Inhibition of Phosphatidylinositol 3-Kinase Enhances Gemcitabine-induced Apoptosis in Human Pancreatic Cancer Cells

Sylvia S. W. Ng, Ming-Sound Tsao, Sue Chow, and David W. Hedley

ABSTRACT

Human pancreatic adenocarcinoma cell lines PK1 and PK8 are resistant to the clinically relevant chemotherapy agent gemcitabine. Cell cycle analysis demonstrated an accumulation of cells in the early S phase during treatment with 20 μM gemcitabine, consistent with its mode of action as a DNA chain terminator. However, apoptosis was evident only in a small percentage of cells. Similar to pancreatic cancers in the clinic, PK1 and PK8 cells carry constitutively active Ki-Ras and overexpress multiple receptor tyrosine kinases. Both genetic abnormalities may potentially up-regulate the activity of the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)/Akt cell survival pathway. The current study examined the relevance of this pathway in the modulation of drug resistance in PK1 and PK8 cells. After exposure to 20 μM gemcitabine for 48 h and in the continuous presence of the drug, treatment with the PI3K inhibitors wortmannin (50–200 nM) and LY294002 (15–120 μM) for 4 h substantially enhanced apoptosis in a concentration-dependent manner as compared with treatment with gemcitabine alone, as determined by the loss of mitochondrial membrane potential and the increase in propidium iodide uptake using flow cytometry. Furthermore, Western blotting showed that the reduction of phosphorylated PKB/Akt levels correlated with the enhancement of gemcitabine-induced apoptosis, suggesting that the PI3K-PKB/Akt pathway plays a significant role in mediating drug resistance in human pancreatic cancer cells. PI3K inhibitors may have therapeutic potential when combined with gemcitabine in the treatment of pancreatic cancers.

INTRODUCTION

Adenocarcinoma of the pancreas is resistant to almost all classes of cytotoxic drugs (1). Currently, the only active agent appears to be the DNA chain terminator gemcitabine (2’-2’-difluorodeoxycytidine). However, even with this drug, the objective response rate is less than 20% in the clinic (2). This broad spectrum of drug resistance is inherent to pancreatic cancer rather than acquired during the course of initially successful chemotherapy (1). Until recently, drug resistance has mostly been attributed to increased expression of detoxification mechanisms such as P-glycoprotein or antioxidants or to alterations in drug-metabolizing enzymes. Although these “classical” mechanisms are expressed in pancreatic cancers, there is no compelling evidence that their levels are greater than those seen in more responsive cancers (3–5). Hence, they are unlikely to explain the high level of drug resistance seen in pancreatic cancer patients.

PI3K, a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit, is capable of phosphorylating phosphoinositides at the 3-position of the inositol ring, generating phosphatidylinositol-3-phosphate, phosphatidylinositol-3,4-biphosphate, and phosphatidylinositol-3,4,5-triphosphate (6). Plasma membrane localization and subsequent activation of PI3K can occur by binding of its p85 subunit to phosphorylated tyrosine residues such as those on activated receptor tyrosine kinases (7) or by binding of its p110 subunit to constitutively active, membrane-bound Ras (8, 9). The relative contribution of Ras and p85 to PI3K activation appears to vary between cell lines and receptors (10). PI3K has been demonstrated to phosphorylate the serine/threonine kinase PKB/Akt (6, 11), which, in turn, translocates to the nucleus, where it is believed to regulate the transcription of genes important in mediating cell survival (12–14). In addition, it has been reported that PKB/Akt can phosphorylate and inactivate BAD, a proapoptotic member of the Bcl-2 family (15–17). There is good evidence that several Bcl-2 family proteins act to mediate the release of cytochrome c from mitochondria, a key event activating the late stages of apoptosis. This is associated with alterations in the mitochondrial inner and outer membranes, including the loss of MMP (18), which can be detected using flow cytometry.

It is well documented that certain genetic abnormalities occur at very high frequencies in pancreatic cancers. These include activating Ki-Ras mutations (19, 20) and overexpression of multiple receptor tyrosine kinases (21–23), both of which may potentially up-regulate the activity of the PI3K-PKB/Akt cell survival pathway. Elevated levels of phosphorylated PKB/Akt can then protect cells from undergoing apoptosis induced by cytotoxic drugs and contribute to drug resistance. The current study investigated the significance of this pathway in mediating drug resistance and the effects of PI3K inhibitors on gemcitabine treatment in human pancreatic cancer cells.

MATERIALS AND METHODS

Cell Lines. Human pancreatic adenocarcinoma cell lines PK1 and PK8 were previously obtained from Dr. Masao Kobari (Sendai, Japan). They have activated Ki-Ras mutations and p16 deletion (24), overexpress receptor tyrosine kinases, and show typical morphological features of human pancreatic cancer when grown as xenografts in immune-deprived mice (25). Another tumor suppressor gene, p53, is mutated in PK1 but not in PK8 as determined by DNA sequencing. Cells were maintained as monolayer cultures at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% FCS and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B). The doubling times of PK1 and PK8 were approximately 32 and 24 h, respectively. Experiments were performed using cells in the exponential phase of growth.

Drug Treatments. For flow cytometric analysis, cells were treated with the drug vehicle (±1% DMSO) or 20 μM gemcitabine (2’-2’-difluorodeoxycytidine; Eli Lilly & Co., Indianapolis, IN) for 48 h or with the same concentration of gemcitabine for the same duration followed by wortmannin (50–400 nM) or LY294002 (15–120 μM) for 4 h in the continuous presence of gemcitabine. The concentration and duration of gemcitabine treatment were chosen based on preliminary studies examining its effects on cell cycle inhibition and induction of apoptosis. For Western blotting, cells were treated with similar concentrations of wortmannin or LY294002 alone for 4 h or with gemcitabine (20 and 40 μM) alone for 48 h before harvest. Wortmannin and LY294002 were purchased from Biomol (Philadelphia, PA). All compounds were dissolved in methanol and added at 1 μL/ml to culture media which contained 1% DMSO.
DMSO at a stock concentration of 10 mM, stored at −20°C, and added to cell cultures at a final concentration of ≤1% DMSO, with appropriate solvent additions to control cultures. All experiments were performed in triplicate.

Flow Cytometry. Cell cycle distribution was analyzed by incubating cells with the vital DNA-specific dye Hoechst 33342 at 10 μM for 30 min at 37°C. For detection of apoptosis, MMP was measured using the cyanine dye DiICl(5), whose cationic and amphipathic nature allows its concentration in the energized, negatively charged mitochondria. Generation of ROI was assessed using the weakly fluorescent dye carboxy-DCFDA. This molecule passively diffuses into cells, where it is cleaved by intracellular esterases forming carboxy-dichlorofluorescin. The oxidation of carboxy-dichlorofluorescin by ROI results in a green fluorescence. DiICl(5) and carboxy-DCFDA were made up as 4 and 5 mM stock solutions in 100% ethanol, respectively. Surface membrane integrity was determined by cell permeability to the DNA stain PI. Cells showing loss of MMP and PI leakage were defined as apoptotic. Briefly, cells were trypsinized and resuspended at 1 × 10^6 cells/ml in medium, stained for 25 min at 37°C with 40 nM DiICl(5) and 5 μM carboxy-DCFDA and then stained with 10 μg/ml PI, and incubated for an additional 5 min. All fluorescence probes were purchased from Molecular Probes (Eugene, OR). Flow cytometry measurements were performed using an Epics Elite cell sorter (Coulter, Miami, FL) equipped with HeCd, Ar, and HeNe lasers emitting at 325, 488, and 633 nm, respectively. The HeCd laser was used to excite Hoechst 33342 with emission collected at 440 nm. DiICl(5) was excited by the HeNe laser with fluorescence collected at 675 nm. The Ar laser was used to excite carboxy-DCFDA and PI with fluorescence collected at 525 and 640 nm, respectively.

Fluorescence Microscopy. Drug-treated cells were trypsinized and loaded with 40 nM DiICl(5) and 10 μM Hoechst 33342 for 30 min at 37°C. They were then mounted on glass slides and imaged at ×63 using an Olympus BX50 fluorescence microscope fitted with a Xillix MicroImager (Xillix, Vancouver, British Columbia, Canada). On excitation, DiICl(5) and Hoechst 33342 produced red and blue fluorescence, respectively. Apoptosis was determined by the identification of apoptotic nuclei with Hoechst 33342 and low DiICl(5) fluorescence.

Western Blot Analysis. PKB/Akt phosphorylation was used as the end point for PI3K activation. Briefly, cells (1 × 10^6) were washed twice in ice-cold PBS and then incubated with 300 μl of lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM NaVO_4, 0.1 mM benzamidine, 5 μg/ml leupeptin, and 5 μg/ml aprotinin] for 5 min on ice. Whole cell lysates were clarified by centrifugation at 15,000 rpm for 15 min at 4°C. Samples were heated in SDS sample buffer for 5 min at 95°C, run on 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Xymotech, Ontario, Canada) using the Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Membranes were blocked overnight at 4°C with 10% nonfat milk in TBST [10 mM Tris (pH 7.6), 150 mM NaCl, and 0.5% Tween 20] and then exposed to a primary antibody specific for PKB/Akt phosphorylated at serine 473 (New England Biolab, Mississauga, Ontario, Canada) for 1 h at room temperature. Secondary antibody containing the horseradish peroxidase detection system for chemiluminescence was used as recommended by the manufacturer (New England Biolab).

RESULTS

Effects of Gemcitabine on Pancreatic Cancer Cell Lines. Gemcitabine at 20 μM produced a build-up of cells in early S phase (Fig. 1A) and a 1.5–2-fold increase in the generation of ROI (Fig. 1B). In addition, correlated dot plots of MMP versus ROI show a loss of MMP and an increase in ROI in gemcitabine-treated cells (Fig. 1B). Fig. 1C presents correlated dual parameter plots of cell count and PI uptake versus MMP, demonstrating that gemcitabine alone induced a small but distinct population of cells with low MMP and increased PI uptake relative to the vehicle control. PI uptake in low MMP cells was observed to be a biphasic phenomenon. Cells with high PI fluorescence have a disrupted surface membrane, whereas other low MMP cells could still substantially exclude PI. Both PK1 and PK8 cells behaved similarly in this and all other experiments.

Effects of PI3K Inhibitors on Gemcitabine-induced Apoptosis. Wortmannin (50–400 nM) and LY294002 (15–120 μM), when added to gemcitabine-treated PK1 and PK8 cells for 4 h, significantly increased the population of low MMP cells in a concentration-dependent manner (Fig. 2, A and B; Fig. 3, A and B). Enhancement of gemcitabine-induced loss of MMP was not observed when LY294002 was used at concentrations similar to those of wortmannin (data not shown). The highest concentrations of wortmannin and LY294002 alone did not affect MMP (Fig. 2, A and B). In addition, following treatment with gemcitabine and wortmannin or LY294002, the low MMP and PI-positive populations increased in parallel (Fig. 2, A and B; Fig. 3, C and D).

To further confirm that cells with low MMP were apoptotic, fluorescence microscopy with Hoechst 33342 and DiICl(5) dual labeling was used to examine the nuclei and energized mitochondria, respectively. Cells with low mitochondrial staining intensity were observed to exhibit nuclear features of apoptosis such as nuclear fragmentation (Fig. 4, arrows).

Levels of Phosphorylated PKB/Akt in Response to PI3K Inhibitors. In both pancreatic cancer cell lines, phosphorylated PKB/Akt was detectable under normal growth conditions in the presence of 10% FCS. Wortmannin (50–400 nM) and LY294002 (15–120 μM) significantly reduced the levels of phosphorylated PKB/Akt in PK1
and PK8 cells in a concentration-dependent fashion (Fig. 5, A and B). LY294002 used at concentrations similar to those of wortmannin failed to block PKB/Akt phosphorylation (data not shown). Treatment of cells with gemcitabine alone for 48 h did not alter phosphorylated PKB/Akt levels (Fig. 5C). The levels of total PKB/Akt were not changed by any of the drugs (Fig. 5, A–C).

DISCUSSION

Our results demonstrate that inhibition of the PI3K-PKB/Akt cell survival pathway enhances gemcitabine-induced apoptosis in PK1 and PK8 human pancreatic cancer cells. Gemcitabine at 20 μM caused minimal apoptosis in these cells, indicative of their drug-resistant nature. However, flow cytometric analysis showed that gemcitabine is able to induce cell cycle perturbations with a decrease in the G2–M-phase peak and a build-up of cells in the G1/early S-phase, consistent with its mechanism of action as an inhibitor of DNA elongation. Gemcitabine treatment was also associated with increased reactive oxygen generation, indicative of oxidative stress. It is therefore apparent that gemcitabine accumulates at a sufficient concentration in the cells to inhibit its nuclear target and that drug resistance is not simply due to failure of drug uptake or metabolism. These findings suggest that the suppression of apoptosis after gemcitabine exposure may be a relevant resistance mechanism in these cell lines.

Previous reports from our laboratory (26, 27) and others (28, 29) have demonstrated that oxidative stress occurs in response to cytotoxic agents and that this is temporally related to the loss of mitochondrial inner membrane potential (MMP). The release of mitochondrial components can trigger the mitochondrial permeability transition (MPT), leading to the activation of apoptosis signals.

Fig. 2. Correlated dual parameter plots of cell count and PI uptake versus MMP. After 48 h of gemcitabine (Gem.) treatment and in the continuous presence of the drug, PK1 cells were exposed to increasing concentrations of wortmannin (Wort.; A) or LY294002 (LY.; B) for 4 h. PK1 cells were also treated with the highest doses of wortmannin (Wort.; A) or LY294002 (LY.; B) alone (i.e., in the absence of gemcitabine) for 4 h.

Fig. 3. Percentage (mean ± SE) of PK1 (■) and PK8 (▲) cells with low MMP after treatment with wortmannin (A) or LY294002 (B). Percentage (mean ± SE) of PK1 (○) and PK8 (△) cells showing PI leakage after treatment with wortmannin (C) or LY294002 (D).
drial mediators of apoptosis such as cytochrome c and apoptosis-inducing factor is associated with the loss of MMP (30-33). It has been suggested that a primary event responsible for mediator release is the opening of a multiprotein pore complex in the inner mitochondrial membrane termed the permeability transition (18). However, cytochrome c release can also occur in early apoptosis before the loss of MMP (32). Although it remains unclear which of these mechanisms predominates, the loss of MMP appears to be a universal event during apoptosis in mammalian cells. There is evidence suggesting the physical association of the mitochondrial inner and outer membranes with Bcl-2 and related family members (33). Expression of Bcl-2 was reported to prevent the loss of mitochondrial integrity and enhance the survival of cells exposed to tumor necrosis factor (28, 33). Furthermore, it has been shown that activated PKB/Akt can phosphorylate the proapoptotic protein BAD, hampering the ability of BAD to heterodimerize with Bcl-2 or Bcl-XL and resulting in suppression of apoptosis (16). It seems logical to speculate that inhibition of PI3K and subsequent phosphorylation of PKB/Akt would promote apoptosis. Indeed, wortmannin and LY294002 significantly enhanced the loss of MMP and increased PI uptake in a concentration-dependent fashion in human pancreatic cancer cells exposed to gemcitabine, with the former being more potent than the latter. Fluorescence microscopy illustrated that cells with low MMP show nuclear fragmentation, confirming the apoptotic nature of cell death. The PI3K inhibitors used alone did not affect MMP and PI uptake, indicating their nontoxic nature.

Western blotting revealed that wortmannin and LY294002 block PKB/Akt phosphorylation in PK1 and PK8 cells in a concentration-dependent manner. The reduction of phosphorylated PKB/Akt levels correlated with the enhancement of gemcitabine-induced apoptosis.

Fig. 4. Fluorescence microscopic image. PK1 cells treated with 20 μM gemcitabine for 48 h and 100 nM wortmannin for an additional 4 h were stained with DiIC(5) (red fluorescence) and Hoechst 33342 (blue fluorescence) to visualize MMP and nuclear integrity, respectively. Low MMP cells exhibit typical nuclear features of apoptosis (arrows).

Fig. 5. Western blotting. Phospho-PKB and PKB levels in PK1 cells after treatment with wortmannin (A) or LY294002 (B) alone for 4 h or with gemcitabine (C) alone for 48 h.
Although both agents have been shown to inhibit other enzymes in addition to PI3K, such inhibition usually occurs at much higher concentrations than those used in our experiments. Wortmannin at 20–2000 nm appears to be specific for PI3K and fails to inhibit PKA, protein kinase A, protein kinase C, and protein kinase G (34). However, it remains possible that wortmannin at the concentrations used in this study may inhibit DNA-dependent protein kinase, a member of the PI3K family (35, 36), and contribute to promoting gemcitabine-induced apoptosis. Phosphorylation of PKB/Akt was apparent in both pancreatic cancer cell lines under basal conditions, suggesting the possibility of constitutive activation of PI3K mediated by mutated Ki-Ras and/or increased activity of signal transduction pathways downstream of receptor tyrosine kinases that are overexpressed in these cell lines.

To summarize, we conclude that wortmannin and LY294002 enhance apoptosis induced by gemcitabine in in vitro drug-resistant human pancreatic cancer cells. Our findings suggest that PI3K inhibitors may have therapeutic potential when used in combination with cytotoxic agents in reversing drug resistance in pancreatic cancer patients. The in vivo activity of wortmannin and LY294002 is currently being investigated.

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