Molecular Evidence for Increased Antitumor Activity of Gemcitabine by Genistein In vitro and In vivo Using an Orthotopic Model of Pancreatic Cancer


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Abstract

Soy isoflavone genistein exhibits growth inhibitory activity against human pancreatic cancer cell lines. We previously reported the potential of genistein to augment chemotherapeutic response of pancreatic cancer cells in vitro. In the present study, we investigated whether genistein pretreatment could be used as a novel strategy for gemcitabine-induced killing in vitro and enhanced antitumor activity in vivo using an orthotopic tumor model. We conducted our studies using paired isogenic human pancreatic cancer cell line with differences in metastatic behavior (COLO 357 and L3.6pl). In vitro studies were done to measure growth inhibition and degree of apoptotic cell death induced by either genistein alone, gemcitabine alone, or genistein followed by gemcitabine. Our results show that pretreatment of cells with genistein for 24 hours followed by gemcitabine resulted in 60% to 80% growth inhibition compared with 25% to 30% when gemcitabine was used alone. The overall growth inhibition was directly correlated with apoptotic cell death irrespective of the metastatic potential of cells. Several genes that are known to inhibit apoptosis and contribute to chemoresistance such as nuclear factor-κB (NF-κB) and Akt were assessed to investigate the basis for the observed chemosensitizing effects of genistein. Genistein potentiated the gemcitabine-induced killing by down-regulation of NF-κB and Akt. In contrast, Akt and NF-κB were found to be up-regulated when pancreatic cancer cells were exposed to gemcitabine alone, suggesting the potential mechanism of acquired chemoresistance. In addition to in vitro results, we show here for the first time, that genistein in combination with gemcitabine is much more effective as an antitumor agent compared with either agent alone in our orthotopic tumor model. But most importantly, our data also showed that a specific target, such as NF-κB, was inactivated in genistein-treated animal tumors and that gemcitabine-induced activation of NF-κB was completely inhibited in animal tumors treated with genistein and gemcitabine. These results provide strong molecular in vivo evidence in support of our hypothesis that inactivation of NF-κB signaling pathway by genistein could also abrogate gemcitabine-induced activation of NF-κB resulting in the chemosensitization of pancreatic tumors to gemcitabine, which is likely to be an important and novel strategy for the treatment of pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma is a highly aggressive malignant disease, which is currently treated with limited success and dismal outcomes using conventional therapeutics including chemotherapy and irradiation. Currently, it is ranked as the fourth most common cause of cancer related mortality in the United States and other industrialized countries (1). Despite therapeutic advances, the prognosis of patients with pancreatic ductal adenocarcinoma is extremely poor, with a median survival of 6 months (2). In recent years, novel strategies for sensitizing pancreatic tumor cells with naturally occurring dietary chemopreventive compounds have gained considerable attention because of their beneficial effects in overcoming intrinsic tumor cell resistance to apoptosis (3). We previously reported that genistein augments therapeutic efficacy of cytotoxic chemotherapeutic agents (4). As a corollary to our previous study, we report herein the potential of genistein to augment gemcitabine-based chemotherapy for pancreatic cancer. Furthermore, to rule out the possibility of pancreatic adenocarcinoma cell specificity, we extended our studies using paired isogenic pancreatic cancer cell lines (COLO 357 and L3.6pl) differing in their metastatic behavior. Genistein (4',5,7-trihydroxyisoflavone) is an isoflavone having a heterocyclic diphenolic structure similar to estrogen and is present in dietary items such as soy (5). Genistein has been reported to exert potent antitumor activity in a variety of human cancer cell lines in vitro and in several xenograft models with minimal or no toxicity to nonmalignant human cells (6, 7). Intracellularly, genistein has been shown to inhibit protein tyrosine kinase (8), topoisomerase I and II (9), protein histidine kinase, and 5α-reductase (10). Additionally, previous reports from our laboratory have shown that genistein regulates genes related to the control of cell proliferation, cell cycle, apoptosis, oncogenesis, transcription regulation, angiogenesis, and cancer cell invasion and metastasis (11–15). Although gemcitabine monotherapy (2',2'-difluorodeoxycytidine), a deoxycytidine analogue, or its combination with other agents has become standard chemotherapy for the treatment of advanced pancreatic cancer (16–19), the outcome is very disappointing. A recently published study involving combination of gemcitabine and monoclonal antibody targeting epidermal growth factor receptor revealed marginal effects on survival and objective tumor response against advanced pancreatic cancer in a multicenter phase II trial (20).
Therefore, further improvements in chemotherapeutic response in pancreatic cancer are urgently needed.

Because most human pancreatic tumors show high levels of activated Akt, a serine/threonine protein kinase that mediates survival signaling and also confers resistance to conventional therapeutics (21, 22), targeting Akt, could potentially be an effective therapeutic approach. Consistent with this notion, in vitro studies showed that Akt activation inhibits gemcitabine-induced apoptosis and that the addition of Akt inhibitors could enhance apoptosis (23, 24). The mechanism by which Akt stimulates cell survival is not fully understood; however, recent studies have shown that activation of Akt leads to the activation of a series of survival factors, including the activation of nuclear factor-κB (NF-κB), arming cancer cells to resist induction of apoptosis (25, 26). It has been shown that many conventional cancer chemotherapeutic agents such as vinblastine, vincristine, daunomycin, doxorubicin, camptothecin, cisplatin, and etoposide activate NF-κB (27, 28), thereby resisting apoptosis which results in poor clinical outcome for patients with pancreatic cancer. Preclinical studies in vitro from our laboratory have shown that genistein acts as a double-edged sword, inactivating NF-κB and Akt activity, resulting in the induction of apoptosis and, at the same time, abrogating de novo or acquired chemoresistance (25, 29). Based on our results and others, we hypothesized that dietary genistein may block multiple intracellular signaling pathways that are known to confer a high degree of chemoresistance by pancreatic cancer cells, thereby abrogating either de novo or acquired chemoresistance. Although search for the development of alternative gemcitabine schedules and chemotherapy combinations continues, here we report our preclinical observations in support of our hypothesis that better cell killing is feasible by presensitizing pancreatic cancer cells with genistein during gemcitabine-induced killing. These results are primarily due to inactivation of NF-κB signaling in vitro, and most importantly, in vivo using our orthotopic model of pancreatic tumors.

Materials and Methods

Cell culture. Human pancreatic carcinoma cell lines, COLO-357 and L3.6pl, were obtained from M.D. Anderson Cancer Center (Houston, TX). The human pancreatic L3.6pl cells were established from COLO 357 cells by injecting them into the pancreas of nude mice. Hepatic metastases were procured from American Type Culture Collection (Rockville, MD) supplemented with 10% fetal bovine serum. BxPC-3 cells were procured from American Type Culture Collection (Rockville, MD) and were split regularly before attaining 70% to 80% confluency. BxPC-3 cells were treated with 25 μmol/L of gemcitabine for an additional 72 hours. Thus, for a single-agent treatment, cells were exposed to gemcitabine for 96 hours and gemcitabine for 72 hours. The effect of genistein pretreatment on cell viability was examined by the MTT assay method as stated above.

Quantification of apoptosis by ELISA. The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis in COLO 357 and L3.6pl cells with different treatments according to the manufacturer's protocol. Briefly, COLO 357 and L3.6pl cells were treated with 25 μmol/L gemcitabine for 24 hours and then exposed to a chemotherapeutic agent, 25 μmol/L of gemcitabine, for an additional 72 hours. For single-agent treatment, COLO 357 and L3.6pl cells were treated with gemcitabine for 96 hours and gemcitabine for 72 hours. After treatment, the cytoplasmic histone DNA fragments from COLO 357 and L3.6pl cells with different treatments were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC).

DNA ladder analysis for detecting apoptosis. COLO 357 and L3.6pl cells were treated with 25 μmol/L of gemcitabine for 24 hours and then exposed to 25 nmol/L of gemcitabine for additional 72 hours. After treatment, cellular cytoplasmic DNA from COLO 357 and L3.6pl cells with different treatments was extracted using 10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 15 minutes at 13,000 × g to separate the fragment DNA (soluble) from intact chromatin (nuclear pellet). The supernatant from the lysate was treated with RNase, followed by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, gels were stained with ethidium bromide and the DNA was visualized under UV light and photographed.

Protein extraction and Western blot analysis. The pancreatic cancer cells COLO 357 and L3.6pl were plated and allowed to attach for 36 hours. Genistein was directly added to cell cultures at 30 μmol/L concentration and incubated for 48 hours followed by the addition of gemcitabine. Control cells were incubated in the medium containing an equivalent concentration of Na2CO3. After 24 hours of incubation with gemcitabine, the cells were harvested in PBS and whole cell lysate was prepared by suspending the cells in 200 μL of lysis buffer [1 mol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Triton X-100; 0.1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 μg/mL leupeptin, 2 μg/mL aprotinin]. The cells were disrupted by sonication and the total proteins were extracted by centrifuging the tubes at 4°C for 30 minutes at maximal microtube speed to remove debris. For immunoblotting, each extract was prepared as above and an equivalent to 50 μg total proteins was separated on SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with specific antibodies. Detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit according to manufacturer's instructions. The same membrane was reprobed with the anti-β actin antibody, which was used as an internal control for protein loading.

Cell growth inhibition by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. COLO 357 and L3.6pl cells were seeded at a density of 3 × 104 cells per well in 96-well microtiter culture plates. After overnight incubation, medium was removed and replaced with fresh medium containing different concentrations of genistein (0-100 μmol/L) diluted from a 10 mmol/L stock. On completion of 72 hours of incubation, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) were added to each well and incubated further for 2 hours. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in 100 μL of isopropanol. The plates were mixed for 30 minutes on a gyratory shaker, and absorbance was measured at 595 nm using a plate reader (TECAN, Durham, NC).

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Analysis of cytochrome c release. COLO 357 cells were plated at a density of $5 \times 10^5$ cells in 100-mm dish and allowed to attach overnight. Genistein and gemcitabine were added in the sequence as described above. Following termination of incubation period, cells were collected and washed once with ice-cold PBS, and gently lysed using Dounce homogenizer in 400 $\mu$L of ice-cold lysis buffer [250 mmol/L sucrose, 1 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 7.2), 1 mmol/L DTT, 10 mmol/L KCl, 1.5 mmol/L MgCl$_2$, and protease inhibitors, Complete Cocktail from Roche Molecular Biochemicals, Indianapolis, IN]. Lysate was centrifuged at 500 $\times$ g for 5 minutes at 4℃. The supernatant containing mitochondria was collected and centrifuged again at 10,000 $\times$ g for 10 minutes to obtain cytosolic extract and the pellet (fraction containing mitochondria). The pellet was resuspended in 50 $\mu$L of homogenizing buffer and used for detecting mitochondrial cytochrome c.

Electrophoretic mobility shift assay. Nuclear extracts were prepared according to the method described by Chaturvedi et al. (31). Briefly, respective treatment cells were washed with cold PBS and suspended in 0.15 mL of lysis buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 2 $\mu$g/mL leupeptin, 2 $\mu$g/mL aprotinin, and 0.5 mg/mL benzamidine]. The cells were allowed to swell on ice for 20 minutes and then 4.8 $\mu$L of 10% NP40 were added. The tubes were then vigorously mixed on a vortex mixer for a few seconds and centrifuged in a microfuge. The nuclear pellet was resuspended in 30 $\mu$L of ice-cold nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 2 $\mu$g/mL leupeptin, 2 $\mu$g/mL aprotinin, and 0.5 mg/mL benzamidine] and incubated on ice with intermittent mixing. The tubes were then centrifuged for 5 minutes in a microfuge at 4℃, and
the supernatant (nuclear extract) was collected in cold Eppendorf tubes and stored at −70°C for later use. The protein content was measured by bicinchoninic acid method.

Electrophoretic mobility shift assay (EMSA) was done by incubating 5 μg of nuclear proteins with IB-Dye-700 labeled NF-κB oligonucleotide. The incubation mixture included 2 μg of poly(deoxyinosinic-deoxyuridylic acid) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotide on 8.0% native polyacrylamide gel using buffer containing 50 mmol/L Tris, 200 mmol/L glycine (pH 8.5), and 1 mmol/L EDTA and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1. Equal protein loading was ensured by immunoblotting 10 μg of nuclear protein with anti-retinoblastoma antibody.

**Experimental animals.** Female nude mice (ICR-SCID) were purchased from Taconic Farms (Germantown, NY). The mice were housed and maintained under sterile conditions in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the NIH. The mice were used in accordance with Animal Care and User Guidelines of Wayne State University under a protocol approved by the Institutional Animal Care and Use Committee. The mice received Lab Diet 5021 (Purina Mills, Inc., Richmond, IN).

**Orthotopic implantation of tumor cells.** COLO 357 and L3.6pl cells were harvested from subconfluent cultures after a brief exposure to 0.25% trypsin and 0.2% EDTA. Trypsination was stopped with medium containing 10% fetal bovine serum. The cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of a single cell with >90% viability were used for the injections. The pancreas of anaesthetized mice was exposed through a midline laparotomy incision and by retraction of the spleen. Cells (2 × 10⁷) in 20 μL PBS were injected into the parenchyma of the pancreas with a 27-gauge hypodermic needle and a Hamilton syringe. The abdominal wound was sutured using a 5.0 chromic gut suture in a running fashion. Based on our previous experience with this model, we found a tumor take rate approaching >90%.

**Experimental protocol.** Mice were randomized into the following treatment groups (n = 7): (a) untreated control; (b) only gemcitabine (80 mg/kg body weight), once every other day (i.v. injection); (c) genistein, everyday orally for 10 days; and (d) genistein and gemcitabine, following schedule as for individual treatments. All mice were killed on day 3 following last dose of treatment, and their body weight was determined. The pancreas was fixed in formalin and embedded in paraffin. Another part was rapidly frozen in liquid nitrogen and stored at −70°C. H&E staining confirmed the presence of tumor(s) in each pancreas.

**Tumor tissue nuclear protein extraction and electrophoretic mobility shift assay.** Tissues were minced and incubated on ice for 30 minutes in 0.5 mL of ice-cold buffer A composed of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L DTT, 0.1% IGEPAL CA-630, and 0.5 mmol/L PMSF. The minced tissue was homogenized using a Dounce homogenizer (Kontes Co., Vineland, NJ) followed by centrifuging at 5,000 × g at 4°C for 10 minutes. The crude nuclear pellet was suspended in 200 μL of buffer B [20 mmol/L HEPES (pH 7.9); 25% glycerol, 1.5 mmol/L MgCl₂; 420 mmol/L NaCl, 0.5 mmol/L DTT; 0.2 mmol/L EDTA; 0.5 mmol/L PMSF; and 4 μmol/L leupeptidin] and incubated on ice for 30 minutes. The suspension was centrifuged at 16,000 × g at 4°C for 30 minutes. The supernatant (nuclear proteins) was collected and kept at −70°C until use. The protein concentration was determined using the bicinchoninic acid assay kit with bovine serum albumin as the standard (Pierce Chemical Co., Rockford, IL). Anti-retinoblastoma immunoblotting with nuclear protein was done as loading control.

**Statistical analysis.** Data are represented as mean ± SD for the absolute values or percent of controls as indicated in the vertical axis legend of Figs. 1 and 6. The statistical significance of differential findings between experimental groups and control was determined by Student’s t test as implemented by Excel 2000 (Microsoft Corp., Redmond, WA). Ps < 0.05 were considered statistically significant.

**Results**

**Effect of genistein and gemcitabine on cell proliferation.** To test the effect of genistein on cell growth, pancreatic cancer cells were treated with increasing concentrations of genistein (0-100 μmol/L) for 72 hours. As shown in Fig. 1A, cell growth was inhibited by genistein treatment in a dose- and time-dependent manner. In COLO 357 cells, treatment with 10, 25, 50, and 100 μmol/L of genistein for 72 hours resulted in 95%, 71%, 42%, and 33% of cell growth relative to control, respectively. Similarly, treatment of L3.6pl cells resulted in 71%, 37%, 23%, and 15% of cell growth, respectively, relative to control when exposed to identical concentrations of genistein for similar period of time. These results indicate that genistein was overall an effective inhibitor of pancreatic cancer cell growth as a single agent, and L3.6pl cells were more sensitive to genistein compared with parental COLO 357 cells. We subsequently evaluated the effect of gemcitabine on cell growth in vitro and found that gemcitabine was effective in inhibiting cell growth with equal potency in both cell lines tested (Fig. 1B). Subsequent studies were undertaken to examine whether or not cells pretreated with genistein were more sensitive to the cytotoxic effect of gemcitabine.

**Genistein potentiates growth inhibition induced by gemcitabine in COLO 357 and L3.6pl cells.** We assessed the effect of pretreatment and cotreatment of a combination of genistein and gemcitabine on cell viability by MTT assay. For these studies, cells were either pretreated with genistein (25 μmol/L) alone or in combination with a single dose of gemcitabine (25 mmol/L), and viable cells were evaluated at 96 hours posttreatment by MTT assay. The dose used here was chosen based upon a preliminary dose escalation study done by us. We found that treatment with either genistein or gemcitabine alone for 96 hours resulted in only 25% to 30% loss of viability of COLO 357 and L3.6pl cells. However, pretreatment with genistein for 24 hours followed by treatment with gemcitabine resulted in the loss of 60% to 80% of viable cells in both cell types investigated (Fig. 1C). Similarly, treatment with genistein plus gemcitabine simultaneously was also effective (data not shown).
but pretreatment of cells with genistein was more effective in gemcitabine-induced killing. These results suggests that the combination of genistein with low therapeutic doses of gemcitabine elicits significantly greater inhibition of cancer cell growth compared with either agent, suggesting that lower toxic side effects are likely to occur in normal cells. Inhibition of cell growth and viability as assessed by MTT could also be due to the induction of apoptotic cell death induced by genistein and gemcitabine. We therefore investigated whether gemcitabine in combination with genistein could induce more apoptosis compared with either agent alone.

Genistein sensitizes COLO 357 and L3.6pl cells to apoptosis induced by gemcitabine. We observed induction of apoptosis in pancreatic cancer cells treated with either genistein (25 μmol/L) or gemcitabine (25 nmol/L) alone. Relative to single agents, genistein pretreatment followed by gemcitabine treatment induced much more apoptosis in both the cell lines as shown by both histone DNA ELISA (Fig. 1D) as well as DNA ladder analysis (Fig. 2). These results are consistent with cell growth inhibition studies by MTT, suggesting that the loss of viable cells by genistein and gemcitabine is partly due to the induction of an apoptotic cell death mechanism. Collectively, the above results clearly suggest that the enhanced cell growth inhibition and induction of apoptosis by gemcitabine in genistein-pretreated cells is not cell type specific, because we observed similar effects in both the cell lines tested, and also supports our previous observation in BxPC-3 cells (4).

Genistein inhibits nuclear factor-κB DNA-binding activity. Consistent with earlier reports, constitutively active NF-κB DNA-binding activity was found in nuclear extracts from BxPC-3 as well as...
as L3.6pl cells, whereas very weak basal NF-κB DNA-binding activity was detected in COLO 357 cells (Fig. 3A). The specificity of the band was confirmed by supershift using p65 antibody. Furthermore, as a corollary to previous reports from our laboratory indicating the potential of genistein to abrogate constitutive and inducible NF-κB in prostate cancer cells, we analyzed whether genistein can abrogate the basal constitutively active NF-κB in these cells. To evaluate the effect of genistein in COLO 357 and L3.6pl cells, semiconfluent cells were treated with varying concentrations (0, 10, 25, and 50 μmol/L) of genistein for 72 hours. As shown in Fig. 3B, incubation with 50 μmol/L genistein for 72 hours resulted in a decrease in NF-κB DNA-binding activity in L3.6pl cells. These results are consistent with our findings using BxPC-3 cells as reported earlier (4), which clearly suggest that genistein is effective in down-regulating NF-κB DNA-binding activity. We found no alterations in the nuclear protein content of retinoblastoma, which was used as protein loading control.

Genistein abrogates nuclear factor-κB activation induced by gemcitabine. Next, we analyzed whether gemcitabine could induce NF-κB DNA-binding activity and whether inactivation of NF-κB by genistein could abrogate chemoresistant phenotype of COLO 357 and L3.6pl cells, resulting in more pronounced gemcitabine-induced apoptosis. First, we analyzed dose and time response to gemcitabine in the induction of NF-κB in COLO 357 cells. For this, nuclear extracts were prepared from COLO 357 cells treated with incremental doses of gemcitabine for up to 6 hours and analyzed for NF-κB DNA-binding activity by EMSA. Our results showed that relative to untreated control, gemcitabine (25 mmol/L) treatment induced NF-κB DNA-binding activity as early as 4 hours in COLO 357 cells (Fig. 3C). Furthermore, pretreatment of cells with 30 μmol/L genistein for 48 hours abrogated gemcitabine-induced activation of NF-κB DNA-binding activity (Fig. 3D, left). Under similar assay conditions, we also assessed the NF-κB activation in L3.6pl pancreatic cancer cells expressing constitutively active NF-κB DNA-binding activity in response to gemcitabine. A dose escalation study with gemcitabine revealed modest up-regulation of constitutive NF-κB DNA-binding activity at 100 nmol/L concentration of the drug after 48 hours of treatment. Thus, a low concentration of gemcitabine may not be sufficient to effectively up regulate NF-κB from its basal level in this cell line. Moreover, we tested whether treatment of L3.6pl cells with 30 μmol/L genistein for 48 hours could abrogate gemcitabine-induced NF-κB DNA-binding activity. Our results showed that gemcitabine-induced NF-κB activity could be inhibited by genistein pretreatment in L3.6pl cells (Fig. 3D, right). These results show that genistein not only down-regulates NF-κB DNA-binding activity in unstimulated conditions but also inhibits gemcitabine-induced NF-κB activity, which could be the molecular mechanism of gemcitabine-induced cell death in genistein-pretreated cells. We found similar results in BxPC-3 cells (Fig. 4A and B). These observations provide strong evidence that gemcitabine induced NF-κB, and its down-regulation by genistein could be a common phenomenon in pancreatic cancer cells.

Genistein augments apoptosis signaling by gemcitabine in COLO 357 and L3.6pl cells. In an attempt to explore the mechanism of enhanced apoptotic process induced by treatment of cells with genistein and gemcitabine, we assessed the levels of caspase-3, PARP, antiapoptotic Bcl-2, Bcl-XL, and changes in the status of Akt phosphorylation in COLO 357 and L3.6pl cells (Fig. 5). Initial experiments were done to determine the optimal treatment schedule and dose of individual agents. Cells were pretreated with genistein (30 μmol/L) for 48 hours before addition of 25 mmol/L (for COLO 357) or 100 mmol/L (for L3.6pl) gemcitabine for 24 hours. Our results showed that whereas treatment of these cells with gemcitabine alone had no change in procaspase-3 level, their combination with genistein pretreatment substantially reduced the expression level of procaspase-3 as determined by Western immunoblotting.

PARP is a substrate for caspase activity and a reliable marker of apoptosis. Although each of the agents by themselves showed no PARP cleavage in COLO 357 and L3.6pl cells to a detectable level, combination of gemcitabine with genistein resulted in PARP cleavage. Furthermore, based on our results showing strong caspase activity and PARP cleavage by genistein and gemcitabine combination, we assessed cytochrome c release into cytosol by immunoblotting mitochondrial extract and mitochondria-free cytosolic extract. A significant decrease in the mitochondrial cytochrome c level in the combination treatment was observed compared with each agent alone (Fig. 5B). Results for antiapoptotic Bcl-2 and Bcl-XL proteins also showed down-regulation in the combination group relative to single-agent treatment and untreated control. Analysis for p-Akt (Ser173) revealed an up-regulation by gemcitabine treatment that was down-regulated by genistein pretreatment and evident in both the cell types. These data provide insight into mechanistic basis of chemosensitization by genistein pretreatment.

Genistein augments in vivo therapeutic effect of gemcitabine on primary tumor. Two sets of independent experiments were done to investigate the therapeutic utility of genistein and gemcitabine combination in SCID mice bearing orthotopically implanted pancreatic tumor cells, COLO 357 and L3.6pl. A dose of 1 mg genistein/d/mouse was selected for oral administration,
whereas gemcitabine dose was based on previously published reports (80 mg/kg body weight, i.v., and every other day for a total of three injections). For in vivo experiment, 28 mice were divided into four groups as described in Materials and Methods. In an effort to establish the efficacy of a single-agent treatment compared with combinations, we determined the mean tumor weight in all treated groups. In both set of experiments, administration of genistein by gavage treatment resulted in nonsignificant reduction in the mean tumor weight compared with placebo treatment after 13 days as described in Materials and Methods. Genistein treatment did not cause any weight loss of the animals, suggesting that genistein does not induce any deleterious effect under the present experimental conditions. Analysis of the average pancreas weight in the different treatment groups showed that single modality treatment with either genistein or gemcitabine alone in mice harboring COLO 357 cells caused 13% and 27% reduction in tumor weight, respectively, compared with control (Fig. 6A). Under identical experimental conditions, combination of genistein and gemcitabine treatment showed significant decrease (75%, $P < 0.01$) in tumor weight compared with untreated control, genistein alone-, or gemcitabine alone-treated group. To further investigate the therapeutic potential of genistein and gemcitabine combination in metastatic pancreatic cancer cell line L3.6pl, a second set of experiments was done including the same treatment groups as used in the first set of experiments. The profile of pancreas weight harvested at termination of the experiment revealed identical trends as the former set of experiments, showing that combination of genistein and gemcitabine was significantly better in lowering total pancreas weight than either no treatment or only genistein or gemcitabine treatment (Fig. 6B). Under the current experimental conditions, we did not observe any metastasis in the liver when examined at autopsy, suggesting that the number of cells implanted and the time course was not sufficient to allow metastatic tumor growth.

Nevertheless, our study documents, for the first time, that combination of genistein and gemcitabine can be given safely to inhibit pancreatic tumor growth.

Nuclear factor-κB DNA-binding activity in vivo. We subsequently asked the most important question whether treatment of animals with genistein, gemcitabine, or their combination could effectively target a specific signaling molecule such as NF-κB in tumor tissues. Our results clearly show that NF-κB was down-regulated by genistein, but most importantly, that the gemcitabine-induced activation of NF-κB DNA-binding activity was abrogated when gemcitabine was given together with genistein (Fig. 6C). These in vivo results were similar to our in vitro findings, suggesting that the inactivation of NF-κB is at least one of the molecular mechanisms by which genistein potentiates gemcitabine-induced antitumor activity in our experimental animal model.

Discussion

Despite rapid advances in diagnostic and operative techniques, pancreatic cancer remains one of the most difficult human malignancies to treat, which is partly due to the advanced stage of the disease and de novo chemoresistant behavior to cytotoxic chemotherapeutic agents and/or radiotherapy. In recent years, this problem has been addressed by combinatorial approach. Several randomized studies have shown significant increase in patient response rate by the use of combinations of different class of chemotherapeutic agents, but the major problem is due to treatment associated high toxicity with no added benefit in significant overall survival (32–34). However, these limitations could be overcome by the use of rational chemotherapeutic combinations, in which toxic agents are used in lower doses, and the efficacy of treatment is complemented by using a nontoxic agent that has a different mechanism of action. Based on this rationale, in the present study, we used genistein, a nontoxic...
flavonoid compound, in combination with a commonly used chemotherapeutic agent, gemcitabine, to test its efficacy against two isogenic pancreatic cancer cell lines differing in their metastatic potential. This preclinical study documents that sensitization of cancer cells could be achieved by genistein during gemcitabine-induced killing, as shown by more pronounced cell death compared with single-agent treatment, and this effect was independent of metastatic behavior of pancreatic cancer cells.

Genistein has been shown to synergize with cisplatin and docetaxel, documented by enhanced antiproliferative effect on BxPC-3 pancreatic cancer cells (4). We have recently shown that genistein has a strong modulatory effect on a number of crucial mitogenic pathways in human prostate cancer cells and is effective in inhibiting tumor xenograft in nude mice (6,11). In this study, we found that genistein pretreatment enhanced significant tumor cell killing compared with either agents alone. This observation is of high significance because 60% to 80% growth inhibition could be achieved using the same doses of gemcitabine that produce only 25% to 30% growth inhibition when used alone. Inhibition of cell growth was also correlated with apoptotic cell death.

Gemcitabine-induced apoptosis in cells pretreated with genistein was mediated by caspase pathway as evidenced by DNA fragmentation and PARP cleavage, the latter designated as sensitive marker of caspase-induced apoptosis. In fact, many cancer-preventive and chemotherapeutic agents have been shown to activate the apoptosome pathway that involves the release of cytochrome c from mitochondria, which then oligomerizes with procaspase-9 leading to formation of active caspase-9 that activates downstream executioner caspases such as caspases-3 and caspases-7 (35). In accordance with these reports, our results are consistent showing decreased levels of procaspase-3 and cleavage of the PARP concomitant with a decrease in mitochondrial cytochrome c. Furthermore, pretreatment of cells with caspase inhibitor totally abolished PARP cleavage, further supporting the role of caspase activation during gemcitabine-induced apoptosis of pancreatic cancer cells. Moreover, antiapoptotic molecules, such as Bcl-2, Bcl-xL, and p-Akt, were substantially down-regulated by pretreatment with genistein followed by gemcitabine treatment, and the inactivation of these molecules could be due to inactivation of NF-κB during sensitization of pancreatic cancer cells to gemcitabine-induced killing. Together, these observations suggest that genistein strongly sensitizes pancreatic cancer cells to gemcitabine-induced apoptosis.

Our results also showed that gemcitabine alone could activate NF-κB, resulting in reduced apoptosis supporting the notion that NF-κB activation inhibits apoptosis. We also found that genistein inhibits NF-κB in COLO cells as well as in other cancer cells (25, 29). In addition, our in vitro results showed that genistein alone or genistein pretreatment followed by gemcitabine treatment abrogates NF-κB activation and increases apoptotic index, suggesting...

Figure 6. A, flow chart representation of in vivo experimental design and treatment schedule. B, changes in the isolated pancreatic tumor weight revealing in vivo therapeutic efficacy of genistein and gemcitabine treatment as deduced from pancreas weight. Student's t test was used to explore the statistical significance between the data sets. C, gel shift assay for NF-κB done on randomly selected tumor tissues obtained from each treatment groups of animals. Bottom, loading control by Western immunoblotting of retinoblastoma protein in the nuclear extract.
that inhibition of NF-κB by genistein is mechanistically associated with sensitization of pancreatic cancer cells to apoptotic cell death. Interestingly, these in vitro results (such as antitumor activity and inactivation of NF-κB) were recapitulated in vivo using the orthotopic mouse model which provides a scientific rationale for therapeutic exploitation of our strategy for the treatment of patients with pancreatic cancer. Collectively, these results provide strong molecular evidence in support of our hypothesis that genistein pretreatment could be useful to sensitize pancreatic cancer cells to gemcitabine-induced killing. These results are also relevant in the context of clinical course of pancreatic adenocarcinoma, which is characterized by an early propensity to metastasize and a high risk for disease recurrence following resection. The remarkable ability of genistein and gemcitabine within a therapeutic range to induce apoptosis opens new and novel avenues by which our strategy could provide therapeutic benefit for patients diagnosed with unresectable pancreatic adenocarcinoma as well as for those patients who have undergone resection but are at a high risk for disease recurrence. It is of interest to note that a report by Bushby et al. (36) showed that up to 27 μmol/L of genistein in human plasma could be achievable after genistein supplementation at a dose of 16 mg/kg, suggesting the bioavailability of genistein from supplements.

In conclusion, our current findings are consistent with the hypothesis that it is possible to enhance chemosensitivity of pancreatic cancer cells by pretreatment with genistein, which is mediated by inactivation of NF-κB DNA-binding activity leading to apoptotic cell death. However, further mechanistic studies could be useful to fully support our strategy for the treatment of patients with pancreatic tumors.

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References

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