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Effects of genistein and daidzein on the cell growth, cell cycle, and differentiation of human and murine melanoma cells[1]

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Abstract

Genistein and daidzein are two major isoflavonoids in dietary soybean that have inhibition effect on the cell growth of different tumor cell lines. We previously reported the anti-tumor activities of genistein and daidzein in human co1on tumor (HCT) cells and their different ability to enhance the activation of murine lymphocytes. In the present study, the effect of genistein and daidzein on the cell growth, cell cycle progression, and differentiation of murine K1735M2 and human WM451 cel1s was investigated. It was found that genistein could inhibit the cell growth of two metastatic melanoma cell lines, murine Kl735M2 and human WM45l in a dose-dependent manner. Flow cytometry showed that genistein could cause arrest of both Kl735M2 and WM45l at G_2/M phase, while daidzein increased the cell numbers at S phase, decreased the cell numbers at G_1 phase. Detection of melanin and morphological observation showed that genistein can induce Kl735M2 and WM45l to produce dendrite-like structure and produce more melanin by 80%. In contrast, daidzein only retarded the growth of K1735M2 and did not induce differentiation in either K1735M2 or WM451. These results suggest that genistein and daidzein in soybean can inhibit certain malignant phenotype of melanoma via different mechanisms and be potential medical candidates for melanoma cancer therapy.

Keywords: Soybean; Daidzein; Genistein; Melanoma; Cell growth; Cell cycle; Differentiation

1. Introduction

Isoflavonoids are among the most promising potential anticarcinogenic compounds in dietary soybeans. Epidemiological studies indicate that high consumption of soybean is associated with a lower incidence of breast and prostate cancer in Asian people. Genistein and daidzein are the two major isoflavonoids in soybean, and were thought to play important roles in cancer prevention [1,2]. Previous studies have demonstrated that genistein and/or daidzein has inhibition effects on the growth of leukemia, breast and prostate cancer [3–7]. Our earlier studies showed that genistein and/or daidzein could inhibit the growth of human colon tumor (HCT) cells [8]. However there are evidences that genistein and daidzein displayed different character in mediating the behavior of malignant cells despite that there existed only nuance between their structures [8,9]. The biochemical mechanisms behind the effects of genistein have been reported to include its interaction with the estrogen receptor, its action as an antioxidant, inhibition of DNA topoisomerase, and inhibition of tyrosine protein kinases activities [10–14]. The exact mechanism by which such effects are induced is, however, largely unknown.

Incidence of malignant melanoma has approximately doubled each decade for the last 50 years. Major advances have been made not only in basic research but also in clinical management. The antitumoral effect of soybean isoflavonoids on the melanoma cells has recently been recognized. It was found that genistein could arrest human melanoma cells at G2 or G2/M and mouse melanoma cells at both G1 and G2 [15–18]. These conflicting results made us explore further their different mechanisms on different melanoma cells. In this study, two highly metastatic melanoma cells, mouse Kl735M2 and human WM45l cells were selected which their isolation and metastatic activity were previously described [19–20].

With the treatment of single or multiple differentiation modulating agents, many cancer cells can be induced to

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[1] The abbreviations used are: BSA, bovine serum albumin; FBS, fetal bovine serum; ECM, extracellular matrix; DMSO, dimethyl sulfoxide; PTK, protein tyrosine kinase.

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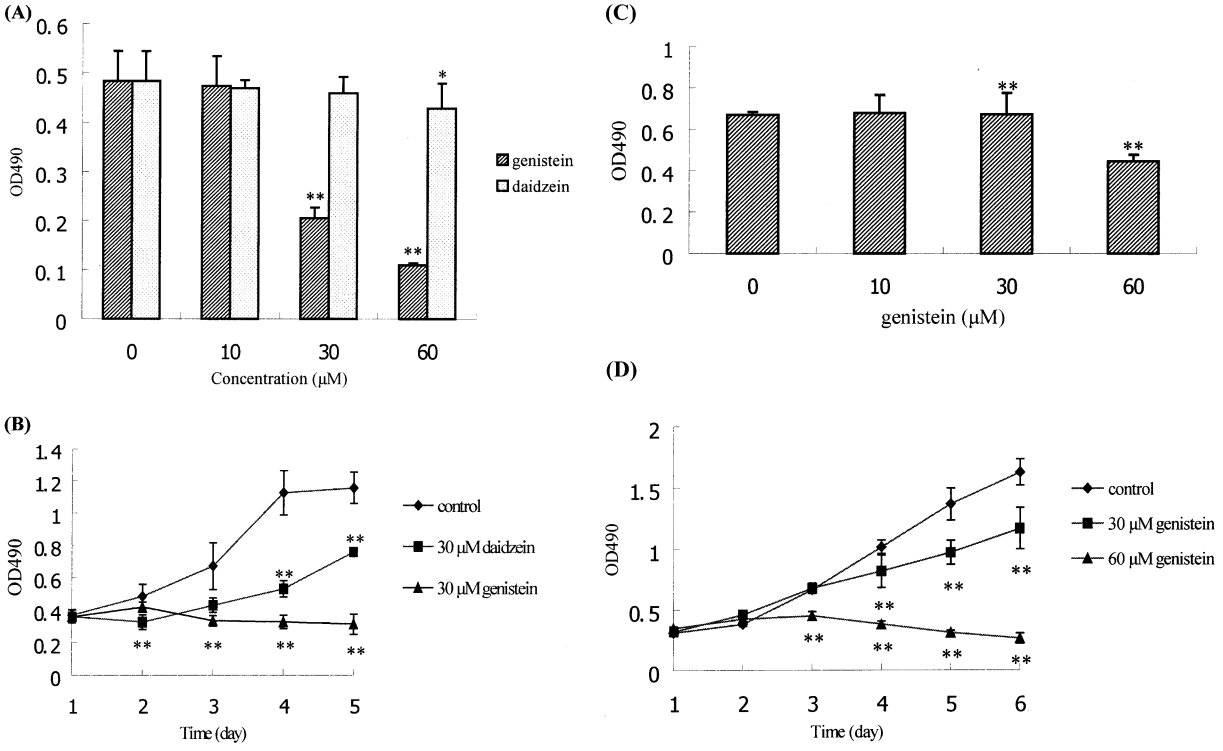

Fig. 1. Effects of genistein and daidzein on cell growth of melanoma (A), (B) K1735M2 and (C), (D) WM451 cells. Cells in logarithm growing period were treated with genistein or daidzein. Medium is refreshed every 2 days. After incubation several days, cell numbers were counted by 3-(4,5-dimethylthiazolyl)-2,5-diphynyltetrozolium bromide colorimetric assay. (A), (C) 10–60 μM for 3 days in the dose-response study; (B), (D) with 30 μM in the time-course study. The OD490 values of the control treatment are 0.484 and 0.371 in (A) and (B); 0.671 in (C); 0.309, 0.382, 0.669, 1.020, 1.371 and 1.631 in (D). The values represent the means ± SD (n = 6). *, $p < 0.05$ vs. control; **, $p < 0.01$ vs. control.

terminal cell differentiation in vitro. The control of tyrosinase activity appears to be the pivotal factor in melanogenesis regulation. It has been shown that genistein can inhibit the tyrosine protein kinases activities [10], so in this study, we checked whether it can induce cell differentiation in melanoma cells. Melanin formation and dendrite-like structure are quantifiable markers of melanoma cells differentiation, and these markers are characteristic of cell differentiating melanocytes. By use of K1735M2 and WM451 cell lines as inducing differentiation models, we studied the differentiating effect of genistein on the two kinds of melanoma cells which enabled us taken further insight into the functional mechanisms of isoflanvonoids.

2. Materials and methods

2.1. Cell lines and cell culture

The highly metastatic mouse me1anoma cell line Kl735M2 was derived from a large nonpigmented spontaneous lung metastasis produced by the Kl735 parent tumor growing at s.c. site [19] and generously provided by Dr. I. J. Fiddler (M. D. Anderson Cancer Center, Houston, TX) and was maintained in MEM with 5% FBS and antibiotics (l00 units of penicillin/ml, and l00 mg streptomycin/ml) [1].

The human melanoma cell line WM45l was obtained from Chinese Medicine University (Beijing, China) and was maintained in medium RPMIl640 containing l0% FBS and antibiotics (l00 units of penicillin/ml, and l00 mg streptomycin/ml).

2.2. Chemicals and reagents

Genistein, daidzein, human plasma fibronectin and mouse collagen IV were purchased from Gibco BRL (Grand Island, NY). The purity of genistein and daidzein was >98%. The two isoflavonoids were dissolved in dimethyl sulfoxide (DMSO) and diluted to the final concentration in media before use. All the controls were containing 40μM DMSO in media. Media MEM, RPMIl640 and fetal bovine serum were purchased from Hyclone (Logan, UT).

2.3. In vitro growth inhibition assay

The effect of genistein and daidzein on growth of melanoma cells was determined using modified procedure as described previously [21]. Murine Kl735M2 and human

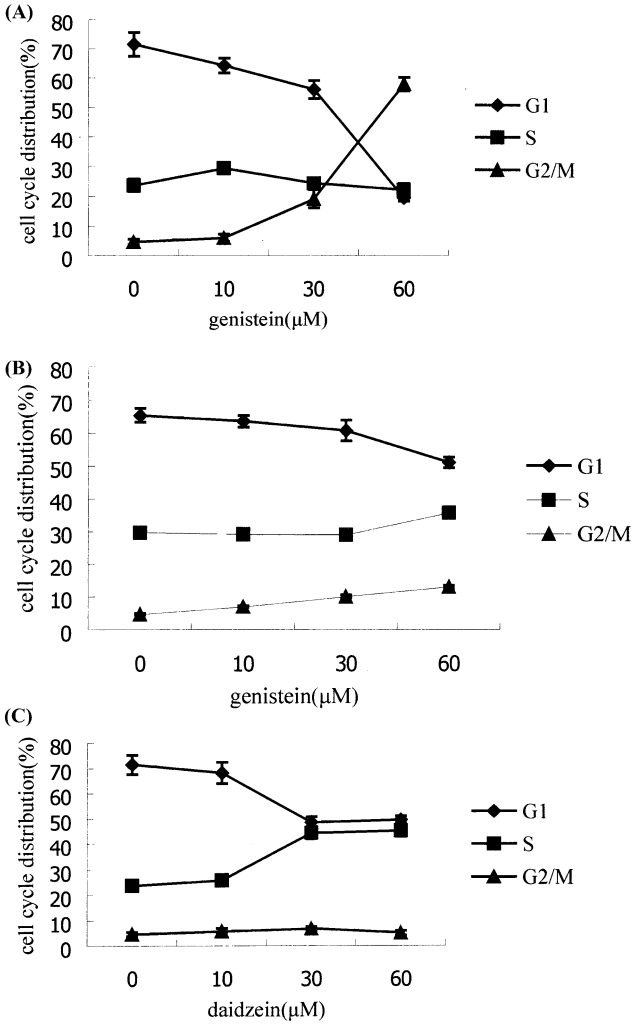

Fig. 2. Effects of genistein and daidzein on cell cycle distribution of melanoma (A), (C) K1735M2 and (B) WM451 cells. Cells have been grown in different concentration of genistein and daidzein for 3 days, then analyzed by flow cytometry as described in Materials and Methods. The data are representative example for duplicate tests. (◆) G1 phase; (■) S phase; (▲) G2/M phase.

WM45l cells were maintained as monolayer cultures in MEM with 5% FBS and antibiotics (100 units of penicillin/ml and l00 mg streptomycin/ml) and RPMIl640 with l0% FBS and antibiotics (l00 units of penicillin/ml, and 100 mg streptomycin/ml), respectively. The cel1s were inoculated in 5% CO2 atmosphere. The *in vitro* studies were complete with $0.5 \times l0^4$ cells/ml plated into 96-well flat-bottom microplates (Costar Corp), treated with genistein and daidzein of desired concentration when cell began to grow exponentially. After incubation for several days, 20 μl of 3-(4,5-driethy-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT 5 mg/ml) was added to each well, and the cells were cultured for another 4 hr. Then the supernatants were removed and the formazan crystals were disso1ved by the addition 200 μl DMSO. The plate was then read on a microplate reader (Bio-RAD, model 550) at 490 nm. The growth assay was conducted six times.

2.4. Cell cycle assay by flow cytometry

Kl735M2 and WM451 cells ($l \times 10^7$) were cultured in the presence of genistein and daidzein at various concentrations for 72 hr. Then approximately 10^6 cells were harvested with trypsin/EDTA. After a washing, the cells were fixed by the addition of 0–70% ethanol. Cells were then treated with 100 μg/ml RNase-A and were incubated 30 min at 37. After labeled with propidium iodide, the cells were then analyzed by a flow cytometer (Partec, CCA-II) with as described previous1y [22].

2.5. Cell differentiation assay

The induction of differentiation and mature phenotype were characterized by the formation of dendrite-like cellular protrusions and the increase in melanin content. In culture, cells in exponential phase were treated with l0, 30 and 60 μM genistein or daidzein. After 24-hr incubation, Kl735M2 cells were observed morphologically using microscope (Nikon). And after 4-day incubation, WM45l cells were analyzed for changes in morphology and melanin content (ng/μg protein) [16]. Briefly, 5×10^6 cells were routinely used to measure melanin content. Cells were centrifuged at 2500 rpm for l5 min and the resulting pellet was dissolved in 0.5 ml of 1N NaOH. Melanin concentration was calculated by OD_{475} and comparison with a standard curve of synthetic me1anin. Melanin contents above 5 ng/μg protein were re1iably detectable. Protein concentration was determined by Bradford assay using BSA as a standard.

2.6. Statistical analysis

The results were expressed as the mean ± SD and accompanied by the number of tests. Student's t-test was performed to determine the level of significance between groups and a value of $P < 0.01$ was considered to indicate a statistically significant difference.

3. Results

3.1. Different effects of genistein and daidzein on melanoma cells growth

As shown in Fig. l, genistein significantly inhibited the growth of murine Kl735M2 and human WM45l in a dose-dependent manner. Cells can hardly grow in the presence of 30 μM genistein (for Kl735M2, Fig. 1(A), (B)) or 60 μM genistein (for WM451, Fig. 1(C), (D)). In contrast, 30 μM daidzein can only retard the growth of Kl735M2 cells (Fig. 1(A), (B)). When control cel1s has been in stagnate phase, cells treated with daidzein were still in growth with a much slower speed. Similarly, treated with 30 μM daidzein exhibits no obvious effect on WM45l growth (data not shown).

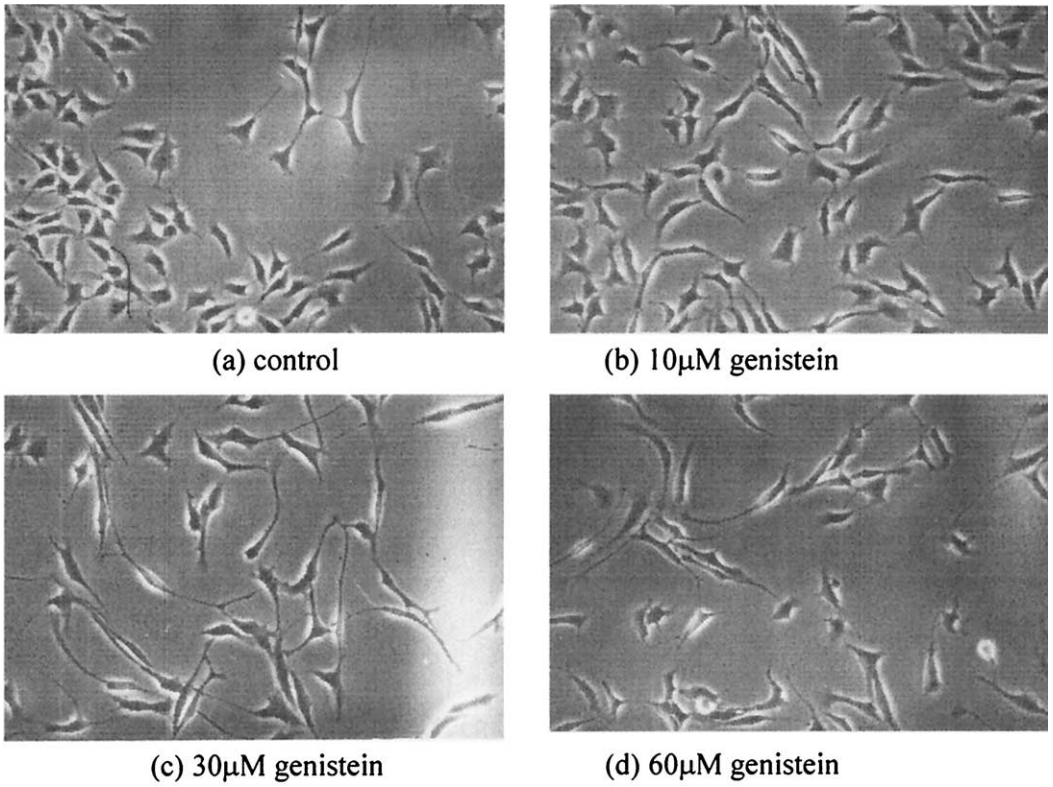

Fig. 3. Dendrite-like structure formation in melanoma K1735M2 cells treated for 24 hr with (a) control, (b) 10 μM, (c) 30 μM, and (d) 60 μM genistein. The morphology of cells was photographed by Nikon FX-35A camera.

3.2. G2/M arrest by genistein and cell cycle alteration by daidzein

To determine whether genistein and daidzein treatment of me1anoma cells resulted in alteration of cell cycle progression, the cell cycle patterns of the melanoma Kl735M2 and WM451 cel1s were examined by flow cytometric analysis. Genistein extensively arrested me1anoma Kl735M2 and WM451 cel1s in G_2/M phase in a does-dependent manner (Fig. 2(A), Fig. 2(B)). At the concentration of 60 μM, much more Kl735M2 cells (58.1%) were stationed in G2/M phase and much less cells (19.6%) in G1 phase compared with the control. Also at the concentration of 60 μM, the WM451 cells in G2/M phase were increased to about 2.7 times compared with the control and the cells in S phase were primarily constant. Daidzein exhibited a different effect on the cell cycle distribution. As shown in Fig. 2(C), daidzein increased the number of cells in S phase, decreased the cells in Gl phase and the number of cells in G2/M phase was maintained the same.

3.3. Inductive effect of genistein on melanoma cells differentiation

The differentiation status of melanoma cells was monitored by the formation of dendrite-like cellular protrusions. The treatment of cells with l0, 30 and 60 μM genistein caused the Kl735M2 to form dendrite-like structure (Fig. 3). These dendrite-like structures became progressive1y longer in accordance with the increase of incubation time and genistein concentration. Similar phenomenon was also observed for WM51 cells after 4-day incubation (pictures not shown). However, we cou1 days not find such phenomena with daidzein. Additionally, we a1so found a dose-dependent increase in me1anin content after genistein treatment. The melanin in WM45l had nearly doubled after treatment of 60 μM genistein for 4 days (Fig. 4). These results indicated that genistein effectively induced melanoma ce1ls to differentiate in vitro.

4. Discussion

Among other biochemical and physiological activities, the most remarkable character of soy isoflavonoids, mainly genistein and daidzein, are their inhibition ability to the growth of various malignant cell lines except that a few negative results of genistein have been reported [23–25]. In the present studies, we demonstrated that genistein significantly inhibited the cell growth of murine Kl735M2 and human WM45l cells *in vitro*. Recently, our research provided direct evidences that daidzein at high dose is able to enhance mu1tiple immunologic functions in mice [9,26]. Another study has shown genistein and/or daidzein have

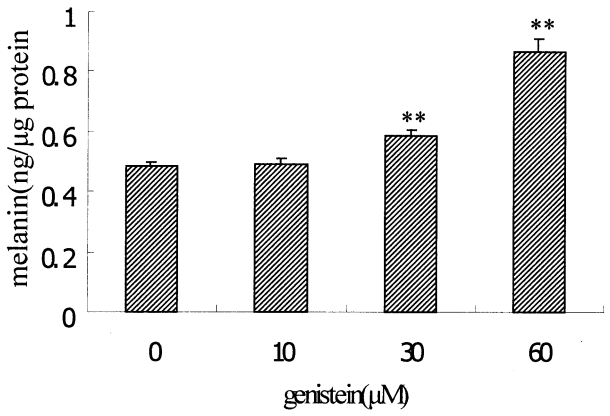

Fig. 4. Melanin induction in melanoma WM451 cells by genistein. After 4-day incubation, cells were analyzed for changes in melanin content by OD_{475}. The results are presented as ng melanin/μg protein. The melanin value for the control treatment is 0.481. The values represent the means ± SD (n = 3). **, $p < 0.01$ vs. control.

effect on the membrane characteristics of HCT cells, including membrane fluidity density of cell surface charge, and the conformation of membrane proteins [8]. It was a1so proposed that genistein functioned by altering cell cycle distribution [27,28]. Our present studies demonstrated that genistein cou1 days induce a G2/M cell cycle arrest in K1735M2 and WM451 in a dose-dependent manner. This result was consistent with the study of Matsukawa Y. [15]. Besides, genistein could induce Kl735M2 cells undergoing terminal differentiation. More dendrite-like structures were observed with the increasing of genistein concentration from l0 μM to 60 μM. Thus, we provided experimental evidences that the anti-tumor effect of genistein was associated with cell cycle arrest and morphological changes.

On the other hand, daidzein only retarded the growth of Kl735M2 and displayed no obvious inhibition effect on WM45l in spite of its structural similarity with genistein. Daidzein was also found to be able to influence Kl735M2 cell cycle progression, but the facture of its function was different from that of genistein. The reason of the difference may lie in that genistein possesses the hydrogen bond of the 5-hydroxy1 group with the 4-keonic oxygen and therefore is more hydrophobic than daidzein. However, the precise molecular mechanisms by which genistein and daidzein display different effects on the cell growth and change the cell cycle progression of melanoma cells, as discovered in this paper are still unclear. This paper may provide a basis on which further detailed studies on their mechanisms are merited.

In summary our studies demonstrated that genistein and/or daidzein exerted multiple suppressive effects on melanoma cells, including growth inhibition, cell cycle arrest, and induction of cell differentiation. Although daidzein may exerts its anti-melanoma effect in a mechanism different from that of genistein, these evidences suggest both of them can be potential candidate for melanoma therapy. These discoveries provided a basis for further investigation of genistein and daidzein for treating and preventing melanoma.

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