Genistein induces cell apoptosis in MDA-MB-231 breast cancer cells via the mitogen-activated protein kinase pathway

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A B S T R A C T

Genistein, an isoflavonoid present in soybeans, exhibits anti-carcinogenic effects. Several studies have shown that genistein inhibits cell proliferation and triggers apoptosis in human breast cancer cells. In this study, we assessed the role of the MEK-ERK cascade in the regulation of genistein-mediated cell apoptosis in MDA-MB-231 cells. The results indicate that genistein, in a concentration-dependent manner, suppresses the protein levels of MEK5, total ERK5, and phospho-ERK5, effects that are consistent with inhibition of cell growth and induction of apoptosis. Exposure of these cells to genistein results in a concentration-dependent decrease in NF-κB/p65 protein levels and DNA-binding activity of NF-κB. Genistein down-regulates Bcl-2 and up-regulates Bax. NF-κB binding sites are present in the promoter of Bcl-2, suggesting that genistein might inhibit the expression of Bcl-2 through down-regulation of NF-κB.

Exposure of MDA-MB-231 cells to genistein results in cleavage of caspase-3 and induction of caspase-3 activity in a concentration-dependent manner. Genistein inhibits NF-κB activity via the MEK5/ERK5 pathway; it also inhibits cell growth and induces apoptosis. In conclusion, inhibition of the MEK5/ERK5/NF-κB pathway may be an important mechanism by which genistein suppresses cell growth and induces apoptosis.

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1. Introduction

Genistein, the major isoflavonoid contained in soybeans, has various activities, including estrogenic, growth-promoting, and anti-carcinogenic effects (Peterson and Barnes, 1991; Fotésis et al., 1995; Zhou et al., 1998). Epidemiological studies, as well as work performed with animal models (Messina et al., 1994; Shao et al., 1998), suggest that it has a chemopreventive effect for breast, colon, and skin tumors.

Genistein kills cancer cells in culture by causing apoptosis. In breast cancer cells, the effects of genistein are dependent on the estrogen receptor (ER) status. Although genistein at low concentrations (<10 μM) is growth stimulatory in ER-positive breast cancer cells (Fioravanti et al., 1998; Zava and Duwe, 1997; Hsieh et al., 1998), it is growth suppressive at all concentrations in ER-negative cell lines (Monti and Sinha, 1994).

Signaling mediated by mitogen-activated protein kinases (MAPKs) is involved in the regulation of cell proliferation and in the response of cells to DNA damage (Schaeffer and Weber, 1999). On the basis of their sequence similarities and the nature of their upstream activators, MAPKs are grouped into four subfamilies: ERK1/2, JNK/SAPK, p38 and ERK5. MAPKs are activated by a variety of stimuli (Chang and Karin, 2001). The final signal commonly includes transcription of target genes, leading to a cellular response such as proliferation, apoptosis, or cycle progression (Rubinfeld and Seger, 2005).

Although the role of several MAPKs has been actively investigated, the function of MEK5 and its downstream molecule, ERK5, is not adequately defined. ERK5 contributes to cell proliferation stimulated by EGF (Kato et al., 1998). Activation of ERK5 prevents apoptosis in endothelial cells and inhibits caspase-3 activity (Pi et al., 2004). Reducing ERK5 levels with a specific small hairpin RNA 5 (shERK5) reduces cell viability and sensitizes cells to death receptor-induced apoptosis (Garaude et al., 2006). These studies show that ERK5 is important for cell proliferation and survival.

Since MAPK families influence cell survival and apoptosis, we determined the effect of genistein on ERK5, particularly as it participates in genistein-induced cell apoptosis. Our data demonstrate for the first time, to our knowledge, that, in human breast cancer cells, inhibition of the MEK5/ERK5/NF-κB pathway may be an important mechanism for growth suppression and induction of apoptosis by genistein.
2. Materials and methods

2.1. Reagents and immunochemicals

Genistein, methyl thiazolyl tetrazolium (MTT), propidium iodide, and triton-X-100 were obtained from Sigma (USA). Leibovitz’s L-15 medium and trypsinase were from Gibco (USA). Newborn fetal calf serum was obtained from PAA Laboratories (Austria). ApoAlert Annexin V-FITC and ApoAlert Caspase Colorimetric Assay kits were from Clontech (USA); the NE-PER nuclear extraction kit was from Pierce (USA).

The antibodies against cyclin B1 and caspase-8 were from NeoMarkers (USA). Antibodies against Bcl-2, Bax, and ERK5 were from Stressgen (Canada). Antibodies against caspase-3, cyclin-dependent kinase 1 (Cdk1), Cdc25C, NF-κB/P65, and phospho-ERK5 were from Santa Cruz Biotechnology (USA). Antibodies against MEK5 were from BD Biosciences Pharmingen (USA), and antibodies against actin were from Boster Corporation (China). The rabbit polyclonal antibodies and horseradish peroxidase conjugates of anti-rabbit and anti-mouse immunoglobulin G were from BioRad Laboratories (USA). Enhanced chemiluminescence (ECL) detection reagents were obtained from Amersham (USA).

2.2. Cell culture

Human breast cancer cells, MDA-MB-231, obtained from the American Type Culture Collection (Rockville, MD), were routinely maintained in Leibovitz’s L-15 medium, pH 7.3, supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum (FCS) and were grown at 37 ºC. Genistein, dissolved in ethanol, was added directly to the culture media at different concentrations. The same concentration of ethanol was present in all cultures.

2.3. Cell growth inhibition studies by MTT assay

Experiments were accomplished with 96-well plates containing Leibovitz’s L-15 medium supplemented with 10% FCS. The numbers of viable cells after exposure to genistein were determined by the MTT assay. Initially, cells were seeded at a plating density of 6 x 10^4 cells/well in 200 μl of medium, and were then cultured for 24 h to allow their adhesion to the plate. Before preincubation, the culture medium was changed to the experimental medium supplemented with genistein (5, 10, or 20 μM). After 48 h, cells were incubated with MTT (0.5 mg/ml) at 37 ºC for 4 h and then with DMSO at room temperature for 1 h. The plates were read at 490 nm on a scanning multi-well spectrophotometer. Data points represented the mean values for eight wells.

2.4. Quantitative apoptotic cell death assay

Apoptosis induction in control (ethanol-treated) or genisten-treated MDA-MB-231 cells was assessed by use of an Annexin V-FITC kit. Cells were exposed to desired concentrations of genistein or ethanol for 48 h, and the floating and adherent cells were collected. Pooled cells were washed with the binding buffer supplied by the manufacturer. Approximately 5 x 10^5 cells were suspended in 200 μl of binding buffer and mixed with 5 μl of Annexin V-FITC and 10 μl of propidium iodide. After 15 min of incubation in the dark, cells were analyzed with a flow cytometer.

2.5. Caspase-3 activity assay

For assay of caspase-3 activity, MDA-MB-231 cells were exposed to ethanol (control) or genistein for 48 h. Both floating and adherent cells were collected and suspended in 50 μl of lysis buffer supplied by the manufacturer. After 10 min of incubation on ice, the cell lysate was cleared by centrifugation at 20,000g for 3 min. Caspase-3 activity was determined according to the manufacturer’s instructions.

2.6. Determination of NF-κB activity

NF-κB activity was determined by use of a TransAM kit from Active Motif North America (USA), according to the manufacturer’s instructions. Nuclear fractions were prepared by use of an NE-PER nuclear extraction kit from Pierce (USA), according to manufacturer’s instructions. Briefly, nuclear extracts from control and genistein-treated MDA-MB-231 cells were added to 96-well plates pre-coated with the oligonucleotide containing an NF-κB consensus sequence (5'-GGGACTTCCC-3'). Following incubation at room temperature for 1 h to facilitate the binding, a primary antibody that recognizes only activated NF-κB/p65, was added to each well. The absorbance was read at 450 nm by use of an ELISA plate reader. This assay is specific for NF-κB/p65 activation and is more sensitive than electrophoretic mobility shift assays.

2.7. Western blotting

The MDA-MB-231 cells were plated on culture dishes and allowed to attach for 24 h, followed by the addition of genistein (5, 10, or 20 μM) and incubation for 48 h. Control cells were similarly incubated in the medium with ethanol. After incubation, the cells were washed twice with ice-cold PBS and then scraped off in 0.2 ml of buffer (20 mM HEPES, pH 6.8, 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg/ml okadaic acid, 1 mM DTT, 0.4 M KCl, 0.4% Triton X-100, 10% glycerol, 5 μg/ml leupeptin, 50 μg/ml PMSF, 1 mM benzamidine, 5 mg/ml aprotinin, and 1 mM Na orthovanadate) and incubated on ice for 30 min, followed by centrifugation at 12,000 rpm for 20 min. The supernatant was stored at −70 ºC. Protein concentrations were measured by use of the BCA protein assay (Pierce, Rockford, IL). After this determination, all preparations were diluted to an equal concentration of protein, boiled for 5 min, and then proteins were separated by 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes, which were probed with the primary antibodies overnight at 4 ºC. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature before enhanced chemiluminescence (Amersham Biosciences) and exposure to film. Actin was used to normalize for protein loading. All experiments were performed at least twice with similar results.

2.8. Statistical analyses

Data are expressed as means ± SD. Statistical differences were analyzed using one-way ANOVA. A value of P < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Genistein inhibited proliferation and induced apoptosis in MDA-MB-231 cells

MDA-MB-231 cells were chosen as a model system because they exhibit highly metastatic and aggressive behavior in vivo compared with other human breast cancer cell lines. In order to determine the effect of genistein on cell proliferation, MDA-MB-231 cells were exposed to increasing concentrations of genistein. After 48 h, cell proliferation was determined by the MTT assay. A dose-dependent inhibition of cell growth was observed at concentra-
tions of 5–20 μM (Fig. 1A). In all preparations, the concentration of ethanol was 0.1%. Inhibition of cell proliferation could be attributable to the induction of apoptosis in these cells, as reported earlier (Shim et al., 2007).

The apoptosis-inducing effect of genistein was investigated by flow cytometric analysis of MDA-MB-231 cells stained with Annexin V and propidium iodide. Exposure of cells to genistein (5, 10, or 20 μM) for 48 h resulted in an accumulation of apoptotic cells (Fig. 1B). The induction of apoptosis was in a dose-dependent manner.

Caspases are aspartate-specific, cysteine proteases that mediate apoptosis. Caspases are sequentially activated by cleavage of their inactive, pro-caspase forms. Based on our results showing apoptosis induction by genistein in MDA-MB-231 cells, we conducted additional assays involving caspase-3 cleavage to confirm the apoptotic response of genistein. Exposure of MDA-MB-231 cells to genistein resulted in reduction of pro-caspase-3 and cleavage of caspase-3 in a dose-dependent manner; in contrast, caspase-8 showed little or no change after exposure to genistein (Fig. 2), we did not detect any cleaved caspase-8.

Apoptosis induction by genistein was further established by determining its effect on caspase-3 activity in MDA-MB-231 cells (Fig. 3). Caspase-3 activity increased in genistein-treated cells and remained elevated for the duration of the experiment.

3.2. Genistein inhibited MEK5/ERK5/NF-κB pathway in MDA-MB-231 cells

In most cellular systems, the MEK5/ERK5 cascade promotes proliferation and survival. In order to assess whether the MEK5/ERK5 signaling pathway is involved in genistein-induced anti-proliferation and apoptosis, MDA-MB-231 cells were exposed to increasing concentrations of genistein for different time intervals.
and the effect on levels of MEK5, total ERK5, and phospho-ERK5 proteins was determined by immunoblotting. Representative blots are shown in Fig. 4A and B. Immunoblotting revealed that genistein caused a reduction in the protein levels of MEK5, total ERK5, and phospho-ERK5 in a dose- and time-dependent manner, results that are consistent with inhibition of cell growth and induction of apoptosis by genistein. These observations demonstrate that the MEK5/ERK5 pathway is involved in cell survival.

In breast cancer, there is increased NF-κB activity, which is believed to enhance cell survival by inhibiting apoptosis. The most abundant form of NF-κB is the heterodimer of p50 and p65. The p65 subunit is responsible for transcriptional activity, resulting in the expression of NF-κB-responsive target genes. Employing western blot analysis, we investigated the effect of genistein (5–20 μM, 48 h) on the levels of constitutively expressed NF-κB/p65 nuclear protein in MDA-MB-231 cells. In these cells, genistein caused a concentration-dependent decrease in NF-κB/p65 nuclear protein levels (Fig. 5A). The effect of genistein on DNA-binding of NF-κB was also determined (Fig. 5B). The NF-κB activity in the nuclear fraction was inhibited by exposure of cells to genistein for 48 h, indicating that genistein inhibits NF-κB activation.

4. Discussion

MAPK cascades are involved in cellular responses to environmental stimuli. In most cellular systems, the MEK5/ERK5 cascade is thought to promote proliferation and survival. MEK5 functions through activation of its immediate downstream target, ERK5. Studies with intact animals indicate that ERK5 supports vascular endothelial viability (Hayashi et al., 2004). Reducing ERK5 levels with a specific small hairpin RNA 5 (shERK5) reduces cell viability and sensitizes cells to death receptor-induced apoptosis (Garreauet al., 2006). In the present study, genistein suppressed the protein levels of MEK5, total ERK5, and phospho-ERK5 in a dose-dependent manner, effects that are consistent with its inhibition of cell growth and induction of apoptosis. These results demonstrate that the MEK5/ERK5 pathway is involved in cell survival. Increased apoptosis may occur due to loss of ERK5.

Genistein can inhibit cell proliferation and induce cell apoptosis in many cell systems. Induction of cell apoptosis only is one of the effects after treatment with genistein. We also found that genistein inhibits cell proliferation through G2/M cell cycle arrest in MDA-MB-231 cells (data not shown). Thus, we observed the high inhibition of cell proliferation and the low induction of cell apoptosis in MDA-MB-231 cells with genistein treatment.

The transcription factor NF-κB, a known target of ERK5, is involved in cell survival and apoptosis (Liptay et al., 2003). We proposed that NF-κB in breast cancer cells functions by participating in proliferative pathways and by regulating cell death signals. The results show that exposure of cells to genistein results in a concentration-dependent decrease in NF-κB/p65 protein levels and DNA-binding activity of NF-κB in MDA-MB-231 cells. Others have reported that, in MDA-MB-231 cells, genistein inhibits NF-κB DNA-binding and abrogates EGF-induced NF-κB activation via the Akt pathway (Gong et al., 2003). In breast cancer cells, NF-κB activation induces transcription of several anti-apoptotic genes (Mayo et al., 1997), whereas inactivation of NF-κB by genistein contributes to increased apoptosis (Li et al., 2005). Thus, a possible mechanism for the induction of apoptosis by genistein may involve suppression of NF-κB function and subsequent inhibition of anti-apoptotic stimuli.

The Bcl-2 family of proteins is involved in apoptosis (Adams and Cory, 1998). Bcl-2 protects cells from apoptosis, whereas Bax inhibits cell growth and induces apoptosis. We and others found that genistein down-regulates NF-κB and Bcl-2 and up-regulates Bax and that NF-κB binding sites are present in the promoter of
Bcl-2, suggesting that genistein inhibits the expression of Bcl-2 through down-regulation of NF-κB (Baxa and Yoshimura, 2003).

Caspases are cysteine proteases involved in the process of apoptosis. Caspase-3, a cell death protease, is activated in response to various apoptotic stimuli (Thornberry and Lazebnik, 1998). In the present study, cleavage of procaspase-3 and induction of caspase-3 activity occurred following exposure of MDA-MB-231 cells to genistein, suggesting a caspase-dependent mechanism is involved in cell death.

The results of the present study indicate that inhibition of the MEK5/ERK5/NF-κB pathway by genistein is an important mechanism for growth suppression and for induction of apoptosis in human breast cancer cells. The work provides new insights into the molecular mechanisms of genistein in these cells.

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