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Genistein-induced apoptosis is mediated by endoplasmic reticulum stress in cervical cancer cells

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Abstract. – OBJECTIVE: Genistein, a major isoflavone found in soybeans, exhibits anti-cancer activity. Endoplasmic reticulum (ER) stress is known to be implicated in apoptosis induced by anti-cancer drugs. This study aimed to characterize the role of ER stress in genistein-induced apoptosis in cervical cancer.

MATERIALS AND METHODS: HeLa cells were treated with genistein or/and 4-phenylbutyric acid. Cell viability and apoptosis were evaluated by MTT assay and flow cytometry. Protein levels were detected by Western blot analysis.

RESULTS: Genistein suppressed the viability of HeLa cells in a dose dependent manner. In addition, genistein caused apoptosis in HeLa cells in a dose dependent manner. Genistein triggered ER stress in HeLa cells, as indicated by the upregulation of glucose-regulated protein 78 (GRP78) and CHOP expression. Furthermore, ER stress inhibitor 4-phenylbutyric acid alleviated genistein-induced apoptosis and ER stress in HeLa cells.

CONCLUSIONS: Our results suggest that ER stress contributes to genistein-induced apoptosis in cervical cancer cells, and genistein is a promising agent for cervical cancer therapy.

Key Words:
Genistein, Endoplasmic reticulum stress, Apoptosis, CHOP.

Introduction

Cervical cancer, a gynaecological malignant disorder, is a commonly diagnosed cancer and a leading cause of cancer death in the female worldwide[1]. The death rate of cervical cancer has declined significantly in recent decades, largely due to screening for the prevention and early detection[2]. However, the disease is normally detected at late stage due to inadequate screening. Moreover, invasive cervical cancer remains a serious problem in the clinical. Therefore, the development of effective therapies for cervical cancer is highly needed.

Genistein (5,7,4'-trihydroxyisoflavone), a multifunctional isoflavones, has shown anti-cancer potential[3]. Epidemiological study suggested that soybean products, the major source of dietary genistein, have the potential to reduce cancer risk[4,5]. Both *in vitro* and *in vivo* studies showed that genistein could inhibit the growth, survival, metastasis and angiogenesis, while induce the apoptosis of various cancer cells, which may be crucial for its anti-cancer action[6-9].

Accumulating evidence indicates that endoplasmic reticulum (ER) is involved in apoptotic signaling pathways[10]. In addition, the anticancer activity of many drugs has been linked to the induction of apoptosis via the activation of ER stress responses[11,12]. ER stress is caused by physiological and pathophysiological conditions, which disturb ER function with the accumulation of the misfolded proteins and alterations in the calcium homeostasis, leading to diverse diseases[13]. In response to such conditions, ER triggers unfolded protein response (UPR) that attempts to restore ER homeostasis. As a result, GRP78, also referred as the immunoglobulin binding protein (BiP), is released from the ER stress sensors IRE1α (inositol-requiring transmembrane kinase and endonuclease 1α), PERK (protein kinase-like ER kinase), and ATF6 (activation of transcription factor 6) to support protein folding[14]. However, excessive or prolonged ER stress may result in apoptosis. Yeh et al[15] reported that ER stress mediated genistein induced apoptosis in human hepatocellular carcinomas. However, it is unknown whether ER stress mediates genistein-induced apoptosis in cervical cancer. Therefore, in this

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study we aimed to investigate the role of ER stress in genistein-induced apoptosis in cervical cancer cells.

Materials and Methods

Cell Cluture and Treatment

Human cervical cancer HeLa cells were provied by Heilongjiang Cancer Institute, and maintained in RPMI 1640 medium at 37°C in 5% CO_2 atmosphere. HeLa cells were treated with genistein and 4-phenylbutyric acid (4-PBA) (all purchased from Sigma, St. Louis, MO, USA) when appropriate. Cells treated with dimethyl sulfoxide (DMSO) were used as the controls.

Cell Viability Assay

HeLa cells were plated at 4×10^3 cells/well in 96-well plates, and then the cells were exposed to genistein at 37°C for 48 h. Subsequently, 10 μl of 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml from Sigma) was added to each well and the cells were incubated at 37°C for additional 4 h. After removal of the culture medium, 100 μl DMSO was added to each well. In the end, the optical density (OD) at 490 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). The following formula was used: relative percentage of cell viability = (OD of the experimental sample/OD of the control group) ×100%.

Apoptosis Analysis by Annexin V-FITC Staining

Apoptosis was measured with Annexin V-FITC apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, HeLa cells were resuspended in 100 μl 1×binding buffer at a concentration of 1×10^6 cells/ml, then incubated with 5 μl Annexin V-FITC and 5 μl PI for 15 min at room temperature in the dark. Afterwards, the cells were analyzed by FACSC auto flow cytometer immediately. The data were analyzed with Cell Quest software.

Western Blot Analysis

HeLa cells were lysed in lysis buffer supplemented with protease inhibitor cocktails (Sigma). The supernatants were collected by centrifugation at 12,000 g for 5 min and the total protein concentration was determined by the bicinchoninic acid method. 30 μg proteins were loaded on a 10% SDS-polyacrylamide gel for each lane. After electrophoresis, the proteins were transferred to PVDF membranes (Roche Diagnostics, Mannheim, Germany), which were incubated with the antibodies against GRP78, caspase 3, cleaved caspase 3, PARP, cleaved PARP, CHOP, and β-actin (all purchased from Cell Signaling Technology, Beverly, MA, USA) for 2 h. Next, the membrabes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 h. Finally, the membranes were incubated with chemiluminescent reagent (EZ-ECL, Beit-Haemek, Israel) to detect immunopositive bands.

Statistical Analysis

All data were expressed as mean±SD and analyzed by one-way analysis of variance (ANOVA) followed by a LSD post hoc test. $p<0.05$ was considered as statistically significant.

Results

Genistein Decreased the Viability of HeLa Cells

To investigate the effect of genistein on the viability of cervical cancer cells, HeLa cells were treated with various concentrations of genistein for 48 h. MTT assay demonstrated that genistein significantly inhibited HeLa cell viability in a dose dependent manner (Figure 1).

Genistein Induced the Apoptosis of HeLa Cells

To determine whether genistein inhibits the viability of HeLa cells via the induction of apoptosis, HeLa cells were treated with various concentrations of genistein for 48 h and apoptotic cells were detected by flow cytometry. We found that genistein induced apoptosis in HeLa cells in a dose-dependent manner (Figure 2A). Moreover, genistein increased the levels of cleaved caspase 3 and cleaved PARP in HeLa cells in a dose-dependent manner (Figure 2B). These results indicate that genistein induces the apoptosis of HeLa cells.

Genistein Induced ER Stress in HeLa Cells

ER stress has been implicated in genistein incuced apoptosis in human hepatocellular carcinomas[15]. Next, we detected the effect of genistein on GRP78, a molecular marker of ER stress, and C/EBP homologous protein (CHOP), a transcrip-

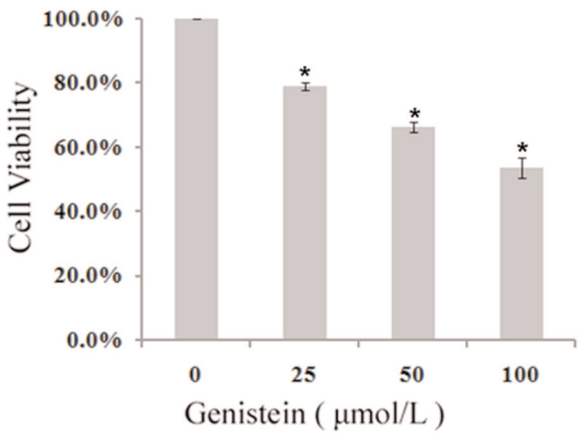

Figure 1. Effect of genistein on HeLa cell viability. HeLa cells were treated with genistein for 48 h, and then the cell viability was determined by MTT assay. Data were expressed as mean ± SD for 3 independent experiments in which each treatment was repeated in 5 wells. $^{*}p<0.05$ *vs.* control.

tion factor involved in ER stress-induced apoptosis. Western blot analysis showed that genistein increased the protein levels of GRP78 and CHOP in a dose-dependent manner in HeLa cells (Figure 3). These data suggest that genistein could trigger ER stress in HeLa cells.

ER Stress Contributed to Genistein-induced Apoptosis in HeLa Cells

To confirm the role of ER stress in apoptosis induced by genistein, HeLa cells were pre-treated with 15 μmol/L 4-PBA followed by treatment with 100 μM genistein. As shown in Figure 4A, genistein-induced upregulation of GRP78 and CHOP was significantly inhibited by PBA (Figure 4A). In addition, 4-PBA significantly attenuated genistein induced PARP cleavage in HeLa cells (Figure 4A). Furthermore, 4-PBA inhibited genistein induced apoptosis in HeLa cells (Figure 4B). Collectively, these results indicate that PBA could reduce genistein-induced ER stress and apoptosis. suggesting that activated ER stress is closely related to genistein-induced apoptosis in HeLa cells.

Discussion

A wide variety of plant-derived bioactive agents have shown anti-tumor activity and been developed as potential chemo-preventive and chemotherapeutic agents for various cancers[16,17]. Among these agents, genistein has attracted considerable attention due to its beneficial activities to inhibit tumor development[18,19]. The inhibitory effect of genistein on cervical cancer has been demonstrated[20]. Moreover, genistein enhanced the radiosensitivity and chemosensitivity of cervical cancer cells[21,22]. However, the detailed mechanisms by which genistein exhibits anti-cancer effects in cervical cancer are poorly understood. In this study,

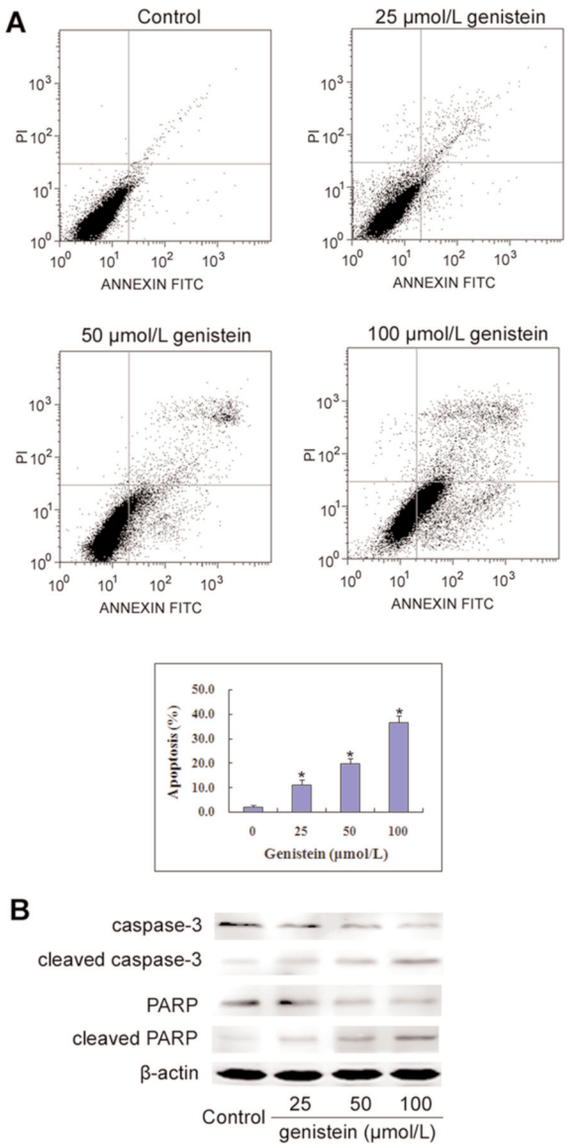

Figure 2. Effect of genistein on HeLa cell apoptosis. ***A,*** HeLa cells were treated with the indicated concentrations of genistein for 48 h and then the percentage of apoptotic cells was determined by flow cytometry. Data were expressed as mean ± SD for 3 independent experiments. $^{*}p<0.05$ vs. control. ***B,*** The cleavage of caspase-3 and PARP was detected by Western blot analysis. Shown were representative blots from three independent experiments with similar results. β-actin served as loading control.

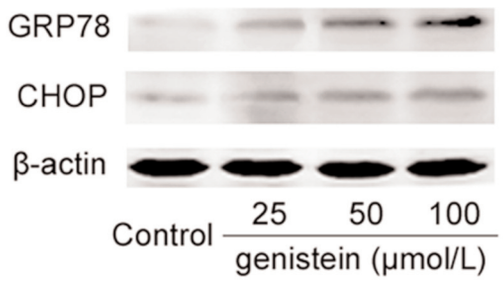

Figure 3. Genistein increased the expression of GRP78 and CHOP. HeLa cells were incubated with genistein for 48 h and then the levels of GRP78 and CHOP were analyzed by Western blot analysis. Shown were representative blots from three independent experiments with similar results. β-actin served as loading control.

for the first time we demonstrated that genistein-induced apoptosis was mediated by activating ER stress in cervical cancer cells.

The anti-cancer activity of anticancer agents is frequently associated with their capability to trigger the apoptosis of tumor cells[23]. Previous studies demonstrated that genistein induced the apoptosis of many tumor cell lines[6,8,9]. In agreement with these studies, we found that genistein induced the apoptosis of HeLa cells in a dose dependent manner. Moreover, we showed that genistein increased the amounts of cleaved caspase-3 and cleaved PARP, providing further evidence that genistein has the capability to induce apoptosis in HeLa cells.

ER is the principal site for protein synthesis and folding, lipid biosynthesis and calcium storage, and plays crucial roles in the maintenance of cell homeostasis and survival[24]. However, excessive or prolonged ER stress can trigger apoptosis and ultimately terminate damaged cells[24]. ER stress is involved in genistein-incuced apoptosis in human hepatocellular carcinomas[15]. Here we investigated whether ER stress plays a role in genisten-induced apoptosis of cervical cancer cells. We concluded that genisten-induced apoptosis was mediated, at least in part, by genistein-induced ER stress in HeLa cells, based on the following evidences: (1) genistein significantly elevated the expression of GRP78, a molecular marker of ER stress; (2) genisten strongly induced the expression of CHOP, an important mediator of ER stress-induced apoptosis[25]; (3) ER stress inhibitor 4-PBA effectively decreased genisten-induced GRP78 and CHOP expression; (4) 4-PBA attenuated genisten-induced apoptosis in HeLa cells.

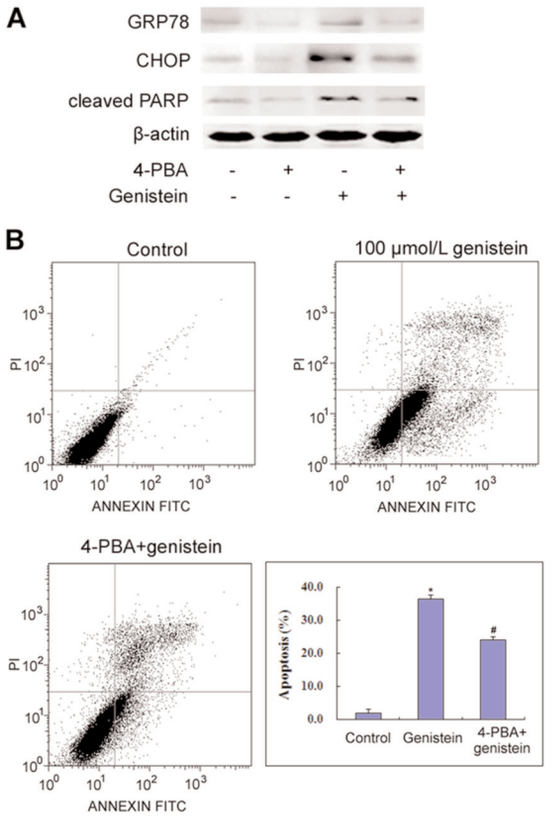

Figure 4. PBA attenuated genistein induced HeLa cell apoptosis. HeLa cells were pre-treated with PBA (15 μmol/L) for 24 h and then treated with or without 100 μmol/L genistein for 48 h. ***A***, The levels of GRP78, CHOP, and cleaved PARP were analyzed by Western blot analysis. Shown were representative blots from three independent experiments with similar results. β-actin served as loading control. ***B***, Apoptotic cells were detected by flow cytometry. Data were expressed as mean ± SD for 3 independent experiments. $^{*}p<0.05$ *vs.* control, $^{\#}p<0.05$ *vs.* cells treated with genistein.

Conclusions

ER stress contributes to genistein-induced apoptosis in cervical cancer cells. Pharmacological agents such as genistein that effectively augment cancer cell apoptosis via the activation of ER stress would show promise for anti-cancer therapy.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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