Genistein Potentiates the Antitumor Effect of 5-Fluorouracil by Inducing Apoptosis and Autophagy in Human Pancreatic Cancer Cells

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Abstract. Background: Although 5-fluorouracil (5-FU)-based combination chemotherapy (i.e. FOLFIRINOX) has demonstrated effectiveness against pancreatic cancer, novel therapeutic strategies must be developed to increase the therapeutic window of these cytotoxic agents. Genistein is a soy-derived isoflavone with pleiotropic biological effects that can enhance the antitumor effect of chemotherapeutic agents. Materials and Methods: To understand how genistein potentiates the antitumor effects of 5-FU, we examined apoptosis and autophagy in MIA PaCa-2 human pancreatic cancer cells and their derived xenografts. Apoptosis was evaluated using DNA fragmentation assays, and western blots of poly(ADP ribose)polymerase and caspase-3. Meanwhile, autophagy was evaluated using western blots of microtubule-associated protein light chain 3 (LC3)-I/II, fluorescent microscopy observation of green fluorescent protein-LC3B puncta formation, and acidic vesicular organelle formation using acridine orange staining. Tumors from animal treatment studies were examined for apoptosis and autophagy using the TdT-mediated dUTP nick-end labeling assay and immunohistochemical staining of LC3B, respectively. Results: We observed that genistein increased 5-FU-induced cell death through increased apoptosis, as well as autophagy. The increased autophagy was accompanied by decreased B-cell lymphoma 2 (Bcl2) and increased beclin-1 protein levels. Animal treatment studies supported these observations. The combination of 5-FU and genistein significantly reduced final xenograft tumor volume when compared to 5-FU-alone by inducing apoptosis as well as autophagy. Conclusion: Genistein can potentiate the antitumor effect of 5-FU by inducing apoptotic as well as autophagic cell death. These results demonstrate the potential of genistein as an adjuvant therapeutic agent against pancreatic cancer.

Pancreatic ductal adenocarcinoma (PDAC) remains an unsolved healthcare dilemma in the United States and the world (24). One of the primary treatments of PDAC is chemotherapy because more than 80% of patients present with unresectable or metastatic disease (23). Recently, FOLFIRINOX, the combination of 5-fluorouracil (5-FU), leucovorin calcium, oxaliplatin, and irinotecan, has been applied in clinical settings as a first-line treatment for metastatic PDAC (6). While the antitumor effect of this regimen is promising, treatment-related toxicity remains a concern. In the ACCORD II study, researchers found that severe neutropenia and thrombocytopenia events were significantly higher with FOLFIRINOX when compared to gemcitabine-alone (45.7 vs. 18.7%, 9.1 vs. 2.4%, respectively) (6). 5-FU is one of the principle agents responsible for both the therapeutic effect and the toxicity of the regimen (7, 21). Therefore, novel methods are required to enhance drug efficacy of 5-FU while limiting further toxicity.

Genistein, a soy-derived isoflavone, exhibits multiple biological effects against various types of cancer when studied using in vitro and in vivo models (2, 3, 13, 14, 19). Several studies found that genistein can potentiate the antitumor effects of chemotherapeutic agents (e.g. gemcitabine, cisplatin and oxaliplatin) by modulating the apoptotic pathway (1-3). Furthermore, recent studies demonstrate that genistein stimulates autophagy (11, 18). Autophagy is a degradation process in which cytosolic proteins and organelles are sequestered into autophagosomes and degraded by lysosomes (15). Traditionally, autophagy has been considered to be a survival response during stressful conditions by which cancerous cells avoid apoptotic death through lysosomal degradation of damaged proteins.
Recent evidence, however, suggests that autophagy may also promote cell death through unintended degradation of essential cellular components and excessive self-digestion (17, 22). There is little data, however, on the influence of genistein on 5-FU-based treatment of pancreatic cancer cells.

In the present report, we describe how genistein modulates 5-FU-induced apoptosis and autophagy in human pancreatic cancer cells. Our results suggest that genistein potentiates the anticancer effects of 5-FU by promoting both apoptotic and autophagic cell death.

Materials and Methods

Cell lines and reagents. The MIA PaCa-2 human pancreatic cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated in an incubator at 37˚C with 5% CO2.

Genistein, 5-FU, acridine orange, thiazolyl blue tetrazolium bromide (MTT), and an antibody against β-actin (used as protein loading control) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroquine was purchased from Invitrogen (Grand Island, NY, USA) and z-VAD-fmk (a pan-caspase inhibitor) from Abcam (Cambridge, MA, USA). Antibodies against B-cell lymphoma (Bcl2), poly(ADP-ribose) polymerase (PARP), caspase-3, microtubule-associated protein light chain 3 (LC3B), and beclin-1 were purchased from Cell Signaling Technology (Boston, MA, USA).

MTT assay for cell proliferation. Cell viability was evaluated using the MTT assay, as described previously (5). After treatment with 5-FU, genistein, z-VAD-fmk and chloroquine, MTT was added to each well, and the optical density (OD) of each well was measured at 570 nm using a microplate reader (FLUOstar Omega, Cary, NC, USA). The OD570 in untreated cells was taken as 100% viability. Each experiment was performed in triplicate.

Detection of acidic vesicular organelles. Acridine orange staining was performed to detect acidic vesicular organelles as described previously (16). Briefly, cells were seeded onto eight-well chamber slides (2×104 cells/well) and incubated for 24 h. After treatment, cells were stained with acridine orange (1 μg/ml) for 15 min at 37˚C. Cells were washed with PBS and evaluated under a fluorescence microscope.

Apoptosis detection. After treatment, DNA fragmentation in cell lysates was analyzed by the Cell Death Detection ELISA kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s instructions. Absorbance was measured at 405 nm using a plate reader. The apoptotic index was calculated as an enrichment factor.
that is, the ratio of the result compared with the no-treatment control set arbitrarily at 1.0. Each experiment was performed in triplicate.

In vivo human pancreatic cancer xenograft study. We performed an animal experiment using MIA PaCa-2 human pancreatic cancer cells in a subcutaneous xenograft model. MIA PaCa-2 cells were resuspended in sterile 50% growth factor–reduced Matrigel (BD Biosciences, Bedford, MA, USA) in PBS. Cells (2×10⁶ cells/100 μl) were injected subcutaneously into the right flank of female nude mice aged 4-6 weeks (MD Anderson Cancer Center ERO, Houston, TX, USA). When the estimated tumor volume reached 100 mm³, mice were randomly assigned to four treatment groups (four mice per group): no treatment, genistein, 5-FU, and the combination of 5-FU and genistein. In treatment groups, mice were given 5-FU (60 mg/kg, intraperitoneally) and/or genistein (1.3 mg, intraperitoneally) every four days for 21 days. Mice were sacrificed 29 days after initiation of treatment, and the final tumor volume was calculated using the formula 0.5 × (length) × (width) × (thickness) of tumor.

All animal experiments were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and were conducted in accordance with institutional and national regulations (IACUC protocol # 01-11-00133).

Immunohistochemical staining and TdT-mediated dUTP nick end labeling assay (TUNEL) assay. Tissues from subcutaneous xenograft tumors were embedded in paraffin and then cut into 4-μm-thick sections at the clinical core laboratory of MD Anderson. Apoptosis was determined by TUNEL assay with an ApopTag peroxidase in situ apoptosis detection kit (Chemicon Int., Temecula, CA, USA) according to the manufacturer’s instruction. Three microscopic fields at ×400 magnification were evaluated from each section. The number of stained cells per visual field were recorded. Immunohistochemical staining for LC3B was performed using a Lab-Vision 480-2D immunostainer (Thermo Fisher Scientific, Fremont, CA, USA). All images were captured using an Olympus DP72 camera and its CellSens software through an Olympus BX51 microscope at a magnification of ×400 (Olympus America Inc.).

Statistical analysis. Data are presented as mean±standard deviation (SD). Statistical analyses were performed by using analysis of variance or Student’s two-tailed t-test using GraphPad Prism 6.0 software (GraphPad software Inc., San Diego, CA, USA). Differences with p<0.05 were considered statistically significant.

Results

Genistein enhances the antitumor effect of 5-FU in vitro. MIA PaCa-2 human pancreatic cancer cells were exposed to 5-FU (100 μM), genistein (100 μM), alone or in combination, for 72 h. The combination of 5-FU and
Figure 3. Induction of cytotoxic autophagy with the combination of 5-fluorouracil (5-FU) and genistein. a: Expression of microtubule-associated protein light chain 3 (LC3), beclin-1, and B-cell lymphoma 2 (Bcl2) after treatment was detected by western blot assay. β-Actin was used as the loading control. b: Cells were transiently transfected with green fluorescent protein-LC3 and treated with 5-FU, genistein, or both. Images were captured using fluorescence microscopy (magnification, ×200). c: After treatment with the indicated concentrations of 5-FU, genistein, or their combination, cells were stained with acridine orange (1 μg/ml) for 15 min at 37˚C, and images were captured using fluorescence microscopy (magnification, ×200). d: Cells were treated with 10 μM or 20 μM chloroquine (CQ) for 72 h. Cell viability was detected by the MTT assay, and the optical density at 570 nm in untreated control cells was taken as 100% viability. e: The change in expression of LC3 was detected by western blot assay. f: Cells were treated with the combination of 5-FU and genistein with or without 10 μM CQ. Cell viability was detected by the MTT assay. *p<0.05; n.s.=not significant.
Genistein resulted in a significant induction of cell death compared to the use of genistein or 5-FU alone (Figure 1a). We also observed an increase in apoptosis of MIA PaCa-2 cells after similar treatments. Relative to single agents, their combination significantly increased apoptosis as shown in the DNA fragmentation ELISA assay (Figure 1b). These results suggest that the increase in cell death induced when genistein was combined with 5-FU was due, in part, to a higher rate of apoptosis.

Genistein enhances 5-FU-induced apoptosis. To better understand the 5-FU-induced apoptosis, we treated MIA PaCa-2 cells with 5-FU (100 μM), genistein (100 μM), or their combination for 72 h. Western blot detected increased expression of cleaved PARP and caspase-3 in the cells treated with the combination of 5-FU and genistein compared to cells treated with 5-FU-alone (Figure 2a). To confirm whether apoptotic cell death was the main cause of overall cell death induced by the combination, we treated
MIA PaCa-2 cells with the combination plus the pan-caspase inhibitor z-VAD-fmk (10 μM). This concentration of z-VAD-fmk (10 μM) had a minimum cytotoxic effect when used alone but was sufficient to inhibit the combination treatment-induced apoptosis (Figure 2b-2d). The addition of z-VAD-fmk prevented 47.1% of the cytotoxic effect of the treatment (75.1% vs. 28% cytotoxicity), but it did not prevent 28% of the cytotoxic effect of the combination treatment (Figure 2b). These results suggested that the combination of 5-FU and genistein resulted in cell death through apoptosis as well as another mechanism. Based upon previous studies (4), we hypothesized that autophagy was the method of non-apoptotic cell death in cells exposed to 5-FU and genistein.

Figure 5. Induction of apoptosis and autophagy in a subcutaneous pancreatic tumor xenograft model. a: TdT-mediated dUTP nick end labeling assay using the ApopTag peroxidase in situ apoptosis detection kit and immunohistochemical staining with antibody to microtubule-associated protein light chain 3B (LC3B) were performed to show induction of apoptosis and autophagy. b: Numbers of TUNEL-positive cells per visual field were recorded at ×400 magnification in three microscopic fields. c: Apoptosis represented by DNA fragmentation was detected using tumor lysate. Each experiment was performed in triplicate and data are presented as mean±standard deviation. *p<0.05.
Genistein enhances 5-FU-induced autophagy. To investigate the role of autophagy, we exposed MIA PaCa-2 cells to 5-FU and genistein and examined the autophagy-related proteins, LC3-II and beclin-1. Western blots detected an increase in expression of both LC3-II and beclin-1 (Figure 3a), which suggests autophagy. GFP-LC3 puncta and acidic vesicular organelle formation, a marker of autophagy, were increased after exposure to 5-FU and to 5-FU combined with genistein (Figure 3b and c). Together, these results demonstrate that genistein increased 5-FU-induced autophagy. As a next step, we investigated whether the enhanced autophagy after exposure was cytoprotective or cytotoxic. After first determining that 10 μM of the autophagy inhibitor chloroquine had minimal cytotoxic effects when used alone (Figure 3d), we found that pancreatic cancer cells exposed to this concentration of chloroquine accumulated LC3-II, which indicated successful inhibition of autophagy (Figure 3e). MTT assay was then performed after cells were exposed for 72 h to 5-FU alone or in combination with genistein in the presence or absence of 10 μM chloroquine. This assay demonstrated that the cytotoxic effect of 5-FU alone was increased in the presence of chloroquine, which suggests that 5-FU-induced autophagy was cytoprotective. Conversely, chloroquine reduced the cytotoxic effect of the 5-FU and genistein combination from 76% to 57% (Figure 3f). This important finding indicates that autophagy induced after cells were exposed to the combination of 5-FU and genistein was cytotoxic rather than cytoprotective.

Genistein potentiates in vivo therapeutic effect of 5-FU. To further test for genistein potentiation of the anticancer properties of 5-FU, we performed an animal experiment using the same cell line in a murine xenograft model (Figure 4a). The growth-inhibitory effects of 5-FU were significantly enhanced when combined with genistein, with significantly smaller tumors in this group compared to 5-FU or genistein alone (Figure 4b and c). Analysis of tumor lysates using TUNEL and DNA fragmentation assays demonstrated a significant increase in 5-FU-induced apoptosis when combined with genistein (Figure 5a-c). Additionally, immunohistochemical staining demonstrated a qualitative increase in LC3B expression when 5-FU treatment was combined with genistein, which was consistent with our in vitro data.

Discussion

In the current study, in vitro and in vivo experiments both demonstrated that the combination of 5-FU and genistein had superior antitumor effects against human pancreatic cancer cells when compared with both 5-FU and genistein alone. Although genistein itself did not induce apoptosis and autophagy, it significantly enhanced 5-FU-induced apoptosis and autophagy, as evidenced by the associated decrease in Bcl2 and increase in beclin-1 protein levels. Experiments using chloroquine to inhibit autophagy support the concept that genistein enhanced 5-FU-induced autophagy and caused autophagic cell death. To our knowledge, this is the first study to have shown that genistein can enhance 5-FU-induced autophagy and trigger autophagic cell death in human pancreatic cancer cells.

We demonstrated that the combination of 5-FU and genistein induced autophagic cell death by significantly altering the expression of two important molecules, Bcl2 and beclin-1, which regulate autophagy. Bcl2 is an anti-apoptotic protein overexpressed in various types of cancer, and it contributes to treatment resistance by inhibiting chemotherapy-induced apoptosis (25). Bcl2 is also involved in autophagy inhibition by binding to the BH3 domain of beclin-1 and negatively-regulating the autophagy-promoting beclin-1– vacuolar protein sorting 34 complex (20). It has been reported that mutant proteins of beclin-1 that do not bind to Bcl2 can trigger excessive cellular autophagy and death in the absence of autophagic stimuli (20). Therefore, the down-regulation of Bcl2 and up-regulation of beclin-1 shown here by the combination of 5-FU and genistein provides a reasonable mechanistic explanation of the observed autophagic cell death induction. As shown in the current study, both cytoprotective and cytotoxic autophagy could occur in the same experimental system in response to treatment, and cytoprotective autophagy induced by 5-FU can be converted to cytotoxic autophagy by the addition of genistein. This is similar to what Bristol et al. observed in breast cancer cell lines (4). The spectrum of autophagy in cancer cells is now recognized to include cytoprotective, cytostatic, cytotoxic, and non-protective types, but the molecular mechanisms driving each type are relatively unexplored (10).

In summary, we found that genistein potentiates the antitumor effect of 5-FU by inducing apoptotic, as well as autophagic cell death in human pancreatic cancer cells. These results demonstrate the potential of genistein as an additional therapeutic agent. Future studies will focus on defining the molecular events after tumors are exposed to low-toxicity agents such as genistein in combination with cytotoxic therapies.

Conflicts of Interest

Authors declare no conflict of interests for this article.

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