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Cytotoxicity of flavones and flavonols to a human esophageal squamous cell carcinoma cell line (KYSE-510) by induction of G_2/M arrest and apoptosis

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ABSTRACT

In this study, cytotoxic effects of structurally related flavones and flavonols on a human esophageal squamous cell carcinoma cell line (KYSE-510) were determined, and the molecular mechanisms responsible for their cytotoxic effects were studied. The results of MTT assay showed that flavones (luteolin, apigenin, chrysin) and flavonols (quercetin, kaempferol, myricetin) were able to induce cytotoxicity in KYSE-510 cells in a dose- and time-dependent manner, and the cytotoxic potency of these compounds was in the order of: luteolin > quercetin > chrysin > kaempferol > apigenin > myricetin. Flow cytometry and DNA fragmentation analysis indicated that the cytotoxicity induced by flavones and flavonols was mediated by G_2/M cell cycle arrest and apoptosis. Furthermore, the expression of genes related to cell cycle arrest and apoptosis was assessed by oligonucleotide microarray, real-time RT-PCR and Western blot. It was shown that the treatment of KYSE-510 cells with these compounds caused G_2/M arrest through up-regulation of $p21^{waf1}$ and down-regulation of cyclin B1 at the mRNA and protein levels, and induced p53-independent mitochondrial-mediated apoptosis through up-regulation of PIG3 and cleavage of caspase-9 and caspase-3. The results of western blot analysis further showed that increases of p63 and p73 protein translation or stability might be contributed to the regulation of $p21^{waf1}$, cyclin B1 and PIG3.

1. Introduction

Polyphenolic compounds can be divided into various classes on the basis of their molecular structure, with flavonoids being one of the main groups. The molecular structure of flavonoids consists of two aromatic rings (A ring and B ring), that are linked by a three-carbon bridge (Fig. 1). Depending on their oxidation state and functional groups, flavonoids are further divided in six subclasses: flavones, flavanols, flavanones, flavonols, isoflavones, and anthocyanidins (Beecher, 2003; Ross and Kasum, 2002).

Many studies dealing with flavonoids have focused on their antioxidant properties, but a number of reports in different cell lines, animal models and human epidemiological trials have pointed out an association between intake of dietary flavonoids and reduced risk of cancer, including esophageal cancer (Rossi et al., 2007; Watson et al., 2000; Wenzel et al., 2000). Esophageal cancer has been reported as the ninth most common malignancy and ranks as the sixth most frequent cause of cancer death in the world. It also constitutes 7% of all gastrointestinal cancers and is one of the most lethal of all cancers (Kuwano et al., 2005). There are two main forms of esophageal cancer, each with distinct etiological and pathological characteristics: squamous cell carcinoma (>90% of esophageal malignancies) and adenocarcinoma (<10% of esophageal malignancies) (Kuwano et al., 2005).

Some reports revealed that flavones and flavonols exhibited cytotoxicity in vitro to many human cell lines, including colon cancer cells (Takagaki et al., 2005; Lim do et al., 2007), hepatoma cells (Chiang et al., 2006; Granado-Serrano et al., 2006), prostate carcinoma cells (Gupta et al., 2002; Shukla and Gupta, 2006; Vijayababu et al., 2006), human cervical carcinoma cells (Zheng et al., 2005; Horinaka et al., 2005). However, the precise molecular mechanisms by which flavones and flavonols exert their cytotoxic effects on esophageal cancer cells are poorly studied and understood. In one of our recent study, it had shown that flavones (luteolin, chrysin and apigenin) and flavonols (quercetin, kaempferol and myricetin) can induce cytotoxicity to OE33 cells (a human esophageal adenocarcinoma cell line) by causing G_2/M arrest and inducing apoptosis (Zhang et al., 2008). In this study, cytotoxic effects of these compounds on a human esophageal squamous cell carcinoma cell line

Abbreviations: BAX, BCL2-associated X protein; BTG2, BTG family member 2; DMSO, dimethyl sulfoxide; EB, ethidium bromide; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; 5-FU, 5-fluorouracil; KILLER/DR5, death receptor 5; GADD45, growth arrest and DNA-damage-inducible; MDM2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide; p53AIP1, p53-regulated apoptosis-inducing protein 1; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; PIDD, Leucine-rich and death domain containing; PMSF, phenylmethyl sulfonylfluoride; RNase, ribonuclease; PUMA, Bcl-2 binding component 3; SDS–PAG, sodium dodecyl sulfate–polyacrylamide gel; TBS, Tris buffered saline.

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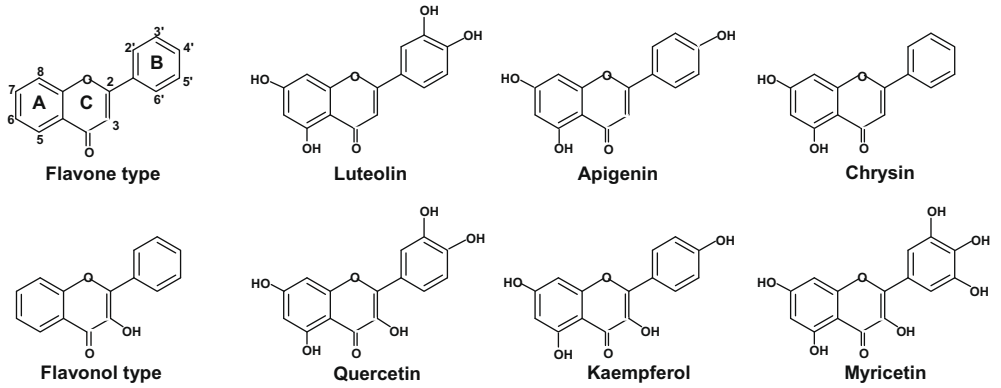

Fig. 1. Chemical structures of flavones and flavonols.

(KYSE-510) were also studied, and molecular mechanisms responsible for their cytotoxic effects were elucidated.

2. Materials and methods

2.1. Materials

Quercetin (>98% of purity), luteolin (>98% of purity), apigenin (>98% of purity), and kaempferol (>98% of purity) were purchased from Nanjing Qing Ze Medical Technology Co., Ltd. (Nanjing, Jiangsu, China). Chrysin (>96% of purity), myricetin (>95% of purity) and MTT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The solubilities of these compounds are >50 mg/ml in DMSO. The following reagents were from Solarbio Science & Technology Co., Ltd. (Beijing, China): PI, EB, DMSO, RNase, and materials for Western blot.

The mono- and poly-clonal antibodies (human reactive anti-$p21^{waf1}$, p53, p63, p73, cleaved caspase-9, and β-actin) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The human reactive antibodies for anti-cleaved caspase-3 and PIG3 were obtained from Abcam Inc., (Cambridge, MA, USA) and Chemicon International Inc., (Temecula, CA, USA), respectively, while the anti-cyclin B1 and cleaved caspase-8 were obtained from Lab Vision Co., (Fremont, CA, USA) and Cell Signaling Technology Inc., (Beverly, MA, USA), respectively. The horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibody were from Zhongshan Gold Bridge Biotechnology Co., Ltd. (Beijing, China).

2.2. Cell culture

The KYSE-510 cell line was obtained from Tianjin Medical University Cancer and Hospital (TMUCIH, Hexi, Tianjin, China). The cell line was established from a well-differentiated esophageal squamous cell carcinoma of a 67-year-old Japanese woman. KYSE-510 cells express p53 mutation. This cell line was cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G and 100 mg/ml streptomycin (all obtained from Solarbio) and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.3. MTT assay

Cytotoxicity was measured by MTT assay. KYSE-510 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 flavones (luteolin, apigenin, chrysin) and flavonols (quercetin, kaempferol, myricetin). 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 μM concentration of each compound for 24, 48 and 72 h. Cells treated with 0.1% DMSO and 100 μM of 5-FU served as a negative control and a positive control, respectively. After incubation for specified time at 37 °C in a humidified incubator, MTT (5 mg/ml in PBS) 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 the wavelength of 570 nm. The effect of each compound on growth inhibition was assessed as percent cell viability where vehicle-treated cells were taken as 100% viable.

2.4. Cell cycle analysis

The cells (70% confluence in 6-well plates) were starved in 1% FBS-containing media for 36 h to arrest them in G_0 phase of the cell cycle, after which they were treated with each compound (80 μM) for 24 h. The cells were trypsinized thereafter, washed twice with cold PBS and then centrifuged. The pellet was resuspended in 75% ethanol for 24 h at −20 °C. The cells were centrifuged at 110*g* for 5 min (dead and apoptotic cells were removed), pellet washed twice with cold PBS, suspended in 500 μl PBS, and incubated with 5 μl RNase (20 μg/ml final concentration) at 37 °C for 30 min. The cells were incubated in the dark with PI (50 μg/ml final concentration) for 1 h and analyzed by flow cytometry (Beckman Coulter Inc., Fullerton, CA, USA). Flow cytometry data were gated according to size to obtain a more homogeneous population in terms of cell size for fluorescence analysis. The parameters for gating were set such that maximum number of data points in all the samples is covered. The following protocol was used: (a) The Forward scatter (FSC) versus Side scatter (SSC) dot plots of the FACS samples were plotted; (b) A rectangular region from ~3–60 U on the SSC scale and a similar range on the FSC scale was chosen; (c) The cells inside this region were gated out and used in the analysis of their fluorescence distribution.

2.5. DNA fragmentation assay

The cells were treated with each compound (80 μM) for 24 h. The Apoptosis DNA Ladder kit (Applygen Technologies Inc., Beijing, China) was used for the detection of DNA fragmentation according to the kit's instructions. Total amount of DNA was resolved over 1.5% agarose gel containing 0.3 mg/ml EB in 1×TAE buffer (pH 8.5, 20 mM Tris-acetic acid, 2 mM EDTA). The bands were visualized and photographed by PhotoDoc-It Imaging System (UVP Inc., San Gabriel, CA, USA).