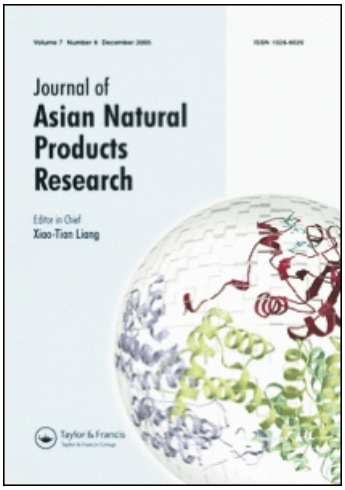

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Effects of Genistein, A Soybean-Derived Isoflavone, on Proliferation and Differentiation of B16-BL6 Mouse Melanoma Cells

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EFFECTS OF GENISTEIN, A SOYBEAN-DERIVED ISOFLAVONE, ON PROLIFERATION AND DIFFERENTIATION OF B16-BL6 MOUSE MELANOMA CELLS

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Genistein, a soybean-derived isoflavone, may contribute to the lower cancer incidence in South Asian countries. In this study, the effects and molecular mechanisms of genistein on growth and differentiation of B16-BL6 mouse melanoma cells were investigated. Genistein suppressed the growth of these melanoma cells. The IC_{50} value is 15.5 μM. On the other hand, genistein induced the changes of cell shape and cytoskeletal network. The cytoskeletal filaments were induced to form a bundle along the direction of elongation of the cells. Moreover, tyrosine phosphorylation levels of cytoskeleton-associated proteins decreased after the cells were exposed to 20 or 30 μM of genistein for 3 days. All these morphological and molecular changes were accompanied by appearance of the differentiated phenotypes. Genistein induced the increase of cellular melanin content, enhancement of tyrosinase activity, and decrease of colonization potentials in soft agar in a time-dependent and dose-dependent manner. The effective concentration was no more than 10 μM after 3 days' exposure. The tumorigenic potentials of B16-BL6 cells in C57BL/6 mouse also decreased after exposure to 20 or 30 μM of genistein for 3 days. When expressions of tumor-related genes were investigated in the differentiation-induced cells, the content of P53 dramatically increased while that of c-Myc protein decreased. Therefore, due to its ability to induce cellular and molecular changes, genistein suppressed the growth and induced differentiated phenotypes in B16-BL6 melanoma cells.

Keywords: Genistein; Anti-cancer effects; Differentiation; Melanoma cells

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INTRODUCTION

Asian diets, such as those consumed in China and Japan, contain large amounts of soybean products. Increasing evidence from both the *in vitro*, animal and epidemiological research suggests that Asian diets are associated with overall low cancer mortality rates, particularly for breast, colon and prostate cancers [1]. Soybeans are therefore recommended as the source for screening compounds which are candidates for prevention of cancers from development and outgrowth [2].

Soybeans are a rich and relatively unique source of isoflavones. It is estimated that 1 g of defatted soybeans contains approximately 2 mg of isoflavones. Genistein (4′,5,7-trihydroxy-isoflavone, Fig. 1), as its β-glucoside genistin, accounts for two-thirds or more of the total soybean isoflavone content, while glycoside conjugates of daidzein and small amounts of glycitin account for the remainder [1]. Genistein drew the initial interest because of its structural similarity to estrogen, suggesting its possible role as estrogen antagonist. But in addition to its inhibitory effect on growth of estrogen-receptor positive MCF-7 cells, genistein also inhibited the growth of estrogen-independent breast cancer cells [3]. In recent years, genistein has drawn wide attention since it was discovered to be a potent inhibitor of protein tyrosine kinases (PTK), such as receptors for epidermal growth factor and platelet-derived growth factor [4]. Because PTK-dependent protein tyrosine phosphorylation plays a crucial role in cell proliferation and transformation, genistein may have important anti-cancer properties. Till now, a great variety of anti-cancer effects of genistein has been defined. For example, besides the inhibitory effects on cancer cell proliferation, genistein can induce apoptosis in leukemia cells [5], suppress growth of vascular endothelial cells and angiogenesis [6], and prevent experimentally-induced cellular transformation or animal cancers [7]. Recently we also found that genistein efficiently suppressed invasion and metastasis of malignant cells [8,9]. Thus, extensive investigation on the anti-cancer properties and molecular mechanisms of

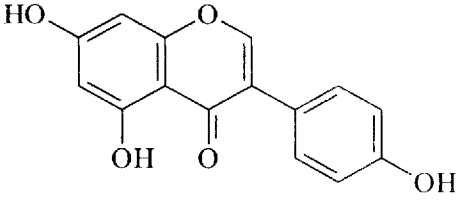

FIGURE 1 Chemical structure of genistein.

genistein should be done, in order to evaluate the possibility of developing genistein as a plant-derived, low toxic and effective anti-cancer drugs.

Here we report the effects of genistein on differentiation status of solid epithelial cancer cells. Derived from B16 mouse melanoma, B16-BL6 cells are highly malignant that they can colonize in the lungs of animal when they are transplanted subcutaneously into C57 BL/6 mouse [10]. After exposure to genistein, we found the induction of differentiated phenotypes in these cells. Mechanism studies revealed changes in cytoskeleton-associated structure and protein tyrosine phosphorylation, and alteration of expressions of tumor-related genes. As a PTK inhibitor, genistein may serve as candidate compound for chemotherapy of solid tumors.

RESULTS

Effects of Genistein on Growth and Morphology of B16-BL6 Cells

Figure 2 depicts that the growth of B16-BL6 cells was suppressed by genistein. The growth rate of the cells was decreased even when exposed to genistein at a lower concentration of 10 μM. At 30 μM, genistein suppressed significantly the growth of B16-BL6 cells in the first 4 days. But when the exposure time prolonged, genistein may be toxic to the cells, as shown by the dramatic decrease in cell number on day 6 (Fig. 2). With MTT assay, the

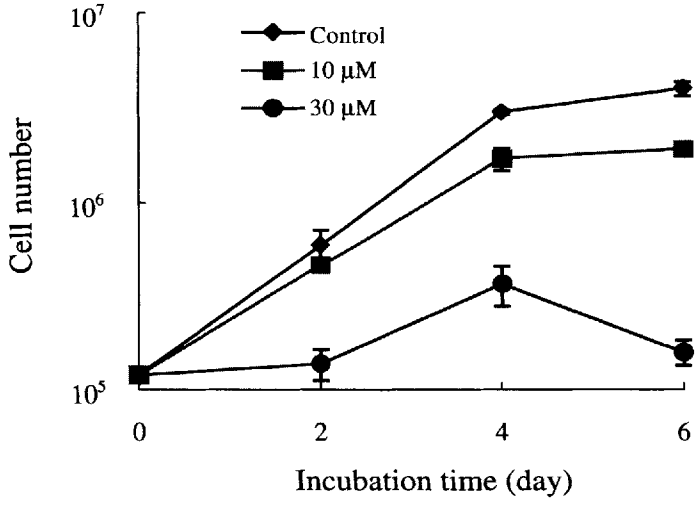

FIGURE 2 Effects of genistein on growth of B16-BL6 melanoma cells.

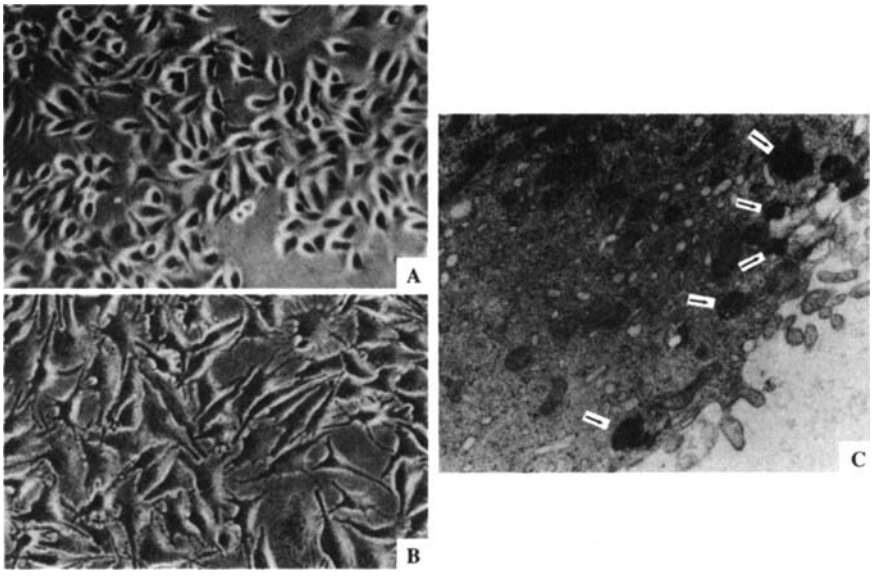

FIGURE 3 Effects of genistein on morphology of B16-BL6 cells. (A) Control cells; (B) The cells exposed in 30 μM genistein for 3 days (×200). The dark particles in the cytoplasms of drug-treated cells were shown as melanosomes (showed by arrows) under electron microscope (C) (×20,000).

IC_{50} value was determined as 15.5 μM or 4.18μg/ml. The cellular morphology changed dramatically when the cell was exposed to genistein. Figure 3 shows the changes of cell shape after 30 μM of genistein treatment for 3 days. The cells were induced to change from a small, multi-angle shape (Fig. 3(A)) to a larger, dendritic one (Fig. 3(B)). In some of the genistein-treated cells, dark yellow particles were observed to be distributed in the cytoplasm, which were proved to be melanosomes under electron microscope (Fig. 3(C)). But the yellow particles or melanosomes were seldom observed in the DMSO-treated control cells. These changes of cellular morphology were in a dose-dependent and time-dependent manner. An enlarged and dendritic cell shape was induced either by 30 μM genistein for 1 day or by 10 μM genistein for 3 days (data not shown).

Effects of Genistein on Cytoskeletal Network

Cytoskeletal network is associated with morphology, adhesion, migration, differentiation and other cellular functions. Figure 4(A) shows that the cytoskeletal filaments in the control cells were distributed around the

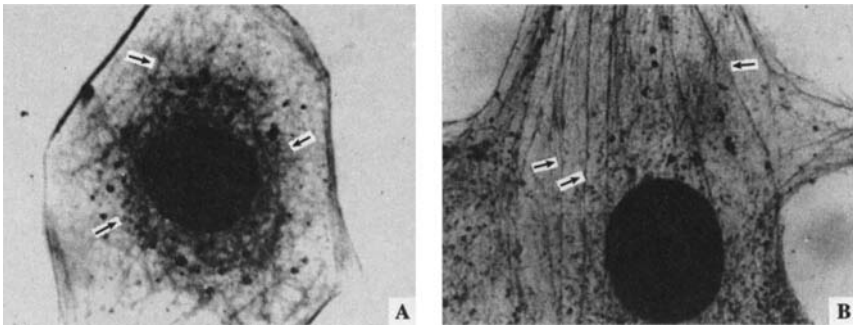

FIGURE 4 Effects of genistein on ctyoskeletal structure (×200). (A) Control cell; (B) The cell treated with 30 μM genistein for 3 days. Arrows show the cytoskeletal filaments.

nucleus in cluster, due to its small and poorly-spread shape. But after the cells were exposed to 30 μM of genistein for 3 days, the cytoskeletal filaments were induced to form a bundle along the direction of elongation of the cell (Fig. 4(B)). The proteins associated with cytoskeletal network were extracted from the cell lysate for electrophoresis resolving analysis. There is no difference in the constituent of cytoskeleton-associated proteins between the control and genistein-treated cells (Fig. 5(B)). However, difference was found in the profiles of tyrosine-phosphorylated proteins. As compared with the control cells, the cytoskeleton-associated protein tyrosine phosphorylation level decreased a little in the 20 μM genistein-treated cells. It was noteworthy that a marked decrease was observed consistently in the phosphotyrosine contents of three proteins with approximate molecular weights of 65, 60 and 53 kD (Fig. 5(A)). After the cells were exposed to 30 μM genistein for 3 days, the contents of phosphotyrosine were found to decrease in not only the above mentioned proteins but also almost in all the tyrosine-phosphorylated cytoskeleton-associated proteins (Fig. 5(B)). These changes in tyrosine phosphorylation pattern of cytoskeleton-associated proteins may contribute to the changes of cytoskeletal filaments and cell shape induced by genistein.

Effects of Genistein on Melanogenesis

The cellular melanin content and tyrosinase activity are two of the biochemical markers for differentiation of malignant melanoma cells. Genistein induced the increase of the cellular melanin content in a dose-dependent and time-dependent manner (Fig. 6). After 3 days' exposure to 10 μM genistein, the cellular melanin content increased about 2.4-fold, while 20 and 30 μM

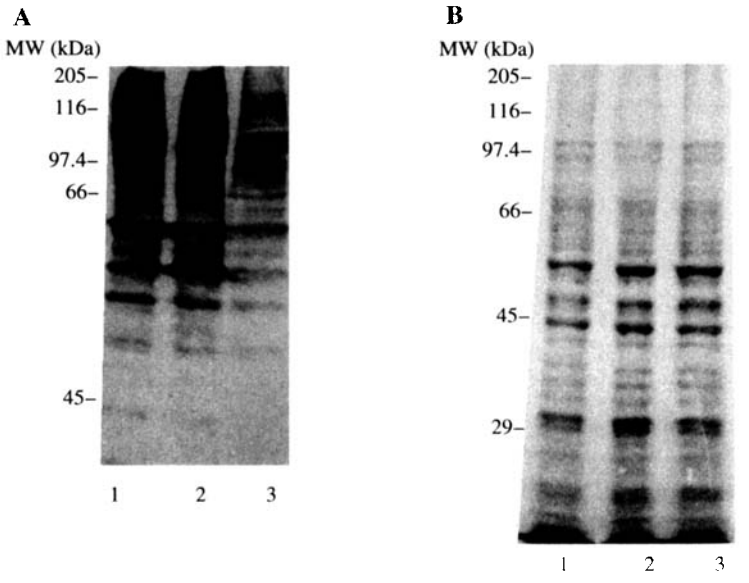

FIGURE 5 Effects of genistein on cytoskeleton-associated protein tyrosine phosphorylation in B16-BL6 cells. Genistein has no effects on the constituent (B) but decreases the phosphotyrosine content (A) of cytoskeleton-associated proteins. Lane 1, extract from control cells. Lane 2, extract from 20 μM-genistein-treated cells. Lane 3, extract from 30 μM-genistein-treated cells. Please see Experimental Section for preparation of cytoskeletal-associated proteins.

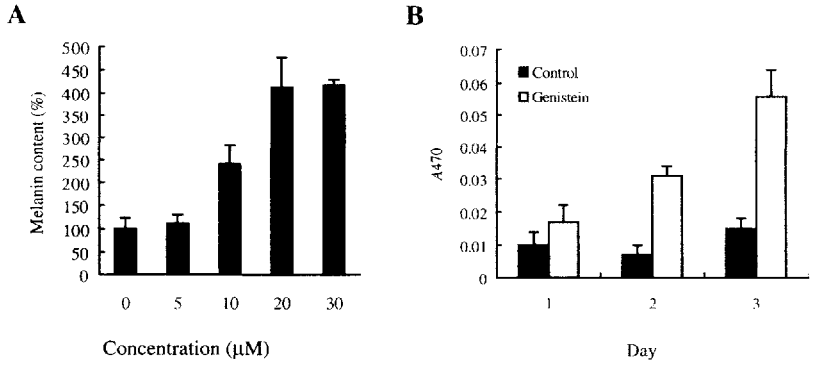

FIGURE 6 Melanogenesis in B16-BL6 mouse melanoma cells induced by genistein in dose-dependent (A) and time-dependent (B) manner. Followed by being treated with genistein, the cells were lysed in NaOH–DMSO. Melanin contents in cell lysates were evaluated by absorbance values at 470 nm wavelength.

TABLE I Effects of genisten on tyrosinase activity in B16-BL6 melanoma cells

Group	*Rate of dopa oxidation* (ΔA_{550}/min/mg *protein*)
Control	2.47 ± 0.65
Genistein	
10 μM	4.58 ± 1.77
20 μM	4.71 ± 0.30*
30 μM	5.00 ± 1.01*

*difference ($p < 0.05$) as compared with control ($n = 3$, Student's *t*-test).

genistein induced about 4-fold increase of the melanin content (Fig. 6(A)). The melanogenesis was induced by genistein after only 1 day's treatment, while the induction of melanogenesis became significant after 2 and 3 days' treatment (Fig. 6(B)). The melanogenesis induced by genistein was consistent with the increase of tyrosinase activity in the drug-treated cells. When the tyrosinase-catalyzed L-dopa oxidation activity in the cell lysate was determined, it was found that the tyrosinase activities were induced to enhance 1 fold in the genistein-treated cells (Table I).

Effects of Genistein on Clonogenecity and Tumorigenicity

Table II indicates that colonization potentials in soft agar decreased dramatically after the cells were exposed to genistein for 3 days. The control B16-BL6 cells formed large amount of colonies in the soft agar. By contrast, only a few of colonies were formed by the genistein-treated cells, suggesting the decrease of anchorage-independent potentials of the drug-treated cells. Similarly, the tumorigenic potentials of the drug-treated cells decreased (Table II). Of the 5 experimental animals injected with the 20 μM- or 30 μM-genistein-treated cells, only 3 were found with overt tumors. The average weight of tumors formed by the 30 μM-genistein-treated cells was approximately 10% of the weight of tumors formed by the control cells. These results suggest the lack of malignancy and aggression of the genistein-treated cells.

Effects of Genistein on Expressions of Tumor-related Genes

To investigate the possible actions on the molecular events in B16-BL6 cells, we adopted Western blot analysis to observe the effects of genistein on expressions of P53, a tumor suppressor, and c-Myc protein, an oncoprotein

TABLE II Effects of genistein on clonogenicity and tumorigenicity of B16-BL6 cells

Group	*Rate of colony formation* (%)	*Average weight of tumors* (g)
Control	48.8 ± 2.4	2.6 + 1.1
Genistein		
10 μM	7.2 ± 2.4*	2.8 ± 0.4
20 μM	2.8 ± 0.4*	0.61 ± 0.68†
30 μM	3.0 ± 0.4*	0.25 ± 0.15†

*significant difference ($p < 0.01$) as compared with control ($n = 3$, Student's t-test)
†difference ($p < 0.05$) as compared with control ($n = 5$, Mann–Whitney U-test).

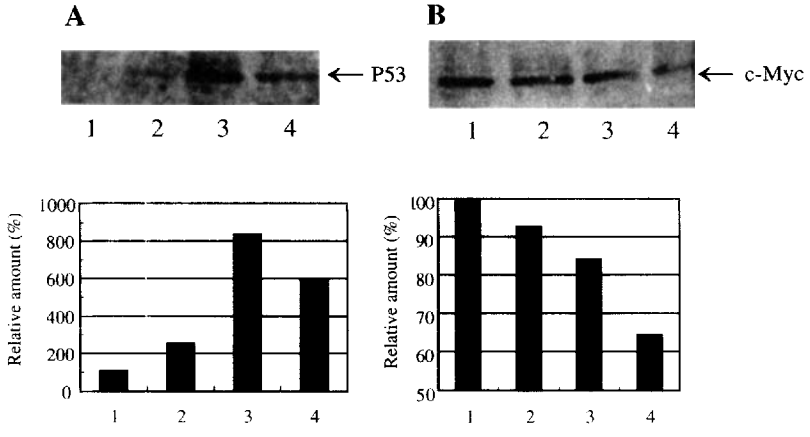

FIGURE 7 Effects of genistein on expressions of tumor-related genes in B16-BL6 cells. The cells were treated with 0 (lane 1), 10 (lane 2), 20 (lane 3) and 30 μM (lane 4) genistein for 3 days. Cell lysates were collected and resolved in SDS-polyacrylamide gel (10%) electrophoresis. Followed by Western blotting onto nitrocellulose membranes, p53 (A) and c-Myc (B) proteins were revealed with monoclonal antibodies and horseradish-conjugated secondary antibody.

associated with poorly-differentiation status of cancer cells. The content of P53 protein in the control cells was much lower, nearly at the undetectable level. But after B16-BL6 cells were exposed to genistein for 3 days, the P53 content was increased (Fig. 7(A)). As compared with the control cells, the P53 content in the cells treated with 10 μM genistein increased one fold, while the contents in the 20 μM- or 30 μM-genistein-treated cells increased 8-fold and 6-fold, respectively. On the other hand, genistein induced the decrease of the cellular c-Myc proteins (Fig. 7(B)), though the extent of decrease was not much larger.

DISCUSSION

It is believed that genistein enriched in soybeans may contribute to the lower cancer incidence in Asian population who consume diet containing higher soy products [1]. Due to its anti-oxidant properties, genistein specifically prevent experimentally induced cellular damage by free radicals or tumor promotors [11,12], and prevent the development of experimental rat mammary cancers [7]. However, we determined to investigate the effects of genistein on cancer cells in order to develop it as a chemotherapeutic agent because of the fact that genistein is food-derived, and thus apparent lack of toxicity and danger to human health. In fact, the plasma levels of genistein can reach the low micromolar per liter range in the higher-soy intake individuals [1]. In the present study, we found genistein suppressed the growth of malignant and highly aggressive mouse melanoma cells with a lower IC_{50} value (15.5 μM). In our other related studies, genistein suppressed the growth of a great variety of cancer cells, particularly the cells derived from solid tumor or resistant to vinblastine and taxol. The IC_{50} values ranged from 13.9 to 39.7 μM (unpublished data). As we can see from the growth curve of the genistein-treated melanoma cells, genistein is cytostatic instead of cytotoxic to cancer cell. But at higher concentrations and with prolonged exposure time, genistein may also be toxic to the cells. These results are consistent with other reports [13].

B16-BL6 cell strain was selected from B16 parent cells by its highly spontaneously metastatic potentials in the syngenic animal [10]. In this paper, we present evidence that genistein efficiently induces the mature phenotypes at relatively lower concentrations in these highly malignant cells. Differentiation in these cells was characterized by the increase of cellular melanin content, enhancement of tyrosinase activity and the morphological changes to dendritic cell shape. It is noted that very few agents were found to efficiently induce the differentiated phenotypes in solid tumor cells like the B16-BL6 cells. At a concentraion lower than the IC_{50} value (10 μM) and with 3 days' exposure time, genistein induced the morphological changes and melanogenesis in the cells. Treated under this condition, the B16-BL6 cells show lower colonization potential in soft agar, suggesting the decrease of malignancy of the cells. The latter was also verified by tumorigenesis experiment in the syngenic C57BL/6 mice. As compared with the heavy tumor burden in the control animals, no overt larger tumor was found in the animals inoculated with the 20 μM- or 30 μM-genistein-treated cells. Larger tumors unfortunately grew in the animals inoculated with the 10 μM-genistein-treated cells, possibly due to the immature and not-well-differentiated status of the

cells. These results suggest that genistein may lower down the malignancy of cancer cells, leading to prevention of solid tumors from outgrowth and thus refraining from the disease induced by uncontrolled growth of tumor. Concluded from this study and our previous discovery of anti-invasive and anti-metastatic effects, we believe that genistein is valuable for more investigation to develop as a new chemotherapeutic drug.

Though extensive anti-cancer properties were defined for genistein, the molecular mechanisms by which genistein exerts its effects remain poorly understood. In addition to its ability to inhibit PTKs, genistein inhibits DNA topoisomerase II [14] and ribosomal S6 kinase [15], which may lead to protein-linked DNA strand breaks [16] and arrest of cell cycle progression [17] of several malignant cell lines. The fact that genistein suppressed the growth of estrogen receptor positive MCF-7 and negative MDA-468 breast cancer cells at almost equal efficiency [3] strongly suggests that genistein does not exert its anti-cancer effects through classical anti-estrogen mechanisms. Several reports hypothesized that at least the differentiation–induction effects of genistein may be due to inhibition of DNA topoisomerase II activity, leading to DNA strand breakage and subsequent expressions of differentiation-associated genes [16]. This hypothesis was supported by observations that DNA damage appeared in the early period of differentiation and no detectable change was found in tyrosine phosphorylation levels of cellular proteins in the genistein-treated leukemia cells [16]. But the IC_{50} value of genistein to inhibit topoisomerase II activity *in vitro* is around 111 μM [18]. Moreover, the effects of genistein on cell proliferation, cell cycle progression and differentiation are not completely the same as that of defined topoisomerase II inhibitors such as doxorubicin. These facts suggest that at least other mechanisms may exist underlying the anti-cancer effects of genistein, for example, suppression of activities of PTKs and functions of downstream signaling elements. We noted that changes of cellular morphology were commonly observed in epithelial cancer cells after exposure to genistein. As shown in our present study, the changes in cellular morphology may be caused by changes of cytoskeletal networks through decreasing the tyrosine phosphorylation levels of cytoskeleton-associated proteins by genistein. Cytoskeleton-associated proteins include a large variety of Triton X-100 insoluble proteins with many more essential cellular functions such as regulation of migration, adhesion and anchorage-dependent growth, and differentiation. The decrease of the tyrosine phosphorylation levels of these proteins may specifically affect these functions. Recently, evidence has been accumulated that cytoskeleton-associated protein tyrosine phosphorylation plays a crucial role in the differentiation control of cancer cells [19,20]. The

decrease of cytoskeleton-associated protein phosphorylation levels may be one of the reasons for differentiation induction by chemotherapeutic agents [21]. As a PTK inhibitor, genistein suppressed cytoskeleton-associated protein tyrosine phosphorylation as shown in this paper. Though the exact PTK target was not defined, genistein may therefore affect the signal tranduction pathways involving in the functions of cytoskeletal networks, and thus alter expressions of differentiation- or proliferation-related genes. This assumption was proved by the observation that genistein induced changes of cellular contents of P53, a tumor suppressor and transcription factor, or c-Myc, another transcription factor involving in the growth and differentiation control. Here we would not discuss the significance of changes of expression patterns of p53 and c-myc genes in cell proliferation and differentiation. But we believed that the effects of genistein on cell proliferation and differentiation can be ascribed to suppression of cytoskeleton-associated protein tyrosine phosphorylation due to its ability to inhibit PTKs.

By the way, it is noteworthy that we did not find genistein to induce changes of protein tyrosine phosphorylation levels in total cellular protein preparation [22]. This observation is consistent with other reports, in which the hypothesis that genistein induces differentiation via inhibition of PTKs was denied [16]. In fact, almost all of the tyrosine-phosphorylated proteins are associated with cytoskeletal networks, and are insoluble in Triton X-100 solution. But with convenient protocols for cell lysis and extraction of total cellular proteins, the cancer cells are incubated in the balanced-salt buffer containing Triton X-100, which leads to incomplete solution of cytoskeleton-associated proteins in the obtained total cellular protein preparation. Thus, investigation using this protein preparation may conceal the true effects of anti-cancer drugs on protein tyrosine phosphorylation.

In conclusion, we have presented evidence here that genistein induce suppression of cell growth and differentiated phenotypes in B16-BL6 mouse melanoma cells. The molecular mechanisms may involve in suppression of cytoskeleton-associated protein tyrosine phosphorylation, which leads to changes in cytoskeletal network, cell morphology and expressions of tumor-related genes.

EXPERIMENTAL SECTION

Chemicals and Reagents

Genistein, white powder (FW = 270.24), was purchased from Sigma Chemical Co., as an agent for treatment of cancer cells, it is dissolved in DMSO to

make a stock solution of 100 μM. The reagents for Western blot analysis were purchased from Promega Corporation. Antibodies to P53 and c-Myc were from Santa Cruz Company. Antibody to phosphotyrosine, Triton X-100, sodium orthovanadate, sodium deoxycholate, bicinchoninic acid solution and other chemicals were all purchased from Sigma Chemical Co.

Cell Culture and Drug Treatment

B16-BL6 mouse melanoma cells, a gift from Dr. I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX, USA), were routinely cultivated in RPMI1640 medium supplemented with 10% calf serum. After being trypsinized and counted, the cells were seeded and incubated in CO_2 incubator. On the following day, genistein was added into the culture medium to the final concentrations as indicated. Solvent DMSO was also added into the medium for control. Growth curve of control and drug-treated cells were plotted according to the cell number counted on day 0, 2, 4, and 6 after genistein exposure, respectively. For determination of 50% inhibition concentration (IC_{50}), 10^3 cells were incubated in 200 μl of medium containing genistein or DMSO in 96-well plate for 5 days, followed by convenient MTT assay [23].

Microscopic Observations

The cells exposed to genistein were observed and photographed under an inverse phase microscope. After being trypsinized and detached from flask, the cells were rinsed with PBS and fixed in 2.5% glutaraldehyde solution. Samples for electron microscopy were prepared with convenient methods. To observe the structural changes in cytoskeleton, the cells were plated on the autoclaved coverslips, and exposed to genistein as above. After being rinsed with PBS, the coverslips were treated with 1% Triton X-100/PBS for 10 min, and then fixed in 2.5% glutaraldehyde/PBS for 30 min. The cells on the coverslips were then stained in 0.2% Coomassie blue R-250 solution. After destaining in methanol–acetic acid–H_2O (46.5 : 7 : 46.5) solution, the cytoskeletal filaments were observed under an Olympus microscope [24].

Cellular Melanin Content Assay

The treated cells were lysed in 1 ml lysis buffer containing 1 M NaOH and 10% DMSO. The absorbance values at 470 nm wavelength were collected to estimate the cellular melanin content [25].

Tyrosinase Activity Assay

Tyrosinase activity in the cell lysate was assayed as described in Ref. [26]. Briefly, the trypsinized cells were lysed by freezing and sonication in 50 mM phosphate buffer (pH 6.9), followed by centrifugation at 12,000×*g* (4°C) for 10 min. Then 60 μl of supernatant was added into pre-warmed cuvette (37°C) containing 200 μl of 5 mM L-dopa, 290 μl of 20.7 mM 3′-methyl-2-benzothiazoline hydrazone and 450 μl of 100 mM phosphate buffer (pH 7.1, containing 4% N,N′-dimethylformamide). The changes in the absorbance values at 550 nm wavelength were recorded for 5 min. After being normalized to the protein content of lysates, the tyrosinase activity was described as changes of the absorbance values at 550 nm in one minute induced by one miligram protein (ΔA_{550}/min/mg protein).

Colony Formation Assay in Soft Agar

After being dissociated from flasks, the treated cells were suspended in RPMI1640 medium supplemented with 0.3% agar and 20% calf serum at a density of 10^3/ml. The cell suspension was rapidly layered on feeder medium containing 0.5% agar in a Φ33 mm dish. The cells were then incubated at CO_2 incubator (37°C) for 10–14 days till colonies appeared.

Tumorigenicity Assay

The treated cells were trypsinized, rinsed, and suspended in PBS at a density of 10^6/ml. The cell suspension 0.2 ml was injected subcutaneously into C57 BL/6 mouse. The growing tumors were weighed 21 days later.

Cytoskeletal Protein Preparation and Tyrosine Phosphorylation Analysis

All the following processes were done at 0°C. The treated cells were lysed in buffer B containing 10 mM Tris-HCl (pH 6.9), 75 mM KCl, 0.1 mM $MgCl_2$, 5 mM EGTA, 0.1 mM Na_3VO_4, 50 mM NaF, 1% Triton X-100 and proteinase inhibitor mixture (10 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM PMSF). The Triton X-100-insoluble cytosol fractions were harvested by centrifugation at 38,000×*g* for 75 min. The pellets enriched in cytoskeleton-associated proteins were resuspended in buffer B minus Triton X-100 and sonicated [20]. Protein concentration of each sample was determined with the BCA assay [27]. Tyrosine phosphorylation of cytoskeleton-associated proteins was revealed with Western blot analysis described as follows,

except using mouse anti-phosphotyrosine antibody PT66 and ProtoBlot Western Blot AP system for detection of tyrosine-phosphorylated proteins.

Western Blot Analysis

The cells were lysed in 400 µl buffer containing 50 mM Hepes (pH 7.0), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 10 mg/ml *p*-nitrophenylphosphate and protein inhibitor mixture on ice for 20 min [28]. After centrifugation at 12,000×*g* for 10 min, the supernatant was collected and determined for protein concentration with BCA assay [27]. The samples containing 50 µg of protein were then resolved with SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane. After being blocked in TBS solution (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 3% BSA, the blots were incubated in antibodies to P53, c-Myc or phosphotyrosine. The P53 and c-Myc proteins were revealed with a horseradish-labelled secondary antibody and the DAB stain. The bands of target proteins on the blot were scanned, and quantified with a laser densitometer.

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