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RESEARCH NOTE

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Induction of Caspase-3 Dependent Apoptosis in Human Ovarian Cancer SK-OV-3 Cells by Genistein

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Abstract The present study was designed to determine how the phytochemical genistein activates caspase-3 to cause cell cycle arrest and apoptosis. When human ovarian cancer SK-OV-3 cells were treated with 200 μM genistein for 24 hr, cell growth decreased significantly ($p<0.05$). Conversely, genistein treatment significantly increased cytotoxicity (measured as lactate dehydrogenase release) under the same conditions ($p<0.05$). To elucidate the mechanism behind the induction of apoptosis by genistein, we studied the cell cycle and caspase-3 activation. When cells were treated with genistein, the population of cells in sub-G1 phase increased by 44.2% compared to untreated cells. Genistein caused decrease in precursor caspase-3, increase in cleaved caspase-3 and a significant increase in caspase-3 activity ($p<0.05$). Therefore, genistein may induce apoptosis via caspase-3 activation. However, high-dose genistein treatment must be viewed with caution because of its potential cytotoxicity.

Keywords: apoptosis, caspase-3, cytotoxicity, genistein, SK-OV-3 cell

Introduction

Genistein (4',5,7-trihydroxyisoflavone) is the predominant isoflavone of legumes such as soybeans, and it occurs in a variety of human foods (1,2). Several epidemiological studies have reported significant correlations between isoflavone consumption and a reduced cancer risk. Moreover, it has been demonstrated that high soybean consumption in Asia is associated with a decreased incidence of cancer compared to other countries with western lifestyles (3,4).

In 1987, it was discovered that genistein is a potent inhibitor of the tyrosine-specific protein kinases of the epidermal growth factor (EGF) receptor (5). Because protein tyrosine kinase inhibitors play a key role in cancer cell growth and apoptosis (6,7) several studies subsequently focused on the pharmacological activities of genistein as a cancer chemopreventive agent with retard to cancer (8-10). Many studies have that genistein significantly inhibits cancer cell growth *in vitro* (11-13) and that this growth inhibition stimulates the signal transduction pathway leading to apoptosis or necrosis.

Nevertheless, the cellular mechanisms underlying the action of genistein-induced cell cycle arrest and apoptosis remain unknown. In this study, we investigated the induction of cell cycle arrest and apoptosis by genistein using human ovarian cancer SK-OV-3 cells.

Materials and Methods

Cells culture and genistein treatment Human ovarian cancer SK-OV-3 cells were purchased from the Korean Cell Line Bank (KCLB, Korea). The cells were routinely maintained in RPMI 1640 (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fatal bovine serum (FBS) and antibiotics (50 U/mL of penicillin and 50 μg/mL streptomycin, Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were treated with either 200 μM genistein or vehicle alone for 24 hr. Genistein was purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO, final concentration 0.1% in medium).

Cell proliferation Cell proliferation was determined using the methyl thiazolyl tetrazolium (MTT) assay. After 24 hr of treatment, 20 μL of 5 mg/mL MTT solution was added to the cells exposed to genistein. Four hr later, 200 μL of DMSO was added to each well to dissolve the resulting formazan crystals and then the absorbance was recorded at 490 nm in a microplate reader (SpectraMax Plus; Molecular Devices Corp., Sunnyvale, CA, USA).

Determination of cytotoxicity Cellular cytotoxicity was evaluated in terms of the activity of lactate dehydrogenase (LDH). LDH activity was determined by the formation of NADH (absorbance 340 nm). Protein concentration was determined using the Bradford reagent (Bio-Rad Lab., Hercules, CA, USA)

Cell cycle distribution Cells were harvested, washed with cold phosphate-buffered saline (PBS), and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol and stored at −20°C for later analysis. The fixed cells were centrifuged at 800×g and washed twice with cold PBS. RNase A (20 μg/mL final concentration) and propidium iodide staining solution (50 μg/mL final concentration) were added to the cells and incubated for 30 min at 37°C in the dark. The cells were analyzed on a FACS Calibur Instrument (BD Biosciences, San Jose, CA, USA) equipped with CellQuest 3.3 Software. ModFit LT 3.1 trial cell cycle analysis software was used to determine

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the percentage of cells in the different phases of the cell cycle.

Immunoblotting assay Cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 7.5) containing protease inhibitor for 1 hr at 4°C. The supernatant was separated by centrifugation, and protein concentration was determined by the Bradford protein assay kit II (Bio-Rad Lab.). Proteins (25 μg/well) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes (0.45 μm). The membranes were blocked with a 1% bovine serum albumin (BSA) solution for 3 hr and washed twice with PBS containing 0.2% Tween-20, and incubated with the primary antibody overnight at 4°C. Antibodies against precursor caspase-3 (35 kDa), cleaved caspas-3 (20 kDa), and β-actin (43 kDa) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and used to probe each membranes. The next day, the immunoreaction was continued with the secondary goat anti-rabbit horseradish-peroxidase-conjugated antibody after washing for 2 hr at room temperature. Specific protein bands were detected by the Opti-4CN Substrate kit (Bio-Rad Lab.).

Statistical analyses All data were expressed as a percentage of the vehicle-treated control cells, which were arbitrarily assigned 100%. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test (SigmaStat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $p<0.05$.

Results and Discussion

To investigate the possible anticancer effects of the phytochemical genistein on human ovarian cancer SK-OV-3 cells, we first examined the anti-proliferative effects of 200 μM genistein using the MTT assay ($p<0.05$, Fig. 1). The proliferation of cells exposed to genistein decreased by 44.9% compared to the control, but genistein exhibited marked cytotoxicity as measured by the release of LDH ($p<0.05$, Fig. 1). This was consistent with our previous reports that genistein inhibits cell proliferation in various cells (14-17). In addition, previously out report demonstrated that chronic administration of a high dose genistein induces cytotoxicity and apoptosis in rat brain (18).

To further scrutinize these results, we analyzed the cell cycle distribution of SK-OV-3 cells treated with genistein using fluorescence-activated cell sorting (FACS) under the same experimental conditions. We sought to determine whether the anti-proliferative activity of genistein would lead to cell cycle arrest. Although genistein treatment for 24 hr caused cell cycle arrest at G2/M phase in a dose-dependent manner (data not shown), we observed a higher percentage of apoptosis in sub-G1 phase SK-OV-3 cells exposed to 200 μM genistein. Compared to the vehicle-

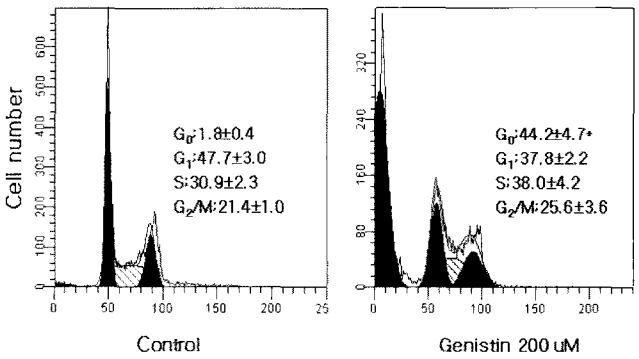

Fig. 2. Effect of genistein on the cell cycle distribution of human ovarian cancer SK-OV-3 cells. *$p<0.05$, significantly different from the vehicle-only group.

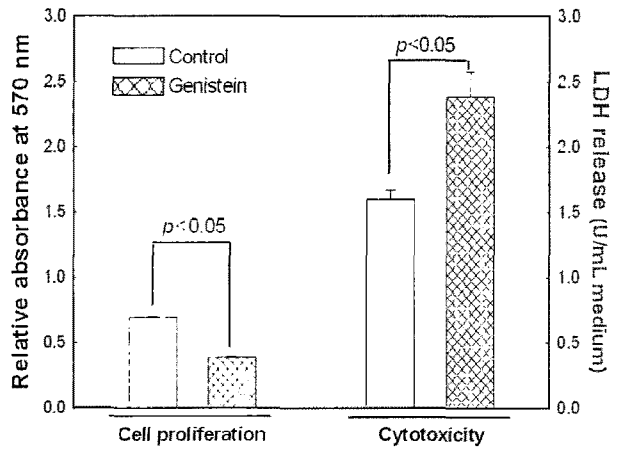

Fig. 1. Cell proliferation and cytotoxicity in human ovarian cancer SK-OV-3 cells exposed to genistein. Cell proliferation and cytotoxicity were analyzed by MTT and LDH release, respectively.

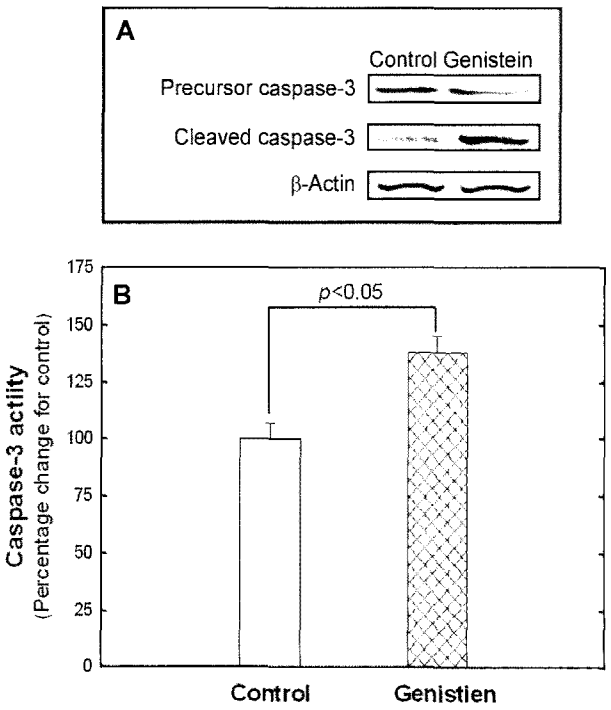

Fig. 3. Effect of genistein on caspase-3 expression (A) and activity (B) in human ovarian cancer SK-OV-3 cells.

treated cells, the apoptotic cells exposed to 200 μM genistein decreased by 44.2% ($p<0.05$), and we thus used a concentration of 200 μM to investigate the apoptotic pathway induced by genistein.

Apoptosis is an important series of events that leading to programmed cell death that is also essential for development and tissue homeostasis. The potential mechanisms involved in the apoptotic process involve a balance between apoptosis induction and apoptosis inhibition. Recently, the regulation of apoptosis has been proposed as a promising target for cancer chemotherapy (19-21), and some studies have reported that genistein induces apoptosis via a caspase-dependent pathway (11,13).

Caspases, which comprise a family of cysteine proteases, are central mediators of the programmed cell death process. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. Caspase-3 is generally synthesized in cells as an inactive zymogen (32 kDa) and requires proteolytic cleavage for activation. It is cleaved on the carboxyl side of 2 specific aspartic acid residues to form 2 mature subunits, p18 (18 kDa) and p12 (12 kDa). In the present study, genistein increased caspase-3 expression remarkably (Fig. 3A), and exposure to 200 μM genistein significantly increased caspase-3 activity by up to 37.8% compared to the controls ($p<0.05$, Fig. 3B).

Our findings suggest that genistein activates caspase-3-dependent apoptotic pathways to cause apoptotic cell death. However, high-dose genistein treatment must be carefully considered due to its potential cytotoxicity.

Acknowledgement

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