

Cell Survival, Apoptosis and AMPK-COX-2 Signaling Pathway of Mammary Tumor Cells after Genistein Treatment Combined with Estrogen

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Abstract

Genistein is an active component of legumes and other related food shown to be associated with prevention of degenerative diseases such as cancer through inducing signaling pathways. Treatment of genistein resulted in the induction of apoptosis in the cultured cancer cells. This induction of apoptosis was demonstrated by the Tunel assay in these cells. Unveiling the potential of genistein in cytotoxicity via apoptosis when it is treated with estrogen can predict the therapeutic capability of genistein in breast cancers in the presence of endogenous estrogen. We have found that apoptosis induced by genistein treatment in the presence of estrogen is agonistic or antagonistic depending on the concentrations and treatment periods applied in MCF-7 breast cancer cells. For the suppression of cell survival, 24 hr of treatment was required to induce a synergistic agonistic response between estrogen and genistein at low concentrations of genistein. After this period, the agonistic pattern of genistein to estrogen disappeared. The decrement of COX-2 expression in MCF-7 cells treated with genistein was accompanied with the activation of AMPK only at a high concentration of genistein. This association between AMPK activation and down-regulation of COX-2 by genistein was dampened in the presence of estrogen. It was also demonstrated that genistein and estrogen regulate cell survival and apoptosis by modulating p53 and caspase-3 in the opposite direction. These results suggest that genistein has the potential to control breast cancer development, and co-treatment with estrogen can cause agonistic or antagonistic action on breast cancer cell control.

Key words: genistein, genistein cancer prevention in presence of estrogen, apoptosis, AMPK-COX-2 pathway, apoptotic proteins, MCF-7 breast cancer cells

INTRODUCTION

Genistein is a phytoestrogen of the 'isoflavone' class compounds. This diphenolic compound resembles the structure of estradiol, and the molecular distances between the two-OH groups on the equol nucleus of genistein and those of 17 β -estradiol are similar and OH groups offer strong binding affinity to the estrogen receptor (1). Epidemiological evidence indicates that there are positive associations between chemoprevention and dietary soy consumption. Asian women with high soy intake have a low incidence of breast cancer (2), and the protection by soy intake is lost following adoption of a Western-based diet in the second generation of immigrants to the US (3). On the basis of its potential anti-cancer activity, genistein has been extensively studied in human breast cancer cells. Interference at the level of the estrogen receptor suggested a major role of genistein in the inhibition of tumor-promoting effect of estrogen (4). At concentrations lower than 10 μ M, the growth of MCF-7 cell, an estrogen receptor positive cancer cell

line, was stimulated by genistein, however, genistein did not stimulate the growth of estrogen receptor-negative breast cancer cells (5). The biphasic effect has been attributed to the genistein exerting estrogen-like effects at lower concentrations, but at higher levels, genistein might act as an estrogen-antagonist (6-10). Genistein has been known to inhibit neoplastic cell proliferation by blocking cell cycle progression in the G₂/M phase (11). In recent years, the role of genistein containing isoflavones in breast cancer has become controversial, since, in contrast to the possible beneficial effects, some animal studies and limited human data suggest that genistein might have breast tumor-promoting effects (12, 13). Considering the wide use of soy-based dietary supplements and food products, there is a need for clarification of the impact of this phytochemical on breast cancer risk in women at high risk of the disease and on the survival of breast cancer patients. The aim of the present study was to evaluate the effects of genistein on cancer cell survival to provide background in-

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formation on antagonistic or agonistic activities of genistein to estrogen in estrogen treated mammary cancer cells. Here, we have hypothesized that the treatment conditions by genistein might induce apoptosis in MCF-7 breast cancer cells, and this anti-proliferation effect might exert either agonistic or antagonistic effects when genistein is treated with estrogen.

MATERIALS AND METHODS

Cell culture

The MCF-7 human breast cancer cell line was purchased from ATCC (Gaithersburg, MD). The cells were cultured in RPMI1640 medium containing 10% FBS, 100 mg/L streptomycin, and 100 U/mL penicillin.

Cell proliferation assay

Cell proliferation was assessed by the MTT method. Cells were seeded in 24-well plates containing the test compounds for indicated dose or time dependently, and then incubated with 30 μ L MTT solution (5 mg/mL in PBS) for 2 hr at 37°C. Optical densities of the solutions, in each well, were determined by an ELISA reader.

Tunel assay

Apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling method. The commercially available *in situ* death detection kit (product of Boehringer, Ingelheim, Germany) was used to detect DNA fragmentation.

Western blotting

Cells were lysed with ice-cold lysis buffer, including 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NAF, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, and 1 μ g/mL pepstatin. Solubilized proteins were centrifuged at 13,000 $\times g$ in a microfuge (4°C) for 5 min, and supernatant protein was collected. The cell lysates were separated on SDS-PAGE and then electro-transferred onto a nitrocellulose membrane (Scheicher & Schuell, Dassel, Germany). Antibodies (p-Acc, pAMPK, COX-2, p53, caspase-3 and β -actin) were purchased from Cell Signaling Technology.

RESULTS

Effects of genistein and estrogen on MCF-7 cell growth

We evaluated the effects of genistein and estrogen on the growth of MCF-7 cells. A dose-dependent growth inhibition was observed when the cancer cells were treated with genistein (0~200 μ M) for 24 or 48 hr (Fig. 1). Treatment of MCF-7 cells with genistein and estro-

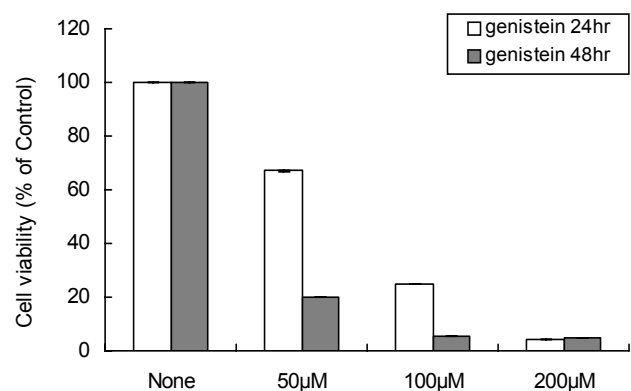


Fig. 1. Cell survival after 24 and 48 hr treatments with control and genistein in MCF-7 cancer cells. MCF-7 breast cancer cells were exposed to DMSO, 50, 100 and 200 μ M genistein for 24 or 48 hr and cell survival was measured by MTT assay.

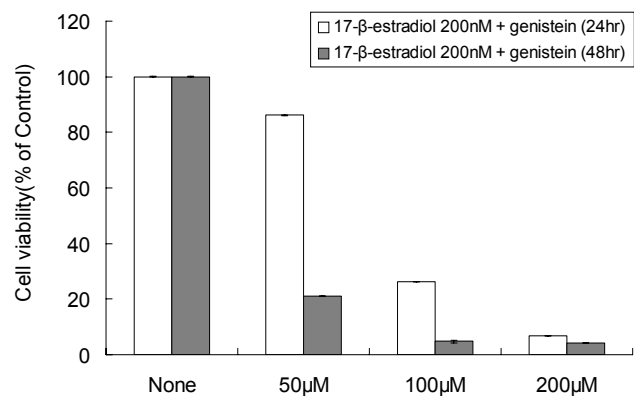


Fig. 2. Cell survival after 24 and 48 hr treatment with control and genistein in presence of estrogen in MCF-7 cancer cells. MCF-7 breast cancer cells were exposed to DMSO, 50, 100 and 200 μ M genistein in the presence of 200 nM 17- β -estradiol 24 hr and 48 hr, and cell survival was measured by MTT assay.

gen combinations resulted in much less growth inhibition at lower concentrations of genistein and 24 hr treatment (Fig. 2).

Effects of genistein and estrogen on MCF-7 cell apoptosis

The Tunel assay revealed an apoptotic pattern in cells treated with genistein (Fig. 3). The greater apoptotic pattern was shown when genistein was treated with estrogen compared to genistein alone or estrogen alone.

Effects of genistein and estrogen on AMPK-COX-2 signaling pathway in MCF-7 cells

To examine the association of AMPK with the inhibitory effect of genistein on COX-2 regulation, the phosphorylated pattern of Acc, AMPK and the activation of COX-2 were investigated (Fig. 4A and 4B upper). It was found that at the lower concentrations (50 μ M)

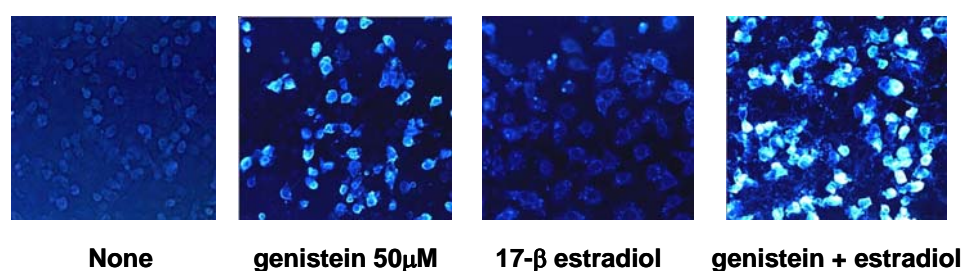


Fig. 3. TUNEL assay results of 24 hr treatment with control, genistein, estrogen and genistein in presence of estrogen in MCF-7 cancer cells. MCF-7 breast cancer cells were exposed to DMSO, 50 μ M genistein, 200 nM 17- β -estradiol and 50 μ M genistein in the presence of 200 nM 17- β -estradiol 24 hr, and cell apoptosis was measured by the TUNEL assay.

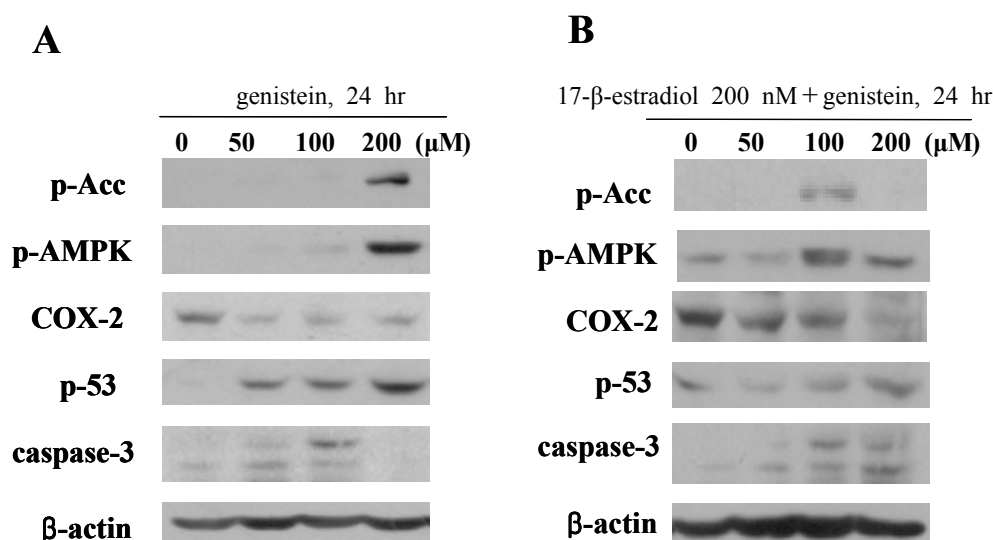


Fig. 4. Expression of p-ACC, AMPK, COX-2, p53 and caspase-3 following treatment with control, genistein and genistein in the presence of estrogen in MCF-7 cancer cells for 24 hr. MCF-7 breast cancer cells were exposed to DMSO, 50, 100 and 200 μ M genistein in the presence of 200 nM 17- β -estradiol 24 hr. Western blotting was performed as described in Materials and Methods.

of genistein the activation of AMPK did not appear to be important in down-regulation of COX-2 by genistein. At the higher concentrations of 200 μ M genistein the activation of AMPK was associated with the down-regulation of COX-2. In the presence of estrogen, COX-2 down-regulation was accompanied by a weaker activation of AMPK compared to the genistein treatment alone in MCF-7 cells.

Effects of genistein and estrogen on the expression of p53 and caspase-3 in MCF-7 cells

As shown in Fig. 4A and 4B, lower and Fig. 5A and 5B, genistein increased the levels of p53 and caspase-3 at the high concentrations of genistein treatment, and the genistein treatment in the presence of estrogen revealed a weaker expression of these proteins. However, this decreased pattern of p53 and caspase-3 in the presence of estrogen disappeared when the cells were treated for 48 hr.

DISCUSSION

In this study, the effects of genistein in the presence of lower concentrations of estrogen on cell survival, apoptosis, AMPK-COX-2 pathway and expressions of apoptotic index proteins were investigated and it was found that genistein behaved slightly differently depending on the genistein concentrations used or incubation periods. Estrogen has been suggested as a promoter of breast cancer for its cell proliferative effect (14) and an antiapoptotic agent (15). Genistein induced apoptosis at 100 μ M and estrogen could reduce this apoptotic activity of genistein after 48 hr treatment as shown with the MTT assay. However, this tendency was not evident at 24 hr treatment of genistein in the presence of estrogen. The stimulated COX-2 expressions were observed with co-treatment with genistein and estrogen at 24 hr incubation, and a decrement in COX-2 expression occurred at high concentration of genistein (200 μ M). However,

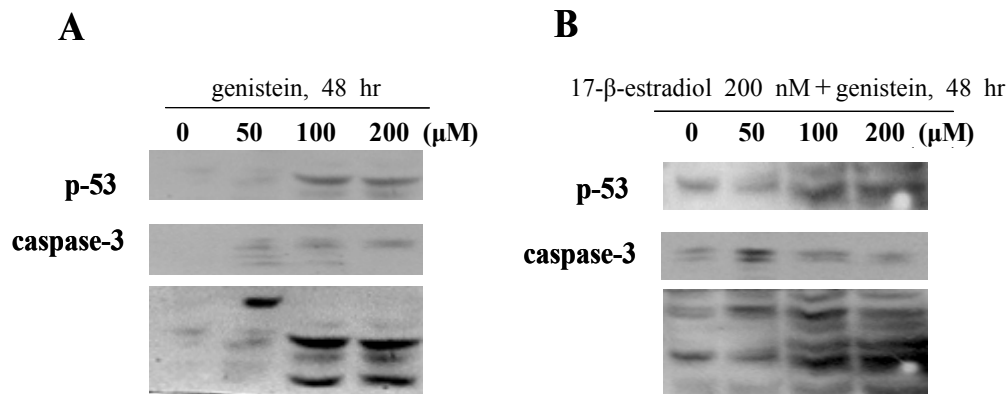


Fig. 5. Expression of p53 and caspase-3 following treatment with control, genistein and genistein in the presence of estrogen in MCF-7 cancer cells for 48 hr. MCF-7 breast cancer cells were exposed to DMSO, 50, 100 and 200 μM genistein in the presence of 200 nM 17-β-estradiol for 48 hr. Western blotting was performed as described in Materials and Methods. In this figure, the uniformity of protein used in each sample was not compared with beta-actin, but the whole patterns of protein display was used.

this tendency disappeared at 48 hr treatment of genistein and estrogen. The activation of AMPK appeared to be involved in COX-2 regulation, and higher rate of apoptosis occurred in conditions of high concentrations of genistein treatment. A decreased expression of p53 by co-treatment of genistein and estrogen was noticed, suggesting that agonistic activity of genistein on apoptosis with estrogen was the result of p53 regulation by estrogen and genistein. This study indicates that genistein has the potential to inhibit the proliferation of tumor cells through an apoptotic mechanism. The presence of relatively low concentrations of estrogen might hinder this anti-proliferatory property of genistein, especially with mild doses of genistein. Many studies have demonstrated that phytochemicals present in naturally occurring plants play an important role in the control of carcinogenesis (16,17). Recently, great attention has been focused on the controlling of cell survival or death through modulating intracellular signaling pathways by edible phytochemicals (18). In our previous work, phenolic phytochemicals such as EGCG and resveratrol also have been shown to activate apoptotic signaling, possibly modulating apoptosis related gene expressions (19,20). Our data demonstrate the AMPK activation and COX-2 inhibition with phytochemical treatments is important in apoptosis when the breast cancer cells were treated with high dose of genistein. However, in the present study there exists a possibility that the concentrations of genistein used were too high to cause not only apoptosis but also cell cytotoxicity. Therefore, the conditions of using lower concentrations of genistein used in this study might be applied to clearly examine the effect on apoptosis excluding any effect on cell cytotoxicity.

In this study, it was also demonstrated that genistein

modulates apoptotic markers such as p53 and caspase-3. There exists persuasive evidence that genistein and estrogen regulates apoptosis by modulating these proteins (21-24).

Breast cancer is the most alarming cancer in females, which often exerts uncontrolled growth and invades other organs (25-27) and; therefore, the finding of strategies for breast cancer prevention and treatment remains an imperative goal to overcome the deadly consequences of malignant tumors. Apoptosis is not in control in many human tumors including breast cancers, and therefore it is considered to be an important target in naturally occurring chemopreventive and chemotherapeutic agents. Accumulating evidence suggests that these agents induce apoptosis through the regulation of various protein kinases, which can activate intracellular signaling pathways (28,29). The importance of AMPK-COX-2 signaling pathway has been emphasized in previous publication (30).

We have investigated the potential of genistein to influence cell growth and apoptosis when it is treated with estrogen in MCF-7 breast epithelial cancer cells. Genistein resulted in the inhibition of cell growth and induction of apoptosis in tested breast cancer cells. The effect of genistein in combination with estrogen on induction of apoptosis showed that the initial agonistic activity of genistein to estrogen was abolished by further treatment for 48 hr. These results suggest that genistein has a potential to control breast cancer development and co-treatment with estrogen can cause agonistic or antagonistic action on this breast cancer control. However, the observed agonistic or antagonistic activity of genistein to estrogen with a time-dependent trend requires further investigation to test the efficacy of genistein in the prevention of breast cancers.

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