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Preferential Cytotoxic Effect of Genistein on G361 Melanoma Cells Via Inhibition of the Expression of Focal Adhesion Kinase

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Resistance to the induction of apoptosis is a possible mechanism by which tumor cells can survive anti-neoplastic treatments. Melanoma is notoriously resistant to anti-neoplastic therapy. Previous studies have demonstrated focal adhesion kinase (FAK) overexpression in melanoma cell lines. Given its probable role in mediating resistance to apoptosis, many researchers have sought to determine whether the downregulation of FAK in melanoma cells would confer a greater sensitivity to anti-neoplastic agents. Genistein is a known inhibitor of protein-tyrosine kinase (PTK), which may attenuate the growth of cancer cells by inhibiting the PTK-mediated signaling pathway. This present study was undertaken to investigate the effect of genistein on the expression of FAK and cell cycle related proteins in the G361 melanoma cell line. Genistein was found to have a preferential cytotoxic effect on G361 melanoma cells over HaCaT normal keratinocytes. Genistein decreased the expression of 125 kDa phosphotyrosine kinase and the FAK protein in particular. Genistein treatment did not affect the expression of p53 in G361 cells in which p21 is upregulated. The expression of cyclin B and cdc2 was downregulated by genistein treatment. Taken together, our data indicate that genistein induces the decreased proliferation of G361 melanoma cells via the inhibition of FAK expression and regulation of cell cycle genes. This suggests that the use of genistein may be a viable approach to future melanoma treatments.

Key words: Focal adhesion kinase, Genistein, Melanoma cell

Introduction

Melanoma cells require adhesion through integrin receptors for their survival and growth. Attachment to the extracellular matrix suppresses apoptosis in these cells [1]. Clusters of integrins, occurring in focal adhesion contact sites, interact with the matrix during cellular attachment. Focal adhesion is not only important for attachment, but also essential to subsequent cell spreading and motility [2]. Integrin-matrix interactions regulate cell growth and apoptosis by initiating signal transduction pathways [3]. FAK is a major signaling mediator, the activation of which requires both integrin attachment and cell spreading [4].

It has been shown that Focal adhesion kinase (FAK) is overexpressed in breast, colon, and thyroid cancers [5-7], whereas normal tissues express little detectable FAK. The overexpression of FAK in tumors is likely to affect three functions as follows: motility, adhesion, and survival. FAK is thought to play a role in adhesion-mediated survival because overexpression of a constitutively activated form of FAK in Madin-Darby canine kidney cells has been shown to confer resistance to apoptosis following loss of adherence [8]. FAK overexpression in Chinese hamster ovary (CHO) cells cau-

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sed an increase in migration [9], suggesting that FAK may play a role in motility of CHO cells. Although these experiments were performed in normal cells, they raise the possibility that tumor cells upregulate FAK expression in order to leave their tissue of origin, invade their surrounding stroma, and migrate into new environments. However, FAK overexpression has also been demonstrated in preinvasive tumors [10], suggesting a role for FAK in tumors that occurs before development of anchorage-independent growth potential.

Low rates of breast, colon, and prostate cancers have been reported in Asian countries, such as China and Japan, relative to the United States [11]. Epidemiological studies have suggested that a diet rich in isoflavonoids may play an important role in cancer prevention [12]. Genistein is an isoflavone [13], believed to be a metabolite of soy produced by the gut floral bacteria [14]. Genistein has a heterocyclic, diphenolic structure similar to estrogen [15] and was shown to be a potent inhibitor of epidermal growth factor receptor tyrosine kinase [16]. The protein products of approximately one-half of the known oncogenes have been shown to be membrane-bound receptors with tyrosine kinase activity or intracellular proteins undergoing or catalyzing tyrosine phosphorylation [17]. The anticancer effects of soy products could be attributed to genistein [18] perhaps by modulating tyrosine kinase activity. Genistein has also been shown to be an inhibitor of angiogenesis in vitro [19], which plays an important role in tumor growth and metastasis. It also inhibits DNA topoisomerases I and II in vitro [20-21] and ribosomal S6 kinase [22] and induces differentiation of cancer cell lines [23]. Genistein was shown to induce cell cycle progression arrest at the G_2/M phase and induced apoptosis in breast, gastric and prostate cancer cell lines [24-26]. Therefore, this study was undertaken to investigate the effect of genistein on the expression of FAK and cell cycle related proteins in G361 melanoma cell line, comparing with normal HaCaT keratinocytes.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated companies: genistein, dimethyl sulfoxide (DMSO), leupeptin, aprotinin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT), Ponceau S were purchased from Sigma (St Louis, MO). ECL western blotting detection reagents and Hybond nitrocellulose membrane were from Amersham international (Buckinghamshire, UK).

Antibodies

Monoclonal antibodies (MoAb) mouse anti-human FAK (05-1139) and PY20 (05-947) were purchased from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal anti-human p53 (SC-99), p21 (SC-53393), cdc2 (SC-54) and cyclin B1(SC-7393), and 14-3-3γ (SC-25276) were from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase labelled anti-mouse IgG were purchased from Amersham Biosciences (UK).

Cell culture

The G361 human melanoma cell line was purchased from ATCC (Rockville, USA). Cells were maintained at 37℃ with 5% CO_2 in air atmosphere in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 μg/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and supplemented with 10% FBS. Cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS.

Cell viability assay

The viability of cultured cells was estimated by MTT assay. In the MTT assay, cells were placed in a 96-well plate and incubated for 24 h. Then cells were treated with various concentrations of genistein for 24 h. And then, the cells were treated with 1 mg/ml of MTT in growth medium. Cells were incubated at 37℃, 5% CO_2 for 4 h. The medium was aspirated and the formazan crystals, which are formed from MTT by NADH-generating dehydrogenases in metabolically active cell, were dissolved in 200 μl DMSO. Cell viability was evaluated in comparison to the control culture (taken as 100%) by measuring the intensity of the blue color (OD at 570 nm) by a multi-well reader (Quant, Bio-Tek, Highland Park, USA). The assay was performed in triplicate.

FAK antisense oligonucleotide treatment

The FAK antisense oligonucleotide was 5'-TTT-CAA-CCA-GAT-GGT-CAT-TC-3'. Oligonucleotides were added at 300 nM, with 3 μl of lipofectin (Invitrogen, Life Technologies, Inc., Carlsbad, California, USA) per ml of OptiMEM I medium (Life Technologies, Inc.) per 100 nM oligonucleotide, and incubated for 4 h. Subsequently, the cells were washed and complete medium was added for 20 h. The control sample was incubated for 4 h in OptiMEM I and lipofectin, but

no oligonucleotides.

Western blot analysis

For protein analysis, cells were lysed with RIPA buffer (10 mM Tris/HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride) on ice for 1 h. Lysate were clarified by centrifugation at 12,000 revolution per min for 20 min at 4℃, and then the supernatant was obtained. The protein contents of the lysate were determined using the Bio-Rad Protein Assay (Bio-Rad laboratoris Hercules, CA). The 20 μg protein was mixed with equal volume of electrophoresis buffer (10 mM Tris/HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). After heating, the protein was resolved on polyacrylamide SDS gels and transferred to nitrocellulose membrane. After transfer, the membranes were blocked with blocking reagent (5% non-fat milk in distilled water) for 1 h and then the membranes were incubated with primary antibody. The membranes were incubated for 1 h with the corresponding secondary antibody, diluted in the above blocking reagent. After three final washes, the membranes were treated with chemiluminescence reagent. All the procedures were done at room temperature.

Statistical Analysis

Three independent experiments were performed triplicates. The results of treated and control groups were compared for statistical significance ($p<0.05$) using paired T-test statistical method by SPSS (SPSS version 18 for windows, SPSS Inc, USA).

Results

The cytotoxic effect of genistein on G361 cell viability

Genistein preferentially had a significant time-dependent inhibitory effect on the viability of G361 cells compared with HaCaT. (Fig. 1). The cytotoxic effect of 50 μM genistein significantly decreased the proliferation of G361 cells, the viability of HaCaT cells was slightly affected by genistein treatment in the course of time.

Effect of genistein on 125 kDa tyrosine phosphorylation of G361 and HaCaT cells

In order to demonstrate that G361 cells are much more sensitive to genistein than normal HaCaT cells, we measured the expression of 125 kDa phosphotyrosine kinase in both cells after genistein treatment (Fig. 2). Because genistein is known to be a inhibitor of protein tyrosine kinase. In the case of G361 cells, a small quantity of phosphotyrosine kinase was expressed and it was almost disappeared at 16 h and 24 h by genistein. Relatively to G361 cells, HaCaT cells expressed more quantity of 125 kDa phosphotyrosine kinases, which were slightly decreased by genistein treatment.

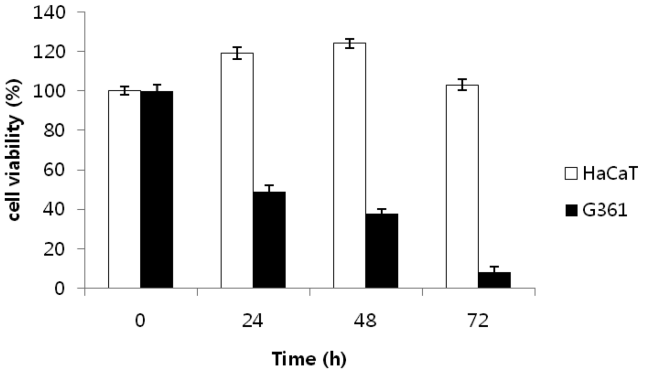

Fig. 1. Preferential cytotoxic effects of genistein treatment on the viability of G361 melanoma cells. Cells were treated with 50 μM genistein for indicated time periods and cell numbers were measured by MTT assay (G361, 24-72 h $p<0.05$). Four independent assays were performed and data shown are the mean ±SD of the means obtained from triplicates of each assay.

Effect of genistein on FAK expression in G361 and HaCaT cells

FAK is the most representative protein among 125 kDa phosphotyrosine kinase proteins. Thus, we investigated the expression of FAK in two cells treated with genistein (Fig. 3). Genistein treatment resulted in the decreased FAK protein

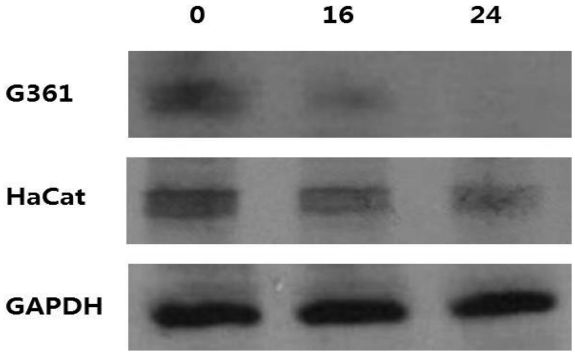

Fig. 2. Inhibition of 125 kDa tyrosine phosphorylation by the genistein. After two cell lines were treated with 50 μM genistein for 16 and 24 h, cells were lysed and separated on SDS-PAGE, transferred to nitrocellulose membrane and reacted with anti-phosphotyrosine antibody.

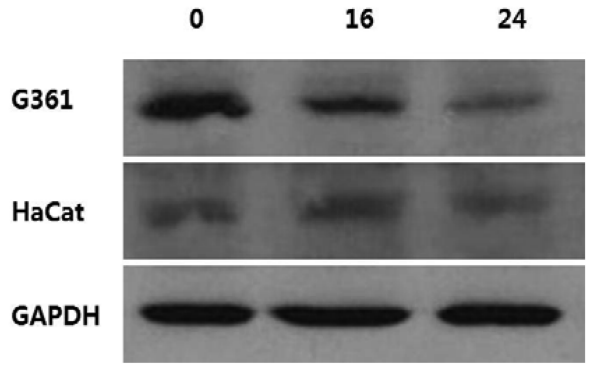

Fig. 3. Inhibition of the expression of FAK protein by the genistein. After two cell lines were treated with 50 μM genistein for 16 and 24 h, cells were lysed and separated on SDS-PAGE, transferred to nitrocellulose membrane and reacted with anti-FAK antibody.

levels found on Western blots performed. The expression level of FAK of G361 cells was higher than in HaCaT cells, dislike the expression pattern of 125 kDa phosphotyrosine kinases.

Effect of FAK antisense oligonucleotide on the viability of melanoma cells

In order to examine that FAK takes the responsibility for different sensibility of these cells to genistein, FAK antisense oligonucleotide was treated to both cells. Treatment of FAK antisense oligonucleotide significantly decreased the viability of G361 cells compared with HaCaT, which were rarely affected by FAK antisense oligonucleotide (Fig. 4).

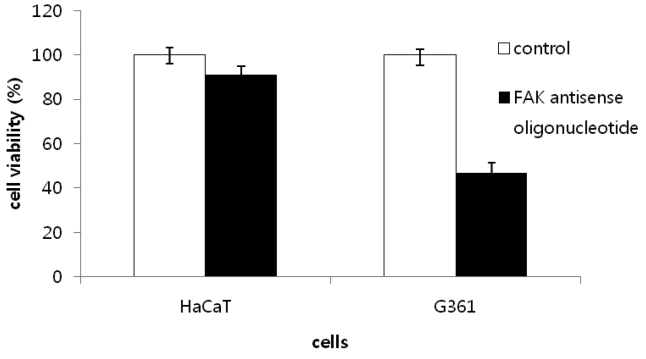

Fig. 4. Effect of FAK antisense oligonucleotide on the viability of G361 and HaCaT cells. After two cell lines incubated with FAK antisense oligonucleotid for 24 h, cell numbers were measured by MTT assay (G361, 24 h; $p<0.05$). Four independent assays were performed and data shown are the mean ±SD of the means obtained from triplicates of each assay.

The regulation of cell cycle related proteins

Next we investigated the modulation of cell cycle regula

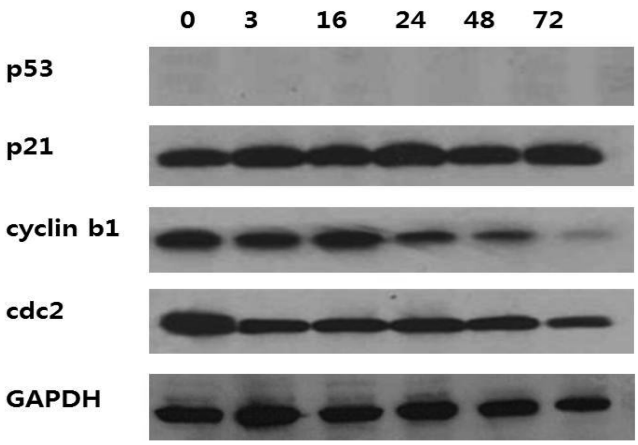

Fig. 5. Western blot analysis of cell cycle regulation proteins in G361 melanoma cells treated with 50 μM genistein. The expression of p21 were increased up to 24 h, whereas the expression level of cdc2 and cyclin B were decreased time-dependently. The expression of p53 was not observed.

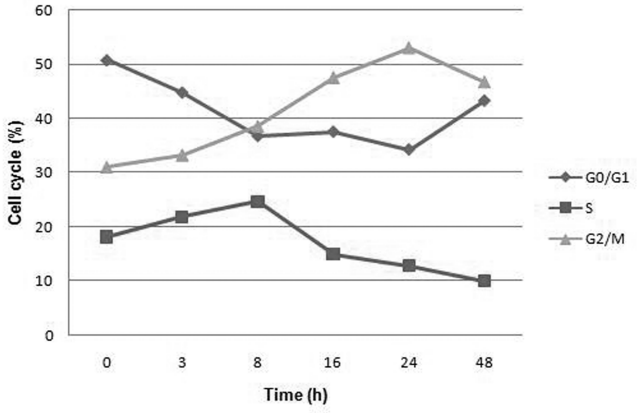

Fig. 6. The kinetic analysis of the effect of 50 μM genistein on G361 cell cycle progression. The increase in the G2/M phase cell percentage was shown.

tory proteins in G361 cells by genistein treatment. The expression of p53 was not observed at indicated time after genistein treatment. While the expression of p21 was increased up to 24 h, the down-regulation of cdc2 and cycline B was detected in time-dependent manner (Fig. 5). When cell cycle arrest at specific phase was examined, genistein induced the cell cycle arrest at G_2/M phase (Fig. 6).

Discussion

Resistance to induction of apoptosis may be a mechanism by which tumor cells survive during anti-neoplastic treatments [27]. Melanoma is notoriously resistant to anti-neop-

lastic therapy, with only one Food and Drug Administration (FDA)-approved chemotherapeutic agent (dacarbazine), and no better than 25% response rates for any given agent [28].

Previous studies have demonstrated FAK overexpression in melanoma cell lines [29-30]. Recently, using immunohistochemical staining, Smith *et al.* (2005) reported that FAK was overexpressed in 89 samples of primary human melanoma, and the level of expression on all of the tumors was greater than the level of expression in melanocytes in the adjacent normal epidermis [31]. Given its probable role in mediating resistance to apoptosis, they sought to determine whether the downregulation of FAK in melanoma cells would confer a greater sensitivity to anti-neoplastic agents. FAK is required for cell survival in adhesion-dependent cells, and autophosphorylation of a major tyrosine site is needed to perform this function [32]. Cell attachment induces FAK autophosphorylation on Tyr-397, and this allows the SH2 domains of the Src family and other kinases to bind. Src then phosphorylates at least five Tyr residues in FAK. Phosphorlyation of some of these residues leads to the activation of the mitogen-activated protein kinase cascade [33]. Therefore, FAK is thought to have various functions, ranging from the regulation of focal adhesion turnover to the prevention of apoptosis [34]. Moreover, inhibition of FAK expression causes apoptosis in several human tumor cell lines including melanoma [35].

Genistein is a known inhibitor of protein-tyrosine kinase (PTK), which may attenuate the growth of cancer cells by inhibiting PTK mediated signaling pathway [36-37]. PTKs are known to play key roles in carcinogenesis, cell growth and apoptosis [38]. It has been reported that genistein is a potent inhibitor of cell proliferation, oncogenesis and clonogenic ability of animal and human cells [39]. Experiments have shown that genistein inhibits growth of cancer cells including leukemia, lymphoma, neuroblastoma, gastric, breast and prostate cancer cells [24-26,40] .

This study demonstrates that genistein has a preferential cytotoxic effect on G361 melanoma cells compared with HaCaT normal keratinocytes. Genistein decreased not only the expression of 125 kDa phosphotyrosine kinase but also FAK in G361 melanoma cells. We also observed the alteration of cell cycle related proteins. It has been reported in the literature that genistein can cause G_2/M arrest in many other tumor cells [41-43]. Cells progressing through the cell cycle are controlled by the activation of a special family of protein kinases called the cyclin-dependent kinases (CDKs) [44]. For example, at the conclusion of the G_2 phase, CDKs phosphorylate and activate a set of proteins that function to promote mitosis and cytokinesis. The G_2/M phase transition is controlled by the cyclin B-cdc2 complex, which is regulated by phosphorylation [45]. The 15-tyrosine and the 161-threonine residues of cdc2 must be phosphorylated first and, subsequently, the 15-tyrosine residue must be dephosphorylated to activate the cyclin B-cdc2 complex activity. It is possible that genistein, a PTK inhibitor, may inhibit the activity of the PTK and, in turn, may deactivate the cyclin B-cdc2. It may, therefore, disturb the whole process of this phosphorylation-dephosphorylation chain reaction of tyrosine residues of cdc2 kinase at the very beginning, leading finally to G_2/M arrest. Although p21, a CDK inhibitor, has been reported to induce G_1 arrest [46], the accumulated evidence has shown that upregulation of p21 expression may also be associated with G_2/M phase arrest in the cell cycle [42-43]; the latter is consistent with our results. In this study, the p21 level in the genistein-treated G361 cells increased significantly. Taken together, genistein inhibits the proliferation of G361 melanoma cells via suppressing FAK expression and regulating cell cycle gene. These results suggest that genistein may be a good strategy for melanoma treatment.

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