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Increase of Bax/ Bcl-X_L ratio and arrest of cell cycle by luteolin in immortalized human hepatoma cell line

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Abstract

Luteolin is a common constituent of many kinds of fruits and vegetables. It possesses the anti-neoplastic activities against several human cancers, but its activity against hepatocellular carcinoma (HCC) is seldom mentioned. To evaluate the activity against HCC and to provide information about the mechanism, we tested luteolin against five human hepatoma cell lines, namely HepG2, SK-Hep-1, PLC/PRF/5, Hep3B, and HA22T/VGH, with XTT assay and flow cytometry. The results showed that luteolin inhibited PLC/PRF/5, Hep3B and HA22T/VGH at a concentration of 1 μg/ml, but it needed 5 μg/ml to inhibit HepG2 and 10 μg/ml for SK-Hep1 ($P < 0.05$). The inhibitive concentrations of 50% (IC_{50}) of luteolin were between 7.29 μg/ml and 32.59 μg/ml, which were comparable with those of 5-FU (15.35 μg/ml to 32.84 μg/ml). The least effective cell line as affected by luteolin (SK-Hep1) was the most effective one when treating with 5-FU. The least effective cell line as affected by 5-FU (HA22T/VGH) was effectively affected by luteolin. It seemed that luteolin had some complementary activity to 5-FU against these HCC cell lines. The luteolin-treated PLC/PRF/5 cells exhibited typical changes of apoptosis with a characteristic DNA laddering pattern on gel electrophoresis. Luteolin also activated casepase-3, increased Bax protein with a concomitant decrease in Bcl-X_L level. Increase in Bax/ Bcl-X_L ratio and activation of caspase-3 supported the apoptotic finding on gel electrophoresis. Luteolin also induced cell cycle arrest at

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G_0/G_1 phase. We suggested that luteolin might exhibit anti-HCC activity as efficient as 5-FU by the mechanism of not only cell cycle arrest but also apoptosis.

Keywords: Apoptosis; Bax; Bcl-X_L; Cell cycle; Luteolin; PLC/PRF/5

Introduction

Primary hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. HCC often develops in patients with chronic liver diseases associating with hepatitis B (HBV) or hepatitis C (HCV) virus infections. The natural history of this disease without treatment is quickly fatal (Dienstag and Isselbacher, 2001). Several treatment options and surgery have not been able to deal with HCC. There are only limited effective therapeutic modalities now. The prognosis was further worsened by the frequent recurrence. Currently, chemotherapy is not the cornerstone of therapy against HCC due to little efficacy. There is large space to improve the effectiveness of chemotherapy by finding new therapeutic agents. Development of mechanism-based novel agents for its management is needed.

Luteolin is one of the most wildly distributed flavonoids in plants (Matsuda et al., 1995; Williams et al., 1996; Yanoshita et al., 1996; Lin et al., 1997; Tan et al., 1999). It is also a common component of many kinds of fruits and vegetables. Several researchers had found that luteolin had anti-neoplastic activities against several human cancers (Pettit et al., 1996.), including leukemia cell (Post and Varma, 1992; Li et al., 2001), thyroid cancer cell (ARO, NPA, WRO) (Yin et al., 1999a,b), prostate cancer cell (Knowles et al., 2000; Kobayashi et al., 2002), and melanoma cell (OCM-1) (Casagrande and Darbon, 2000). Luteolin could inhibit gastric cancer cell (HGC-27) by cell cycle arrest in G1 stage (Plaumann et al., 1996). However, little was mentioned about its activity against hepatoma before. It was reported that luteolin inhibited HepG2 cell, a HCC cell line, by cell cycle arrest only (Yee et al., 2003). The important observation of this study (Yee et al., 2003) was that luteolin treatment did not result in apoptosis of the hepatoma cell line. In recent years, the regulation of apoptosis has become an area of extensive study in cancer research. Many cancer cells are significantly affected by apoptosis (Ehlert and Kubbutat, 2001; Penn, 2001; Sjostrom and Bergh, 2001; Makin, 2002; Adhami et al., 2003). We wondered the previous conclusion (Yee et al., 2003) that luteolin could not induce apoptosis to inhibit HCC. We assessed the activity of luteolin against five cell lines of HCC, and provided the mechanisms of its antiproliferative effect.

Materials and methods

Reagents and materials

Fetal bovine serum (FBS), penicillin G, streptomycin, amphotericin B and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO BRL (Gaithersburg, MD). Luteolin, [2-(3,4-

Dihydroxy-phenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one], was purchased from Wako Pure Chemical Industeries Ltd. (Japan). Dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide were obtained from Sigma Chemical (St. Louis, MO). XTT was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Bax and Bcl-X_L antibody were obtained from Calbiochem (Cambridge, MA). The stock solutions of luteolin and 5-Flurouracil (5-FU, Sigma) were prepared at a concentration of 2 mg/ml with DMSO. It was then stored at −20°C until use. For all experiments, the final concentrations of the test compound were prepared by diluting the stock solution with DMEM. Control cultures received the carrier solvent (0.1% DMSO).

Cell lines and culture

Different HCC cell lines have different molecular characters. These specific molecular characters might affect the anti-HCC activity of luteolin. Therefore, we used five HCC cell lines, namely HepG2 (American Type Culture Collection [ATCC HB 8065], p53-positive; Rb-positive; Ras-mutated; and HBV-negative cell), Hep3B (ATCC HB 80640, p53-negative; Rb-negative; Ras-normal; and HBV-positive cell), SK-Hep1 (ATCC HTB52, p53-positive; Rb-positive; Ras-normal; and HBV-negative cell), and PLC/PRF/5 (ATCC CRL 8024, p53-negative; Rb-positive; Ras-normal; and HBV-positive cell) (Hsu et al., 1993; Puisieux et al., 1993), and HA22T/VGH (CCRC 60168, HBsAg [+]) (Aspinall et al., 1986) to evaluate the anti-HCC activity of luteolin in response to these molecular characters. Normal embryonic hepatocytes of BALB/C mouse were used as normal cell control. They were maintained in a monolayer culture at 37 °C and 5% CO_2 in DMEM supplemented with 10% FBS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B.

Cell proliferation assay

Inhibition of cell proliferation by luteolin was measured by XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) (Roche, Germany) assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h incubation, the cells were treated with luteolin (0, 1, 5, 10, and 20 μg/ml) for 48 h. 5-FU was used as positive control and 0.1% DMSO (w/w) was used as negative control. 50 μl of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 μl of electron coupling reagent, was then added to each well. After 6 h of incubation, the absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm (Goodwin et al., 1995). Data were calculated as percentage of inhibition by the following formula: inhibition (%) = [100-(ODt/ODs) × 100]%, where ODt and ODs indicated the optical density of the test substances and the solvent control, respectively. The concentration of 50% cellular proliferation inhibition of cancer cells (IC_{50}) of test substances was calculated thereafter. The cytotoxic concentration of 50% (CC_{50}) against normal embryonic hepatocytes of BALB/C mouse was assayed and calculated by the above methods. The selectivity index (SI) was determined by the ratio of the CC_{50} to the IC_{50}.

Assay for cell cycle distribution

To determine cell cycle distribution, 5×10^5 PLC/PRF/5 cells were plated in 60-mm dishes, treated with vehicle alone (0.1% DMSO) or luteolin (7 and 15 μg/ml) for 24 h. Cells were then

collected by trypsinization, and fixed by 70% ethanol. Cell pellets were suspended in 2 μl of 10 μg/ml RNase containing 0.5% Triton (J. T. Baker Inc.) plus the same volume of 20 μg/ml PI, and then incubated in the dark at room temperature for 30 min. Cell suspensions were filtered through a 60-μm mesh filter (Spectrum Medical Industries, CA). Data acquisition and analysis were performed on an EPICS flow cytometer (Coulter Electronics). Data from 10,000 cells were collected for each data file.

Detection of apoptosis

PLC/PRF/5 cells (4×0^6) were treated with vehicle alone (0.1% DMSO) or various concentrations of luteolin for 48 h, and were subsequently collected and lysed by DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM EDTA, and 1% Triton X-100). After centrifugation, the supernatant was incubated overnight with proteinase K (0.1 mg/ml) and then with RNase (0.2 mg/ml) for 2 h at 37 °C. After extraction with phenol-chloroform (1:1), the DNA was separated in a 2% agarose gel and visualized by UV after staining with ethidium bromide.

The activity of caspase-3

The activity of caspase-3 was determined using the CaspACE assay kit (Promega Corporation, Wisconsion). These assays measured the cleavage of a specific colorimetric caspase substrate, DEVD-pNA. pNA (*p*-nitroaniline) is released from the substrate upon cleavage by caspase. Free pNA produces a yellow color that is monitored by a spectrophotometer at 405 nm. The detail analysis procedure was described in the manufacturer's protocol.

Assay for Western blotting

PLC/PRF/5 cells, treated with 0, 7, and 15 μg/ml of luteolin for 24 h, were lysed and the protein concentration was determined by using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). For Western blotting, 50 μg of total cell lysates were subjected to SDS-PAGE. The protein was transferred to PVDF membranes using transfer buffer (50 m*M* Tris, 190 m*M* glycin, and 10% methanol) at 100 V for 2 h. The membranes were incubated with blocking buffer (50 m*M* Tris, 200 m*M* NaCl, 0.2% Tween 20, and 3% BSA) overnight at 4°C. After washing three times with washing buffer (blocking buffer without 3% BSA) for 10 min each, the blot was incubated with Bax or Bcl-X_L antibody for 2−15 h, followed by horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using the enhanced chemiluminescence Western blotting detection system (Amersham, USA).

Statistical analysis

Analysis of variance (ANOVA) was used to test the significance of the results by statistical software of JMP5 (SAS Institute Inc., USA). The least square means Tukey honestly significantly different (HSD) test was used for comparisons of pairs of groups in ANOVA. Difference between pairs of groups with a $p < 0.05$ was considered statistically significant. Data of cell cycle distribution were analyzed with Multicycle software (Phoenix Flow Systems, San Diego, CA).

Results

Luteolin inhibited proliferation of Hepatocellular carcinoma cells

Luteolin and 5-FU showed anti-neoplastic activities against HCC cells compared to the negative control (Table 1). The effect was dose-dependent ($P < 0.0001$). Luteolin inhibited PLC/PRF/5, Hep3B and HA22T/VGH at a concentration as low as 1 μg/ml, but it needed 5μg/ml to inhibit HepG2 and 10μg/ml for SK-Hep1 ($P < 0.05$). PLC/PRF/5 was the most responsive cell line to luteolin, followed by Hep3B, and HA22T/VGH. HepG2 and SK-Hep1 were poorly responsive. The difference of responsiveness between responsive and poorly responsive cell lines was significant ($P < 0.05$). The responsiveness of 5-FU was quite different from that of luteolin. The most responsive cell line was SK-Hep1, followed by Hep3B, HepG2, PLC/PRF/5, and HA22T/VGH sequentially. The least effective cell line as affected by luteolin (SK-Hep1) was the most effective one when treating with 5-FU. The least effective cell line as affected by 5-FU (HA22T/VGH) was effectively response to luteolin. It seemed that 5-FU had some complementary activity to luteolin against these HCC cell lines. Considering all these 5 cell lines, the inhibitory effects were not different ($P = 0.5188$) between luteolin and 5-FU. Luteolin had IC_{50}s against HCCs between 7.29 μg/ml and 32.59μg/ml, which were comparable to those of 5-FU between 15.35μg/ml and 32.84μg/ml (Table 1).

The specific molecular characters of HCC cell lines might affect the anti-neoplastic activity of luteolin. We used multivariate linear regression for factorial analysis to find the effect of these molecular characters on the inhibitory rates of luteolin. Interestingly, p53/HBV ($P < 0.0001$) might significantly affect the activity of luteolin. HCC cell lines with HBV and without p53 were more responsive to luteolin. Ras ($P = 0.4017$) and Rb ($P = 0.0835$) might not affect the anti-HCC activity of luteolin.

Table 1
The inhibitory rate of luteolin and 5-FU against different Hepatocellular carcinoma cells

	Dose (μg/ml)	PLC/PRF/5	Hep3B	HepG2	HA22T/VGH	SK-Hep-1
Control	0.1%MSO	0.33±0.31	0.05±0.04	0.26±0.21	0.24±0.23	0.05±0.04
luteolin	20	89.24±1.54*	57.46±7.06*	37.50±2.06*	45.48±3.76*	30.24±0.95*
	10	64.15±2.33*	44.40±1.75*	29.65±2.71*	34.82±2.02*	14.28±1.04*
	5	43.16±1.98*	32.28±1.95*	19.79±0.89*	35.23±1.68*	2.77±0.47
	1	20.11±2.41*	10.16±2.69*	1.11±0.23	19.51±3.83*	1.52±0.05
*IC50 (μg/ml)		7.29±2.09	15.04±0.17	24.97±0.11	22.78±0.57	32.59±0.68
[a]SI		12.9	6.25	3.76	4.12	2.88
5-FU	20	53.47±0.83*	52.91±1.38*	48.27±2.25*	37.72±1.67*	51.40±3.18*
*IC50 (μg/ml)		17.21±0.86	17.20±0.14	19.22±0.30	32.84±0.92	15.35±0.21
[a]SI		10.88	10.88	9.74	5.7	12.2

[a] SI: The selectivity index = CC_{50}/IC_{50}.

* IC_{50}: The inhibitiory concentration of 50% against different cell types of HCC. The cytotoxic concentration of 50% (CC_{50}) of 5-FU against normal embryonic hepatocytes of BALB/C mouse was 187.2 μg/ml and that of luteolin was 93.94μg/ml. The IC50s of HepG2, HA22T/VGH and SK-Hep-1 were estimated data. The data are the mean ± SD obtained from three independent experiments.

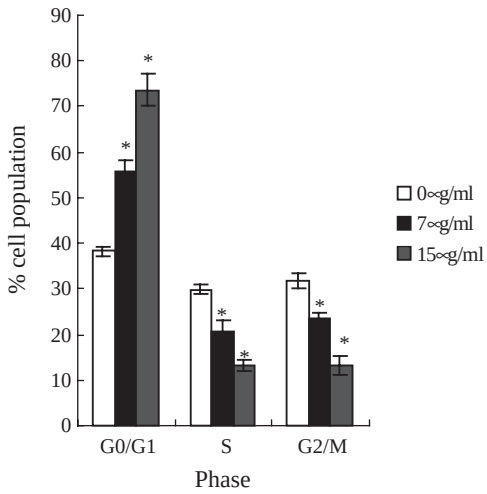

Fig. 1. Effects of luteolin on cell cycle distribution in PLC/PRF/5 cells at 24th hour. PLC/PRF/5 cells following treatment with 0, 7, and 15 μg/ml luteolin for 24 h were fixed and stained with propidium iodide, and cell cycle distribution was then analyzed by flow cytometry. Each value is the mean ± SD of three determinations. (* $p < 0.05$).

Luteolin induced cell cycle arrest at G1 phase

The DNA content analysis exhibited an increase of cell population in G_0/G_1 phase, accompanied with decrease in S_1 phase and G_2/M phase (Fig. 1). This indicated a cell cycle arrest at the G_0/G_1 phase. The effect was dose dependent ($p < 0.0001$).

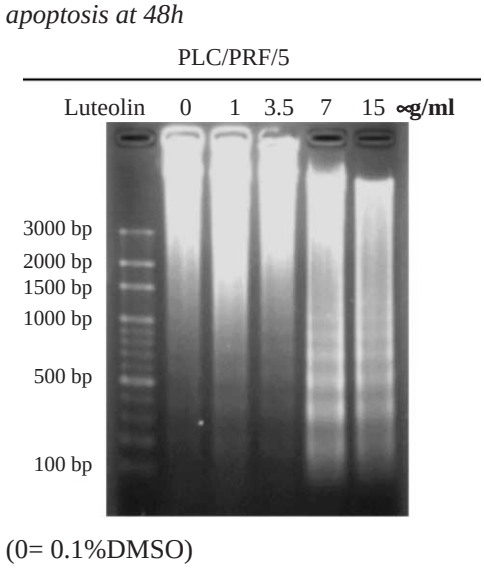

Fig. 2. Induction of apoptosis at 48 h in PLC/PRF/5 cells by luteolin. Cells were treated with various concentrations of luteolin for 48 h. The fragmentation of DNA in luteolin-treated cells was estimated by agarose gel electrophoresis.

Luteolin increased apoptosis with activation of caspase-3 and Bax protein

The agarose gel electrophoresis exhibited dose-dependently a typical apoptotic marker with laddering of DNA bands of about 200 base pairs in length, whereas the control group did not (Fig. 2). To confirm the apoptotic process, the activity of caspase-3 was evaluated (Fig. 3). Luteolin could activate the activity of caspase-3 at the 12th hour of treatment. The effect was markedly increased at the 48th hour.

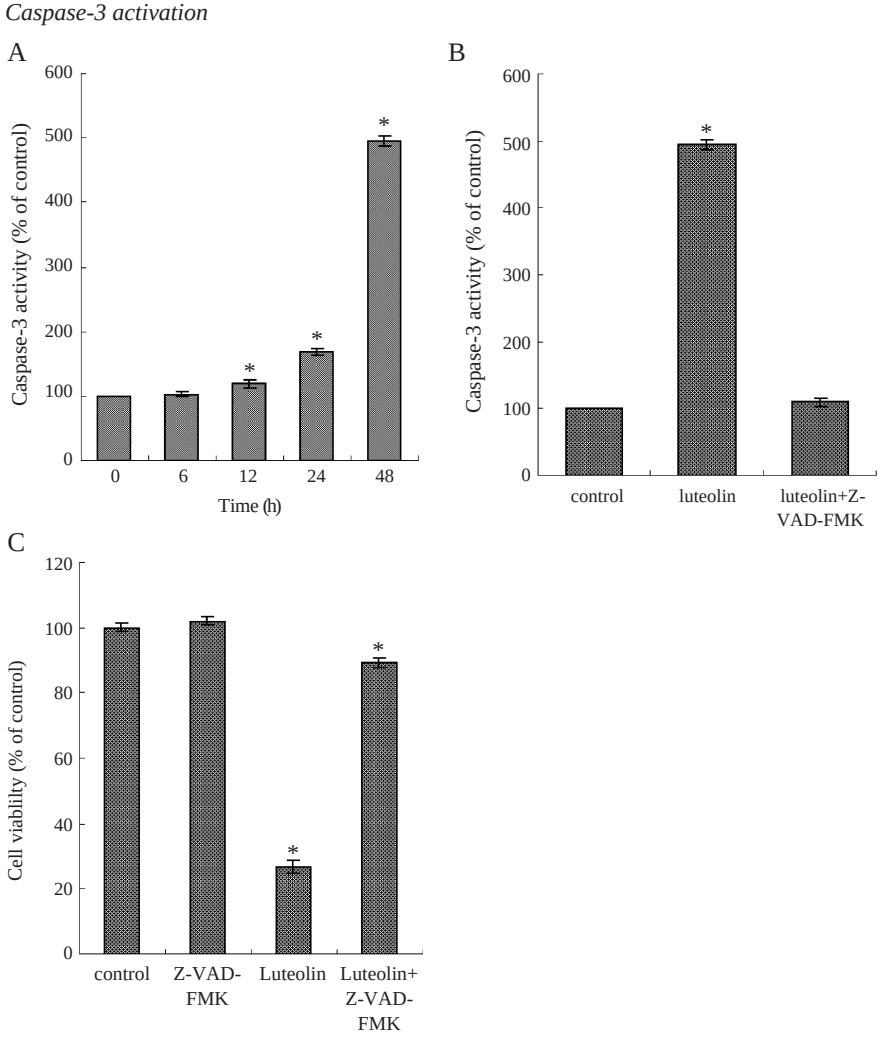

Fig. 3. Effect of luteolin on the activity of caspase-3 and cell viability in PLC/PRF/5 cells. A) The cells were treated with 15 μg/ml luteolin for the indicated time. Protease activity was induced by luteolin as early as 12th hour of treatment; B) For blocking experiments, cells were preincubated with caspase inhibitor, Z-VAD-FMK (50 μM), for 1 h before the addition of 15 μg/ml luteolin. After 48 h treatment, the activation of caspase-3 by luteolin could be completely abolished. C) Pretreatment of 50 μM Z-VAD-FMK could prohibit the effect of luteolin on cell proliferation. Each value is the mean ± SD of three individual determinations. (* $p < 0.05$).

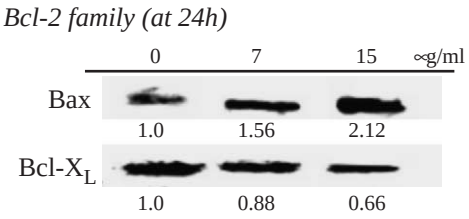

Fig. 4. Effect of luteolin on protein expression of Bax and Bcl-X_L. PLC/PRF/5 cells were treated with 0, 7, and 15 μg/ml of luteolin. The cells were harvested after 24 h. Total cell lysates were separated, and 50 μg of proteins were separated via SDS-PAGE followed by Western blotting analysis and chemiluminescence detection. The values below the figure represent change in protein expression of the bands normalized to β-actin.

Pretreatment with caspase inhibitor before the addition of luteolin could completely abolish this effect. To understand the mechanisms of apoptosis, Bcl-2 family proteins were assessed (Fig. 4). Luteolin clearly increased the expression of pro-apoptotic Bax protein with a concomitant decrease in anti-apoptotic Bcl-X_L level, and thereby increased the Bax/ Bcl-X_L ratio.

Discussion

Impaired apoptosis is a crucial step in the process of cancer development (Cory et al., 1999; Johnstone et al., 2002; Cory and Adams, 2002). Many anticancer agents exert their effect by apoptosis. By targeting the mitochondria, many apoptosis-causing agents trigger the execution phase of apoptosis by the activation of caspases (Allen et al., 1998; Herr and Debatin, 2001; Petak and Houghton, 2001). The Bcl-2 family proteins play a critical role in the induction of apoptosis (Allen et al., 1998; Adams and Cory, 2001; Cory and Adams, 2002). The interaction between pro-and anti-apoptotic proteins of Bcl-2 family integrate the diverse death and survival signals to decide the fate of the cell (Allen et al., 1998; Adams and Cory, 2001; Cory and Adams, 2002). Studies have shown that anti-apoptotic Bcl-2 family proteins form a heterodimer with Bax and might thereby neutralize its pro-apoptotic effects (Oltvai et al., 1993; Chinnaiyan et al., 1996; Srivastava et al., 2001). In addition, anti-apoptotic Bcl-2 family proteins are also known to prevent the release of caspases (Chinnaiyan et al., 1996). The Bcl-2 family proteins regulate the release of cytochrome *c* from the mitochondria into cytosol (Budihardjo et al., 1999; Wang, 2001). The over-expression of anti-apoptotic Bcl-2 family protein, such as Bcl-X_L protein, has been shown to block the release of cytochrome *c* in response to a variety of apoptotic signals (Budihardjo et al., 1999). On the contrary, the pro-apoptotic member of Bcl-2 family proteins, such as Bax protein, promote cytochrome *c* release from the mitochondria (Budihardjo et al., 1999; Wang, 2001). Released cytochrome *c* will activate initiator caspase-9 to activate sequential cascade of caspases. The proteolytic activation of executioner, such as caspase-3, results in the proteolysis of death substrates and subsequent DNA degradation and apoptotic death (Allen et al., 1998; Budihardjo et al., 1999; Kuida, 2000; Adams and Cory, 2001; Cory and Adams, 2002). In this study, we have shown that luteolin treatment to the PLC/PRF/5 cell results in significant increase in the pro-apoptotic Bax protein and decrease in the levels of anti-apoptotic Bcl-X_L protein, thus shifting the Bax/ Bcl-X_L ratio in favor of apoptosis (Fig. 4). Furthermore, we also demonstrated the significant induction of the execution protease of apoptosis, caspase-3 (Fig. 3). Several investigators had reported anti-neoplastic effects of luteolin by

cell cycle arrest in cell lines with wild type p53 gene (Plaumann et al., 1996; Yee et al., 2003). Luteolin could increase the expression of p53 protein and CDK inhibitor p21 (WAF1/CIP1) protein (Yee et al., 2003). Luteolin could down-regulate the expression of CDK4 (Yee et al., 2003). These activities of luteolin could achieve cell cycle arrest in cell lines with wild type p53 gene. Apoptosis was not thought as the mechanism of anti-neoplastic effect of luteolin against HCC with wild type p53 gene before (Yee et al., 2003). However, our experiment clearly demonstrated the typical pattern of apoptosis as DNA laddering on gel electrophoresis in a cell line with mutant p53. We also showed that the activities of caspase-3 and Bax protein were induced by luteolin. These findings supported that luteolin also caused HCC cell death by apoptosis. We found that luteolin had the effect that was most efficient against PLC/PRF/5. However, HepG2 had a relatively poor response. Our result was quite different in apoptosis from that of previous report (Yee et al., 2003). The reasons might be due to (a) the different cell line used. PLC/PRF/5 cell (p53-negative; Rb-positive; Ras-normal; and HBV-positive cell) is quite different from HepG2 cell (p53-positive; Rb-positive; Ras-mutated; and HBV-negative cell) in molecular constituents (Hsu et al., 1993; Puisieux et al., 1993). These differences might affect their susceptibility to luteolin; (b) the different dosage of treatment. HepG2 had relatively poor response to luteolin with a relatively high IC_{50} (24.97μg/ml). It might need higher dose to demonstrate the apoptosis event clearly. Higher dose with prolonged treatment might overcome the resistance and show the apoptotic effect of luteolin against resistant cell lines. These differences might explain why apoptosis was not found in previous report (Yee et al., 2003) with a relatively lower dose of luteolin to treat a resistant cell line.

In this study, luteolin had the anti-HCC activity against several HCC cell lines. Luteolin inhibited PLC/PRF/5, Hep3B and HA22T/VGH at a low concentration, but it needed a higher concentration to inhibit HepG2 and SK-Hep1 (Table 1). It was interesting that the least effective cell line as affected by luteolin (SK-Hep1) was the most effective one when treating with 5-FU, and the least effective cell line as affected by 5-FU (HA22T/VGH) had effective response to luteolin. Luteolin might have some complementary activity to 5-FU against these HCC cell lines. The inhibitive concentrations of 50% (IC_{50}) of luteolin were comparable with those of 5-FU (Table 1). This might suggest that luteolin have comparable clinical responsiveness as 5-FU dose. HCC cell lines with HBV and without p53 were more responsive to luteolin in our experiment. However, we could not firmly state this conclusion yet due to the limited cell lines we used.

Conclusion

Our results demonstrated that luteolin inhibited several cell lines of HCC with comparability to 5-FU. Luteolin increased Bax/ Bcl-X_L ratio and activated caspase-3. Luteolin inhibited HCC cells not only by cell cycle arrest at G_0/G_1 phase but also by apoptosis. The efficacy and mechanisms of luteolin to inhibit HCC were quite different from those of 5-FU. It might be speculated that combination of luteolin and 5-FU could be more effective against HCC than each agent alone.

Acknowledgements

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