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Full Length Research Paper

A possible molecular mechanism of two flavones and two flavonols on the induction of differentiation in a human oesophageal adenocarcinoma cell line (OE33)

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The influences of two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol) selected on induction of differentiation in a human esophageal adenocarcinoma (OE33) cell line were studied in the present work. All compounds selected could be able to inhibit proliferation of OE33 cells in a dose- and time-dependent manner. The inhibitory potency of these compounds on OE33 cells was in the order of: galangin > chrysin > baicalein > kaempferol. OE33 cells treated with these compounds for 24 h showed differentiation characteristics including the differentiation-specific morphological changes, the augmentation of differentiation markers and the inhibition of human telomerase reverse transcriptase. The potency of these compounds on induction of differentiation of OE33 cells was similar to that of them on growth inhibition. To reveal the possible molecular mechanisms by which these compounds modulate proliferation and differentiation in OE33 cells, the alterations of three genes, 14-3-3σ, cyclin B1 and cyclin D1, which are related to proliferation and differentiation of cells, were investigated by real-time polymerase chain reaction (RT-PCR) and Western blot. The results showed that up-regulation of 14-3-3σ and down-regulation of cyclin B1 and cyclin D1 at the mRNA and protein levels were observed in OE33 cells treated with these compounds, indicating that 14-3-3σ, cyclin B1 and cyclin D1 might be target genes of these flavonoids in inducing differentiation of OE33 cells.

Key words: Flavones, flavonols, differentiation, esophageal cancer, molecular mechanism.

INTRODUCTION

Esophageal cancer is one of the most lethal malignancies of all cancer (Tak and Naunheim, 2004). It ranks as the ninth most common malignancy worldwide and occurs at a high frequency in Asia and South America (McCabe and Dlamini, 2005). Esophageal cancer exists in two forms with distinct etiological and pathological characteristics, squamous cell carcinoma (SCC) and adenocarcinoma (Stoner and Gupta, 2001). Although, esophageal SCC remains the predominant type of esophageal malignancy in the most parts of the world, recent reports from North America and Europe confirmed clinical suspicions that adenocarcinomas of the lower esophagus were being seen more frequently (Casson, 1998). One of the major risk factors for esophageal adenocarcinoma is gastroesophageal reflux disease (GERD). The increasing incidence of GERD may be attributed to the changes in lifestyle, dietary habits, and physique (Kuwano et al., 2005). In Barrett's esophagus, the squamous epithelium damaged by reflux esophagitis was replaced by a metaplastic, intestinal-type epithelium predisposed to malignancy. In the context of continued injury from GERD, metaplastic Barrett's mucosa gives rise to esophageal

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Abbreviations: BSA, Bovine serum albumin; **DMSO,** dimethyl sulfoxide; **EB,** ethidium bromide; **EDTA,** ethylenediaminetetraacetic acid; **FBS,** fetal bovine serum; **FITC,** fluorescein isothiocyanate; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; **PBS,** phosphate-buffered saline; **PI,** propidium iodide; **PMSF,** phenylmethyl sulfonylfluoride; **RNase,** ribonuclease; **SDSPG,** sodium dodesyl sulfate–polyacrylamide gel; **TBS,** tris buffered saline.

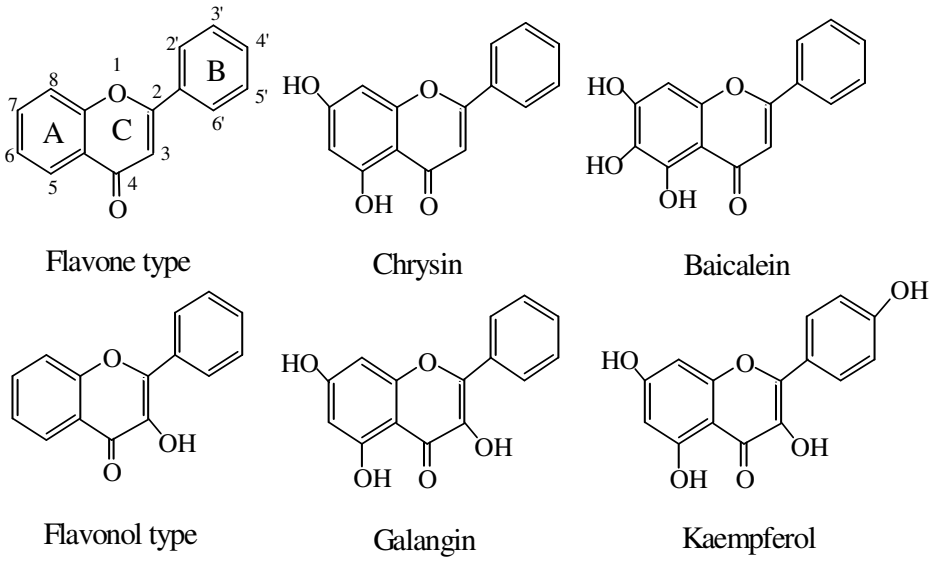

Figure 1. Chemical structures of two flavones and two flavonols selected.

adenocarcinoma (Kuwano et al., 2005).

Flavonoids, chemically being polyphenolic compounds existing in plant foods (Middleton et al., 2000; Ren et al., 2003), are a large group of low molecular weight compounds classified as flavones, flavanols, isoflavones, flavonols, flavanones and flavanonols (López-Lázaro, 2002; Ren et al., 2003; Wach et al., 2007). They are rich in dietary plants such as fruits and vegetables, and also found in medical plants. Herbal remedies containing flavonoids have been used in folk medicine around the world, especially in China (Ren et al., 2003), such as baicalein (isolated from the root of *Scutellaria baicalensis*, which is used as a traditional medicinal herb for centuries) (Yang et al., 2000) and galangin (present at high concentration in propolis, a resinous material produced by the activities of honeybees and in India root, *Alpinia officinarum*, which is common spice and herbal medicine in Asia) (Bestwick and Milne, 2006; Heo et al., 2001). It is well-known that flavonoids have many biological effects and those of particular interest are cancer prevention without affecting normal cells (López-Lázaro, 2002; Neuhouser, 2004). It is suggested in many studies that consumption of vegetables, fruit and beverages of plant origin containing flavonoids is consistently associated with a reduced risk of cancers (Bosetti et al., 2005, 2007; Johnson, 2004; Marchand et al., 2000; Neuhouser, 2004), including esophageal cancer (Johnson, 2004; Rossi et al., 2007). Flavonoids are natural antioxidants and could exert effects such as antioxidants, free radical scavengers, chelators (Havsteen, 2002), and might be involved in preventing free radical induced cytotoxicity and lipid peroxidation in the cells (Zhao and Zhang, 2009). Recent studies revealed that flavonoids might contribute to anti-carcinogenic effects, such as mitigation of oxidative damage, antiproliferative effects, induction of apoptosis and inhibition of malignant transformation (Birt et al., 2001; Ren et al., 2003). Beside these effects, some flavonoids are capable of causing undifferentiated cancer cell lines to differentiate into cells exhibiting mature phenotypic characteristics (Middleton et al., 2000). It was reported that genistein, apigenin, luteolin, quercetin and phloretin could induce differentiation of human acute myelogenous leukemia HL-60 cells into granulocytes and monocytes (Takahashi et al., 1998). In other studies, citrus flavones and flavanones were found also to be active antiproliferative differentiation inducers in human promyelocytic leukaemia cells (HL-60) (Kawaii et al., 1999; Kawaii and Lansky, 2004). Differentiation of human leukemic mast cells also could be induced by two flavonoids, quercetin and kaempferol (Alexandrakis et al., 1999). Genistein was reported to arrest cell growth and induce neuroblastoma (NB) cell differentiation (Brown et al., 1998). Kaempferol was also shown to induce differentiation in partially differentiated KNC colon cancer cells (Nakamura et al., 2005).

Differentiation therapy is best known for hematological malignancies (Aksentijevich and Flinn, 2003; Parmar and Tallman, 2003), to date studies only located in hematological malignancies, prostate, colon and some other cancers. Flavonoids, as a group of differentiation-inducing compounds, are potentially low toxicity to normal cells (Havsteen, 2002; Kawaii and Lansky, 2004; López-Lázaro, 2002). More study is needed to reveal differentiation- inducing effects of flavonoids on cancers and related molecular mechanisms. Based on our previous study results two flavones (baicalein and chrysin) and two flavonols (galangin and kaempferol), their chemical structures are shown in Figure 1 were selected to evaluate their differentiation-inducing effects on human esophageal adenocarcinoma (OE33),

and possible molecular mechanisms responsible for their differentiation-inducing effects were investigated in the present work.

MATERIALS AND METHODS

Baicalein (>98% of purity) and galangin (>98% of purity) were purchased from Shanghai Yousi Biotechnology Co., Ltd. (Shanghai, China). Kaempferol (>98% of purity) were purchased from Nanjing Qingze Medical Technology Co., Ltd. (Nanjing, Jiangsu, China). Chrysin (>96% of purity), and MTT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The following reagents were from Solarbio Science & Technology Co., Ltd. (Beijing, China): PI, EB, DMSO, RNase, BSA, sodium azide and materials for Western blot.

The anti-β-actin antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The human reactive antibody for 14-3-3σ was from Abcam Inc. The anti-cyclin B1 and cyclin D1 were from Lab Vision Co. (Fremont, CA, USA). The horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit antibody and fluorescien-conjugated affinipure goat anti-rabbit IgG (H + L) antibody were from Zhongshan Gold Bridge Biotechnology Co., Ltd. (Beijing, China). Rabbit anti-human c-erbB-2 antibody was from Fuzhou Maixin Biotechnology Development Co., Ltd. (Fuzhou, Fujiang, China).

Cell culture

The OE33 cell line, also known as JROECL33, was obtained from Fourth Hospital of Hebei Medical University (Shijiazhuang, Hebei, China). The cell line was established from the adenocarcinoma of the lower oesophagus (Barrett's metaplasia) of a 73 year old female patient. The OE33 cells which express p53 mutation were cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 mg/ml streptomycin (all obtained from Solarbio) and maintained in monolayer culture at 37°C in an incubator of humidified air with 5% CO_2. Subconfluent cells (80%) were passaged with a solution containing 0.25% trypsin and 0.02% EDTA.

MTT assay

OE33 cells (1×10^4/well) were plated in 96-well plates and incubated for 24 h to allow the cells to attach, before treatment with two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol). Each compound was dissolved in DMSO and made up with the medium so that the final concentration of the vehicle was not >0.1% DMSO. The cells were treated with 10, 20, 40 and 80 µmol/L concentration of each compound for 24, 48 and 72 h. Cells treated with 0.1% DMSO were as a negative control. After incubation for specified time at 37°C in a humidified incubator, MTT (5 mg/ml in PBS) of 10 µl was added to each well and incubated for 4 h after which the plate was centrifuged at 1800 g for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. After careful removal of the medium, 100 µl of buffered DMSO was added to each well, and plates were shaken. The absorbance was recorded on a microplate reader (Bio Rad Laboratories, Hercules, CA, USA) at 570 nm. The effect of each compound on growth inhibition was assessed as percent cell viability (Zhang et al., 2009), where vehicle-treated cells were as 100% viable.

Immunofluorescence assay of differentiation marker

The cells were grown to about 70% confluence in glass bottom microwell dishes used for confocal laser scanning microscopy and were treated with each compound (80 µmol/L) for 24 h. The cultured cells were fixed in paraformaldehyde for 10 min, followed by incubation at 37°C for 30 min with specific rabbit anti-c-erbB-2 and then by incubation at 4°C for 1 h with fluorescence- conjugated goat anti-rabbit IgG (1:100 dilution).

Quantification of differentiation marker

The cells were grown to about 70% confluences in 6-well plates and treated with each compound (80 µmol/L) for 24 h. The cells were harvested by adding a solution of 1 mmol/L EDTA in PBS to remove the cells from the plate, and centrifuged at 1000 g at 4°C for 5 min, washed with 3 ml of PBS + 0.1% sodium azide and 1% BSA, count the cells using a minimum concentration of 1 x 10^6 cells/ml for each sample. Aliquot cells for staining were adjusted into small sample volumes of PBS + 0.1% sodium azide and 1% BSA, proper dilution of anti-c-erbB-2 (1:100 dilution) was added, and incubated on ice for 30 min. To each sample, 4 ml of PBS + sodium azide and BSA was added and mixed gently, centrifuged at 1000 g for 5 min, washed samples with 4 ml of PBS + sodium azide and BSA, later centrifuged samples at 1000 g for 5 min. Resuspended cells in 3 ml PBS + sodium azide, proper dilution of secondary antibody fluorescien-conjugated (1:100 dilution) was added and incubated on ice for 30 min. To each sample, 3 ml of PBS + sodium azide and BSA was added and mixed gently, centrifuged at 1000 g for 5 min. Resuspended cells in 3 ml PBS + sodium azide and BSA to give a final concentration of l x 10^6 to 5 x 10^6/ml. The resuspended cells were analyzed by flow cytometry to determine the percentage of differentiated cells.

Telomerase activity assay

The cells were grown to about 70% confluence in 6-well plates and treated with each compound (80 µmol/L) for 24 h. Two micrograms of total RNA from each sample were subjected to reverse transcription using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China) according to the manufacturer's protocol. Each real-time PCR was carried out in triplicate in a total of 20 µl reaction mixture (2 µl of cDNA, 10 µL of SYBR® Premix Ex Taq™, 0.4 µl of ROX Reference Dye II, 0.5 µl of each 10 mol/L forward and reverse primers, and 6.6 µl of H_2O) on ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers for hTERT cDNA amplification were added to a final concentration of 0.25 µmol/L. Primer for hTERT were listed in Table 1. Actin mRNA was used as an internal control. The amplification reactions were performed with an initial incubation step at 95°C for 10 s followed by 50 cycles of 95°C for 5 s, 60°C for 34 s. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and were normalized to β-actin expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product (data not shown in this paper).

Real-time RT-PCR analysis

The cells were grown to about 70% confluence in 6-well plates and treated with each flavonoids (80 µmol/L) for 24 h. The extraction of RNA and real-time RT-PCR were manipulated as those in the section of telomerase activity assay. Primers used for real-time PCR analysis were presented in Table 1. The PCR program was initiated

Table 1. Primers used for RT-PCR analysis.

Genes	Primer sequence
14-3-3σ	5'-CCT GCG AAG AGC GAA ACC TG-3' 5'-TCA ATA CTG GAC AGC ACC CTC C-3'
cyclin B1	5'-CGC AAA GCG CGT TCC T-3' 5'-AAT GAC TTT TCC AGT AGC TGA AGG TTT-3'
cyclin D1	5'-CGT GGC CTC TAA GAT GAA GG-3' 5'-CCA CTT GAG CTT GTT CAC CA-3'
hTERT	5'- CGG AAG AGT GTC TGG AGC AA-3' 5'- GGA TGA AGC GGA GTC TGG A-3'

by 10 s at 95℃ before 40 thermal cycles, each of 5 s at 95℃ and 34 s at 60℃. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and were normalized to β-actin expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product (data not shown in this paper).

Western blot analysis

The cells (70% confluent) were treated with each compound (80 μmol/L) for 24 h. After which the media was aspirated, the cells were washed with cold PBS and ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4, 0.5% NP-40, 1%Triton X-100, 1 mmol/L PMSF (pH 7.4)] over ice for 30 min. The cells were scraped, and the lysate was collected in a microfuge tube. The lysate was cleared by centrifugation at 14000 g for 15 min at 4℃ and the supernatant (total cell lysate) was used or immediately stored at -80℃. The protein concentration was determined by Gene Quan pro (Amarsham Biosciences, Uppsala, Sweden). Protein (50 μg) from each sample was resolved over 10 to 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% nonfat dry milk; 0.05% Tween 20; in 20 mmol/L TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate rabbit monoclonal (Thermo Scientific, Fremont, CA, US) or polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, US) (1:2000 dilution) in blocking buffer from 2 h to overnight at 4℃, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate (Beijing Zhongshanjingqiao biotechnology Limited Company, Beijing, China) (1:2000 dilution) and detected by chemiluminescence and autoradiography using X-ray film (Applygen Technologies Inc., Beijing, China). Densitometric measurements of the bands in Western blot analysis were performed using LabWorks 4.5 image analysis software (UVP Bioimaging System, USA).

Statistical analyses

All data are expressed as means ±SE from at least three independent experiments. Differences between the mean values of multiple groups were analyzed by ANOVA. Statistical significance was considered at $P<0.05$. The SPSS version 12.0 program was used.

RESULTS

Growth inhibitory effects of two flavones and two flavonols on OE33 cells

Two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol) with chemical structures as Figure 1 were selected in our work to reveal their induced-differentiation on a human esophageal adenocarcinoma cell line (OE33). The potential growth inhibitory effects of two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol) on OE33 cells was studied by MTT assay first. Four compounds all had the ability to inhibit growth of OE33 cells in a dose- and time-dependent manner (Figure 2A), but significant differences were also existed. Galangin was the most active one with an estimated IC50 (50% inhibition of cell growth, 24 h) value of 89 μmol/L (Figure 2B). The potency of the selected compounds on inhibition of OE33 cells was in the order of: galangin (89 μmol/L)>chrysin (90 μmol/L)>baicalein (115 μmol/L)>kaempferol (125 μmol/L).

Morphological differentiation

The four compounds tested not only had the ability to inhibit cell growth, but also to induce differentiation in the OE33 cells. When OE33 cells were treated with four compounds, dramatic morphological changes were observed, including an enlarged, flattened shape, an increased cytoplasmic-to-nuclear ratio and a decreased cell density, which was a morphological feature of differentiated phenotype (Figure 3).

Differentiation induction

C-erbB-2 (also known as HER-2/neu) is a proto-oncogene with extensive sequence homology to epidermal growth factor receptor (EGFR), has intrinsic tyrosine kinase

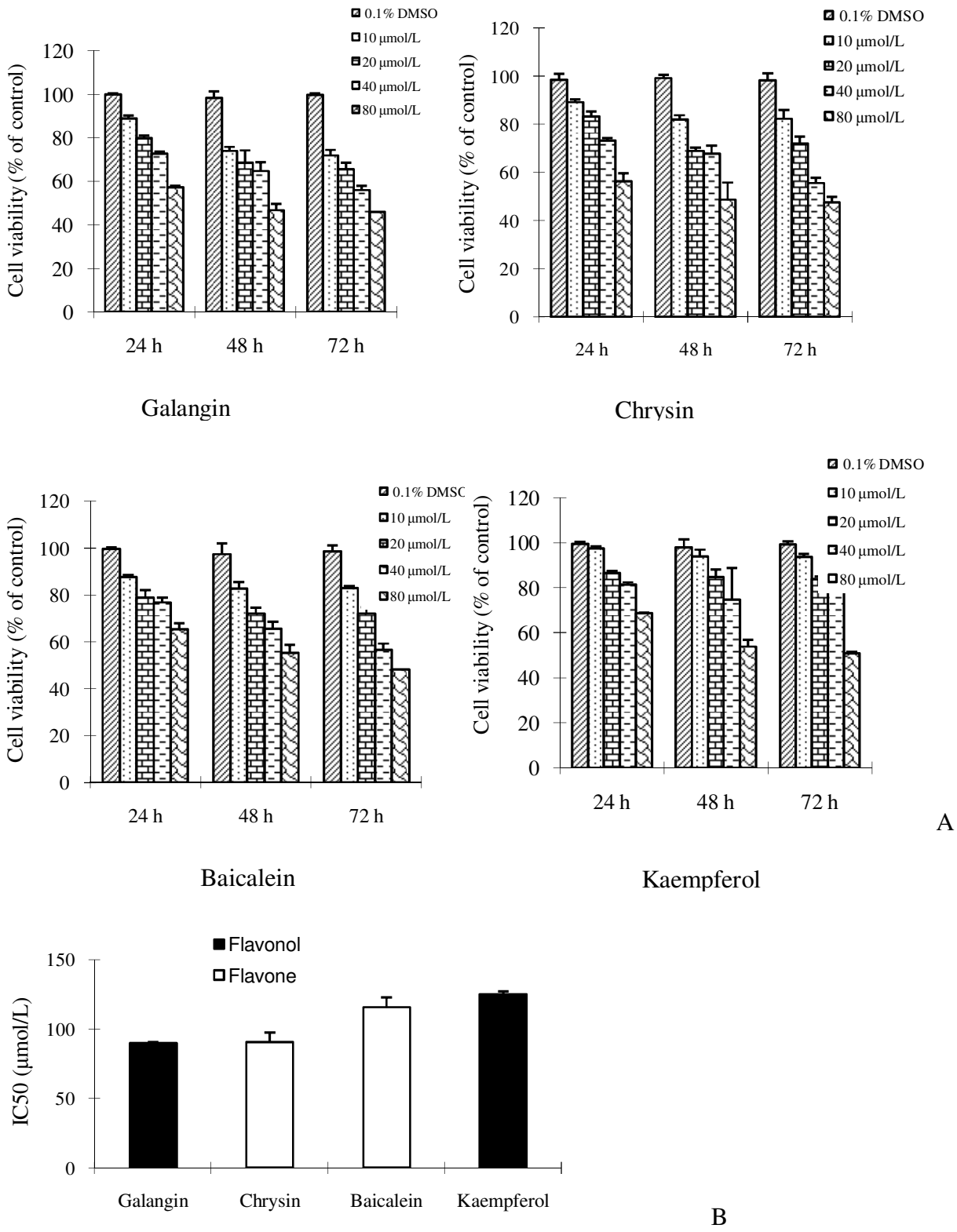

Figure 2. Growth inhibitory effects of two flavones and two flavonols on OE33 cells (A) and comparative effects of them on the growth of OE33 cells (B). The cells were treated with specified concentrations of the selected flavonoids for 24, 48 and 72 h, and growth inhibitory effects were determined by MTT assay. The values were represented as the percentage cell inhibition where vehicle-treated cells were regarded as 100%. The data represents the mean ± SE of three experiments each conducted in triplicate (A).The cells were treated with each compound at 80 µmol/L for 24 h. The values were represented as mean ± SE from three separate experiments each conducted in triplicate (B).

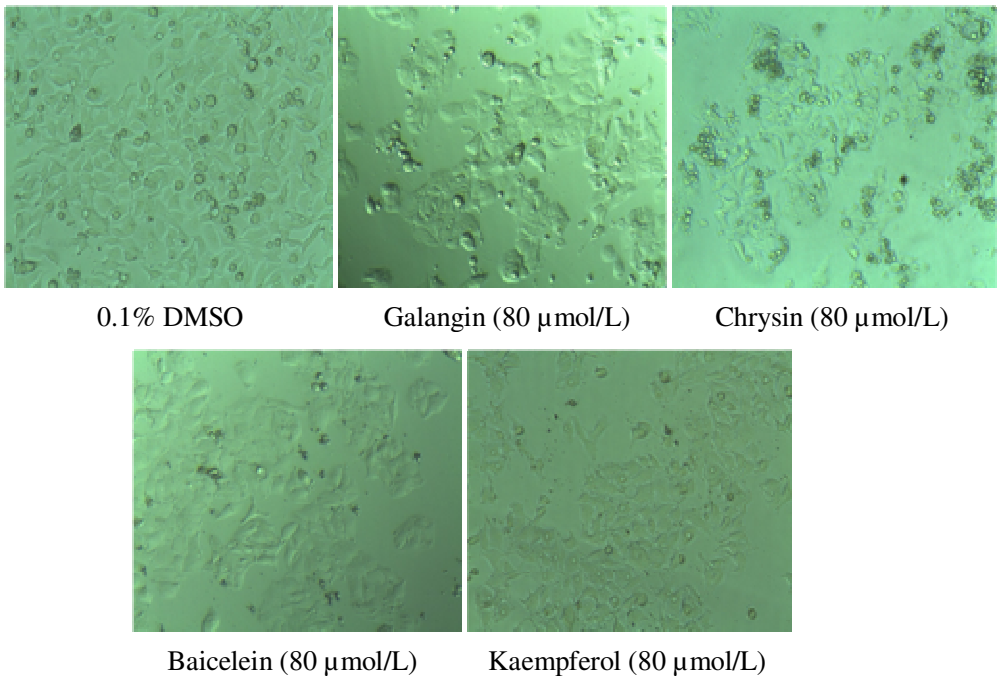

Figure 3. Effects of two flavones and two flavonols on the induction of differentiation in OE33 cells. The cells were treated with each compound at 80 µmol/L for 24 h and showed differentiation phenotype, including an enlarged, flattened shape, an increased cytoplasmic-to-nuclear ratio, and decreased cell density.

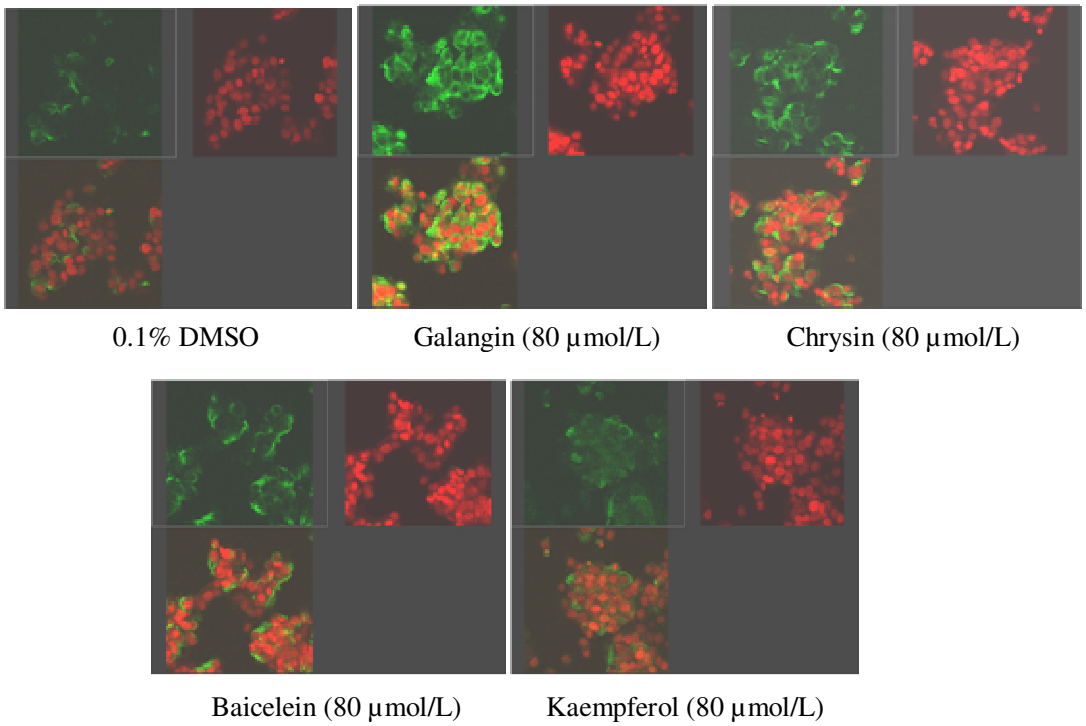

Figure 4. Effects of two flavones and two flavonols on induction of c-erbB-2 expressions in OE33 cells. The cells grown on glass bottom microwell dishs were treated with the selected compound at 80 µmol/L for 24 h. C-erbB-2 positive cells were detected by fluorescein isothiocyanate (FITC)- labeled antibody (top-left) and nuclei reflecting cell density by PI (top-right). The result showed the augmentation of expression of differentiation marker c-erbB-2 in OE33 cells.

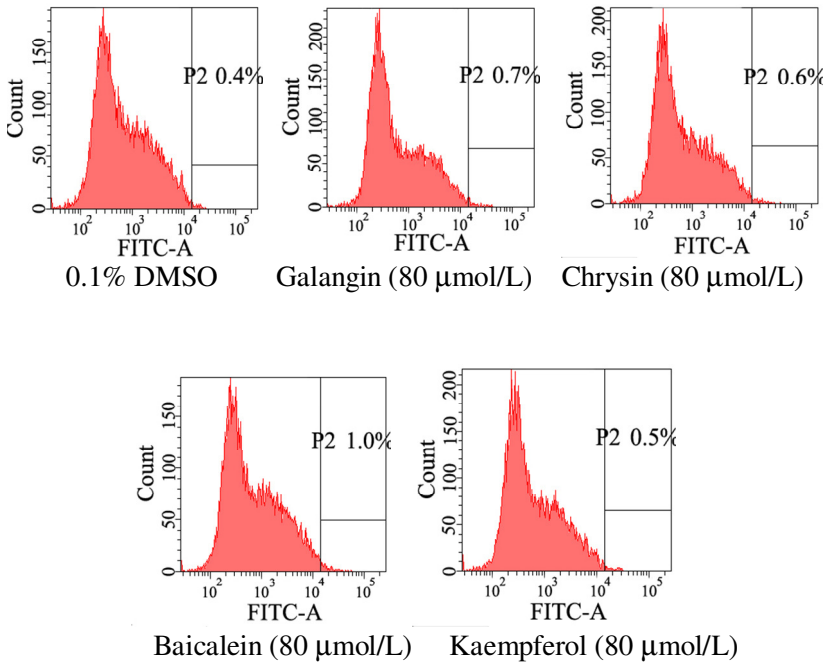

Figure 5. Effects of two flavones and two flavonols on inducting differentiation of OE33 cells.The differentiation induced by 10, 20, 40 and 80 µmol/L of each compound was quantified by flow cytometry with FITC. The analysis result revealed that the two flavones and two flavonols both made an increased level of c-erbB-2 in a dose-dependent manner in gate P2.

activity and plays an important role in regulation of fundamental processes including differentiation (Xia et al., 1997; Selvarajan et al., 2004).

The expression of c-erbB-2 was the greatest in normal tissue, less in dysplastic tissue, and not detected in malignant esophageal adenocarcinoma tissue. Therefore, c-erbB-2 is a differentiation marker of esophagus adenocarcinoma (Sauter et al., 1993). In view of the above-mentioned effects on cell growth and differentiation, it was needed to confirm that the differentiation-inducing effect of the four compounds on OE33 cells. The cells were treated with each of four compounds (80 µmol/L) for 24 h. As judged by an increased number of cells positively stained with anti-c-erbB-2 fluorescein isothiocyanate (FITC) (Figures 4 and 5) by confocal laser scanning microscopy and flow cytometry, four compounds induced expression of differentiation marker (c-erbB-2) and the potency of four compounds on differentiation induction shared similar to that of them on growth inhibition (Figures 5 and 2B).

Human telomerase reverse transcriptase assay

The human telomerase reverse transcriptase (hTERT), as one of the major subunits of telomerase, is specifically activated in most malignant tumors but is usually inactive in normal tussues (Kyo et al., 2000; Liu et al., 2004). The mRNA level of hTERT often closely associated with telomerase activity and has thus been considered as a rate limiting determinant of telomerase activity (Kanaya et al., 1998; Kyo et al., 1999, 2000). The previous study demonstrated that down-regulation of hTERT correlates with cellular differentiation (Pendino et al., 2001; Savoysky et al., 1996; Xu et al., 1999). In order to better understand the precise induction effect of differentiation of the selected compounds on OE33 cells, a differentiation marker, the human telomerase reverse transcriptase, was also investigated. The alterations of hTERT were assayed by real-time RT-PCR assay. Each treatment showed all four flavonoids inhibited the expression of hTERT relative to the vehicle-treated control as shown in Figure 6, the potency was also similar to that of them on growth inhibition. The results indicated that the selected compounds might inhibit OE33 cells growth by inducing differentiation.

Real-time RT-PCR analysis

To verify the alterations of gene expression in OE33 cells treated with four selected compounds at the mRNA level, real-time RT-PCR analysis was conducted for three genes related to differentiation (Table 2). As shown in Table 2, treatments of OE33 cells with each selected compound (galangin, chrysin, baicalein, kaempferol) resulted in

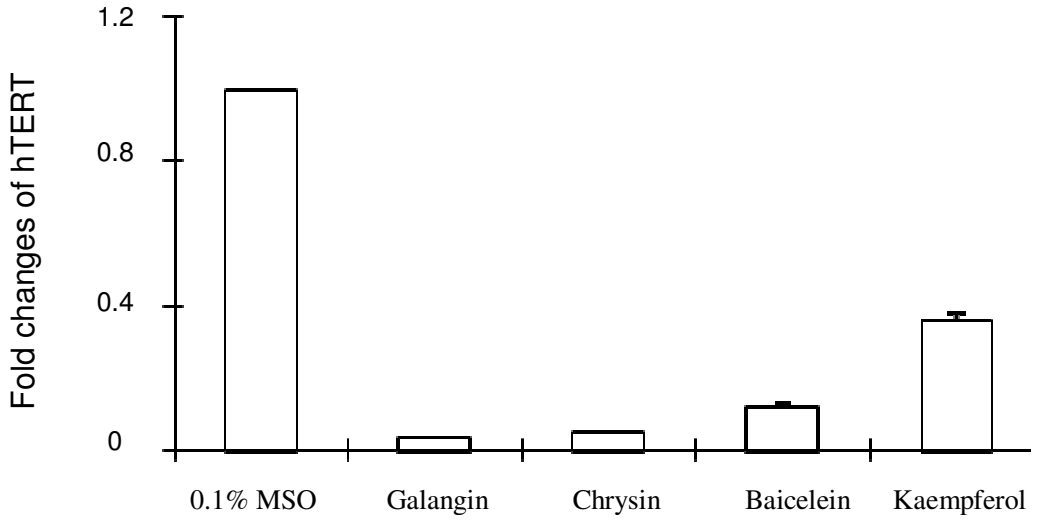

Figure 6. The effects of two flavones and two flavonols on the expression of human telomerase reverse transcriptase (hTERT) by real-time RT-PCR analysis in OE33 cells. The cells were treated with 80 µmol/L concentration of each compound for 24. The cells treated with 0.1% DMSO served as a negative control. The result indicated that each compound inhibited the expression of hTERT.

Table 2. Real-time RT-PCR analysis of comparative effects of two flavones and two flavonols on the regulation of the genes related to differentiation in OE33.

Genes	Galangin	Chrysin	Baicalein	Kaempferol
14-3-3σ	+1.06±0.03[b]	+1.81±0.02[b]	+1.53±0.20[b]	+1.42±0.01[b]
Cyclin B1	-0.79±0.01[c]	+1.56±0.03[b]	-0.85±0.01[c]	-0.98±0.00[c]
Cyclin D1[a]	-0.51±0.02[c]	-0.55±0.01[c]	-0.61±0.01[c]	-0.76±0.06[c]

[a] Bold letters highlight the genes whose expressions were in agreement with the potency of two flavones and tow flavonols on inhibition of KYSE-510 cells, [b] "+" denotes up-regulated expression (fold change), [c] "-" denotes down-regulated expression (fold change).

up-regulation of 14-3-3σ and down-regulation of cyclin B1 and cyclin D1 at the mRNA in OE33 cells. It was suggested that the effects of two flavones and two flavonols on induction of cell differentiation in OE33 cells might be depended on expression levels of these three genes.

Western blot analysis

In order to verify whether the alterations in expression of these three genes at the mRNA level ultimately would lead to the alterations at the protein level, a series of Western blot analysis were conducted.

As shown in Figure 7, the protein levels of cyclin B1 or cyclin D1 in OE33 cells treated with the selected compounds was down-regulated, and the protein level of 14-3-3σ was up-regulated. The potency of the selected compounds on regulation of these three proteins was essentially in agreement with the analysis data in real-time RT-PCR.

DISCUSSION

The growth inhibitory effects of two flavones and two flavonols on OE33 cells

It is well known that flavonoids in the diet reduce the risk of cancer (Garcia-Closas et al., 1999; Knekt et al., 1997; Neuhouser, 2004; Su and Arab, 2002). Many studies had showed that flavonoids had ability to inhibit cell growth of malignant cancers (Veeriah et al., 2006; Wang et al., 2000; Zheng et al., 2005). Therefore, the potential biological effects of the selected flavonoids on OE33 cell growth were investigated. The results showed that the two flavones and two flavonols could inhibit cell proliferation of OE33 in a dose- and time-dependent manner with the potency as: galangin > chrysin > baicalein > kaempferol. The inhibiting effects of these flavonoids on OE33 cell was consistent with these published results, in which flavonoids also were reported to have growth inhibition on other tumor cell lines (Kawaii et al., 1999; Pouget et al., 2001; Wenzel et al., 2000).

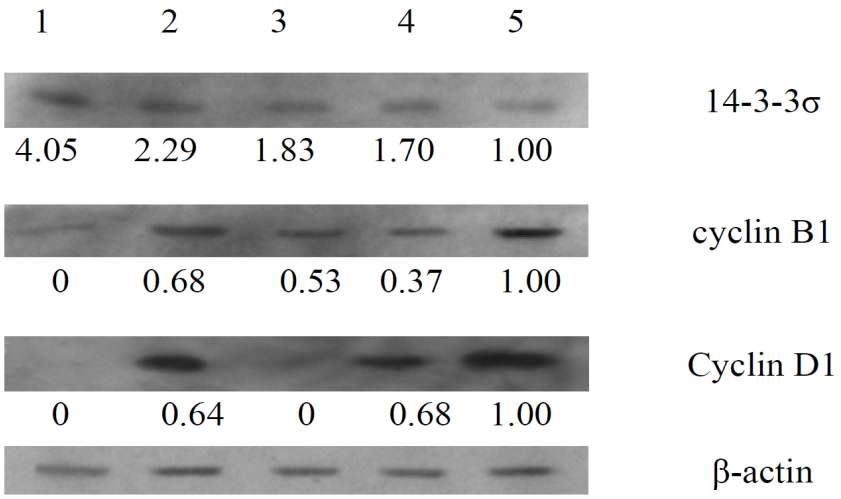

Figure 7. Effects of two flavones and two flavonols on the expression of proteins related to differentiation in OE33 cells. The cells were treated with vehicle (DMSO) only or the selected compound at 80 µmol/L for 24 h and then harvested. Line 1, galangin; Line 2, baicalein; Line 3, chrysin; Line 4, kaempferol; Line 5, 0.1% DMSO. The values below the figure represented changes in protein expression of the bands normalized to β-actin.

Effect of two flavones and two flavonols on the induction of differentiation on OE33 cells

In our previous study, we had found that three flavones and three flavonols could cause G_2/M arrest and induce apoptosis on KYSE-510 (Zhang et al., 2009) and OE33 cells (Zhang et al., 2008). In this process most malignant cells die through an apoptotic mechanism, whereas cells with differentiation potential will survive as a result of selection (Brown et al., 1998). Extending these studies, we here also provide evidence that flavonoids also have the ability to induce undifferentiated cancer cell lines to the cells exhibiting mature phenotypic characteristics. Therefore, we examined the biological effect of flavonoids on OE33 cell differentiation.

The activity of induced-differentiation of flavonoids had been confirmed by their actions on many cancer cell lines, such as human breast squamous MCF-7 and MDA-MB-468 cell lines (Constantinou et al., 1998), human promyelocytic leukemia HL-60 cell line (Kawaii and Lansky, 2004; Lee et al., 2001), human melanoma TVM-A12, M14 and A-375 cell line (Serafino et al., 2004), human prostate carcinoma LNCaP cells (Zi and Agarwal, 1999) and other alimentary tract tumour cells (Gee and Johnson, 2001). However, other studies had showed that curcumin inhibited proliferation, enhanced apoptosis but did not promote differentiation (Balasubramanian and Eckert, 2004). Meanwhile, apigenin could suppress cell differentiation and proliferation but did not enhance apoptosis of keratinocyte (Balasubramanian et al., 2006). Taken together, it could be suggested that flavonoids might have different mechanisms of action on different cells.

Effect of flavones and flavonols on the induction of differentiation morphological on OE33 cells

It was indicated in our study that the four selected compounds were capable of inducing morphological differentiation of OE33 cells, including an enlarged, flattened shape, an increased cytoplasmic-to-nuclear ratio, and a decreased cell density, compared with the negative controls. The phenotype differentiation was also acquired by inducing other human cell lines differentiation, for example, human colon cancer KNC cells with kaempferol were observed dramatic morphological changes, including the appearance of neuron-like and triangular cells (Nakamura et al., 2005). Neuroblastoma N_2A cells treated with genistein also exhibited morphological features of differentiation, dendritic extensions (Brown et al., 1998). Meanwhile, cyanidin-3-O-β-glucopyranoside treatment induced, in a dose- and time-dependent manner, melanoma cell differentiation characterized by a strong increase in dendrite outgrowth similarly to RA, which was a morphological feature of differentiated melanocytes and the first observable parameter of melanoma cell differentiation (Serafino et al., 2004).

Effect of flavones and flavonols on the inducing differentiation marker on OE33cells

C-erbB-2 is located on chromosome 17q21 and encodes a 185 kD transmembran protein that lacks a natural ligand (Schlessinger, 2000; Slamon et al., 1989). Like EGFR, c-erbB-2 can interact with many different cellular proteins and plays an important role in regulation of fundamental

processes such as cell growth, survival and differentiation (Selvarajan et al., 2004; Xia et al., 1997). It was reported that the expression of c-erbB-2 was not detected in malignant esophageal adenocarcinoma tissue, suggesting that c-erbB-2 was a differentiation marker of esophagus adenocarcinoma (Sauter et al., 1993).

Data in our study showed that two flavones and two flavonols restored a differentiated phenotype of OE33 cells as judged by the increased expression of c-erbB-2 by confocal laser scanning microscopy and flow cytometry (Figures 4 and 5). The potency of these flavonoids on induction of differentiation was essentially in agreement with the results of cell growth inhibition analyzed in our study (Figures 5 and 2B). Galangin was the most power compound to induce differentiation of OE33 cells among all four compounds. OE33 cells treated with 80 µmol/L baicalein showed a higher expression of c-erbB-2 than the cells treated with 80 µmol/L galangin. In our study, treating OE33 cells with 80 µmol/L galangin, an initial differentiation response might be followed by cell death. In agreement with our results, other study also proposed that her-2 overexpression was a late event in the formation of esophageal adenocarcinomas (Hardwick et al., 1995). Clearly, more work is warranted to clarify the expression of her-2 in different types and stages of esophageal cancer.

Effect of flavones and flavonols on the expression of human telomerase reverse transcriptase

Human telomerase reverse transcriptase (hTERT) is often detectable in cancerous cells but not in normal somatic cells (Liu et al., 2004) and that expression of hTERT was correlated positively with telomerase activity. Thus, it was suggested that hTERT was the major determinant of telomerase activity (Kraveka et al., 2003; Liu et al., 2004). Previous studies reported that down-regulation of hTERT in human cancer cells lines was linked with the degree of differentiation, such as hepatocellular carcinoma (Kishimoto et al., 1998), human neuroblastoma SK-N-SH and SK-N-AS cell lines (Kraveka et al., 2003), human promyelocytic leukemia HL60 cell lines (Liu et al., 2004;Xu et al., 1999), human hepatoma-derived cell lines, PLC/PRF/5, HuH-7 and JHH-7 (Yasuda et al., 2002), and human teratocarcinoma cells (Liu et al., 2004; Lopatina et al., 2003).

To better understand the precise effects of two flavones and flavonols in inducing differentiation on OE33 cells, the alterations of hTERT expression in OE33 cells were investigated. It was found that two flavones and flavonols had ability to reduce expression of hTERT in OE33 cells, and the potency of these compounds on inhibition of hTERT was also similar to that of them on growth inhibition (Figures 6 and 7). The decreased expression of hTERT also demonstrated the effects of flavones and flavonols in inducing differentiation on OE33 cells. Many previous studies have demonstrated that decreased hTERT expression was associated with the cellular differentiation, such as human promyelocytic leukemia HL60 cell lines (Liu et al., 2004; Pendino et al., 2001; Xu et al., 1996, 1999), hepatocellular carcinoma (Kishimoto et al., 1998), human hepatoma-derived cell lines, PLC/PRF/5, HuH-7and JHH-7 (Yasuda et al., 2002), human neuroblastoma SK-N-SH and SK-N-AS cell lines (Kraveka et al., 2003), human teratocarcinoma cells (Liu et al., 2004; Lopatina et al., 2003).

Possible mechanism related to the differentiation of OE33 cells induced by two flavones and two flavonols

The development of a tissue requires the integration of cell cycle exit with morphological changes culminating in the specialized nature of a differentiated cell (Baldassarre et al., 2000; Zezula et al., 2001). Cell cycle progression in eukaryotes is controlled by a series of proteins named cyclins and they are classified into three groups: G_1 cyclins, an A-type cyclin, and two B-type cyclins (Jong et al., 1999).

Cyclins D are required for progression through G_1, because the CDK4-and/or CDK6-cyclin D complex appears to be necessary for transition through early G_1 (Zi et al., 1998). Some previous studies have showed that down-regulation of cyclin D1 was linked with differentiation (Spinella et al., 1999). Cyclin B is the major controlling cyclin in the G_2 phase of cell cycle. There are two B-type cyclins identified in mammalian cells, cyclin B1and cyclin B2 (Archer et al., 2005). Most studies have focused on the expression and regulation of cyclin B1. Study had demonstrated the association of cyclin B1 repression and the induction of the differentiated phenotype in human colon carcinoma-derived cell lines HT-29 (Archer et al., 2005). However, cell cycle progression is negatively regulated by the CDK-inhibitory proteins (CDKIs), which inhibits cycle progression by binding to the cyclin/cyclin-dependent kinase complex (Graña and Reddy, 1995; Kamb et al., 1994). 14-3-3σ is a negative regulator of cell cycle and is well-known to play an important role in the G_2 checkpoint by sequestering the cdc2/cyclin B1 complex (Hermeking, 2003; Wilker andYaffe, 2004). Recent evidences suggested a crucial role of 14-3-3σ in the control of cell growth and differentiation, such as expression of 14-3-3σ was upregulated upon differentiation in cells of the poststem cell compartment and immortalization of keratinocytes by experimental inactivation of 14-3-3σ (Pellegrini et al., 2001). Moreover, recent studies had revealed the existence of dual-function regulators that could participate in controlling both cell proliferation and differentiation. Certain cell-cycle regulators could directly regulate differentiation separating from their cell-cycle inhibitory functions (Zhu and Skoultchi, 2001). Thus, it was postulated that up-regulation of 14-3-3σ and

down-regulation of cyclin B1 and cyclin D1 might promote differentiation of OE33 cells, independent of any role in regulating cell cycle exit.

Data in our study demonstrated up-regulation of 14-3-3σ and down-regulation of cyclin B1 and cyclin D1 at the mRNA level (Table 2) and protein levels (Figure 7) during differentiation of OE33 cells, which might be a possible molecular mechanism through which flavones and flavonols induce differentiation of OE33 cells. There were several studies about the effect of 14-3-3σ expression on differentiation. Takihara et al. (2000) found that the expression of five 14-3-3 protein isoforms was induced during the retinoic acid (RA)-mediated differentiation of mouse embryonal carcinoma F9 cells. Other study also showed that expression of 14-3-3σ was upregulated upon differentiation in cells of the poststem cell compartment and immortalization of keratinocytes by experimental inactivation of 14-3-3σ (Pellegrini et al., 2001). Meanwhile, Olsen et al. (1995) demonstrated that members of the 14-3-3 family were up-regulated in the differentiated human keratinocytes. Some studies had demonstrated the association of down-regulation of cyclin B1 and induction of differentiation. Archer et al. (2005) had showed that cyclin B1 repression was linked to the differentiation process in colon cancer cells, not merely with growth arrest. Meanwhile, the association of cyclin B1 repression and the induction of the differentiated phenotype in colon cancer cells were similar to that described in other cells (Horiguchi-Yamada and Yamada, 1993; Jahn et al., 1994).

Some previous studies had reported the association of decreased expression of cyclin D1 and induction of differentiation. Spinella et al. (1999) demonstrated that retinoids promoted ubiquitination and degradation of cyclin D1 during retinoid-induced differentiation of human embryonal carcinoma NT2/D1 cells. However, over expression of cyclin D1 had been reported in the TPA-treated HL60, THP-1 and KG-1 cells, which were differentiated into monocytes/ macrophages by TPA treatment (Matsumoto et al., 2006). Similarly, it was reported that over expression of the genes encoding cyclin D1 might also induce differentiation in squamous cell cancer (de Jong et al., 1999). The results of these previous studies also corroborated the postulation that the expression changes of cyclin D1 by flavonoids were depended on the type of cancer cells. In our study, up-regulation of cyclin D1 in the treated OE 33 cells might be explained by this mechanism.

Conclusions

In summary, the differentiation-induced effects of two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol) on a human esophageal adenocarcinoma cell line (OE33) were studied in the present work, and the molecular mechanism responsible for these effects was investigated. Our finding indicated that two flavones and flavonols were able to induce differentiation, as evidenced by neuroendocrine-like morphology, elevated phenotype differentiation, differentiation maker (c-erbB-2), and reduced human telomerase reverse transcriptase (hTERT) in OE33 cells. Additionally, it was also found that up-regulation of 14-3-3σ and down-regulation of cyclin B1 and cyclin D1 at the mRNA and protein levels were observed in OE33 cells treated with these four compounds. It was speculated that 14-3-3σ, cyclin B1 and cyclin D1 might be target genes of two flavones and two flavonols in inducing differentiation. These results might shed new light on molecular mechanisms involved in flavonoid-induced differentiation on other cancer cells.

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