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Induction of apoptosis by luteolin through cleavage of Bcl-2 family in human leukemia HL-60 cells

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Abstract

In our study, luteolin has shown its apoptosis-inducing potent in HL-60 cells with its 76.5% apoptotic ratio of 100 μM treatment. When HL-60 cells were treated with 60 μM of luteolin, DNA ladders were visible at 6 h and increased from 6–12 h after treatment. Luteolin could decrease the mitochondrial membrane potential, trigger cytochrome *c* released to cytosol, and subsequently induce the processing of procaspase-9 and procaspase-3, which were followed by the cleavage of poly-(ADP-ribose) polymerase (PARP) and DNA fragmentation factor (DFF-45). The cleavage of the proapoptotic Bcl-2 proteins, such as Bad and Bax to produce their truncated forms, and the cleavage of the antiapoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-X_L, into their potent pro-apoptotic fragments were detected in our study. From the results, we suggested that the structure of luteolin contributes to its potent in inducing apoptosis in HL-60 cells, and the mitochondrial pathway might play an important role in the luteolin-induced apoptosis. The induction of apoptosis by luteolin may offer a pivotal mechanism for its cancertherapeutic and chemopreventive action.

Keywords: Luteolin; Apoptosis; Caspase; Mitochondria; Bcl-2; Bcl-X_L; Bad; Bax; Cleavage

1. Introduction

Flavonoids are plant secondary metabolites, which are biologically active polyphenolic compounds widely distributed in plant (Crozier et al., 2000). Flavonoids have shown many biological properties that are associated with reduced cancer rates (Birt et al., 2001). They are organized into five classes including flavones, flavonols, flavanones, flavan-3-ols and anthocyanidins, base on their chemical structures. The 3-hydroxyl group may inhibit the capacity of inducing apoptosis, however, the greater the number of 3-hydroxyl groups in the 2-phenyl groups, the greater the effect in recovering the capacity of inducing apoptosis (Wang et al., 1999). Luteolin is one kind of flavone, which exists highly in thyme and also presents in beets, Brussels sprouts, cabbage and cauliflower (Eldridge et al., 2003). Luteolin rich artichoke leaf extract showed a concentration dependent inhibitory activity in several models of oxidative stress, and could protect low-density lipoprotein from oxidation in vitro (Garcia et al., 2000). In addition, luteolin has strong scavenging activity for peroxide radicals (Cotelle et al., 1996; Cai et al., 1997). Luteolin inhibits the effects of tyrosine kinase, an enzyme involved in tumor cell proliferation, and therefore may has potential as a dietary anticarcinogenic agent (Cotelle et al., 1996; Chen et al., 1996, Samejima et al., 1995; Spencer et al., 1999). Report had shown that luteolin is a possible uncompetitive inhibitor of N-acetyltransferase activity in human bladder cancer (Su et al., 2003).

Apoptosis is a normal physiological process or pathological cell death (Kerr et al., 1972), which occurs during maintenance of tissue homeostasis and in the removal of damage cells that break the homeostasis of cell numbers (Arend and Wyllie, 1991). The morphological changes of apoptosis include membrane blebbing, cell shrinkage,

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chromatin condensation, DNA fragmentation, loss of adhesion and rounding (in adherent) (Cho and Choi, 2002). The regulatory factors in apoptosis include death receptor, proteolytic enzymes (such as caspase and calpain), Bcl-2 family members, and so on (Cho and Choi, 2002). There are two main pathways involving apoptotic cell death, One is the interaction of the cell surface receptor, such as Fas and tumor necrosis factor α-1 (TNFα-1) (with their ligands, and the second pathway involved the participation of mitochondria. The mitochondrial pathway is regulated by the antiapoptotic and proapoptotic members of Bcl-2 family (Martin and Green, 1995). The aspartate specific cysteine protease named caspase are present in cells as inactive pro form, and whose active tetramer form are from removing of the prodomain and cleaving between the large subunit and the small one (Martin and Green, 1995). Initiator caspases, such as caspase-8 and caspase-9, trigger apoptosis by cleaving and activating the executioner caspase, such as caspase-3 (Csokay et al., 1997; Zimmermann and Green, 2001). The activation of caspase cascade induces the cleavage of specific cellular substrates and therefore result in the biochemical and morphological changes associated with the apoptotic cell death (Luo et al., 1998). For example, caspase-2, -3, -7, and -9 act on cleaving poly-(ADP-ribose)-polymerase (PARP), and caspase-3 cleaves the inhibitor of caspase-activated deoxyribonuclease (CAD) named iCAD/DFF-45 (DNA fragmentation factor-45), allowing the nuclease to cut the chromatin (Liu et al., 1997).

Mitochondria is an essential role of apoptosis (Green and Reed, 2003). During apoptosis, while antiapoptotic proteins, including Bcl-2 and Bcl-X_L prevent cytochrome *c* release from mitochondria for keeping cell survival (Kluck et al., 1997; Yang et al., 1997), the proapoptotic Bcl-2 proteins, such as Bax Bad, and Bid, play a major role in decreasing the mitochondrial membrane potential ($\Delta\Psi$) and accelerating cytochrome *c* release from intermembrane space into cytoplasm to trigger the downstream molecules of apoptosis (Luo et al., 1998). It has been shown that the antiapoptotic proteins Bcl-2 and Bcl-X_L of mammals can be converted into potent proapoptotic molecules when they are cleaved by caspases during apoptosis (Bellows et al., 2000). In addition, during CTLL-2 cell apoptosis, Bcl-X_L protein was cleaved by caspase 3/CPP32 like proteases to produce two 18 kDa fragments and results in accelerating apoptotic cell death (Fujita et al., 1998). Cleavage of Bcl-2 by Caspase-3 has been shown the releasing of a potent pro-apoptotic fragment (Cheng et al., 1997). The proapoptotic molecule, Bax, exists mainly in cytosol and it can be cleaved by the activated calpains and caspases during drug induced apoptosis (Wood et al., 1998). Moreover, the calpain-induced 18 kDa truncated Bax (tBax) is a more potent inducer of apoptosis than its wild type (Toyota et al., 2003). Therefore, it translocates to the mitochondria and involves in the activation of caspase-9, -2, -3 (Ohtsuka et al., 2004). Bad also cab be cleaved through caspase dependent pathway to generate a 15 kDa truncated Bad (tBad) when apoptosis was induced by transforming growth factor β1 (TGF-β1) (Kim et al., 2002). Studies also indicated that the caspase-3-cleaved Bad is a more potent inducer of cytochrome *c* release than the wild type Bad (Taghiyev et al., 2003).

Although the apoptotic effects of luteolin have been reported (Mittra et al., 2000; Lee et al., 2002), little is known of the mechanisms of luteolin-induced apoptosis. In the present study, we used leukemia cell line HL-60 to elucidate the molecular mechanism of luteolin-induced apoptosis, the apoptotic machinery, and the modulations of several cell death proteins were investigated.

2. Material and methods

2.1. Materials

Luteolin, quercetin, genistein and daidzein were obtained from Versuchsstation Schweizerischer Brauereien, Zürich, Switzerland and dissolved in dimethyl sulfoxide (DMSO) prior to use. The antibodies to Bax and DFF-45 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-caspase-3, and -9, anti-Bad, cytochrome C and PARP antibodies were from PharMingen (San Diego, CA); anti-Bcl-2, Bcl-X_L antibodies were purchased from Transduction Laboratory (Lexington, KY). Secondary antibodies used were fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin G IgG (Chemicon), horseradish peroxidase-conjugated anti-mouse and rabbit IgG. Propidium iodide and rhodamine 123 were obtained from Sigma (St. Louis, MO). Fluorogenic peptide substrates Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) was specific for caspase-3 activity.

2.2. Cell culture

Human leukemia cell lines HL-60 (promyelocytic cells) was obtained from American Type Culture Collection were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640), supplemented with 15% fetal calf serum (Gibco BRL, Grand Island, NY), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-Glutamine (Life Technologies, Grand Island, NY) and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.3. Acridine orange staining assay

Cells (5×10^5) were seeded into 60-mm petri dishes and incubated at 37 °C for 24 h (h). The cells were harvested after treatment for 24 h, and 5 μl of cell suspension was mixed on a slide with an equal volume of acridine orange solution [10 μg/ml in phosphate buffer saline (PBS)]. Green fluorescence was detected between 500 and 525 nm by using an Olympus microscope (Olympus America, Lake Success, NY). Bright-staining condensed chromatin was detected in apoptotic cells.

2.4. Apoptotic ratio analysis

Cell apoptotic ratio was analyzed by flow cytometry as follows. At each time point, cells were harvested, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol for at least 2 h at −20 °C. Fixed cells were washed with PBS, incubated with 1 ml PBS containing 0.5 μg/ml RNase A and 0.5% Triton X-100 for 30 min at 37 °C, and then stained with 50 μg/ml propidium iodide. The stained cells were analyzed by FACScan laser flow cytometer (Becton Dickinson, San Jose, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME).

2.5. DNA extraction and electrophoresis analysis

HL-60 (2×10^5 cells/ml) was harvested, washed with PBS, and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5 mg/ml proteinase K, 50 mM Tris (hydroxymethyl) aminomethane (pH 8.0), 10 mM EDTA at 56 °C for 3 h and treated with RNase A (0.5 μg/ml) for another 2 h at 56 °C. The DNA was extracted by phenol/chloroform/isoamyl alcohol (25/24/1) before loading and analyzed by 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in TBE (Tris-borate/EDTA electrophoresis buffer). Approximately 20 μg DNA was loaded in each well and visualized under UV light and photographed.

2.6. Mitochondrial membrane potential

HL-60 cells were cultured and allowed to reach exponential growth for 24 h before treatment. The cells were harvested for 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h after treatment with 60 μM of luteolin. Loss of mitochondrial transmembrane potential was monitored by flow cytometry. Briefly, HL-60 cells were exposed to luteolin, and the mitochondrial transmembrane potential was measured directly using 40 nM 3,3′ -dihexyloxacarbocyanine (DiOC6) (Molecular Probe Eugene, Oregon, USA). The samples (10^4 events) were analyzed for fluorescence (FL1 detector, filter 430/30 nm band pass) using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Histograms were analyzed using Cell Quest software, and compared with histograms of control untreated cells.

2.7. Cytochrome c release

HL-60 cells were treated with luteolin or vehicle (DMSO) control for indicated times. At the end of the treatment, cells were harvested, washed twice in ice cold PBS, and prepared by resuspending in buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiolthione, 17 μg/ml phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, 2 μg/ml leupeptin [pH 7.4]) and incubated on ice for 30 min. Cells were passed through a needle 20 times. Unlysed cells, and nuclei were pelleted by centrifugation at 750×*g* for 10 min. The supernatant was then spun at 10,000×*g* for 15 min. This pellet was resuspended in buffer A and represents the mitochondrial fraction. The remaining supernatant was centrifugation at 100,000×*g* for 30 min. The supernatant from this final centrifugation represents the cytosolic fraction. Cytochrome *c* release was determined by Western blot as describe later.

2.8. Western blot analysis

HL-60 cells were treated with various concentrations of luteolin for 12 h or 60 μM for indicated times. Cells were harvested and homogenized in a lysis buffer (50 mM Tris–HCl, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.5% NP-40; 0.5 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin; and 0.5 mM dithiothreitol) for 30 min on ice. The cell debris were pelleted by centrifugation at 10,000×*g*, 4 °C for 30 min. The supernatant proteins were measured by bicinchoninic acid assay. 50 μg of total cellular proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% for PARP, DFF-45, and α-tubulin; 12.5% for caspases-3 and caspase-9; 15% for Bcl-2, Bcl-X_L, Bad, Bax, and cytochrome *c*), transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, Arlington, IL), and then probed with primary antibody followed by adding horseradish peroxidase-labeled secondary antibody. The immunocomplexes were visualized by the ECL system (Amersham Life Science, Amersham, Bucks, UK).

2.9. Assay of caspase activity

HL-60 cells were treated with 60 μM luteolin at indicated times and then collected and washed with PBS and resuspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by

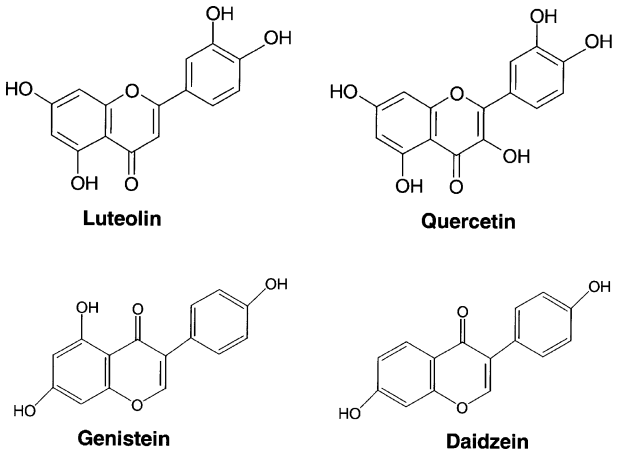

Fig. 1. Chemical structures of flavonoids, (A) luteolin; (B) quercetin; (C) genistein; (D) daidzein.

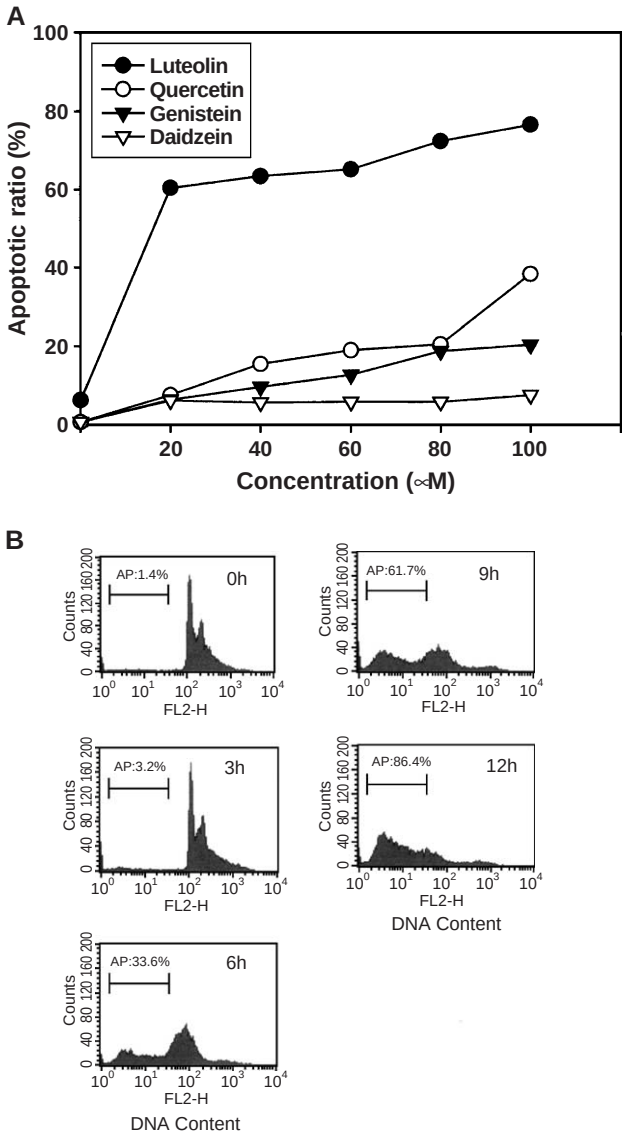

Fig. 2. (A) Effects of flavonoids on apoptotic ratios. HL-60 cells were treated with various concentrations of luteolin, quercetin, genistein and daidzein as indicated for 12 h. The ratios of sub-G1 cells in flavonoids-treated HL-60 cells were analyzed by flow cytometry. Sub-G1 represents apoptotic cells with a lower DNA content. (B) Determination of the effects of sub-G1 cells in control and 60 μM luteolin-treated HL-60 cells at different time periods by flow cytometry.

centrifugation at 4 °C, 12,000×*g* for 20 min. 50 μg of total protein, as determined by bicinchoninic acid assay (Promega), was incubated with 50 μM substrate Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC), Ac-Try-Val-Ala-Asp-AMC (Ac-YVAD-AMC), or Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) at 30 °C for 1 h. The release of AMC (methylcoumaryl-7-amine) was measured by a fluorescence spectrophotometer (Hitachi F-2000) with excitation at 360 and emission at 460 nm. Bcl-2, Bcl-X_L antibodies were purchased from Transduction Laboratory (Lexington, KY). Secondary antibodies used were fluorescein isothiocyanate-labeled rabbit anti-mouse IgG (Chemicon), horseradish peroxidase-conjugated anti-mouse and rabbit IgG. Propidium iodide and rhodamine 123 were obtained from Sigma (St. Louis, MO). Fluorogenic peptide substrates Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) specific for caspase-3 acitvity was purchased from Bachem (King of Prussia, PA).

3. Results

3.1. Flavonoids cause dose-dependent increasing in leukemic cell apoptotic ratio

In the present study, we investigated four structurally related flavonoids: luteolin, quercetin, genistein, and daidzein. The structures of these flavonoids are illustrated in Fig. 1. We tested their effects on apoptotic ratio in human promyelocytic leukemia HL-60 cells with different concentrations of flavonoids. After 12 h of treatment, a sub G1 (sub 2N) DNA peak, which has been suggested to be the apoptotic DNA, was detected, and the percentage of apoptotic cells was analyzed by flow cytometry. As shown in Fig. 2A, luteolin appeared to be potent apoptosis-inducing agent for HL-60 cells, and the apoptotic effects was found to be dose-dependent. The apoptotic effects of luteolin also followed a time-dependent pattern. We also tested the effects of 100 μM luteolin on the apoptotic ratio of HL-60 cells with different time by flow cytometry. The apoptotic effects of luteolin also followed a time-dependent pattern (Fig. 2B), and the percentage of apoptotic HL-60 cells was increased sharply from 3 h (3.2%) to 6 h (33.6%) with 60 μM luteolin treatment. As comparing with other

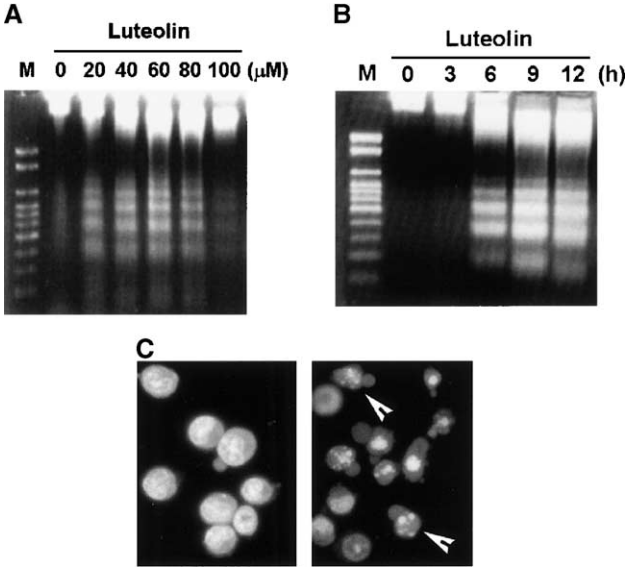

Fig. 3. Induction of DNA fragmentation by luteolin (A) HL-60 cells were treated with various concentrations of luteolin as indicated for 12 h, and (B) HL-60 cells were treated with 60 μM luteolin for indicated time. Internucleosomal DNA fragmentations were analyzed by agarose electrophoresis. M: one hundred base pairs DNA ladder size maker. (C) The chromatin condensation of apoptotic cells induced by luteolin for 12 h. The condensed chromosomes are seen as spots in the nucleus by acridine orange staining; apoptotic cells are shown as white arrowheads.

three flavonoids, luteolin has shown strongest apoptosis-inducing effects on HL-60 cells, thus we further used luteolin to study the mechanism of apoptotic cell death and its effects on caspases and the Bcl-2 family members.

3.2. Luteolin-induced DNA fragmentation of leukemic cell lines

The effect of luteolin on DNA fragmentation, a hallmark of apoptosis, was determined by incubating HL-60 cells with different concentrations of luteolin for 12 h (Fig. 3A). DNA fragmentation appealed at 20 μM of luteolin treatment, and the DNA ladders response was dose-dependent from 20 to 60 μM treatment. When HL-60 cells were treated with 60 μM of luteolin, DNA ladders were visible at 6 h after treatment, and increased from 6 to 12 h (Fig. 3B). Similar with the results of apoptotic ratio, the DNA fragmentation increased sharply at 6 h treatment with 60 μM luteolin indicating that luteolin triggering apoptosis in HL-60 cells. The apoptotic effects of luteolin also followed a time-dependent pattern (Fig. 3B), and the percentage of apoptotic HL-60 cells was increased sharply from 3 h (3.3%) to 6 h (33.6%) with 60 μM luteolin treatment. To characterize the cell death induced by luteolin, we examined the nuclear morphology of dying cells with a fluorescent DNA-binding agent, acridine orange. Within 12 h of treatment with 60 μM luteolin, cell clearly exhibited significant morphological changes and chromosomal condensation, which is indicative of apoptotic cell death (Fig. 3C).

3.3. Disruption of mitochondrial potential and release of cytochrome c

The decreasing of mitochondrial membrane potential ($\Delta\Psi_m$) causes the disruption of the outer mitochondrial membrane and contributes to the release of cytochrome *c*. therefore; the cytochrome *c* release results in the activation of caspase-9 and subsequently leads to apoptosis (Csokay et al., 1997; Zimmermann and Green, 2001). As illustrated in Fig. 4A, a reduction in mitochondrial membrane potential was detected at 30 min and clearly changed at 1.5 h after luteolin treatment. Fig. 4B displayed the cytochrome *c* released into cytosol was detected at, 1, 2, 3, 4, 5, 6 h after

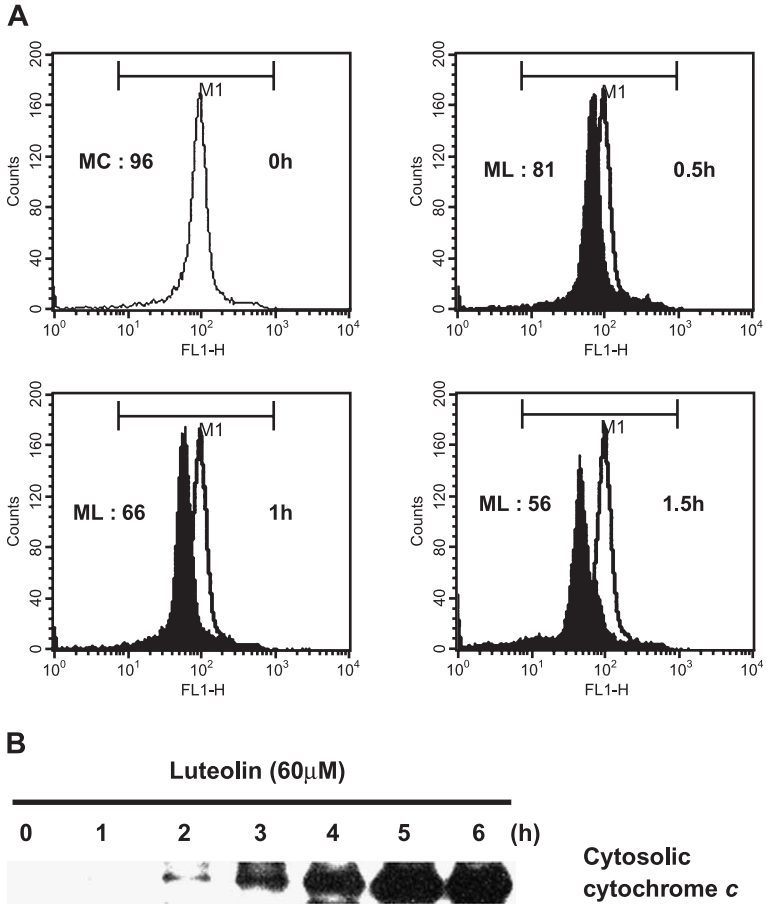

Fig. 4. Decrease of mitochondria membrane potential and release of cytochrome *c* into cytosol. (A) HL-60 cells were treated with 60 μM luteolin for 0.5, 1, 1.5, 2, 2.5, 3 h, and the relative mitochondrial membrane potential ($\Delta\Psi_m$) was measured by fluorescent emission. The MC represented the profile of control cells, and the ML indicated the treated cells. The numbers represented the mean of the relative fluorescent intensity. (B) HL-60 cells were treated with 60 μM luteolin at indicated time periods. Subcellular fractions were prepared as described in Materials and methods, and cytosolic cytochrome *c* was detected by Western blotting.

luteolin treatment relative to gradual decrease of mitochondrial cytochrome *c*. These observations suggested that an apoptosis-inducing mechanism via mitochondria pathway was triggered by luteolin in HL-60 cells.

3.4. Luteolin induced the cleavage and activation of caspase-3 and -9

The caspases are believed to play a main role in causing apoptotic response by cleaving or degrading several cellular substrates (Zimmermann and Green, 2001). For monitoring the enzymatic activity of caspase-1, -3, -9, caspase-1, -3, -9 activity was measured following treatment of HL-60 cells with 60 μM treatment for various time in HL-60 cells. As illustrated in Fig. 5A, both caspase-3 and -9 were time-dependently activated by luteolin, but the data has shown a very low level of caspase-1 activities after luteolin treatment. Luteolin induced a rapid rise in caspase-3 activity to approximately a 37-fold increase and in caspase-9 a 7.5-fold increase after 12 h treatment. Paralleled with the timing pattern of appearance of the DNA ladder, the caspase-3 activity increased from 20 to 40 μM treatment, arose rapidly from 40 to 60 μM treatment, and then dropped down sharply with higher dose treatment (Fig. 5B). Fig. 5C and D showed a gradual decrease in the level of the pro-caspase-3 and pro-caspase-9, respectively, indicating that both caspases were activated after 6 h of luteolin treatment. The cleavage of pro-caspase-9 and pro-caspase-3 were detected at various time points with 6 μM of luteolin treatment, and at various concentrations after 12 h of luteolin treatment in HL-60 cells by western blotting. As shown in Fig. 5C and D, the cleavage of pro-caspase-9 sequentially occurs in a time course- and dose-dependent manner. Caspase-9 activity showed a similar tread as that of the caspase-9 (Fig. 5D). Caspase-9 is activated in the presence of apaf-1, cytochrome c, and dATP. The inactive 68 kDa precursor of pro-caspase-9 was cleaved to generate an active form of 43 kDa subunits (Nicholson and Thornberry, 1997). Caspase-9 is upstream of caspase in a protease cascade that activate caspase-3. Once caspase-9 becomes activated, it will initiate a protease cascade leading to the rapid activation of caspase-3, a major caspase activity in cells undergoing apoptosis (Martin and Green, 1995; Csokay et al., 1997; Zimmermann and Green, 2001). Whereas, caspase-3 is activated by two sequential proteolytic events that cleave the 32 kDa precursor at aspartic acid residue to generate an active heterodimer of 20 and 12 kDa subunits (Liu et al., 1997).

Disruption of mitochondria caused the release of cytochrome *c*, and resulted in the cleavage and activation of caspase-9. Activation of the caspase-3, a central role in many types of apoptosis was associated with the activation of caspase-9 in luteolin-induced apoptosis (Nicholson and

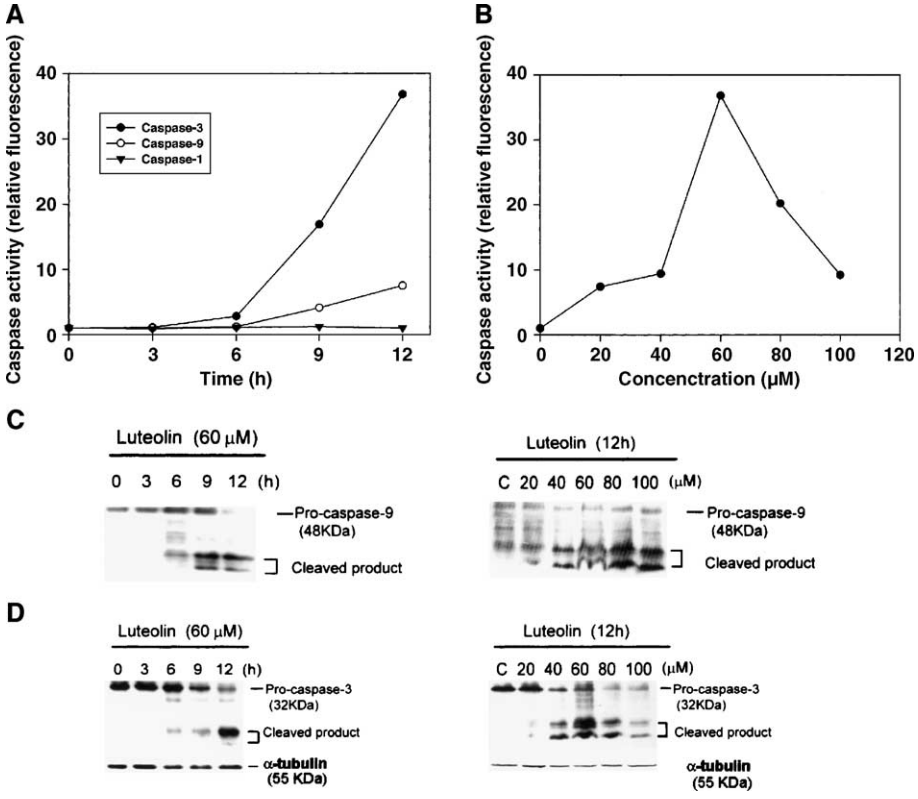

Fig. 5. (A) Activity of caspase-1, -3, and-9 after HL-60 cells were treated with 60 μM of luteolin for indicated time; (B) The effects of various concentrations of luteolin on the activity of caspase-3; (C) Cleavage and activation of caspase-3 resulted the processing of caspase-3 substrates during luteolin-induced apoptosis. (D) Cleavage and activation of caspase-9 resulted the processing of caspase-9 substrates during luteolin-induced apoptosis. The processing of procaspase-3 and -9 were detected by Western blotting analysis using specific antibody against caspase-3 and -9.

Thornberry, 1997; Thompson, 1995). The generation of caspase-9 and the subsequent activation caspase-3 is consistent with DNA fragmentation (Fig. 5C–D).

3.5. The effects of luteolin on PARP and DFF-45

Activation of caspase-3 causes the cleavage of poly-(ADP-ribose)-polymerase (PARP), a hallmark of apoptosis, to produce an 85 kDa fragment during apoptosis (Tewari et al., 1995). To detect the cleavage of PARP following the activation of caspase-3, HL-60 cells were treated with 60 μM of luteolin for various time course. Western blotting analysis has shown that luteolin leads a time dependent proteolytic cleavage of PARP, and results in accumulating of 85 kDa fragment and decreasing of the 116 kDa protein (Fig. 6A).

In apoptotic cells, activation of caspase-activated-DNase (CAD), also a substrate of caspase-3, occurs with the cleavage of DNA fragmentation of factor-45 (DFF-45). Once CAD is activated and released, it can translocate to the nucleus and then degrades chromosomal DNA to produce DNA fragmentation (Enari et al., 1998). To exam the cleavage of DFF-45 by Western blotting, HL-60 cells were treated with different concentrations of luteolin for 12 h and 60 μM of luteolin for various time courses. As shown in Fig. 6B, the cleavage of DFF-45 induced by luteolin progressively increased in a dose and time dependent manner.

3.6. The cleavage of pro-apoptotic and anti-apoptotic members of the Bcl-2 family

The expression of pro-apoptotic and anti-apoptotic members of the Bcl-2 family members regulates the mitochondrial pathway. It is one of the major mechanisms that determine the ultimate fate of cells in the apoptotic process. We examined the expression of pro-apoptotic

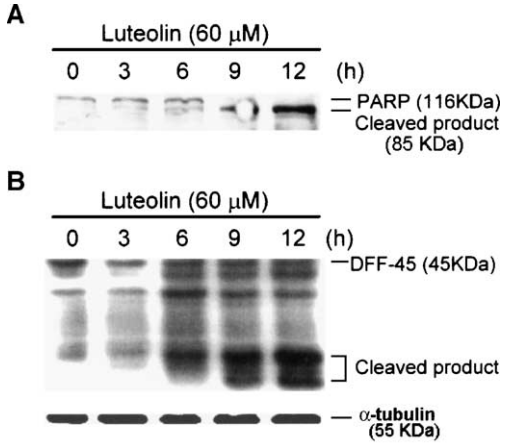

Fig. 6. (A) Time-dependent cleavage of poly-(ADP-ribose) polymerase induced by luteolin. (B) Time-dependent and dose-dependent cleavage of DFF-45 induced by luteolin. The processing of PARP and DFF-45 were detected by Western blotting analysis using specific antibody against PARP and DFF-45.

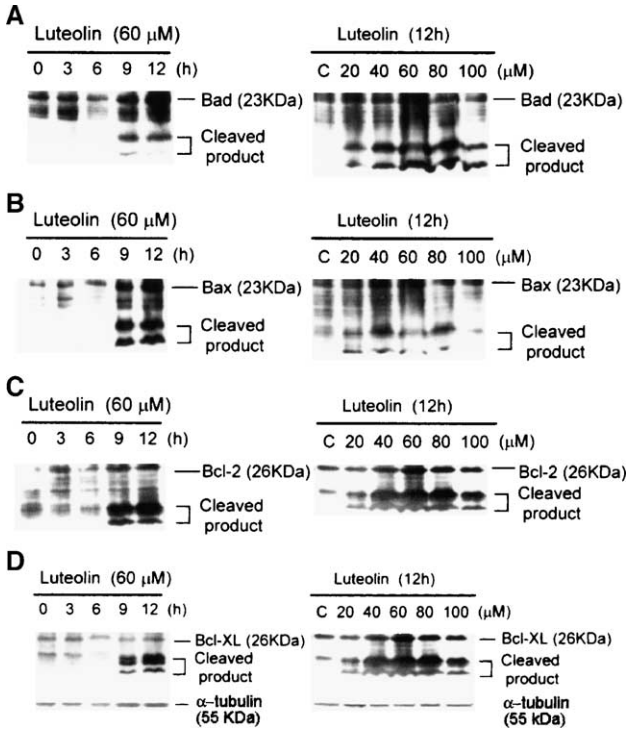

Fig. 7. Effects of luteolin on: (A) Bad; (B) Bax; (C) Bcl-2; and (D) Bcl-X_L expression in HL-60 cells. HL-60 cells were treated with various concentration of luteolin for 12 h and treated with 60 μM luteolin for indicated time. The expressions of Bad, Bax, Bcl-2, and Bcl-X_L were detected by Western blotting analysis using specific antibodies against Bad, Bax, Bcl-2, and Bcl-X_L, respectively.

proteins, Bad and Bax, at different time points and with various concentrations after 12 h of luteolin treatment. As the result in Fig. 7A, there was no visible change of Bad expression, but the cleaved products of Bad were shown after luteolin treatment. It has reported that during drug induced apoptotic cell death, Bax can be cleaved by caspases and calpains, and then it translocated to mitochondria upon introduction of apoptosis (Wood et al., 1998). Similar with the results of the Bad testing, after luteolin treatment, there was also a slight change of Bax expression, but Bax was cleaved to produce small fragments (Fig. 7B).

We next tested the expression of two anti apoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L, with indicated concentrations for 12 h of luteolin treatment. As illustrated in Fig. 7C, there was a slight change of Bcl-2 expression, but a cleaved product showed up and displayed a dose-dependent increase of the cleavage product. There was also slight change of Bcl-X_L expression, and Bcl-X_L was cleaved to produce two small size fragments (Fig. 7D).

4. Discussion

Recent studies have suggested that luteolin may have potential as a dietary anticarcinogen by inhibiting the effects of tyrosine kinase (Cotelle et al., 1996, Chen et al., 1996,

Samejima et al., 1995; Spencer et al., 1999). In this study, we showed the relationship between the structurally related flavonoids and their ability in inducing apoptosis in HL-60 cells. In addition, we also clarified the molecular mechanism and the role of mitochondria and Bcl-2 family members in luteolin-induced HL-60 cell apoptosis (Fig. 8).

From the results of the luteolin-induced DNA fragmentation in HL-60 cells, the 60 μM treatment for 12 h showed the greatest amount of DNA ladder, and then the amount dropped when treated with 80 and 100 μM luteolin. However, the data of apoptotic ratio showed that the luteolin-induced apoptotic effects for HL-60 cells were dose-dependent. We suggested that the decrease of DNA fragmentation of 80 and 100 μM luteolin treatment was due to the secondary apoptosis of HL-60 cells.

The decrease of mitochondrial membrane potential ($\Delta\Psi_m$) after stimulus disrupts outer mitochondrial membrane, and therefore leads to cytochrome *c* release from mitochondria and induces apoptosis (Luo et al., 1998). In our study, we found that the mitochondrial membrane potential of HL-60 cells decreased after luteolin treatment, and the cytosolic cytochrome *c* increased gradually in a time-dependent manner. These results indicated that luteolin has potency on changing mitochondrial membrane potential and triggering cytochrome *c* released to cytosol. Furthermore, the release of cytochrome *c* contributes to the activation of caspase-9 and subsequently causes the activation of caspase-3 (Pan et al., 1999; Csokay et al., 1997; Zimmermann and Green, 2001). The study in ours,

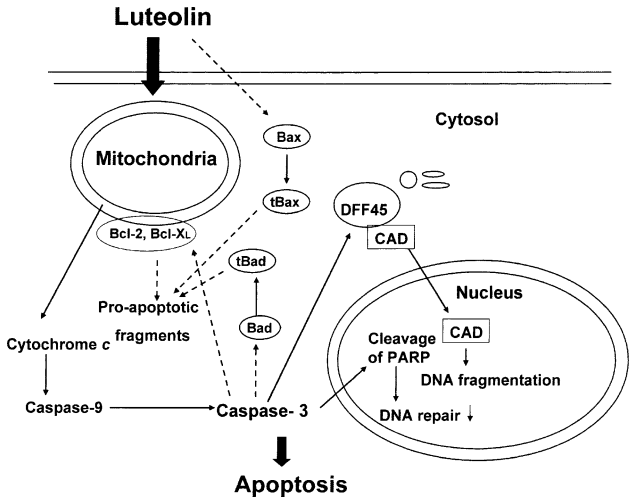

Fig. 8. Schematic representation of action mechanism by which luteolin induced apoptosis in HL-60. Luteolin might decrease mitochondrial membrane potential, cause cytochrome *c* release and result in the activation of caspase-9 and caspase-3. The activated caspase-3 further cleaved DFF-45 and lead to the release of CAD to cleave DNA and the cleavage of PARP to block DNA repair. Luteolin also lead to the cleavage of the proapoptotic Bcl-2 proteins, such as Bad and Bax to produce their truncated forms, and the cleavage of the antiapoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-X_L, into their potent pro-apoptotic fragments, and resulted in further cytochrome *c* release by triggering the translocation the cleavage products to the mitochondria.

cleavage products of caspase-9 and caspase-3 were detected, and the substrates of caspase-3, such as PARP and DFF-45 were also cleaved. The results indicated the existence of mitochondria mediated caspase-3 activity.

Previous studies have shown that the anti-apoptotic proteins, such as Bcl-2 and Bcl-X_L, locate on the outer member of mitochondria and act on maintaining the integrity of mitochondria (Kluck et al., 1997; Yang et al., 1997). However, the transfection studies have expressed that when cleaved by caspase, Bcl-2 and Bcl-X_L proteins are converted into potent pro-apoptotic factors, and they may accelerate apoptosis by amplifying the caspase cascade (Bellows et al., 2000). Previous studies have shown that caspase-3 cleaves Bcl-2 at Asp^{34} and Bcl-X_L at Asp^{61} and Asp^{76} to produce N-terminally truncated proteins (Cheng et al., 1997). The cleavage of Bcl-2 by caspase-3 into a 22-kDa fragment (Hoetelmans et al., 2003) results in loss of BH4 domain, the essential domain for its anti-apoptotic activity (Cheng et al., 1997). Studies have also reported that the truncated Bcl-2 fragment can trigger the release of cytochrome *c* from mitochondria (Bellows et al., 2000). Caspase-3 also involved in cleavage of Bcl-X_L, another anti-apoptotic protein, to two 18 kDa fragments (Fujita et al., 1998). The cleaved C-terminal fragments of Bcl-X_L can increase the sensitivity to apoptosis (Clem et al., 1998).

There were also cleaved fragments of Bax and Bad, two proapoptotic members of Bcl-2 family, detected in present study. Previous studies indicated that the Bad could be cleaved by caspase-3 and become a more potent inducer of cytochrome *c* (Kim et al., 2002; Taghiyev et al., 2003).

From the results of another two proapoptotic Bcl-2 proteins, Bad and Bax, there were also cleaved fragments detected in present study. Previous studies have reported that Bad could be cleaved to the truncated Bad (tBad) by caspase-3 (Taghiyev et al., 2003), and Bax also could be cleaved to a 12 kDa truncated Bax (tBax) by calpain (Wood et al., 1998). Both tBad and tBax, two more potent inducers than their wild type (Wood et al., 1998; Taghiyev et al., 2003), could translocate to out membrane of mitochondria to induce further disturbing of mitochondrial membrane and releasing of cytochrome *c*, and result in the activation of downstream caspases (Ohtsuka et al., 2004).

To sum up, our observation suggested that the structure of luteolin contributed to its potent in inducing apoptosis in HL-60 cells. In addition, the mitochondria mediated pathway that was regulated by the cross-reaction between the caspases and Bcl-2 family members might play a central and important role in the luteolin-induced apoptotic mechanism. In the mitochondria pathway, the decrease of mitochondria membrane potential led to the release of cytochrome *c*, the activation of the downstream caspase cascade, and the cleavage of Bcl-2 family members. Furthermore, the imbalance between the antiapoptotic and proapoptotic proteins of Bcl-2 family devoted to the activation of caspase cascade occurred following the loss of mitochondria membrane potential and further cytochrome *c* release.

In conclusion, our results have clearly demonstrated that luteolin triggered apoptosis might be through the cleavage of the Bcl-2 family members in a dose and time dependent manner in HL-60 cells. These results may provide a potential mechanism for luteolin in cancertherapeutic and chemopreventive functions.

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