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Genistein induces G2/M cell cycle arrest and apoptosis of human ovarian cancer cells via activation of DNA damage checkpoint pathways

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Abstract

Genistein is a major isoflavonoid in dietary soybean, commonly consumed in Asia. Genistein exerts inhibitory effects on the proliferation of various cancer cells and plays an important role in cancer prevention. However, the molecular and cellular mechanisms of genistein on human ovarian cancer cells are still little known. We show that exposure of human ovarian cancer HO-8910 cells to genistein induces DNA damage, and triggers G2/M phase arrest and apoptosis. Furthermore, we also found that checkpoint proteins ATM and ATR are phosphorylated and activated in the cells treated with genistein. It is also shown that genistein increases the phosphorylation and activation of Chk1 and Chk2, which results in the phosphorylation and inactivation of phosphatases Cdc25C and Cdc25A, and thereby the phosphorylation and inactivation of Cdc2 which arrests cells in G2/M phase. Moreover, genistein enhances the phosphorylation and activation of p53, while decreases the ratio of Bcl-2/Bax and Bcl-xL/Bax and the level of phosphorylated Akt, which result in cells undergoing apoptosis. These results demonstrate that genistein-activated ATM-Chk2-Cdc25 and ATR-Chk1-Cdc25 DNA damage checkpoint pathways can arrest ovarian cancer cells in G2/M phase, and induce apoptosis while the cellular DNA damage is too serious to be repaired. Thus, the antiproliferative, DNA damage-inducing and pro-apoptotic activities of genistein are probably responsible for its genotoxic effects on human ovarian cancer HO-8910 cells.

Keywords: Genistein; Apoptosis; DNA damage checkpoint; Ovarian cancer cell

1. Introduction

According to the 2008 American Cancer Society report, ovarian cancer accounts for ~3% of all cancers among women, and ranks second among gynecologic cancer (American Cancer Society, 2008). Currently the standard treatment of this advanced disease involves cytoreductive surgery followed by systematic chemotherapy. Despite advances in treatment, long-term survival rates remain low and an estimated 130,000 deaths per year occur from ovarian cancer worldwide (Spannuth et al., 2008; Visintin et al., 2008).

Epidemiological studies indicate that amount of consumption of soybean is associated in part with a lower incidence of breast, prostate and ovarian cancers in China (Cui et al., 2007; Lee et al., 2003; Zhang et al., 2004). Isoflavonoids are among the most promising potential anticarcinogenetic compounds in dietary soybeans (Kennedy, 1995; Park and Surh, 2004). As a major bioactive phytoestrogen isoflavone found in dietary soybean, genistein (4,5,7-trihydroxyisoflavone) has been investigated extensively for its chemopreventive potential. Both *in vivo* and *in vitro* studies have shown that genistein can induce apoptosis of various cancer cells including leukemia, hepatoma, prostate cancer and breast cancer (Perabo et al., 2008; Sarkar and Li, 2003; Shim et al., 2007; Su et al., 2003; Yamasaki et al., 2007). Genistein exerts inhibitory effects on some human ovarian cell lines *in vitro* (Chen and Anderson, 2001; Choi et al., 2007; Gossner et al., 2007; Novak-Hofer et al., 2008). However, the molecular and cellular mechanisms

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underlying its genotoxicity at high concentration are not fully understood (Klein and King, 2007). In this study we examined the role of DNA damage checkpoint pathways in the genistein-associated genotoxicity on human ovarian cancer cells.

2. Materials and methods

2.1. Materials

Genistein, DMSO, MTT, acridine orange (AO), ethidium bromide (EB), propidium iodide (PI) and monoclonal mouse anti-β-actin antibody were purchased from Sigma–Aldrich (St. Louis, MO, USA). Neocarzinostatin (NCS) and polyclonal rabbit anti-pRad17-Ser645 antibody were generous gifts of Professor Xiao-Fan Wang, Duke University Medical Center. Monoclonal mouse anti-Akt and Bax antibodies, and β-actin antibodies, and polyclonal rabbit anti-Bcl-2, Bcl-xL and Brca1 antibodies and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal rabbit anti-pChk1-Ser345, pH2AX-Ser139, pChk2-Thr68, pBrca1-Ser1524, pCdc25C-Ser216 and pCdc2-Tyr15 antibodies were from Usbio (Swampscott, MA, USA). Polyclonal rabbit anti-pAkt-Ser473, pATM-Ser1981, pATR-Ser428, ATR, P53, pP53-Ser15 and pCdc25A-Thr506 antibodies were purchased from Cell Signaling Technology (Berverly, MA, USA).

2.2. Cell culture and genistein treatment

Human ovarian cancer cell line HO-8910 with low metastatic potential was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% heat-inactivated fetal bovine serum, 100 units/ml ampicillin and 100 μg/ml streptomycin sulfate at 37 °C in a humidified air atmosphere with 5% CO_2. Genistein was dissolved in DMSO 100 mM as a stock solution and freshly diluted to the required level before use. Cells grown in media containing equivalent amount of DMSO without genistein served as control.

2.3. MTT assay

5×10^3 cells/well were plated in 96-well plates for 24 h and treated with of genistein at 1, 10, 25, 50 and 100 μM for 1–3 days. After treatment, cells were washed with PBS and incubated with 100 μl 0.25 mg/ml MTT at 37 °C for 4 h. The MTT solution was removed and incubated with 200 μl Tris-DMSO solution for 30 min to solubilize the MTT-formazan product. Absorbance of the solubilized product was measured at 570 nm using a Kinetic Microplate reader (Molecular Devices, Sunnyvale, CA, USA). At least 3 independent experiments were performed.

2.4. Alkaline comet assay

After treatment with genistein for 4 h, cells were isolated by trypsinization and centrifuged at 1000 rpm at 4 °C for 10 min. Viability of cells was determined by Trypan Blue exclusion test and was >90%. The cells were suspended in 200 μl of 0.5% low melting agarose dissolved in PBS, and layered on a fully frosted slide coated with 0.75% normal melting-point agarose. After treatment with freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris base, 1% Sodium N-lauroyl Sarcosinate, 1% Triton X-100 and 10% DMSO, pH 10) at 4 °C for 1 h, the slides were treated with alkaline buffer (1 mM Na_2-EDTA and 300 mM NaOH, pH 13) for 20 min to allow denaturing and unwinding of the DNA, and then electrophoresed in the same buffer for 30 min. The solution was neutralized with 0.4 M Tris buffer (pH 7.5) and the slides dried at room temperature before being stained with aqueous ethidium bromide (20 μg/ml) and examined under a fluorescence microscope. Undamaged cells appeared as nucleoids and cells with damaged DNA appeared as comets.

2.5. Phase-contrast microscopy analysis and Hoechst 33258 and AO/EB staining

HO-8910 cells and the cells treated with 100 μM genistein were examined under a phase-contrast microscope with a CCD camera (Leica DMIRB, USA). Cells in the process of apoptosis showed significant morphological changes in nuclear chromatin, which could be detected by Hoechst 33258 staining and AO/EB staining. Briefly, after 3 days of treatment, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS, stained with 20 μg/ml Hoechst 33258 for 10 min, and observed under a Leica DMIRB fluorescence microscope. For AO/EB staining, HO-8910 cells and cells treated with 100 μM genistein were collected, suspended in PBS, stained with AO/EB solution (AO 100 μg/ml and EB 100 μg/ml in PBS) and visualized immediately by fluorescence microscopy.

2.6. Flow cytometric analysis

HO-8910 cells and cells treated with 100 μM genistein were harvested and fixed in 70% ethanol at 4 °C overnight. They were centrifuged and resuspended in PBS containing 0.1% Triton X-100, 200 ug/ml RNase A and 50 ug/ml propidium iodide, before being incubated for 30 min in the dark at 37 °C. The distribution of cell cycle phases was analyzed by flow cytometry, with $>10^4$ cells being counted in each sample.

2.7. Western blot

Western blot analysis was done as previously described (Song et al., 2008). HO-8910 cells were treated with 100 μM genistein for 2, 4, 6 and 8 h, or 100 ng/ml NCS for 1 h. The cells were harvested and lysed with a ice-cold lysis buffer for 15 min. Protein concentration of the lysates were determined Protein Assay Kit (Bio-Rad). The lysates (20 μg of protein per lane) were loaded and