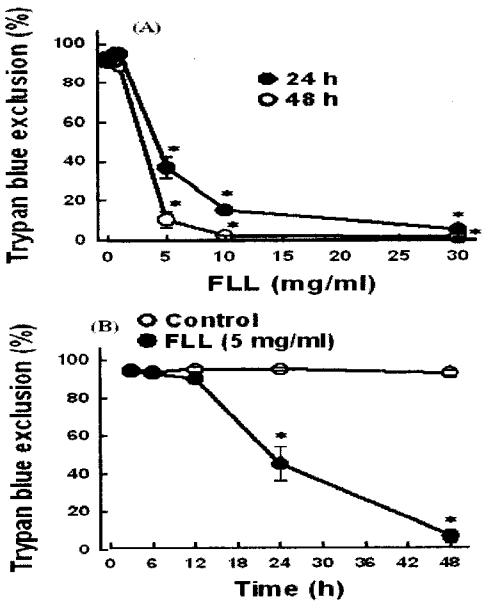

Fig. 2. Effect of *Fructus ligustri Lucidi* (FLL) on cell death. Cells were exposed to various concentrations of FLL for 24 and 48 h (A). Cell death was estimated by trypan blue exclusion assay. Dataaremean± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without FLL. (B) Time dependency of FLL-induced cell death. Cells were exposed to 5mg/ml FLL for various times. Cell viability was estimated by trypan blue exclusion assay. Data are mean± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without FLL.

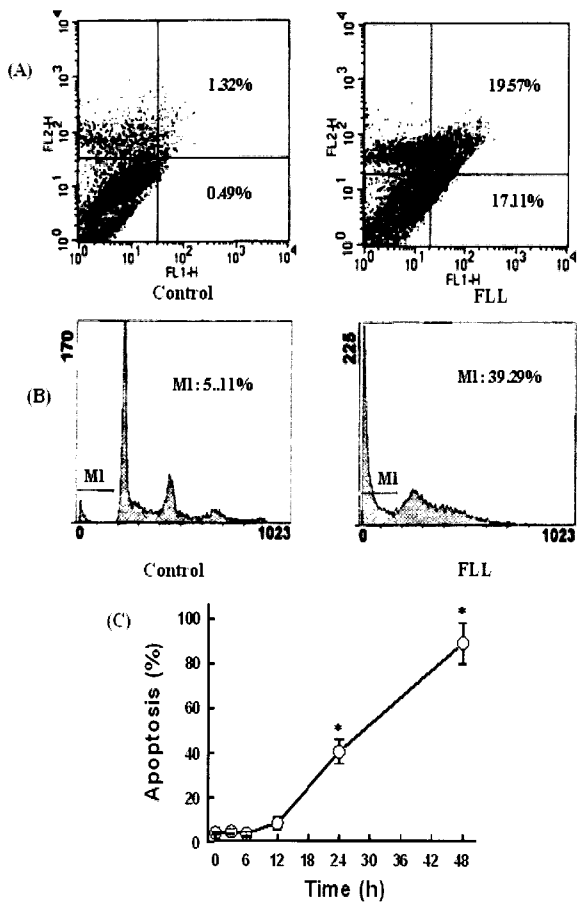

Fig. 3. Effect of *Fructus ligustri Lucidi* (FLL) on apoptosis. Cells were exposed to 5 mg/ml FLL for 24 h. Apoptosis was estimated by Annexin-V binding assay a (A) and cell cycle analysis (B). In Annexin-v binding assay, numbers indicate the percentage of cells in each quadrant. Early apoptotic and late apoptotic cells were shown in right lower and right upper quadrants, respectively. In cell cycle analysis, numbers of M1 gate indicate the percentage of cells with the sub-G1 peak (apoptotic cells).

2. Role of ROS in FLL-induced cell death

To determine whether FLL induces ROS generation in A172 cells, the cells were exposed to FLL and changes in DCF fluorescence were measured by flow cytometry. ROS generation was not altered by FLL, whereas ciglitazone increased ROS generation in a time-dependent fashion(Fig. 4). Since previous studies have showed and ciglitazone stimulates ROS generation in A172 cells[9], we employed as a positive control. FLL-induced loss of cell viability was also not prevented by antioxidants N-acetylcysteine and Trolox(Fig. 4B), indicating that FLL-induced cell death is not associated with ROS generation in A172 cells.

3. Role of survival kinases in FLL-induced cell death

Phosphatidylinositol-3 kinase (PI3K)/Akt and ERK play a pivotal role in cell proliferation, differentiation, and survival[10-12]. If FLL causes down-regulation of these kinases, cell death could be induced. To test this possibility, activity of these kinases was evaluated by detecting their phosphorylation forms. Cells were exposed to 5 mg/ml FLL for various times. FLL induced a rapid decrease in activation of PI3/KAkt, but not ERK (Fig. 5).

To evaluate whether down-regulation of PI3K/Akt is responsible for the FLL-induced cell death, the effect of the PI3K inhibitor was evaluated. The PI3K inhibitor LY984002, but not the ERK inhibitor PD98059, sensitized the cell death induced by FLL. These results suggest that down-regulation of PI3K/Akt is involved in the FLL-induced cell death. To further confirm involvement of PI3K/Akt and ERK in the FLL-induced cell death, the effect of FLL on cell death was examined using cells transfected with constitutively active forms of Akt (caAkt) and MEK (caMEK), the upstream kinase of ERK. Transfection was confirmed by Western blot analysis. In cells transfected with caAkt and caMEK, phosphorylation of Akt and ERK was significantly increased as compared with cells transfected with empty vector (Fig. 6B). Transfection of caAkt, but not caMEK, prevented effectively the FLL-induced cell death(Fig. 6C), suggesting that down-regulation of PI3K/Akt plays a critical role in the FLL-induced cell death.

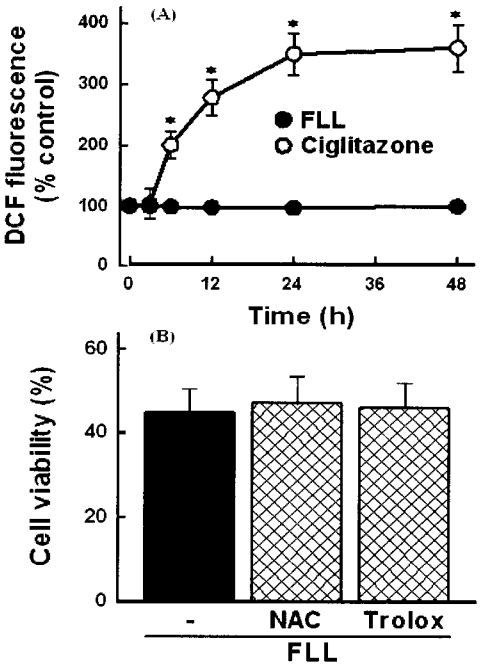

Fig. 4. Role of reactive oxygen species generation in *Fructus ligustri Lucidi* (FLL)-induced cell death. Cells were exposed to 5 mg/ml FLL and 20 mM ciglitazone for various times (A) and the DCF fluorescence intensity was measured by a flow cytometer. Ciglitazone was employed as a positive control. Data in are mean± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without ciglitazone. (B) Effects of antioxidants on FLL-induced cell death. Cells were exposed to 5 mg/ml FLL for 24 hr in the presence or absence of 2 mM N-acetylcysteine (NAC) and 0.5 mM Trolox. Cell viability was estimated by MTT reduction assay. Data are mean± SEM of four independent experiments performed in duplicate.

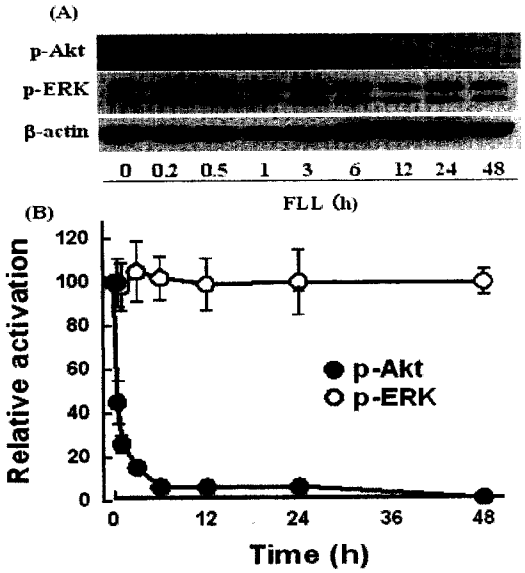

Fig. 5. Effect of *Fructus ligustri Lucidi* (FLL) on activation of Akt and ERK. (A) Representative activation of Akt and ERK. Cells were exposed to 5mg/ml FLL for various times. Activation of Akt and ERK was estimated by Western blot analysis. (B) Activation of Akt and ERK was quantified by densitometry. Data are mean ± SEM of six independent experiments.

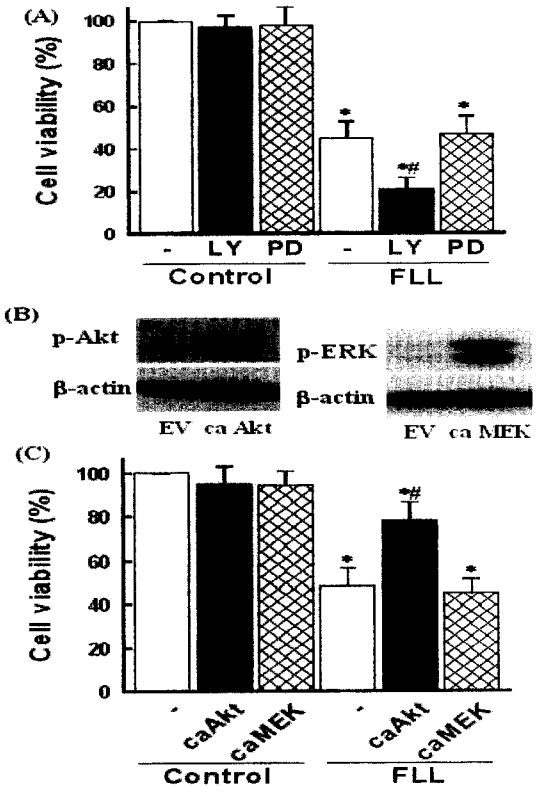

Fig. 6. Effects of modulation of Akt and ERK on *Fructus ligustri Lucidi* (FLL)-induced cell death. (A) Cells were exposed to 5mg/ml FLL for 24 h in the presence or absence of 10 mM LY984002 (LY) and 20 mM PD98059 (PD) Cell viability was estimated by MTT reduction assay. Data are mean ± SEM of four independent experiments performed in duplicate. (B) Cells were transfected with empty vector (EV) and constitutively active forms of Akt (caAkt) and MEK (caMEK). The efficiency of transfection was estimated by Western blot analysis. (C) Effect of transfection of caAkt and caMEK on FLL-induced cell death. Transfection cells were exposed to 5 mg/ml FLL for 24 h. Cell viability was estimated by MTT reduction assay. Data are mean± SEM of four independent experiments performed in duplicate.

4. Role of caspase in FLL-induced cell death

Caspases play a key role during the execution phase in apoptosis and the caspase-3 is one of the executioners of apoptosis[13]. To examine if caspase activation is involved in FLL-induced cell death, activity of caspase-3 was measured by Western blot analysis in cells exposed to FLL for various times. FLL increased the caspase activity after 6 h of treatment as evidenced by degradation of pro-caspase-3 (Fig. 7A). To evaluate if caspase is involved in the FLL-induced cell death, the effect of caspase inhibitors on the cell viability was examined. Cells were exposed to FLL in the presence of the general caspase inhibitor VAD-FMK and the caspase-3 inhibitor DEVD-CHO. The FLL-induced cell death was prevented by these inhibitors (Fig. 7B). These data indicate that FLL induces cell death through a caspase-dependent mechanism.

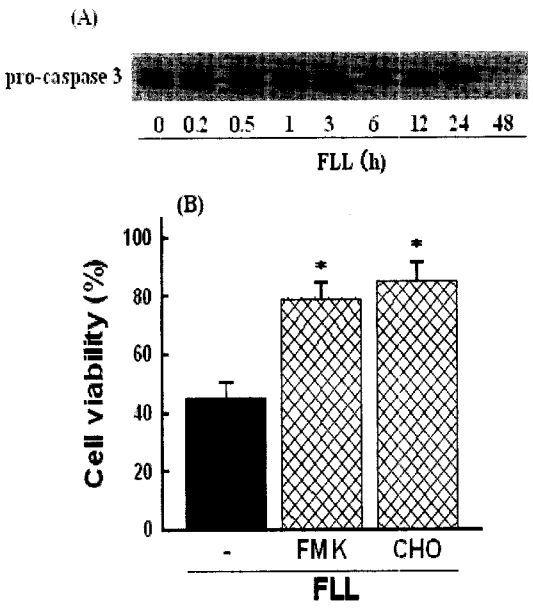

Fig. 7. Role of caspase activation in *Fructus ligustri Lucidi* (FLL)-induced cell death. (A) Representative activation of caspase-3. Cells were exposed to 5 mg/ml FLL for various times and activation of capsase-3 was evaluated from degradation of pro-caspase-3 by Western blot analysis. (B) Effects of caspase inhibitors on FLL-induced cell death. Cells were exposed to 5 mg/ml FLL for 48 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 four independent experiments performed in duplicate. *p<0.05 compared with FLL alone.

Discussion

The present study demonstrated that FLL caused loss of cell viability in a dose-and time-dependent manner (Fig. 1). FLL treatment also resulted in cell death with fashion similar to loss of cell viability (Fig. 2). Annexin-V binding assay and cell cycle analysis showed that FLL-induced cell death was largely attributed to apoptosis (Fig. 3). These data indicated

that FLL induces loss cell viability through apoptotic cell death in human glioma cells.

Flavonoids, the major component of oriental medicinal herbs, behave as an antioxidant[2] or a pro-oxidant generating ROS[14,15]. ROS generation by flavonoids is responsible for cell death in some cancer cells[15,16]. In the present study, FLL did not stimulate ROS generation and the FLL-induced cell death also was not altered by the antioxidants (Fig. 4), indicating that the FLL-induced cell death is not associated with ROS generation.

To explore the underlying mechanism of the FLL-induced glioma cell death, the effect of FLL on activation of PI3K/Akt and ERK was examined. Several lines of evidence described an important role for both kinases in glial survival signaling and glial tumorigenesis[17-19]. The modulation of PI3K/Akt is linked to a wide variety of anti-apoptotic functions[11]. Flavonoids have been known to be a potent inhibitor of PI3K[20,21]. The modulation of the PI3K/Akt signaling may be associated with the FLL-induced cell death. Indeed, Akt activation was down-regulated by FLL (Fig. 5). To clarify the role of PI3K/Akt in the cell death, the effect of FLL on cell viability was examined in the presence of the PI3K/Akt inhibitor. The FLL-induced cell death was increased by the Akt inhibitor (Fig. 6A). To further confirm involvement of PI3K/Akt pathway in the FLL-induced cell death, the effect of FLL on cell death in cells transfected with caAkt. The FLL-induced cell death prevented by transfection of caAkt (Fig. 6C). These data imply that down-regulation of PI3K/Akt pathway plays an important role in the FLL-induced glioma cell death.

It has been reported that ERK is 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[12,22] and activation of ERK is inhibited by flavonoids in vascular smooth muscle cells[23], human epidermal carcinoma cells[24], and neuronal cells[25]. However, in the present study, FLL did not affect activation of ERK (Fig. 5). Treatment of the ERK inhibitor and transfection of caMEK also did not alter the FLL-induced cell death (Fig. 6).

To determine whether FLL induces cell death through a caspase-dependent mechanism, we examined the role of caspase activation in the FLL-induced cell death. In the present study, we observed that treatment of FLL caused activation of caspase-3 and the caspase inhibitors also prevented the FLL-induced cell death (Fig. 7). These data indicate that FLL induces apoptosis through a caspase-dependent mechanism.

In conclusion, the present study demonstrated that FLL results in human glioma cell death through inhibition of PI3K/Akt pathway. Induction of cell death may be a promising therapeutic approach in cancer therapy. Our results suggest that FLL may be considered a potential candidate for both glioblastoma prevention and treatment. Further in vivo studies for anti-cancer effect of FLL are required.

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