

Antioxidant enzymes activity involvement in luteolin-induced human lung squamous carcinoma CH27 cell apoptosis

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Abstract

Luteolin (3',4',5,7-tetrahydroxyflavone) is an active constituent of *Lonicera japonica* (Caprifoliaceae), and has been reported to produce anti-tumor activities. However, the apoptosis-inducing activity of luteolin still remains unknown. Flavonoids have been found to possess prooxidant and antioxidant action. The biological and pharmacological effect of flavonoid may depend upon its behavior as either an antioxidant or a prooxidant. Our experiments found that luteolin-induced CH27 cell apoptosis was accompanied by activation of antioxidant enzymes, such as superoxide dismutase and catalase, but not through the production of reactive oxygen species and disruption of mitochondrial membrane potential. Therefore, the effects of luteolin on CH27 cell apoptosis were suspected to result from the antioxidant rather than the prooxidant action of luteolin.

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1. Introduction

Lung cancer is the world's biggest cancer killer. According to the World Health Organization in 2002, more than 1.3 million new cases were diagnosed that year and during the same period more than 1.1 million people died of lung cancer. In Taiwan, lung cancer is one of the ten leading causes of death. Therefore, we focused our attention on the investigation of the mechanisms of drug-induced lung cancer cell apoptosis. Luteolin is a bio-active flavonoid of *Lonicera japonica* (Caprifoliaceae) and has been demonstrated its effect on various tumors such as human leukemia cell line and pancreatic tumor cells (Ko et al., 2002; Lee et al., 2002). Our previous study has demonstrated that luteolin (50 μM)-induced human lung carcinoma CH27 cell apoptosis is a typical apoptosis that was accompanied by a significant DNA damage (Leung et al., 2005). Luteolin-induced

apoptosis is related to its ability to change the expression of apoptotic markers, such as caspase-3 (caspase-dependent) and apoptosis-inducing factor (caspase-independent) protein expression (Leung et al., 2005). However, the exact mechanisms underlying the biological effects of luteolin-produced anti-cancer effects remain unknown.

Antioxidants, such as fat-soluble vitamin E and more hydrophilic flavonoids, possess free radical scavenging properties. The interaction of these natural antioxidants with reactive oxygen species implicated in inflammation has prompted a number of studies in their effects on the inflammatory pathways (O'Leary et al., 2004; Singh et al., 2005). Recent evidences suggested that vitamin E and its analogues may not only protect cells from free radical damage but also induce apoptotic cell death in malignant cell lines and inhibit tumorigenesis in vivo (Birringer et al., 2003; Stapelberg et al., 2004). Flavonoids are a type of polyphenols that commonly occur in plants, and more than 4000 flavonoids have been found and are frequent components of the human diet. Flavonoids have been found to

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possess prooxidant and antioxidant action, which are on intimate terms with their polyphenolic structure. Some of them have been found to possess antitumoral activity through production of reactive oxygen species and decrease in mitochondrial membrane potential (Pan et al., 2005; Qanungo et al., 2005). Some reports also suggested that phenolic compounds might scavenge free radicals and stimulate apoptosis signaling pathways (Son et al., 2003). However, the signaling pathways of apoptosis induction by antioxidants still remain unknown. Therefore, it seems to indicate that polyphenols-inducing cell toxicity was suspected to result from the prooxidant or the antioxidant action. Since the structure of luteolin contains polyphenolic structure, this study would demonstrate the role that prooxidative or antioxidative action of luteolin play in luteolin-induced CH27 cell apoptosis.

Apoptosis is a major form of cell death and highly regulated process that involves activation of a series of molecular events, leading to cell death. Free radicals are a family of molecules, which modulate several important physiological functions including proliferation and apoptosis. Previous studies reported that reactive oxygen species participated in cancer and apoptosis through inducing DNA damage (Arai et al., 2003; Wu et al., 2004). Recently, many studies also reported that reactive oxygen species participated in apoptosis through inducing loss of mitochondria membrane potential (Lemarie et al., 2004; Ueda et al., 2002). Mitochondrial pathway, which involved in the intracellular reduction/oxidation (redox) state, is important for the induction of apoptosis (Sato et al., 1995; Ueda et al., 1998). Superoxide dismutase (SOD) is a well-known antioxidative enzyme with the activity to convert superoxide into hydrogen peroxide, and at least two types of SOD have been identified. One is mitochondrial Mn SOD, and the other is cytosolic Cu/Zn SOD. Antioxidant enzymes can antagonize initiation and promotion phases of carcinogenesis and they are reduced in many malignancies. The most commonly decreased enzyme is the mitochondrial Mn SOD. Mn SOD is reduced in a variety of tumor cells and has been proposed to be a new type of tumor suppressor gene (Kiningham and Clair, 1997; Mates and Sanchez-Jimenez, 2000). The major purpose of this study was to determine whether luteolin induced CH27 cell death through its antioxidative effect, such as activation of antioxidant enzymes. This study also investigated whether the production of reactive oxygen species and reactive oxygen species downstream cascade including loss of mitochondrial membrane potential or antioxidant effect was involved in luteolin-induced CH27 cell apoptosis.

2. Methods and materials

2.1. Materials

Antipain, aprotinin, chelerythrine, dithiothreitol, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), leupeptin, luteolin (3',4',5,7-tetrahydroxyflavone), pepstatin, phenylmethylsulfonyl fluoride and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chemical Company (St.

Louis, MO); anti-sheep IgG peroxidase-conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA). Cu/Zn SOD and Mn SOD antibodies were purchased from Calbiochem (San Diego, CA). Dimethyl sulfoxide (DMSO) was purchased from Merck (Germany). Enhanced chemiluminescent (Renaissance) detection reagents were obtained from NEN Life Science Products (Boston, MA).

2.2. Cell culture

The human lung squamous carcinoma cell line CH27 were grown in monolayer culture in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD) containing 5% fetal bovine serum (Hyclone, Logan, UT), 100U/ml penicillin and 100 μ g/ml streptomycin (Gibco BRL, Rockville, MD) and 2mM glutamine (Merck, Darmstadt, Germany) at 37 °C in a humidified atmosphere comprised of 95% air and 5% CO₂. When CH27 cells were treated with luteolin, the culture medium containing 1% fetal bovine serum was used. All data presented in this report are from at least three independent experiments showing the same pattern of expression.

2.3. Fluorescence microscopic measurements of reactive oxygen species production

This study used 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA, molecular Probes) to detect intracellular generation of reactive oxygen species. CH27 cells were loaded with 5 μ M CM-H₂DCFDA for 30 min in the dark. After loading, cells were washed with warm PBS (phosphate-buffered saline). During loading, the acetate groups on CM-H₂DCFDA are removed by intracellular esterase, trapping the probe inside the CH27 cells. Cells loaded with CM-H₂DCF were treated with luteolin (50 μ M) and analyzed by fluorescence microscopy. Production of reactive oxygen species can be measured by changes in fluorescence due to intracellular production of CM-DCF (5-(and-6)-chloromethyl-2',7'-dichlorofluorescein) caused by oxidation of CM-H₂DCF. CM-DCF fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

2.4. Measurement of mitochondrial membrane potential and reactive oxygen species production by flow cytometry

For measurements of mitochondrial membrane potential, H460 cells were seeded at a density of 1×10^6 cells onto 10-cm dish 48 h. Cells were treated with various indicated concentrations of luteolin for 8, 16 and 24 h. After incubation, cells were collected, stained with 0.02 μ M DiOC₆ (3,3'-dihexyloxycarbocyanine iodide), a mitochondrial membrane potential-sensitive fluorescent dye, for 20 min at 37 °C and resuspended in PBS. Cellular uptake of DiOC₆ was analyzed by flow cytometer. For measurements of reactive oxygen species production, cells were collected and pretreated for 30 min with 0.4 μ M H₂DCF-DA, a redox-sensitive fluorescent dye, and then incubated with 50 μ M luteolin. After treatment, cells were analyzed by flow cytometer.

2.5. Scavenging activity on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical

Scavenging activity on DPPH radicals by luteolin was assessed as previously described (Son et al., 2003). Briefly, 50 μ l of luteolin containing various concentrations (30, 50 and 80 μ M) was mixed with 1 ml of 0.1 mM DPPH–ethanol solution and 450 μ l of 50 mM Tris–HCl buffer (pH 7.4). After 30 min incubation at room temperature, reduction of DPPH radicals was measured by reading the absorbance at 519 nm. The radical scavenging activity was obtained from the following equation: Radical scavenging activity (%) = $[(OD_{\text{control}} - OD_{\text{sample}}) / OD_{\text{control}}] \times 100$.

2.6. Evaluation of superoxide dismutase (SOD) activity

Adherent and floating cells were collected at the indicated time periods and washed twice in ice-cold PBS. Sonicate the cell pellets in cold 50 mM phosphate buffer, pH 7.8. Samples were then centrifuged for 5 min at 1500 g and 4 °C. The protein concentrations were estimated with the Bradford method (Bradford, 1976). Electrophoresis was performed in 9% polyacrylamide gels without 0.1% SDS (sodium dodecyl sulfate), then stained for SOD activity using nitroblue tetrazolium (Beauchamp and Fridovich, 1971).

2.7. Evaluation of catalase activity

Adherent and floating cells were collected at the indicated time periods and washed twice in ice-cold PBS. Cell lysates were collected as described above. Proteins were separated by electrophoresis through a 9% native PAGE (polyacrylamide gel electrophoresis) gel and detected following a 5-min treatment in 5% methanol, three water rinses, 5 min incubation in 0.03% H₂O₂ and incubation in 1% ferric chloride and 1% potassium ferricyanide solution.

2.8. Protein preparation

Protein was extracted as previously described (Leung et al., 2005). Adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were resuspended in modified RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin and 5 μ g/ml antipain) for 30 min at 4 °C. Lysates were clarified by centrifugation at 1500 g for 30 min at 4 °C and the resulting supernatant was collected, aliquoted (50 μ g/tube) and stored at –80 °C until assay. The protein concentrations were estimated with the Bradford method (Bradford, 1976).

2.9. Western blot analysis

Samples were separated by various appropriate concentrations (12% and 15%) of sodium dodecyl sulfate–polyacrylamide

gel electrophoresis (SDS–PAGE; Bio-Rad, Hercules, CA). The SDS-separated proteins were equilibrated in transfer buffer (Tris–HCl 50 mM, pH 9.0–9.4, glycine 40 mM, 0.375% SDS [Bio-Rad], 20% methanol [Merck]) and electrotransferred to Immobilon-P Transfer Membranes (Millipore Corporation, Bedford, MA). The blot was blocked with a solution containing 5% nonfat dry milk in Tris–buffered saline (Tris–HCl 10 mM, NaCl 150 mM [Sigma]) with 0.05% Tween 20 (TBST; Merck) for 1 h, washed and incubated with antibodies to β -actin (1 : 5000 [Sigma], the detection of β -actin was used as an internal control in all of the data of Western blotting analysis), Cu/Zn SOD (1 : 1000) and Mn SOD (1 : 1000). Secondary antibody consisted of a 1 : 20,000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-sheep IgG. The enhanced chemiluminescent (NEN Life Science Products, Boston, MA) detection system was used for immunoblot protein detection.

3. Results

3.1. Effect of luteolin on intracellular reactive oxygen species in CH27 cells

Reactive oxygen species producing is an important marker of prooxidant activity in polyphenolic compounds-induced apoptosis. In order to demonstrate the role that reactive oxygen species play in luteolin-induced apoptosis, production of reactive oxygen species was examined by using an oxidant-sensitive fluorescent probe, CM-H₂DCFDA. The results showed that treatment with 50 μ M luteolin had no significant effect on the intensity of the DCF signal as compared with those in the control during treatment with luteolin for 0.5, 1 and 2 h (Fig. 1).

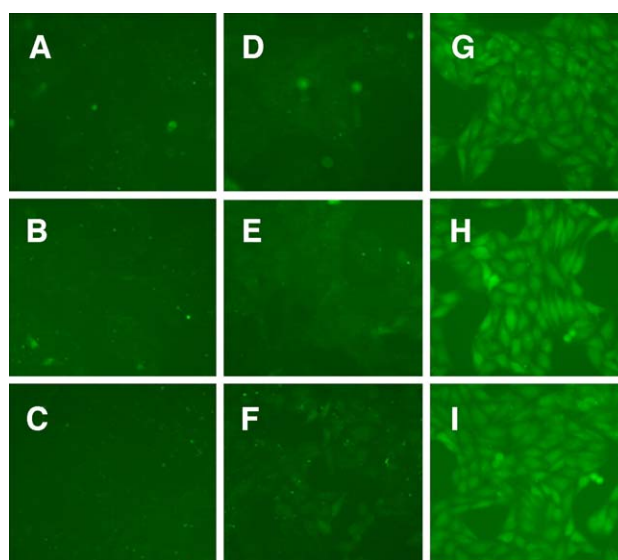


Fig. 1. Effects of luteolin and chelerythrine on reactive oxygen species production in CH27 cells. Cells were cultured for 48 h before drug treatment in 12-well plates. Intracellular levels of reactive oxygen species were examined by 5-H₂DCFDA (5 μ M) for 30 min in the dark, washed and then treated with 0.1% DMSO (A, B and C), 50 μ M luteolin (D, E and F) or 6 μ M chelerythrine (G, H and I) in the presence of 1% serum at 37 °C for 0.5 (A, D and G), 1 (B, E and H) and 2 (C, F and I) h and cells were observed by using fluorescence microscopy. Results are representative of three independent experiments.

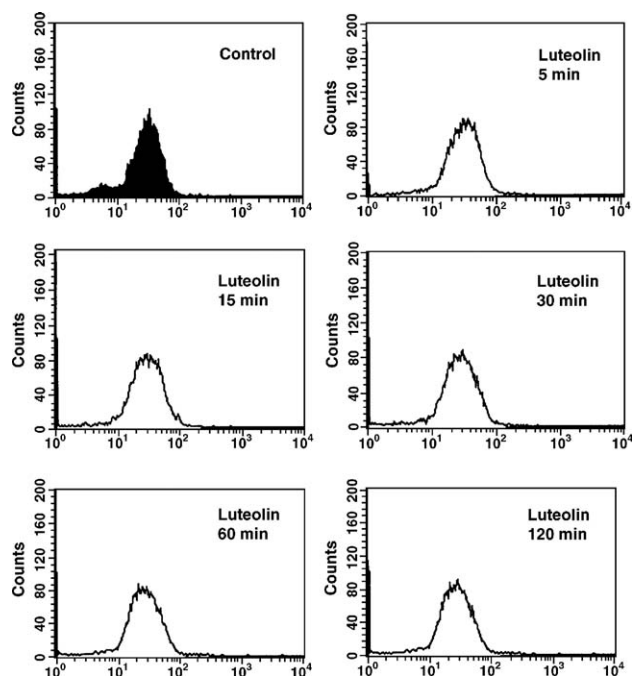


Fig. 2. Effects of luteolin on reactive oxygen species production by flow cytometry. Cells were pretreated for 30min with $0.4\mu\text{M}$ $\text{H}_2\text{DCF-DA}$, a redox-sensitive fluorescent dye, and then incubated with 0.1% DMSO or $50\mu\text{M}$ luteolin. After treatment, cells were analyzed by flow cytometer. Results are representative of three independent experiments.

The DCF signal observed in CH27 cells treatment with $6\mu\text{M}$ chelerythrine (Fig. 1). Chelerythrine was used as a positive control of the generation of reactive oxygen species. Previously study has also demonstrated that reactive oxygen species play an

important role in mediating chelerythrine-induced rapid cardiac myocyte apoptosis. Reactive oxygen species generation was also studied by measuring the H_2DCFDA fluorescence by flow cytometry. Representative flow cytometry histograms are shown in Fig. 2. The concentration of $50\mu\text{M}$ of luteolin was used to induce CH27 cell apoptosis in our previous study (Leung et al., 2005). These results indicated that luteolin showed no effect on the production of reactive oxygen species in CH27 cells.

3.2. Effect of luteolin on mitochondrial membrane potential in CH27 cells

Since the mitochondria membrane potential is an important mitochondrial parameter controlling key cellular processes of prooxidant flavonoids, this study examined the effect of luteolin on mitochondria membrane potential in CH27 cells. The mitochondrial transmembrane potential was investigated with the fluorescent probe DiOC_6 , a mitochondrion-specific and voltage-dependent dye. Representative flow cytometry histograms are shown in Fig. 3. After cells were treated with various indicated concentrations of luteolin for 8, 16 and 24 h, mitochondrial transmembrane potential had no significant change (Fig. 3). Based on above data, luteolin-induced CH27 cell death was independent of reactive oxygen species production and mitochondrial membrane potential disruption.

3.3. DPPH radical scavenging activity of luteolin

Results described above suggested that apoptosis induced by luteolin might not be mediated by its prooxidant activity. Previous studies on polyphenolic flavonoid structures have shown

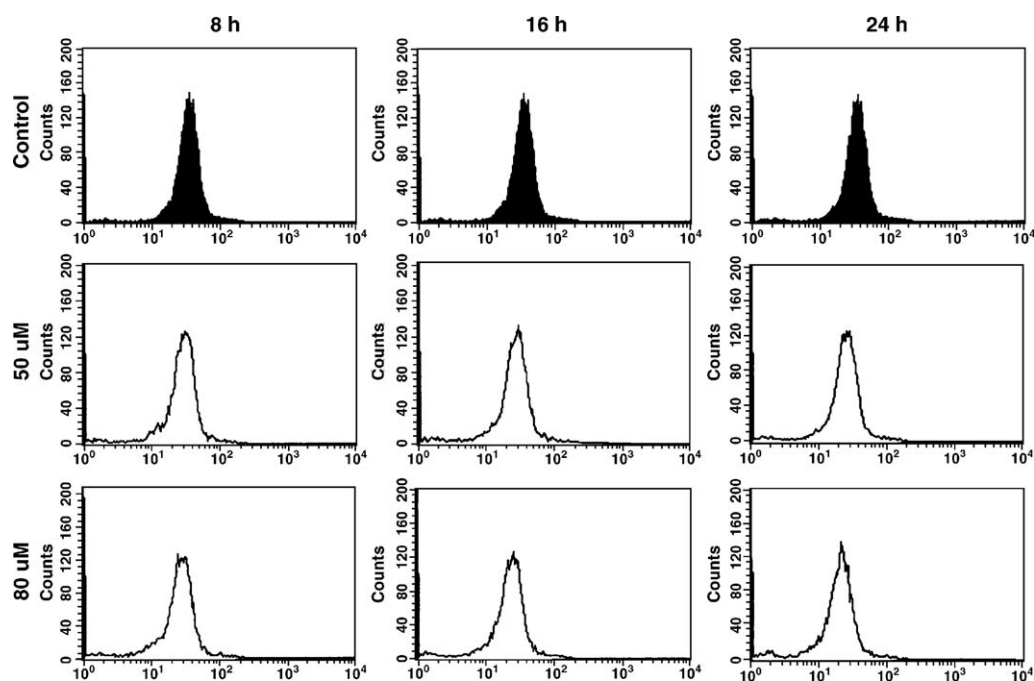


Fig. 3. Effect of luteolin on mitochondrial membrane potential in CH27 cells. For measurement of mitochondrial membrane potential, cells were treated with vehicle alone, 50 or $80\mu\text{M}$ luteolin in the presence of 1% serum at 37°C for 8, 16 and 24h, and then stained with $0.02\mu\text{M}$ DiOC_6 for 20 min at 37°C . The fluorescence intensity was measured by flow cytometer. Results are representative of three independent experiments.

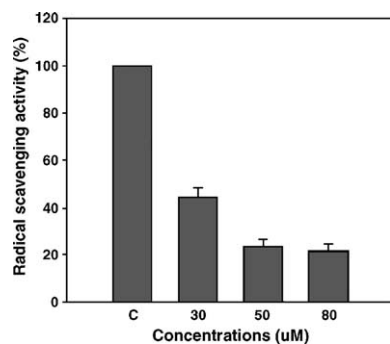


Fig. 4. Free radical scavenging activity of luteolin measured using the DPPH assay. Luteolin was mixed with 0.1 mM DPPH–ethanol solution and 50 mM Tris–HCl buffer. After 30 min incubation at room temperature, reduction of DPPH radicals was measured by reading the absorbance at 519 nm. Results are representative of three independent experiments.

that it is the phenolic hydroxyl groups and their structural arrangements that confer the antioxidant activity, through their H-donating properties. The antioxidant activity of luteolin was evaluated by DPPH free radical scavenging activity. The DPPH radical scavenging activity of luteolin was shown in Fig. 4. Luteolin significantly inhibited the activity of DPPH radicals in a dose-dependent manner (Fig. 4). After treatment with 30 µM of luteolin, DPPH radicals were scavenged by more than 50%.

3.4. Effects of luteolin on superoxide dismutase (SOD) and catalase activity in CH27 cells

This study examined whether luteolin induced changes in the activity of anti-oxidative enzymes, such as SOD and catalase. In this study, native gel analysis was performed to analyze the activity of SOD and catalase. As illustrated in Fig. 5, a time-dependent induction of SOD and catalase activity was detected in luteolin-treated CH27 cells. Our study also demonstrated the

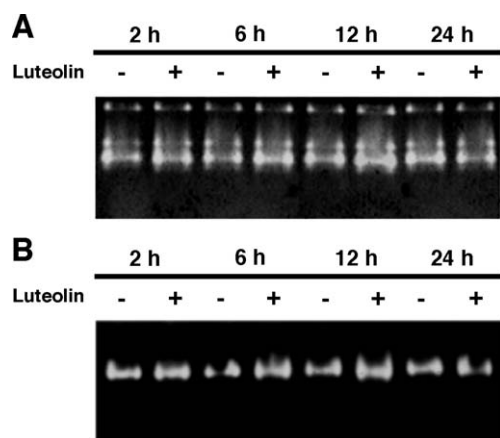


Fig. 5. Effects of luteolin on superoxide dismutase (SOD) and catalase activity in CH27 cells. CH27 cells were treated with luteolin (50 µM) or DMSO (0.1%) for 2, 6, 12 and 24 h. Native gel analysis was performed to analyze the activity of SOD (A) and catalase (B). Proteins were separated by electrophoresis through a 9% native PAGE gel. Activities of SOD and catalase were analyzed as described in Materials and methods. Results are representative of three independent experiments.

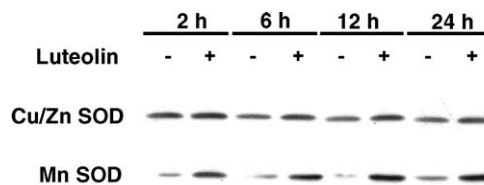


Fig. 6. The effects of luteolin on the protein levels of Cu/Zn SOD and Mn SOD in CH27 cells. The effects of luteolin on the protein levels of Cu/Zn SOD and Mn SOD were detected by Western blot analysis. Cells were incubated with or without 50 µM luteolin in the presence of 1% serum for 2, 6, 12 and 24 h. Cell lysates were analyzed by 12% (Mn SOD) and 15% (Cu/Zn SOD) SDS-PAGE, and then probed with primary antibodies as described in Materials and methods. Results are representative of three independent experiments.

expression of Cu/Zn SOD and Mn SOD protein levels during luteolin-induced apoptosis by Western blotting techniques. The protein levels of Mn SOD and Cu/Zn SOD were increased during treatment with 50 µM luteolin for 24 h (Fig. 6).

4. Discussion

Luteolin has been reported to produce anti-tumor activities, including inhibition of HL-60 cell proliferation, induction of DNA topoisomerase II poisons activities in HL-60 cells and inhibition of invasive activity in MiaPaCa-2 cancer cells (Lee et al., 2004; Sonoda et al., 2004; Yamashita and Kawanishi, 2000). In addition, we previously suggested that luteolin induces DNA damage leading to human lung squamous carcinoma CH27 cell apoptosis (Leung et al., 2005). Luteolin exhibits a wide spectrum of anti-tumor activities, but little is known about its anti-cancer mechanisms.

Recently, many reports indicated that reactive oxygen species participated in cancer cell proliferation and apoptosis (Arai et al., 2003; Wu et al., 2004). Particularly, producing reactive oxygen species participated in the drug-induced cancer cell apoptosis has been receiving increasing attention. In order to demonstrate the role that reactive oxygen species play in luteolin-induced apoptosis, production of reactive oxygen species was examined by using an oxidant-sensitive fluorescent probe, CM-H₂DCFDA. The results showed that treatment with luteolin had no significant effect on the production of reactive oxygen species as compared with those in the control. Several evidences have demonstrated that reactive oxygen species might increase loss in mitochondrial membrane potential during drug-induced cancer cell apoptosis (Lemarie et al., 2004; Tang et al., 2005). Using DiOC₆, a mitochondrion-specific and voltage-dependent dye, luteolin had no significant effect on the loss in mitochondrial membrane potential in the present study. These results indicated that reactive oxygen species production and disruption of mitochondrial membrane potential might not be involved in luteolin-induced cell death in CH27 cells.

Polyphenolic antioxidants are scavengers of free radicals and modifiers of various enzymatic functions. The structure of luteolin is flavonoid and contains two phenolic structures. Therefore, we believe that luteolin-induced CH27 cell apoptosis are closely associated with antioxidant properties of the luteolin. We focused attention on the antioxidant activity of luteolin. In

the present study, the direct approach to examine the scavenging activity of luteolin with DPPH radicals suggested that luteolin contains a strong antioxidant activity. This study also detected the activity of antioxidant enzymes, such as superoxide dismutase and catalase during luteolin-induced CH27 cell apoptosis. In this study, luteolin stimulates a biphasic change in total SOD and catalase activity. SOD and catalase increased in activity over the first 16 h of luteolin treatment, and declined after 24 h. The present study also demonstrated that the protein levels of Mn SOD and Cu/Zn SOD were increased during treatment with luteolin for 16 h. It has been suggested that Mn SOD should be a tumor suppressor gene in several human cancer cells (Kiningham and Clair, 1997; Mates and Sanchez-Jimenez, 2000). The mechanism(s) by which Mn SOD suppresses cancer development is currently unknown. However, expression of this antioxidant enzyme might play a significant role in maintaining cellular redox status. Overexpression of Mn SOD may exert its tumor suppressor activity in part by modulation of specific oncogenes. The relationship between Mn SOD expression, modulation of DNA-binding activity, transcription activation of redox-sensitive oncoproteins and tumor suppressor proteins has been studied recently (Kiningham and Clair, 1997; Offen et al., 1998). An inverse correlation between Mn SOD expression and activity of transcription factors, such as *c-jun*-associated transcription factors, activator protein 1 and c-AMP-responsive element binding protein, was also investigated (Kiningham and Clair, 1997; Offen et al., 1998). Many evidences have suggested that the biological and pharmacological effects of a flavonoid compound should depend upon its behavior as either an antioxidant or prooxidant. Therefore, we believe that luteolin-induced CH27 cell apoptosis is closely associated with antioxidant properties of the luteolin. The overexpression of Mn SOD protein levels may be an important role in luteolin-induced CH27 cell apoptosis.

In summary, our experiments provide evidence that luteolin-induced CH27 cell apoptosis was accompanied by activation of antioxidant enzymes, such as superoxide dismutase and catalase, but not through the production of reactive oxygen species and disruption of mitochondrial membrane potential. Therefore, the effects of luteolin on CH27 cell apoptosis were suspected to result from the antioxidant rather than the prooxidant action of luteolin.

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