MOLECULAR & CELLULAR TOXICOLOGY, Vol. 2, No. 1, 15-20, March 2006

Molecular Effects of Genistein on Proliferation and Apoptosis of MCF-7 Cell Line

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Accepted 12 February 2006

Abstract

Genistein is a potent, plant-derived isoflavone that displays estrogenic activity at low concentrations but inhibits proliferation at high amounts. However, the molecular mechanism of genistein is not completely understood. In the present study, the biphasic effects (estrogenic and antiestrogenic activity) of genistein on the growth of MCF-7 cells were identified. Genistein within a low range of concentration, 1-10 μM, stimulated proliferation, while 50-100 μM caused apoptotic cell death. Additionally, genistein at a low concentration induced estrogen receptor (ER)-mediated gene expression and ER phosphorylation. When pre-treated with PD98059, an MEK inhibitor, ER-mediated gene expression and ER phosphorylation by genistein were noticeably increased. However, the increased gene expression and phosphorylation did not enhance cell proliferation. Moreover, it was observed that ER-mediated signaling performs an important role in the MAPK pathway. The proliferation and apoptosis in genistein-treated MCF-7 cells were partially dependent on the Bcl-2 level. The addition of ICI 182,780, an estrogen receptor antagonist, inhibited Bcl-2 expression induced by genistein. This study suggests that there is a close relationship between Bcl-2 and the ER signaling pathways in MCF-7 cells.

Keywords: Genistein, Bcl-2 MAPK pathway, MCF-7, Estrogenic effect

Genistein (4, 5, 7-trihydroxyisoflavone), a natural flavone found in soy, has been suggested to be responsible for lowering the rate of breast cancer in Asian women[1]. This represents the finding that genistein is associated with cancer prevention. For this reason, genistein has been studied in order to clarify its chemopreventive effect; genistein is a specific inhibitor of protein tyrosine kinases (PTK), topoisomerase II, and angiogenesis[2-5]. Additionally, flow-cytometric analysis showed that genistein almost completely arrested the progression of the cell-cycle at G_2M, and it has a unique effect among the flavonoids[6,7]. Many studies have shown that genistein down-regulates TGF-and inhibits EGF-stimulated growth[8,9].

Genistein, a planar molecule with an aromatic A-ring[10], has a molecular weight similar to those of the steroidal estrogens. It is well known that genistein binds to and acts through ERs, followed by the activation of a number of estrogen-responsive genes, including *pS2* and *c-fos*, *in vitro*[11,12]. Although its binding activity is weaker than that of estradiol, genistein has estrogen-like activity and induces cell proliferation. However, it is not clear whether the mechanism of genistein is similar to that of estradiol. In MCF-7 cells, estradiol activates the tyrosine kinase /p21ras/MAP-kinase pathway with kinetics that are similar to those of peptide mitogens[13]. It has been shown that estrogen activates c-Jun N-terminal kinase (JNK) intracellular signaling molecules and enhances the proliferative capacity of cardiac fibroblasts by acting as an estrogen receptor through the MAPK-dependent pathway[14]. Xenoestrogens, including genistein, can mimic or antagonize the activities of physiological estrogens through the suggested mechanism of binding with ERs. One of the xenoestrogens, zearalenone, also stimulates the growth of the MCF-7 estrogen receptor positive human breast carcinoma cell line. In addition to inducing cell-cycle progression through the MAPK pathway in MCF-7 cells, zearalenone exhibits the properties of an antiapoptotic agent by increasing the survival of MCF-7 cell cultures undergoing apoptosis caused by serum withdrawal[15]. According to these reports, we supposed that genistein is also associated with the MAPK pathway.

It has been previously reported that genistein exhibits a biphasic effect on MCF-7 cells, stimulating proliferation at low concentrations but inhibiting it at high concentrations[10,16]. Thus, we also investigated the effects on MCF-7 cells treated with high con-

centrations of genistein. Accumulating evidence suggests that steroid hormones regulate apoptosis by Bcl-2[17]. The Bcl-2 family of proteins localizes to mitochondria and regulates the permeability of the mitochondrial outer membrane to these apoptotic factors[18]. Inhibition of Bcl-2 expression by anti-sense oligonucleotide and dexametasone has been shown to promote apoptosis and increase sensitivity to chemotherapy-induced apoptosis. Furthermore, overexpression of Bcl-2 prevents apoptosis in response to a variety of stimuli[19-21]. Stable overexpression of Bcl-2 resulted in protection of MCF-7 cells against apoptosis and inhibition of growth by antiestrogen.

In the present study, we have characterized the estrogenic activity of genistein in ER-positive MCF-7 human breast cancer cells to understand the estrogenic effects and molecular mechanism of the actions of genistein. We also examined the correlation between MAPK activation and the genistein-induced signaling pathway. Furthermore, investigations were carried out to determine the involvement of Bcl-2 expression in the genistein-induced biphasic effects.

Genistein shows potent estrogenic activity[11,16], whereas it inhibits cell growth by tyrosine kinase inhibition and cell arrest at cell cycle checkpoints[9]. It was also recently reported that genistein affects cells in a concentration-dependent manner[22].

Effect of Genistein on MCF-7 Cells

We established the concentrations of genistein that increase or decrease the growth of MCF-7 cells. Cell proliferation was measured by an E-screen assay to examine and compare the effects of genistein and 17-estradiol (E2). E2 induced proliferation of MCF-7 BUS cells. A low concentration (1-10 μM) of genistein also induced dose-dependent cell proliferation. However, a high concentration (50-100 μM) of genistein results in apoptosis (Fig. 1). To elucidate the cell death, Poly- (ADP-ribose)-polymerase (PARP) cleavage, which serves as a marker of cells undergoing apoptosis, was measured. It was detected according to the presence of an 85 kDa band that reflects a proteolytic fragment of the 116 kDa PARP proteins (Fig. 2). At low concentrations, genistein did not induce PARP cleavage; 50 μM treatment, however, showed a light band of the 85 kDa proteolytic fragment. When treated with 100 μM of genistein, the band intensity of the 116 kDa and 85 kDa fragment was increased (Fig. 2). From this result, it was confirmed that high concentrations of genistein induce apoptosis on MCF-7 cells; it is believed that this is the cause of its chemopreventive effect. Therefore, genistein has two functions, estrogenic and anti-estrogenic effects, which both operate in a dose-dependent manner.

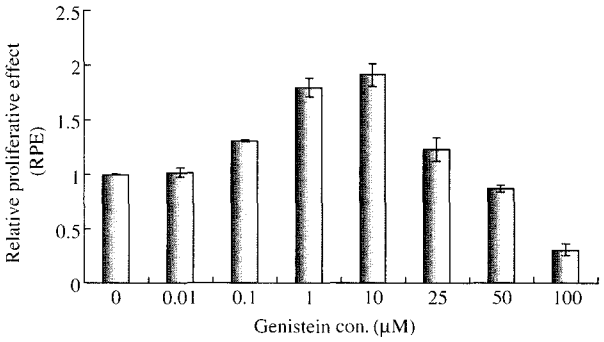

Fig. 1. Effect of the genistein on MCF-7 cells. Cells were cultured in the presence of various concentration of genistein for 4 days. Cell growth was determined by MTT assay. The results (mean ± SD, n=3) are expressed relative to cells grown without genistein. Genistein at 1-10 μM stimulated proliferation in MCF-7 cells, on the other hand, at 50-100 μM caused cell death.

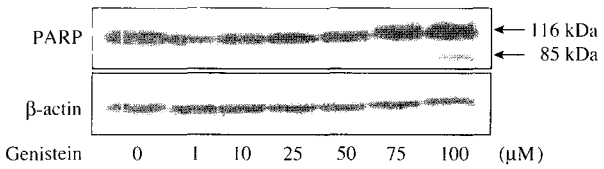

Fig. 2. The induction of apoptosis by genistein. Cleavage of PARP (116 kDa) to 85 kDa fragment is indicative of apoptosis. MCF-7 cells were treated with various concentration of genistein for 48 hr. The proteolytic cleavage of PARP was detected by western immunoblot analysis. Genistein with high concentration at 50-100 μM caused proteolytic cleavage of PARP. Apoptotic cell death was induced in cells treated with genistein at high concentration, but not at low concentration.

Effects of MAPK Phosphorylation in Genistein-Treated MCF-7 Cells

Estradiol and some other chemicals have estrogenic activity and regulate cell proliferation through their activation of the MAPK pathway[13]. MAPK families play an important role in complex cellular programs, such as proliferation, differentiation, development, transformation, and apoptosis. At least three MAPK families have been characterized: extracellular signal-regulated kinase (ERK), Jun kinase (JNK/SAPK), and p38 MAPK. Therefore, in order to know whether cell proliferation and apoptosis by genistein is related to the MAPK signal transduction pathway, we examined the effect of ICI 182,780, an ER-antagonist, on MAPK phosphorylation. In both control and genistein-treated samples, ERK, p38, and JNK MAPK were phospho-

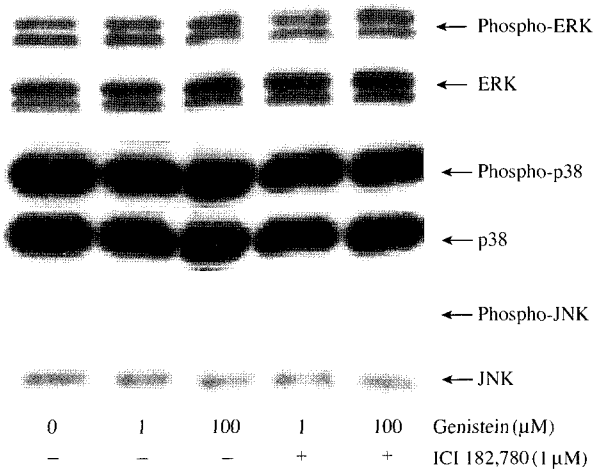

Fig. 3. Effect of ICI 182,780 (ER-antagonist) on MAPK phosphorylation in genistein-treated MCF-7 cells. MCF-7 cells were pre-treated with 1 μM ICI 182,780 for 1 hr prior to genistein stimulation. After 20 min of stimulation, cells were lysed. Total and phosphorylated levels of MAPKs were visualized by western blot analysis. Pretreatment with ICI 182,780 did not affect phosphorylated levels of MAPKs.

rylated, which supports constitutive activation in MCF-7 cells (Fig. 3)[23]. Additionally, we treated ICI 182,780 for inhibition the ER-pathway by genistein. It inhibits estrogen mechanism through ER functional changes, such as nuclear localization hindrance, and ER dimerization. Since gene transcriptional regulation by ERs is inhibited by ICI 182,780, it was determined that MAPK activity was not directly induced by ERs.

Influence of MAPK Inhibitors on ER Expression and Phosphorylation in MCF-7 Cells Treated with a Low Concentration of Genistein

It is well known that 17-estradiol activates the MAPK pathway and is involved in the adaptive process which results in enhanced DNA synthesis and cell proliferation in the absence of exogenous estrogen[13,24]. Since the above results showed constitutive activation of MAPK regardless of the genistein-induced ER pathway, we investigated ER expression and phosphorylation by genistein after the inhibition of MAPK. To observe the ER activation pattern, MCF-7 cells were pretreated with 10 μM PD98059 and SB203580, MAPK inhibitors, followed by 10 μM genistein treatment for 30 min. The cells were then subjected to Western blot analysis. ER expression levels were not altered by the inhibition of ERK phosphorylation. However, phosphorylation of ER was induced by genistein and PD98059 (Fig. 4A).

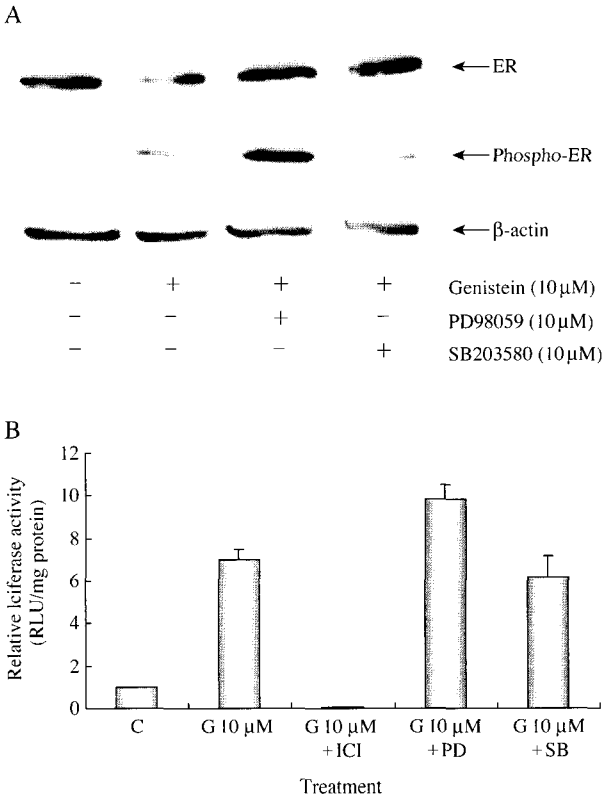

Fig. 4. Influences of MAPK inhibitors on ER phosphorylation and gene expression. A. Cells were preincubated with 10 μM PD98059 and 10 μM SB203580 for 1hr before treatment with 10 μM genistein. After incubation for 30 min, levels of ER expression and phosphorylation were detected by western blot analysis. Inhibition of the ERK phosphorylation caused to increase the levels of ER phosphorylation at a low concentration of genistein. B. Cells were preincubated with 10 μM PD98059, SB203580 and 1 μM ICI 182,780 for 1 hr before treatment with 10 μM genistein. After incubation for 24 hr, ER-mediated gene expression was analyzed by luciferase reporter gene assay. Inhibition of the ERK activation caused to increase considerably for ER-mediated gene expression in a low concentration of genistein-treated MCF-7 cells.

These results demonstrated that the suppression of ERK phosphorylation induced ER activation.

Additionally, we investigated whether the increase of ER activity by PD98059 resulted in ER-mediated gene expression. This was measured by luciferase reporter gene assay with estrogen receptor-positive human MCF-7 breast cancer cells that were stably transfected with a luciferase reporter plasmid, ERE-LUC[25]. High concentrations of genistein did not affect ER-mediated gene expression or MAPK inhibition (data not shown). However, at low concentrations of genistein, the luciferase activity was increased about 7-fold, and with ICI 182,780 treatment, it

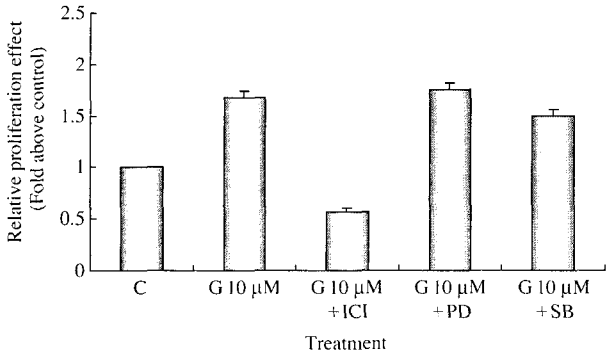

Fig. 5. Influences of MAPK inhibitors on cell proliferation in genistein-treated MCF-7 cells. Cells were preincubated with 10 μM PD98059, SB203580 and 1 μM ICI 182,780 for 1h before treatment with 10 μM or 100 μM genistein. After incubation for 4 days, cell proliferation was measured by MTT assay. Inhibition of the ERK-mediated pathway did not affect in genistein-induced cell proliferation.

was completely inhibited. Interestingly, when ERK phosphorylation was inhibited by PD98059, the luciferase activity was increased about 9-fold (Fig. 4B). From this, we confirmed that, in MCF-7 cells, the inhibition of ERK activity by genistein induced ER phosphorylation and ER-mediated gene expression (Fig. 4A). This suggested that ERK inhibition can cause the activation of the ER-mediated pathway for cell survival. Therefore, we measured cell proliferation under the same conditions.

Influence of MAPK Inhibitors on Cell Proliferation in Genistein-Treated MCF-7 Cells

In order to measure the cell proliferation in the presence of PD98059, we performed an MTT assay. As can be seen from Fig. 5, we were unable to observe significant differences between genistein alone or with inhibitor treatments. This shows that ERK activity is not an essential component of ER-mediated proliferation. While ICI 182,780, an ER antagonist, inhibited cell proliferation at a low concentration of genistein, treatment with MAPK inhibitors, PD98059 and SB203580, had no significant effect on MCF-7 cells. The increase of ER phosphorylation and ER-mediated gene expression by inhibition of ERK did not induce cell proliferation, although the ER pathway plays an essential role in cell proliferation. E2 induces cell proliferation under the condition of growth-arrest by inhibitors of mitogenic activity; this proliferation occurs through an MAPK-independent pathway[26]. Our studies demonstrate that the cell proliferation induced through the ER-mediated pathway by genistein; these results are supported by the findings of Lobenhofer *et al.* (2000).

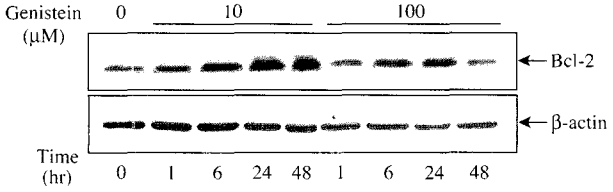

Fig. 6. Expression of Bcl-2 levels in MCF-7 cells after treatment with genistein . Cells were treated with genistein at 10 μM and 100 μM for the times indicated. After harvested, the Bcl-2 levels were assessed by western immunoblot analysis. At low concentration of genistein, levels of Bcl-2 were increased. At a high concentration, however, Bcl-2 levels were decreased.

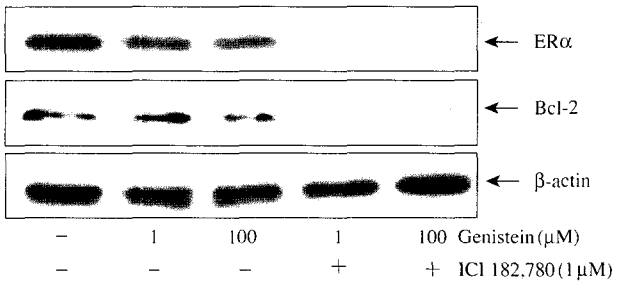

Fig. 7. Effects of ICI 182,780 on Bcl-2 expression in genistein-treated MCF-7 cells . The Bcl-2 protein levels were assessed by western blot analysis. Pretreatment with estrogen receptor antagonist, ICI 182,780 inhibited Bcl-2 expresssion following genistein stimulation. Changes of Bcl-2 expression by ICI 182,780 had strong resemblance to pattern of ER expression inhibited by ICI 182,780 in genistein-treated MCF-7 cells.

Expression of Bcl-2 Levels in MCF-7 Cells upon Treatment with Genistein

At a high concentration of genistein, the viability of MCF-7 cells was decreased. We confirmed the apoptotic death by Poly-(ADP-ribose)-polymerase (PARP) cleavage assay[27]. Several groups also reported that genistein induces apoptosis by delaying the cell-cycle as a result of the inactivation of Bcl-2[28,29]. This provides evidence that Bcl-2 inhibits apoptosis by genistein[30]. We therefore investigated the Bcl-2 expression levels at both low and high concentrations of genistein. Bcl-2 expression was increased in presence of a low concentration of genistein, but it was decreased at a high concentration of genistein (Fig. 6). As can be seen from Fig. 6, Bcl-2 plays an important role in the dose-dependent effect seen in MCF-7 cells. Cell proliferation and apoptosis coincided with

Bcl-2 expression at low and high concentrations of genistein, respectively.

In MCF-7 cells, the effects of estrogens on the regulation of apoptosis involve the coordinated activation of signaling events and Bcl-2 expression[17]. To elucidate whether Bcl-2 regulated through the ER-mediated pathway, cells were pretreated with 1 μM of pure-antiestrogen ICI 182,780 for an hour, and were then treated with low and high concentrations of genistein; Bcl-2 expression was then evaluated by Western blotting. This showed that, at both low and high concentrations of genistein, the Bcl-2 level was also suppressed by ICI 182,780 (Fig. 7)[31]. This finding suggested that the expression of Bcl-2 is mediated by ERs and is related to cell proliferation and apoptosis by genistein.

Discussion

In conclusion, we proved that the biphasic effect of genistein was related to Bcl-2 expression regulated by an ER-mediated pathway in MCF-7 cells. Additionally, we suggest that MCF-7 cells, which are sensitive to estrogen, activate an ER-mediated pathway for survival when MAPK signaling was inhibited by genistein.

Methods

Cell Lines and Culture Conditions

MCF-7 human breast cancer cells were purchased from American Type Culture Collection (ATCC, USA). The MCF-7 (ERE-LUC) cell is a MCF-7 cell derivative containing the ER-controlled gene and regulates the expression of the luciferase reporter gene. It was obtained from Dr. Suh, Pohang University of Science and Technology, Republic of Korea. MCF-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) with phenol red (Gibco, Rockville, USA). The culture medium was supplemented with 10% fetal bovine serum (HI-FBS) (Gibco) and 1% antibiotic-antimycotic (Gibco). Both cell lines were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2.

E-screen Assay

MCF-7 cells were seeded at 5×10^3 cells/well in 96-well plates. The medium was replaced by an experimental medium (phenol red-free DMEM/F12 supplemented with 5% CD FBS) after 24 hr incubation in the growth medium. The treated plates were incubated for 6 days at 37°C in 5% CO_2 in the cell culture incubator, and MCF-7 cell proliferation was then determined by MTT assay.

Western Blot Analysis

The cells were cultured in 60-mm plates and allowed to attach for 24 hr by genistein treatment. To harvest the cells, they were washed twice with PBS and scraped from culture dishes using lysis buffer containing 50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM EDTA, 1 mM dthiothreitol, 0.1 mM sodium orthovanadate, 1 mM benzamidine, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 μg/mL pepstatin, and 1% Triton X-100, pH 7.3. Cell lysates were centrifuged at ~ 10,000 × g for 15 min. Total protein concentrations were then determined, and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was transferred to a nitrocellulose membrane and incubated with mouse or rabbit IgG horseradish peroxidase conjugate for 1 hr at 25°C. Proteins were visualized by an enhanced chemiluminescence detection kit (Santa Cruz, CA, USA). Equivalent loading and protein transfer were confirmed by Western blot using anti-β-actin antibody (Sigma-Aldrich, Deisenhofen, Germany).

Luciferase Reporter Gene Assay

MCF-7 cells were seeded at 3×10^4 cells/well in 12-well plates. To lower the luciferase activity induced by background levels of estrogen and estrogenic compounds in the maintenance medium, cells were incubated for three days in experimental medium consisting of phenol red-free DMEM/F12 supplemented with 5% CD FBS after 24 hr incubation in the growth medium. The cells were treated for 24 hr with the test compounds, and were then assayed for luciferase activity using a commercial detection kit (Promega, Baltimore, USA).

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