Genipin, a constituent of Gardenia jasminoides Ellis, induces apoptosis and inhibits invasion in MDA-MB-231 breast cancer cells

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Abstract. Genipin, a constituent of Gardenia jasminoides Ellis, is used in the treatment of hepatic disorders and inflammatory diseases in traditional medicine. Although mounting evidence suggests an anti-tumor activity of genipin in several cancer cell systems, the inhibitory effect of genipin on the growth of breast cancer cells has not been reported yet. The present study aimed to investigate the anti-proliferative activity of genipin in MDA-MB-231 human breast cancer cells. Herein, we showed that genipin efficiently induced apoptosis in MDA-MB-231 cells by the down-regulation of Bcl-2, up-regulation of Bax and proteolytic activation of caspase-3. Activation of JNK and p38 MAPK also increased by genipin. Importantly, genipin significantly inhibited invasive and migratory phenotypes of MDA-MB-231 cells. Taken together, this study demonstrates that genipin induces apoptosis and inhibits invasive/migratory abilities of highly invasive MDA-MB-231 human breast cancer cells, suggesting a potential application of genipin as a chemopreventive agent that may prevent or alleviate metastatic breast cancer.

Introduction

Genipin, an aglycone derived from an iridoid glycoside called geniposide present in fruit of Gardenia jasminoides Ellis, has long been used as a traditional oriental medicine for the treatment of hepatic disorders and inflammatory diseases (1). Genipin exerts anti-inflammatory, anti-angiogenic, anti-proliferative and hepatoprotective activities in several cell lines including murine macrophage cells, human umbilical vein endothelial cells, human prostate cancer cells, and human hepatocarcinoma cells (2-5). Genipin has been shown to inhibit growth of human leukemia and prostate cancer cells (5,6). However, anti-proliferative activity of genipin in breast cancer cells is poorly understood. The present study aimed to investigate the inhibitory effect of genipin on the growth of MDA-MB-231 human breast cancer cells.

Materials and methods

Reagents. Genipin, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Sigma-Aldrich Inc., MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA).

Cell lines. MDA-MB-231 cells were cultured, as previously described (12). Cells were cultured in DMEM supplemented with 10% FBS and 100 U/ml of penicillin-streptomycin.

MTT assay. MDA-MB-231 cells (3x10^4 cells/well) cultured in a 96 well-plate were treated with various concentrations of genipin (0, 50, 100, 500, 1000 µM) for 24 h. Control cells were treated with dimethyl sulphoxide (DMSO) equal to the highest percentage of solvent used under the experimental conditions. Briefly, 25 mg/ml of 0.5% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to the media and the cells were further incubated for 4 h. After the supernatant (100 µl) was replaced with the same volume of DMSO, absorbance was measured at 540 nm with an ELISA reader (EL 800, Bio-Tek Instruments Inc., Winooski, VT). The percentage

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of surviving cells was defined as the relative absorbance of treated versus untreated cells.

**Immunoblot analysis.** Immunoblot analysis was performed as described previously (13). Anti-p38 MAPK, anti-phospho-p38 MAPK, anti-phospho-ERK1/2, anti-ERK1/2, anti-JNK, and phospho-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Bcl2, anti-Caspase 3, anti-Bax antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-β-actin antibody was purchased from Cell Signaling Technology.

**In vitro invasion assays.** In vitro invasion assay was performed using 24-well Transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA). The lower side of the filter was coated with type I collagen, and the upper side was coated with Matrigel (Collaborative Research, Lexington, KY). Cells were placed in the upper part of the Transwell plate, incubated for 17 h, fixed with methanol, and stained with hematoxylin for 10 min followed briefly by eosin. The invasive phenotypes were determined by counting cells that migrated to the lower side of the filter under microscopy at x400. Thirteen fields were counted for each filter, and each sample was assayed in triplicate.

**In vitro migration assay using Transwell.** An in vitro migration assay was performed using a 24-well Transwell unit with polycarbonate filters, as previously described (13).

**Flow cytometric analysis.** For flow cytometry, MDA-MB-231 cells were grown in six-well plates and incubated for 24 h, and then treated with 200 µM genipin. After 24 h, cells were harvested and washed twice with PBS. Cells (1x10^5) were double stained with FITC-conjugated annexin V and propidium iodide for 15 min at room temperature in 1X binding, and then analyzed using a flow cytometer (Beckman Coulter, Fullerton, CA).

**Results**

**Genipin inhibits cell growth and induces apoptosis in MDA-MB-231 cells.** In order to investigate the effects of genipin on the growth of MDA-MB-231 breast cancer cells were treated with various concentrations of genipin. As shown in Fig. 1A, treatment of cells with genipin for 24 h inhibited growth of MDA-MB-231 cells in a dose-dependent manner, with an IC_{50} value of 327 µM. To investigate whether the genipin-induced growth inhibition involves apoptosis, FACS analysis was conducted. Treatment with 200 µM genipin for 24 h resulted in a marked increase in annexin-positive apoptotic cells (11.14%) compared with the control cells (4.6%), demonstrating that genipin induced apoptosis of MDA-MB-231 cells (Fig. 1B). These results imply that the observed growth inhibitory effect of genipin may be due to the induction of apoptotic cell death.

**Genipin regulates apoptosis-related proteins.** To evaluate the molecular mechanisms underlying the genipin-induced
apoptosis of MDA-MB-231 cells, we determined the protein levels of two key apoptosis-linked gene products, Bcl-2 and Bax. Cells were treated with various concentrations of genipin for 24 h. Expression of the anti-apoptotic oncoprotein Bcl-2 was decreased, whereas the death-promoting Bax expression was increased in MDA-MB-231 cells treated with genipin in a dose-dependent manner (Fig. 2A, left and center). These data demonstrate that genipin induced apoptosis by down-regulation of an anti-apoptotic protein Bcl-2 and up-regulation of a pro-apoptotic protein Bax in MDA-MB-231 cells.

Caspases are crucial mediators of apoptosis that depend on proteolytic activation of their pro-caspase forms to enzymatically active forms (14). The level of pro-caspase-3 was dose-dependently decreased by genipin treatment, suggesting that genipin induced the activation of caspase-3 in MDA-MB-231 cells (Fig. 2A, right). We then performed a kinetic study to examine the effect of genipin on apoptosis-related proteins. As shown in Fig. 2B (left), treatment with 200 µM genipin caused a time-dependent decrease of Bcl-2 expression. In contrast, the expression level of Bax was increased 3 h after genipin treatment and decreased back to the basal level after 24 h. Pro-caspase-3 levels were decreased in a time-dependent manner, suggesting that genipin may enhance the proteolytic activation of caspase-3 (Fig. 2B, right). These results imply that genipin-induced apoptosis may be mediated by Bcl-2, Bax, and caspase-3 in MDA-MB-231 cells.

Genipin activates p38 MAPK and JNK in MDA-MB-231 cells. Activation of mitogen-activated protein kinases (MAPKs) are important intermediates in the signal-transduction pathway regulating cell proliferation and apoptosis (15-18). Two members of the MAPK superfamily, JNK and p38 MAPK, were shown to be activated in the apoptotic responses of cells (6,19,20). To examine the effect of genipin on the activation of these signaling molecules, MDA-MB-231 cells were treated with various concentrations of genipin. As shown in Fig. 3A, activation of p38 MAPK was increased by genipin treatment in a dose-dependent manner. Phosphorylated JNK was markedly increased by 50 µM genipin and decreased back to the basal level at 200 µM genipin. In contrast, activation of ERKs was not increased by the same treatment. A kinetic study showed that p38 MAPK and JNK were activated by genipin in a time-dependent manner, while phosphorylated ERKs were not affected (Fig. 3B).

Genipin inhibits invasion and migration of MDA-MB-231 cells. To investigate the effects of genipin on invasion and migration of MDA-MB-231 cells, we performed in vitro invasion and migration assays. As shown in Fig. 4, invasive and migratory abilities of MDA-MB-231 cells were significantly inhibited by genipin treatment in a dose-dependent manner. The results clearly demonstrate that genipin effectively inhibited the invasive and migratory phenotype of breast cancer cells.
Cancer chemoprevention using natural products that inhibit the development of invasive cancer has emerged as a powerful strategy against cancer (21,22). Bioactive compounds and phytochemicals from plants have been shown to suppress early and late stages of carcinogenesis and can consequently inhibit proliferation of malignant cancer cells (23-25). In the present study, we showed that genipin efficiently inhibited the growth of MDA-MB-231 human breast cancer cells. Given that *G. jasminoides* has been widely used as an oriental medicine for many years (26), any potential adverse effect for anti-cancer agents would not be significant. Our findings suggest a potential role of genipin in regulation of proliferation, invasion and migration of highly invasive MDA-MB-231 human breast cancer cells.

Since apoptosis is arguably the most potent natural defense against cancer (7), efforts have been made to develop strategies that trigger apoptosis in malignant cancer cells. Apoptotic cell death plays an important role in breast cancer. The level of Bcl-2 expression correlates with breast cancer both *in vitro* and *in vivo* (27,28). Our study demonstrates that genipin induces apoptosis in human breast cancer cells with a prominent decrease of Bcl-2, preventing apoptosis by blocking the release of cytochrome C from mitochondria (29). Bax, which directly induces cytochrome C release from mitochondria and consequently triggers caspase activation (30), was increased by genipin treatment. Consistent with these reports, our results suggest that genipin induces apoptosis in human breast cancer cells with a prominent decrease of Bcl-2, preventing apoptosis by blocking the release of cytochrome C from mitochondria (29). Bax, which directly induces cytochrome C release from mitochondria and consequently triggers caspase activation (30), was increased by genipin treatment. Consistent with these reports, our results suggest that genipin may proteolytically activate pro-caspase-3. These results suggest that genipin-induced apoptosis of breast cancer cells is mediated by Bcl-2 and Bax.

Signaling mediated by MAPKs is involved in the regulation of cell apoptosis (15). Among the MAPK pathways, JNK and...
p38 MAPK pathways are generally activated by stress agents, and implicated as key regulators of stress-induced apoptosis (6,31). Apoptosis induced by chemotherapeutic drugs was mediated by the p38 MAPK-caspase signaling pathway in human pancreatic cancer cells (32). Consistent with these reports, our results demonstrated that genipin activated p38 MAPK and JNK, but not ERKs, in MDA-MB-231 cells, suggesting the involvement of p38 MAPK and JNK in genipin-induced apoptosis in human breast cancer cells. The effect of genipin on the activation of MAPK family members varies in different cell systems. Genipin was shown to induce apoptosis through the activation of JNK, but not p38 MAPK, in hepatocarcinoma and prostate cancer cells (3,5). Genipin also induced the activation of JNK in rat hepatoma and HeLa cells (3,33). Further investigation on the association of signaling pathways with apoptotic cell death induced by genipin needs to be performed.

Breast cancer is considered the most commonly diagnosed type of cancer and the second most common cause of cancer-related death among women (34). Metastasis, a characteristic of highly malignant cancers with poor clinical outcome, is one of the major causes of mortality in breast cancer patients. Therefore, anti-tumor agents that may inhibit invasion and migration of breast cancer cells have been extensively pursued in many laboratories, including ours (35,36). Although many studies have reported the anti-tumor activity of genipin in various cancer cells, there is a limited amount of information on the anti-invasive activity of genipin. The present study clearly demonstrated that genipin inhibits invasion and migration of the highly invasive MDA-MB-231 human breast cancer cell line in a dose-dependent manner.

Taken together, the present study showed that genipin inhibits cell growth and the invasive/migratory phenotypes of human breast cancer cells. Given that metastatic breast cancer is one of the most lethal malignancies in women, our novel findings suggest a potential application of genipin as a chemopreventive agent for breast cancer patients.

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