**Flavonoids in *Ginkgo biloba* fallen leaves induce apoptosis through modulation of p53 activation in melanoma cells**

**HYE-JUNG PARK** and **MOON-MOO KIM**

Department of Chemistry, Dong-Eui University, Busan 614-714, Republic of Korea

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**Abstract.** The aim of the present study was to examine the apoptotic effect of flavonoids in methanol extracts of *Ginkgo biloba* fallen leaves (MEGFL) on melanoma cells. *Ginkgo biloba* is a deciduous castle chaplain and its leaves include various types of flavonoids such as flavonol-O-glycosides. *Ginkgo biloba* is known to have therapeutic properties against a number of diseases such as cerebrovascular diseases, blood circulation disease and hypertension. In the present study MEGFL exhibited a higher cytotoxic effect on melanoma cells than *Ginkgo biloba* leaves (MEGL). It was also found that MEGFL induced apoptotic cell death which was characterized by DNA fragmentation. During the cell death process following treatment with MEGFL, the expression of a variety of death-associated proteins including p53, caspase-3, caspase-9, cytochrome c and Bax were analyzed in the cytosol of melanoma cells. MEGFL significantly increased the expression levels of caspase-3, caspase-9 and p53 in a dose-dependent manner. Our results indicate that MEGFL induced apoptotic cell death by increasing the expression of cell death-associated proteins in melanoma cells.

**Introduction**

Although medical technology has experienced great advances, to date cancer still remains an unsolved major health issue. In recent years, the most promising chemotherapy for the treatment of cancer is known to induce apoptosis. Cell mutations and their unrepaired DNA damage not only adversely affect the surrounding normal cells but also initiate diseases such as cancer (1). For escaping these risks, cells operate self death systems such as apoptosis. Cancer cells have the ability to evade signal stimulation inducing apoptosis. Therefore, apoptosis induction may represent a new therapeutic strategy for the treatment of cancer. Apoptosis is proceeded by various death-associated proteins such as the caspase family, the Bcl-2 family, mitogen-activate protein kinase (MAPK) and tumor-suppressor genes such as p53. The caspase family is divided into initiator caspases and effector caspases (2). When killer T cells detect damaged cells, procaspase-8 is activated by CFasL/D95L a death ligand that binds to the surface of death receptors such as Fas, tumor necrosis factor receptor-1 (TNFR-1) and TNFR-2 (3). Caspase-8, an initiator caspase, induces the break-down of proteins, the actin cytoskeleton and lamin protein, a protein of the nuclear membrane, by activation of caspase-3 and other effector caspases, and then causes cell death through DNA fragmentation (2,4,5). In contrast, the mitochondrial apoptotic pathway is initiated by the release of cytochrome c from mitochondria into the cytosol by external death stimulation (6). The apoptosome formed by binding the cytochrome c to the complex of apoptotic protease activation factor-1 (Apaf-1) and procaspase-9 activates capase-9 and caspase-3, then finally resulting in apoptosis. This mitochondrial pathway is controlled by the Bcl-family (7) that includes both proapoptotic factors and antiapoptotic factors. In normal cells, the antiapoptotic factors inhibit the release of cytochrome c from mitochondria. However, once external death signals are transmitted to them, they cause the release of cytochrome c. When DNA damage is not successfully repaired, it induces apoptosis through activation of Bax, a proapoptosis factor as previously described (2). Hypoxia and a malnutrition condition strongly induce apoptosis as cancer cells proliferate at a rapid rate. Moreover, cancer cells evade apoptosis through downregulation of proapoptotic factors and upregulation of antiapoptotic factors. Thus, apoptosis is a valuable target for the development of anticancer drug since apoptotic cells form apoptotic bodies that are removed by macrophages proceeding inflammation.

*Ginkgo biloba* is deciduous castle chaplain, and numerous studies have reported the physiological effects of *Ginkgo biloba* leaves. The major classes of constituents in *Ginkgo biloba* leaves include flavonoids, diterpenes, sesquiterpenes, polyphenol, organic acid, and polycaccharide (8). Among these, the major components in *Ginkgo biloba* are flavonoids such as quercetin, kaempferol, rutin and robinin. In particular, most of the physiological effects of *Ginkgo biloba* are the result of flavonoids and terpenes. The extract of *Ginkgo biloba* leaves has been used as a therapeutic agent for ischemic stroke, ischemic heart disease and atherosclerosis, and has been reported to have antioxidant, memory and blood circulation effects (9). Particularly, the extract of *Ginkgo biloba* leaves was found to...
inhibit amyloid-β fibril formation and activate caspase-3 and was demonstrated to have an Alzheimer's disease protective effect through repressing the apoptosis of neuronal cells (10). In contrast, other studies have found that kaempferol contained in extracts of Ginkgo biloba leaves induced the apoptosis of pancreatic cancer cells (11). However, the physiological effects of Ginkgo biloba fallen leaves has not been investigated to date. Therefore, the aim of this study was to investigate the potential of Ginkgo biloba fallen leaves as a cancer therapeutic agent by studying the inductive effect of apoptosis on a murine melanoma cell line.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, penicillin/streptomycin/amphotericin (10,000 U/ml, 10,000 g/ml and 2,500 g/ml, respectively) and fetal bovine serum (FBS) were obtained from Gibco-BRL, Life Technologies (Grand Island, NY, USA). B16F1 (ATCC no. CRL-6323) cells were purchased from the American Type Culture Collection (ATCC). MTT reagent, agarose, and other materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Extract preparation. For producing the methanol extracts of Ginkgo biloba fallen leaves, air-dried Ginkgo biloba leaves (MEGL) and fallen leaves (MEGFL) underwent extraction with 95% methanol, respectively. The solvent was evaporated in vacuo to yield 50.0 g of MEGFL as a dark brown solid material. MEGFL (1 g) was suspended in 10 ml of methanol, and was subjected to membrane (0.45 µm) filtration. The extracts were dissolved in DMSO for this study.

Spectrophotometric determination of flavonoid. The total flavonoid content in the MEGFL was determined according to a modified version of the Folin-Ciocalteau method (12) using phloroglucinol as the standard. Samples were diluted taking into account the measurable range of the spectrophotometer (a 0.005-mL aliquot of extract of soluble phenolics was mixed with 0.495 ml water). A 0.1-mL aliquot of the diluted sample was mixed in a test tube with 1.0 ml of 1 N Folin-Ciocalteau reagent. The mixture was allowed to stand for 3 min following addition of 2.0 ml of 20% Na₂CO₃. The samples were incubated in the dark at room temperature for 45 min and centrifuged at 1,600 x g for 8 min. The optical density (OD) of the supernatant was measured at 730 nm using a GENios® microplate reader (Tecan Austria GmbH, Austria). The total flavonoid content was calculated using a standard plotted graph and was expressed as a percentage.

Cell line and culture. The cell lines were separately grown as monolayers in 5% CO₂ and at 37°C in a humidified atmosphere using appropriate media supplemented with 5% FBS, 2 mM glutamine and 100 g/ml penicillin-streptomycin. DMEM was used as the culture medium for the B16F1 cell line. Cells were passaged 3 times a week by treatment with trypsin-EDTA.

MTT assay. Cytotoxic levels of MEGFL were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The B16F1 cell line was grown in 96-well plates at a density of 5 x 10⁴ cells/well. After 24 h, the cells were washed with fresh medium and were treated with MEGFL at 1, 2, 4, 6, 8, 10, 50 and 100 µg/ml. After 48 h of incubation, the cells were re-washed, and 20 µl of MTT (5 mg/ml) was added and incubation was carried out for 4 h. Finally, DMSO (150 µl) was added to solubilize the formazan salt formed, and the amount of formazan salt was determined by measuring the OD at 540 nm using a GENios® microplate reader (Tecan Austria GmbH). The relative cell viability was determined by the amount of MTT converted into formazan salt. The viability of the cells was quantified as a percentage compared to the control (OD of treated cells/OD of blank x 100) and the dose response curves were developed. The data are expressed as the means from at least three independent experiments, and P<0.05 was considered to indicate a statistically significant result.

Reducing power. The reducing power of MEGFL was determined using a method described previously (13). The absorbance of this mixture was measured at 700 nm. The level of reducing power was calculated by the absorbance and expressed as a percentage: Reducing power = (OD of MEGFL/OD of blank) x 100.

DNA oxidation. Genomic DNA was extracted from the B16F1 cells using a standard phenol/proteinase K procedure. The purity of genomic DNA was spectrophotometrically determined at 260/280 nm. DNA oxidation mediated by the Fenton reaction was determined by a method described elsewhere (14). One hundred microliters of the DNA reaction mixture was prepared by adding pre-determined concentrations of the test sample (or the same volume of 10% FBS/DMEM with Fenton reaction as the control group), 200 µM final concentration of FeSO₄, 2 mM final concentration of H₂O₂ and 50 µg/ml final concentration of genomic DNA in the same order. Then the mixture was incubated at room temperature for 30 min, and the reaction was terminated by adding a final concentration of 10 mM of EDTA. An aliquot (20 µl) of the reaction mixture containing ~5 µg of DNA was electrophoresed on 1% agarose gel for 30 min at 100 V. The gels were then stained with 1 mg/ml ethidium bromide and visualized by UV light using AlphaEase® gel image analysis software (Alpha Innotech, San Leandro, CA, USA). The protective effect of MEGFL was quantified as a percentage compared to the blank group (density of the genomic DNA band treated with MEGFL/density of the intact genomic DNA band x 100).

DNA ladder assay. For the DNA fragmentation analysis, the cells were treated with different concentrations of MEGFL. DNA was isolated from the cells, as follows. Cells were washed twice in phosphate-buffered saline (PBS), resuspended in lysis buffer (10 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Triton X-100) and incubated at 50°C for 1 h. RNase A was added to a final concentration of 0.5 mg/ml, and incubation was continued at 50°C for 1 h. Samples were then extracted with phenol-chloroform-isomyl alcohol (25:24:1). High molecular weight DNA was then pelleted at 13,000 x g for 10 min, and the low molecular weight DNA in the supernatant was removed and precipitated overnight in two volumes of ice-cold ethanol at -70°C.
Analyses of protein expression using western blot analysis. Western blotting was performed according to standard procedures. Cells treated with different concentrations of MEGFL were lysed with RIPA lysis buffer (Sigma Chemical Co.). Cell lysates were resolved on a 4-20% Novex® gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane and blocked with 10% skim milk. The primary antibodies including p53, p-p53, Ac-p53, p-p21, caspase-3, caspase-9, Bcl-2, cytochrome c, β-actin and their secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used to detect the respective proteins using a chemiluminescent ECL assay kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Protein bands were visualized using AlphaEase® gel image analysis software (Alpha Innotech).

Statistics. Data were analyzed using the Student's t-test for paired data (comparison of the control group and MEGFL). Data are expressed as means ± SD from three independent experiments. P<0.05, P<0.01 and P<0.001 are indicative of statistically significant results, and are indicated in the figures and legends.

Results

Flavonoid contents in MEGL and MEGFL. Flavonoids are produced entirely by polymerization of phloroglucinol, which is a product of the acetate-malonate pathway, also known as the polyketide pathway. Following extraction with methanol, harvest yields of MEGL and MEGFL were 13.40 and 26.22%, respectively, from starting material at dry weight basis. MEGFL consisted of 5.50±0.21% of flavonoids at dry weight basis. In contrast, the methanol extract of Ginkgo biloba leaves consisted of 1.87±0.17% of flavonoids at dry weight basis.

Effect of MEGFL on cell viability. To investigate the cytotoxic effect of MEGFL, MTT assay was carried out in the B16F1 cell line. The cells were treated with MEGFL at the indicated doses for 48 h. MEGFL exhibited a cytotoxic effect in a dose-dependent manner in the B16F1 cell line (Fig. 1A). MEGFL at 100 µg/ml inhibited cell viability by 80% (P<0.001). However, MEGL did not exhibit any cytotoxicity at a concentration <10 µg/ml. It was found that MEGL at a concentration >50 µg/ml showed cytotoxicity in B16F1 cells (Fig. 1B).

Reducing power of MEGFL. The reducing ability of a compound generally depends on the presence of a reducing agent which exhibits antioxidative potential by breaking the free radical chain, donating a hydrogen atom. Vitamin C at 100 µg/ml significantly displayed reducing power compared with the blank group (P<0.001) (Fig. 2). MEGFL significantly showed reducing power in a dose-dependent manner. In particular, it was observed that MEGFL increased the reducing power by 60% compared with the blank group without MEGFL treatment.

Inhibition of radical-mediated DNA oxidative damage by MEGFL. In a subsequent experiment, genomic DNA was isolated from B16F1 cells to study the protective effect of MEGFL against DNA oxidative damage induced by hydroxyl radical. The genomic DNA of the control group was completely degraded by the hydroxyl radical produced by the
Fenton reaction, compared with the blank group without the Fenton reaction (Fig. 3). Treatment with MEGFL at 10 µg/ml or more significantly inhibited the oxidative damage of DNA (P<0.001). The DNA damage was inhibited by 80% in the presence of MEGFL at 10 µg/ml, compared with the control group treated with the same volume of 5% FBs/DMEM instead of the test sample with the Fenton reaction. However, it was found that MEGFL at 1 µg/ml could not clearly protect the oxidative damage of DNA by the hydroxyl radical.

**Effect of MEGFL on DNA fragmentation.** To investigate the effect of MEGFL on DNA fragmentation, DNA of B16F1 cells treated with MEGFL was observed using the technique of DNA electrophoresis. Etoposide at 100 µM was used as a positive control for DNA fragmentation. DNA of cells treated with etoposide was cleaved compared to the blank group without any stimulation (Fig. 4). Furthermore, MEGFL had an inductive effect on DNA fragmentation in a dose-dependent manner and showed apoptosis at a concentration of MEGFL >6 µg/ml.

**Effect of MEGFL on the protein expression levels of caspase-3 and caspase-9.** During the apoptosis process, caspases play a key role in protein cleavage. To investigate the effect of MEGFL on the protein expression levels of caspase-3 and caspase-9, western blot analysis was carried out in B16F1 cells. Etoposide at 100 µM was used as a positive control. The protein expression levels of caspase-3 and caspase-9 were significantly increased in the presence of etoposide and MEGFL at concentrations from 1 to 4 µg/ml compared with the blank group (Fig. 5). However, MEGFL decreased the protein expression levels of caspase-3 and caspase-9 in the presence of MEGFL at 6 µg/ml.

**Effect of MEGFL on the protein expression levels of p53, phosphorylated p53, acetylated p53 and phosphorylated p21.** When DNA damage occurs, p53 is activated to repair DNA or to induce apoptosis. The activation of p53 is controlled by p53 phosphorylation and acetylation. In addition, p53, a transcription factor, binds to the promoter of p21. p21 is closely associated with cell cycle arrest. As shown in Fig. 6, consistent with the results shown in Fig. 5, the protein expression levels of p53,
The aim of the present study was to investigate the potential of MEGFL to induce apoptosis through modulation of p53.

Discussion

Our findings revealed that MEGFL inhibits cell proliferation by disturbing progression of the cell cycle. p53, a transcription factor binding to the promoter of p21, is a normal short-lived protein that is maintained at low levels, yet p53 is transiently accumulated when serious DNA damage occurs in cells. p53 modulates the cell cycle through induction or inhibition of WAF1, AFN and MDM (22,23). When DNA damages are induced, the cell cycle is arrested and p53 is activated by disturbing progression of the cell cycle. p53, a transcription factor, is not only a necessary process to ensure body shape in ontology but also a protective reaction against a detrimental influence on healthy cells surrounding diseased cells when found to be seriously damaged (15,16). In contrary with necrosis, apoptosis does not release toxic substance out of cells. Therefore, it was suggested that apoptosis can be used as a target with which to treat cancer (17). In the present study, an MTT assay was initially carried out to investigate the effect of MEGFL on induction of apoptosis. Consequently, MEGFL caused cell death of the B16F1 cell line. MEGFL exhibited apoptosis at a much lower concentration when compared with other plant extracts. We carried out various characteristic experiments concerning apoptosis to support that cell death was the result of the apoptosis induced by MEGFL. Apoptosis exhibits DNA laddering through cut linker region between nucleosome as 180-200 base pair length while necrosis causes random DNA cleavage. In order to examine the DNA ladder effect of MEGFL, a DNA ladder assay was performed. Etoposide, a well-known positive control, and MEGFL clearly indicated DNA laddering compared with the blank group without treatment. Especially, MEGFL caused marked DNA laddering at 6 µg/ml or more. This result was associated with the tendency of protein expression related to apoptosis. However, most cells were dead in the presence of MEGFL at 6 µg/ml. Apoptosis is initiated by various proteins such as the caspase family and Bcl-2 family (18,19). The caspase family, cysteine-dependent aspartate-specific protease, is divided into initiator caspases and effector caspases. During the cell death process, caspase-8, -9 and -10, initiator caspases, transmit apoptotic signals, and caspase-3, -6 and -7, effector caspases, exert the effect to degrade protein (4,20). Our results suggest that MEGFL activated caspase-9 through cleavage of procaspase-9 in the presence of MEGFL. Furthermore, the protein expression level of caspase-3 was increased in the presence of MEGFL. In general, the apoptosome is a complex formed with Apaf-1 that is activated by cytochrome c released from mitochondria into the cytosol. It subsequently activates caspase-3 leading to apoptosis. Both the expression levels of cytochrome c and Bcl-2, an antiapoptotic factor, were not altered at a nontoxic concentration of MEGFL. p-p21 arrests the cell cycle not only through inhibition of cyclin D/CDK4 and cyclin E/CDK2 complexes in early G1 phase but also inhibiton of cyclin A/CDK2 complex prior to the S phase/G2 phase transition (2,21). MEGFL highly increased the protein expression level of p-p21 (Ser146) at nontoxic concentrations. Especially, MEGFL caused marked DNA laddering at 6 µg/ml or more. This result was associated with the tendency of protein expression related to apoptosis. It is hypothesized that MEGFL caused apoptosis by induction of Bax (24). Stability and site-specific DNA-binding activity of p53 are associated with phosphorylation and acetylation of p53 (25). The phosphorylation of p53 is caused by protein kinase C (PKC) at Ser378 and by casein kinase 2 (CK2) at Ser392. The carboxy-terminal region of p53 phosphorylated p53 (p-p53 and Ser15), acetylated p53 (Ac-p53 and Lys373-382) and phosphorylated p21 (p-p21 and Ser146) were increased in the presence of etoposide and MEGFL at a range from 1 to 4 µg/ml. MEGFL treatment at concentrations >6 µg/ml caused a reduction in the protein expression levels. These results suggest that the altered expression levels of p53 and p-p21 are related to cell death.
p53, including phosphorylation by CK2 and PKC kinases and truncation of the last 30 residues, modulate the ability of p53 to bind its recognition site through the central sequence-specific DNA-binding domain (23,26). On the other hand, acetylation of p53 is formed by histone acetyl transferases (HATs) such as p300 and PCAF. The p300 acetylates Lys382 in the carboxy-terminal region of p53, whereas PCAF acetylates Lys320 in the nuclear localization signal. Acetylations at either site enhance sequence-specific DNA binding site of p21 (27). Our findings revealed that MEGFL induced activation of p21 through modulation of p53, CDK inhibitor. Furthermore, our results suggest that MEGFL induced apoptosis by inhibiting cell proliferation as a result of disturbing progression of the cell cycle and increasing the expression levels of caspase-3 and caspase-9. In addition MEGFL has an antioxidant effect through enhancing reducing power and DNA protection. Therefore, this study provides evidence that MEGFL has the potential to cause apoptosis induction, and represents a new therapeutic strategy for the treatment of cancer.

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