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Effects of Genistein on Cell Cycle and Apoptosis of Two Murine Melanoma Cell Lines

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Abstract: The effects of genistein on several tumor cell lines were investigated to study the effects of genistein on cell growth, cell cycle, and apoptosis of two murine melanoma cell lines, B16 and K1735M2. These two closely related murine melanoma cell lines, however, have different responses to the genistein treatment. Genistein inhibits the growth of both the B16 and K1735M2 cell lines and arrests the growth at the G2/M phase. After treatment with 60 μmol/L genistein for 72 h, apoptosis and caspase activities were detected in B16 cells, while such effects were not found in K1735M2. Further tests showed that after genistein treatment the protein content and mRNA levels of p53 increased in B16, but remained the same in K1735M2. The protein content and mRNA levels of $p21^{WAF1/CIP1}$ increased in both cell lines after treatment. The results show that genistein might induce apoptosis in B16 cells by damaging the DNA, inhibiting topoisomerase II, increasing p53 expression, releasing cytochrome c from the mitochondria, and activating the caspases which will lead to apoptosis.

Key words: genistein; melanoma cells; cell cycle; apoptosis; p53; p21

Introduction

Genistein has been identified as one of the main isoflavonoid components in soybeans, which may play a prominent role in cancer prevention[1]. Epidemiological studies suggest that high consumption of soybeans is associated with lower incidence of breast and prostate cancer in Asians[2-4]. Previous studies have shown that genistein can inhibit the growth of leukemia, breast, and prostate cancer *in vivo* and *in vitro*[5-10]. Recent studies indicated that genistein could also be used at a chemopreventative cure for melanoma[11]. Genistein inhibited melanoma cell growth both *in vivo* and *in vitro*, promoted differentiation *in vitro* and reduced melanoma metastasis in mice[12,13].

The mechanism of the anticancer effects of genistein has been widely studied. Genistein might act as a protein tyrosine kinase inhibitor[14], topoisomerase II inhibitor[15], and S6 kinase inhibitor[16]. Genistein also has antioxidation and anti-angiogenesis effects as well as estrogenic activities[17,18]. Genistein has been demonstrated to arrest the cell cycle in breast, lymphocytes, and leukemic cancer cell lines, and induce apoptosis in human breast cancer cells[18]. However, there is a little information on the effects of genistein on melanoma cells[19].

Previously we found that genistein and daidzein could cause arrest of human and murine melanoma cells at the G2/M phase[20]. In a continuation of previous work[21-23], the present study explores the

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different effects of genistein on two closely related murine melanoma cells and the mechnisms behind these effects. The experimental evidence suggests that the difference is due to the cell cycle distribution, apoptosis, and $p21^{WAF1/CIP1}$ induction. These results may shed light on elucidating the mechanism of genistein's anti-melanoma effects.

1 Materials and Methods

1.1 Reagents

Genistein, dimethylsulfoxide (DMSO), and 3(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium-bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). DMEM, PRIM-1640, trypsin, fetal bovine serum (FBS), and some other culture reagents were purchased from Hyclone (Logan, UT, USA). The mouse monoclonal anti-p53 antibody and anti-p21 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

1.2 Cell cultures

The murine melanoma cells K1735M2 (a gift from Dr. I. J. Fidler, M.D., Anderson Cancer Center, TX, USA) were cultured in DMEM medium (high glucose), pH 7.3. The murine melanoma cells B16 (obtained from the Medical School of Peking University, Beijing, China) were cultured in PRIM-1640 medium, pH 7.3. Both cell cultures were supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. Cells were grown at 37℃under 5% CO_2 atmosphere. Culture media were changed every 2-3 days. When they reached confluence, the cells were dissociated by 0.05% trypsin-0.02% ethylenediamine tetraacetic acid (EDTA) and replated at 1:5 dilution.

1.3 MTT assays

The inhibition of cell proliferation by genistein was determined by use of MTT assays, which monitored the numbers of cells based on the reduction of MTT by the mitochondrial dehydrogenases present in viable cells. Briefly, cells were plated into 96-well tissue culture dishes at a density of 1×10^3 cells/well in 180 μL medium. After being plated, cells were allowed to attach for 24 h. Genistein was added using DMSO as the vehicle, with a maximum DMSO concentration of 1%. Incubation with genistein continued for the desired time, and then 20 μL of MTT (2 mg/mL) was added to each well. After incubation at 37℃ for 4 h, the supernatants were removed and the formazan crystals were dissolved by adding 200 μL DMSO. The plate was then read on a microplate reader at 490 nm. Experiments were conducted in triplicate.

1.4 Cell cycle progression analysis

Cells were typsinized, washed with phosphate buffered saline (PBS) and fixed with 70% ethanol. The fixed cells were spun down and resuspended in PBS at a concentration of 1×10^6 cells/mL and incubated with ribonuclease A (RNase A) at a final concentration of 3000 units/mL in 37℃ for 30 min, then filtered through a nylon mesh of 300 screen meshes. The cell suspension was stained by propidium idide (PI) before being measured by flow cytometry.

1.5 Detection of apoptosis

Apoptosis was determined by the dual-color flow cytometric (FCM) procedure with staining of fluorescein isothiocyanate (FITC)-Annexin-V and PI. FITC-Annexin-V was used to probe the appearance of phosphotidylserine (PS) residues on the outer leaflet of the plasma membrane as previously described[2]. Since necrotic cells also expose PS indicating the loss of membrane integrity, the cells were simultaneously stained with PI. This allowed differentiation of the necrotic and apoptosis populations. Cells were prepared according to the manufacturer's instructions. Briefly, approximately 5×10^5 cells per experimental condition were harvested, washed with cold PBS twice, and resuspended with 200 μL binding buffer. Cells were then incubated for 15 min at 37℃ or for 30 min at 4℃ in the dark, in the presence of FITC labeled at Annexin-V and PI. Another 300 μL binding buffer was added to the cell suspension. The cells were then analyzed by flow cytometry.

1.6 Detection of caspase-3 and caspase-9 activiation

This assay used the ApoAlert® caspase-3 colorimetric assay kit and the ApoAlert® caspase-9/6 fluorescent assay kit (clonTech, CA, USA) to detect the activation of caspase-3 and caspase-9 of K1735M2 and B16,