Wortmannin Inhibits PKB/Akt Phosphorylation and Promotes Gemcitabine Antitumor Activity in Orthotopic Human Pancreatic Cancer Xenografts in Immunodeficient Mice

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ABSTRACT

Pancreatic cancer is resistant to almost all classes of cytotoxic agents. Gemcitabine seems to be the current drug of choice. We have recently reported that inhibition of the phosphatidylinositide 3-kinase-protein kinase B (PKB/Akt) cell survival pathway by wortmannin enhances gemcitabine-induced apoptosis in cultured human pancreatic cancer cells (1). The present study investigated the effects of wortmannin on orthotopic human pancreatic cancer xenografts implanted in severe combined immunodeficient mice. Animals were given single i.v. bolus injections of 0.175, 0.35, or 0.7 mg/kg of wortmannin and killed at 0.5, 1, 2, or 4 h after treatment. Phosphorylated PKB/Akt levels in tumor tissues were measured by fluorescence image analysis. Wortmannin was found to inhibit PKB/Akt phosphorylation in a time- and dose-dependent manner, reaching a plateau at 4 h and at 0.7 mg/kg. The levels of phosphorylated PKB/Akt were maximally decreased by ~50% relative to the vehicle control. Subsequently, the extent of apoptosis in tumors treated with gemcitabine or wortmannin alone or in combination was determined using terminal deoxynucleotidyl transferase-mediated nick end labeling assay and computerized image analysis. Orthotopic tumors exposed to 80 mg/kg gemcitabine for 48 h and then 0.7 mg/kg wortmannin for 4 h showed a 5-fold increase (P = 0.002) in apoptosis compared with those treated with each agent alone and with the vehicle control. The combination treatment also significantly (P < 0.001) inhibited tumor growth. Taken together, our findings support the potential of phosphatidylinositide 3-kinase inhibitors as adjuncts to conventional chemotherapy in the treatment of pancreatic cancer.

INTRODUCTION

Pancreatic adenocarcinoma is the second most common gastrointestinal malignancy and the fifth leading cause of cancer death in North America (2). Surgery remains the sole curative option. However, fewer than 15% of patients are resectable, and recurrence after a successful pancreatectomy occurs in the majority of these patients (3). Chemotherapy or radiotherapy alone or in combination have a limited effect on the overall survival of patients with pancreatic cancer (2, 4). The current drug of choice seems to be the deoxycytidine analogue gemcitabine (2’-2’-difluoro deoxycytidine), despite its <20% objective response rate in the clinic (5). Therefore, the development of more effective treatment is urgently needed to improve quality of life and reduce mortality.

Several genetic abnormalities, which include activating Ki-ras mutations (6, 7) and overexpression of multiple receptor tyrosine kinases (8–10), occur at very high frequencies in pancreatic cancers. PI3K,3 a downstream effector of Ki-ras and receptor tyrosine kinases (11–14), activates PKB/Akt (15, 16), which, in turn, has been shown to mediate cell survival via the regulation of numerous proteins such as glycogen synthase kinase-3β (17), BAD (18), nuclear factor κB and Bel-XL (19). Elevated levels of phosphorylated PKB/Akt can protect cells from undergoing apoptosis induced by cytotoxic drugs and contribute to drug resistance. Given that the most prevalent genetic aberrations found in pancreatic cancers are capable of up-regulating the activity of the PI3K-PKB/Akt cell survival pathway, we hypothesize that its perturbation by pharmacological inhibitors may have therapeutic potential.

The fungal metabolite wortmannin and the synthetic compound LY294002 are the two currently known inhibitors that show fairly high specificity for PI3K. Wortmannin binds to the p110 subunit of PI3K, noncompetitively and irreversibly inhibiting (IC50, 2–4 nm) the enzyme (20). It has been shown that wortmannin at 100 nm causes 95% inhibition of PI3K and that the concentration required to induce apoptosis of cells maintained in growth factors correlates closely with that required for PI3K inhibition (21). There are limited studies reporting the in vivo antitumor

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3 The abbreviations used are: PI3K, phosphatidylinositide 3-kinase; PKB, protein kinase B; SCID, severe combined immunodeficient; MTD, maximum tolerated dose; EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; IOD, integrated optical density.
action of wortmannin (22, 23); however, it is unclear whether the observed effects were caused by inhibition of PI3K and subsequent PKB/Akt phosphorylation. We have recently demonstrated that PI3K inhibitors suppress PKB/Akt phosphorylation and enhance gemcitabine-induced apoptosis in human pancreatic adenocarcinoma cell lines (1). The current study examined the in vivo effects of wortmannin on PKB/Akt phosphorylation and apoptosis enhancement in orthotopic human pancreatic cancer xenografts implanted in SCID mice.

MATERIALS AND METHODS

Orthotopic Model

Human pancreatic adenocarcinoma cells PK1 were obtained from Dr. Masao Kobari (Sendai, Japan). Their genetic characteristics have been described previously (1, 24). All animal experiments were done in accordance with institutional guidelines for animal welfare. In a preliminary study, PK1 cell suspensions were injected both s.c. and orthotopically into SCID mice at various time points. This dose of wortmannin was reported to be the MTD in 0.7 mg/kg wortmannin via the tail vein and killed at various time points. This dose of wortmannin was reported to be the MTD in 0.7 mg/kg wortmannin via the tail vein and killed at various time points.

Surgical Instrument Co., Inc.), respectively. The animals were anesthetized with isoflurane (Janssen Pharmaceutica, North York, Ontario, Canada). An incision was made in the upper left abdomen, and the pancreas was exposed. Tumor pieces were attached to the pancreas using 4-0 chromic gut suture (Roboz Surgical Instrument Co., Inc., Rockville, MD). The pancreas was allowed to recover.

Immunohistochemistry

Five-μm-thick sections of paraffin-embedded, formalin-fixed s.c. and orthotopic tumor tissues were stained with H&E for histological examination. For detection of phosphorylated PKB/Akt, EGFR, Met/HGFR, and Her2/neu, sections were stained with a polyclonal antibody specific for PKB/Akt phosphorylated at serine 473 (1:10; New England Biolabs, Mississauga, Ontario, Canada), a monoclonal anti-EGFR antibody (1:100; Zymed Laboratories, Inc., San Francisco, CA), polyclonal anti-Met/HGFR, and anti-Her2/neu antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Antigens were visualized using the streptavidin-biotin-peroxidase method.

Treatment Protocols

Gemcitabine was obtained from Eli Lilly & Co. (Indianapolis, IN) and snap-frozen in liquid nitrogen for subsequent immunofluorescence staining. In a third series of experiments, tumor-bearing SCID mice were randomly assigned to four groups (n = 4 each). Each group was given i.v. bolus injections of either the drug vehicle (≤1% DMSO), 80 mg/kg of gemcitabine for 48 h, 0.7 mg/kg of wortmannin for 4 h, or 80 mg/kg of gemcitabine for 48 h and then 0.7 mg/kg wortmannin for 4 h. These schedules were based on in vitro experiments treating PK1 cells with the two drugs (1). Harvested tumors were snap-frozen in OCT (Miles, Inc.) in liquid nitrogen and subsequently processed for TUNEL assay.

Quantification of Phosphorylated PKB/Akt

Western Blot Analysis. PKB/Akt phosphorylation was used as the end point for PI3K activation. Briefly, organs and tumors were homogenized in 500 μl of lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 0.1 mM NaVO₃, 0.1 mM benzamidine, 5 μg/ml leupeptin, and 5 μg/ml aprotinin]. Homogenates 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 (Bio-Rad Laboratories, Mississauga, Ontario, Canada) using the Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories). 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 Biolabs) 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 Biolabs).

Indirect Immunofluorescence. Because the tumors were interspersed with variable amounts of necrotic and nonmalignant tissues, a quantitative immunofluorescence technique was developed to examine phosphoPKB/Akt levels in viable tumor areas. Three serial sections (5 μm thick) were obtained from each frozen tumor, mounted on glass slides, and then fixed in 4% paraformaldehyde. The first section was stained with H&E for transmitted light microscopy. The remaining sections were incubated with a rabbit polyclonal anti-phosphoPKB/Akt (serine 473) antibody (1:10; New England Biolabs) or a sheep polyclonal anti-PKB/Akt (1:50; Upstate Biotechnology, Lake Placid, NY) for 1 h. A Cy3-conjugated donkey antirabbit IgG and a Cy5-conjugated donkey antitubulin IgG (1:200; Molecular Probes, Eugene, OR) were used as secondary antibodies for phosphoPKB/Akt and PKB/Akt, respectively. Control samples exposed to secondary antibodies alone showed no specific staining. All sections were counterstained with the DNA-specific dye diamidinophenyl indole (Molecular Probes) at 1 μg/ml to outline the nuclear area.

Computerized Image Analysis. Composite digital images of the entire H&E sections were captured using a color CCD video camera (Sony DXC 970 MD) mounted on a Zeiss Axioskop transmitted light microscope fitted with an auto-stage and linked to a MicroComputer Image Device (Imaging Research, Inc., St. Catherine, Ontario, Canada). These images were used to select representative areas of viable tumor tissues for analysis of the fluorescence markers. Viable tumor
tissues labeled with the anti-phosphoPKB/Akt or anti-PKB/Akt antibodies were examined using a second MicroComputer Image Device image analysis system equipped with a Xillix MicroImager (Xillix, Vancouver, British Columbia, Canada) mounted on an Olympus BX50 reflected fluorescence microscope. Each individual field was obtained using a ×40 objective. Twenty random 0.149-mm² fields of the same tumor and four tumors/group were analyzed. The Cy3- and Cy5-conjugated secondary antibodies were excited using 540 nm and 620 nm bandpass filters with emissions collected at 610 nm and 700 nm, respectively. For phosphoPKB/Akt staining, the nuclear image obtained with DAPI was used to create a mask, which was then overlaid on the Cy3 fluorescence image, so that only nuclear phosphoPKB/Akt fluorescence was measured. It has been shown that phosphoPKB/Akt translocates to the nucleus and regulates the transcription of genes important in mediating cell survival (25–27). For PKB/Akt staining, total Cy5 fluorescence was measured. The fluorescence intensities of Cy3 and Cy5 were expressed as mean IODs, which are measures of the amounts of labeled phosphoPKB/Akt and PKB/Akt, respectively, in the tumor sections.

Apoptosis Assay
Five serial sections (5 µm thick) were obtained for each frozen tumor, mounted on glass slides, and then fixed in 4% paraformaldehyde. The first section was processed for H&E staining. TUNEL assay was performed on the remaining four sections using the ApopTag Red kit according to the manufacturer’s instructions (Intergen Co., Purchase, NY). Tissue sections processed in the absence of terminal deoxynucleotidyl transferase served as negative controls. The rhodamine-conjugated secondary antibody was excited using a 540 nm bandpass filter with emission collected at 610 nm.

Using the fluorescence image analysis system described above fitted with a QuantaX cooled CCD camera (Photometrix, Inc., Tucson, AZ), tiled images of tumor sections subjected to TUNEL assay were acquired using a ×20 objective. Four sections of the same tumor and four tumors/group were analyzed. Tumors were traced manually with reference to the parallel H&E sections so as to exclude edges, necrotic, and nonmalignant tissues from analysis. Apoptotic nuclei, often consisting of clusters of discrete nuclear fragments, could be readily defined using image analysis criteria so as to reject artifacts. The extent of apoptosis in each tumor, expressed as proportional area, was calculated from the sum of the TUNEL-positive pixel area divided by the total viable tumor area.

Antitumor Activity Study
PK1 cells (10⁷) were injected into the pancreas of SCID mice. Four weeks after tumor implantation, the mice were assigned to one of the following four treatment groups (n = 10 each): (a) vehicle control; (b) gemcitabine, biweekly treatment 80 mg/kg/injection; (c) wortmannin, biweekly treatment 0.35 mg/kg/injection; (d) gemcitabine plus wortmannin, with gemcitabine on Monday and Thursday and wortmannin on Wednesday and Saturday. All groups received treatment via i.p. injection. Mice in all groups were killed after 5 weeks of treatment. Orthotopic tumors were harvested and weighed.

Statistics
All values are presented as mean ± SE. Comparisons between control and treatment groups were made with ANOVA and then Dunnett’s test, with P < 0.05 as the criterion for statistical significance.

RESULTS

Characterization of PK1 Orthotopic Xenografts. Microscopic tumors were detected <1 month after the injection of PK1 cell suspensions into the pancreas (Fig. 1A). All SCID mice developed palpable tumors (100% take rate) in the s.c. and orthotopic sites within 2 months. Weight loss was observed in mice bearing orthotopic but not s.c. tumors. Ascites and metastases were not evident at the time of death. Tumors at both sites were poorly differentiated carcinoma with similar growth rates. Whereas the orthotopic tumors demonstrated focal glandular cell differentiation, their s.c. counterparts displayed a squamous differentiation; also, phosphorylated PKB/Akt was detected at much higher level in the former than the latter (Fig. 1B). Immunohistochemical staining revealed high levels of EGFR and Met/HGFR, and moderate levels of Her2/neu expression in the orthotopic tumors (Fig. 1C). Therefore, orthotopic xenografts propagated by suturing tumor pieces onto the pancreas were used in all subsequent experiments.

In Vivo Effects of Wortmannin on PKB/Akt Phosphorylation

Normal Tissues. Fig. 2A shows representative Western blots of lung, heart and brain homogenates from SCID mice treated with single i.v. bolus injections of 0.7 mg/kg wortmannin and killed at 0.5, 1, 2, or 4 h. No mortality or acute toxicity in animals given this dose of wortmannin for 24 h was observed in our pilot studies (data not shown). Phosphorylated PKB/Akt was detected in all three organs under basal conditions. In the lungs, the levels of phosphorylated PKB/Akt were markedly decreased, with the effect being evident at 0.5 h and persisting up to 4 h. However, inhibition of PKB/Akt phosphorylation was less prominent and more transient in the heart; the levels of phosphorylated PKB/Akt at 4 h after wortmannin treatment were comparable with those in the vehicle control group. Phosphorylated PKB/Akt levels in the brain remained relatively constant. Illustrated in Fig. 2B are representative Western blots of organ homogenates from SCID mice treated with increasing i.v. doses (0.175, 0.35, and 0.7 mg/kg) of the drug for 4 h. PKB/Akt phosphorylation was inhibited only in the lungs at the highest dose of wortmannin. Lower doses failed to reduce phosphorylated PKB/Akt levels in the organs. Similar results were obtained from organs of four different animals killed at each time point and at each dose. Total PKB/Akt levels in the three organs were not altered by wortmannin (data not shown). These results demonstrate that i.v. administration of wortmannin is able to inhibit PKB/Akt phosphorylation in normal tissues in vivo.

Pancreatic Carcinoma Xenografts. The levels of phosphorylated PKB/Akt in cryostat sections of vehicle control and treated tumors were determined from immunofluorescence measurements. Bar graphs of the intensities of phosphorylated PKB/Akt staining expressed as mean IODs versus the duration and dose of wortmannin treatment are illustrated in Fig. 3. A and B, respectively. PKB/Akt phosphorylation in
ortotopic tumors was inhibited by wortmannin in a time- and dose-dependent manner, reaching a plateau at 4 h and at 0.7 mg/kg. Total PKB/Akt levels were not different between control and treated tumors (data not shown). The levels of phosphorylated PKB/Akt were significantly decreased by a maximum of ~50% \( (P < 0.05) \) relative to the vehicle control. Furthermore, Western blotting performed on tumor tissues also demonstrated similar trends of reduction of phosphorylated PKB/Akt levels with increasing doses and duration of wortmannin treatment (data not shown).

**Effects of Wortmannin on Gemcitabine-treated Orthotopic Tumors.** On the basis of the effects of wortmannin on PKB/Akt phosphorylation, we then tested its potential in enhancing gemcitabine toxicity in vivo. A composite image of a TUNEL-labeled orthotopic tumor treated with 80 mg/kg i.v. gemcitabine for 48 h and then 0.7 mg/kg i.v. wortmannin for 4 h

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![A. H&E staining showing a microscopic tumor in the pancreas of a mouse injected orthotopically with PK1 cell suspensions. B, phosphorylated PKB/Akt immunohistochemical staining of orthotopic and s.c. tumors. C, immunohistochemical staining of orthotopic tumors for epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (Met/HGFR), and Her2/neu (Her2/neu).](image)
is illustrated in Fig. 4A. TUNEL-positive cells in tumor sections presented with fragmented nuclei (Fig. 4A, close-up) and were observed throughout the entire tumor. The central area was necrotic. Proportional area, calculated from the sum of the TUNEL-positive pixel area divided by the total viable tumor area, represents the extent of apoptosis in the tumors. Values of proportional area were not significantly different among the four parallel sections from the same tumor (e.g., 0.023 ± 0.002 for one untreated tumor), indicating high intratumoral reproducibility of this parameter. As shown in Fig. 4B, gemcitabine (80 mg/kg for 48 h) or wortmannin (0.7 mg/kg for 4 h) alone did not produce significant increases in apoptosis compared with the vehicle control. However, the extent of apoptosis was significantly increased by 5-fold (P < 0.002) in tumors treated with gemcitabine and wortmannin in combination.

**In Vivo Inhibition of Tumor Growth.** Four, three, and three deaths were noted in the vehicle control, gemcitabine-, and wortmannin-treated groups, respectively, before the end of the 5-week treatment period because of large tumors. Conversely, all mice receiving gemcitabine and wortmannin in combination were alive and exhibited a healthier appearance. Orthotopic tumors were dissected free of surrounding normal tissues and weighed. As shown in Fig. 5, gemcitabine alone and in combination with wortmannin significantly reduced tumor weights by 1.4- and 5-fold (P < 0.001) relative to the vehicle control, respectively. The further decrease in tumor weights observed in the combination treatment group was significantly different from the gemcitabine monotherapy group (P < 0.001). Wortmannin alone failed to inhibit tumor growth.

**DISCUSSION**

Our results demonstrate for the first time that the prototype PI3K inhibitor wortmannin suppresses PKB/Akt phosphorylation and promotes apoptosis induced by gemcitabine in orthotopic human pancreatic cancer xenografts. More importantly, the increase in apoptosis associated with the combined use of gemcitabine and wortmannin was translated into greater inhibition of tumor growth. The differences in growth pattern observed and the higher levels of phosphorylated PKB/Akt detected at the orthotopic site of implantation are consistent with previous reports (28, 29) showing the importance of trophic factors in tumor growth and the clinical relevance of the orthotopic model in drug evaluation.

Schultz et al. (22) have reported previously that wortmannin given p.o. at the MTD of 0.75 mg/kg inhibits tumor growth by >60% in the human BxPC-3 pancreatic carcinoma xenograft. Furthermore, LY294002 has been shown to suppress the growth of human ovarian carcinoma xenografts (30). Whether or not the observed in vivo antitumor action was caused by PI3K inhibition remains to be elucidated. In the present study, SCID mice bearing orthotopic tumor xenografts were given increasing doses of i.v. wortmannin and killed at various time points. Because high levels of PKB/Akt expression have been detected in the lungs, heart, and brain (31–33), Western blotting was performed on homogenates of these organs to determine whether wortmannin possesses any in
vivo activity. Phosphorylated PKB/Akt was found in all three organs under basal conditions. The blockade of PKB/Akt phosphorylation in the lungs lasted longer than that in the heart. Possible explanations include more rapid metabolism of wortmannin or higher basal levels of PTEN/MMAC1, a negative regulator of the PI3K-PKB/Akt pathway (34, 35), in the latter. The observation that phosphorylated PKB/Akt levels in the brain remained fairly constant suggests the inability of wortmannin to cross the blood brain barrier.

The effects of wortmannin in orthotopic tumors were studied using a quantitative immunofluorescence technique applied to viable tumor tissues in cryostat sections. Phosphorylated PKB/Akt levels were reduced in orthotopic tumors treated with i.v. wortmannin in a time- and dose-dependent manner. However, PKB/Akt phosphorylation was only inhibited by ~50%, as compared with >90% in PK1 monolayer cultures (1). The lower activity of wortmannin in the orthotopic model might be attributed to its unfavorable pharmacokinetic profile or to the development of wortmannin-resistant PI3K in vivo, as recently suggested (23). Alternatively, activation of PKB/Akt in the orthotopic model might occur via PI3K-independent pathways such as those involving p38 and MAPKAP kinase 2 (36).

We have recently shown that wortmannin significantly increases apoptosis in gemcitabine-pretreated PK1 cells (1). Having established the ability of wortmannin to suppress PKB/Akt phosphorylation, we therefore asked if gemcitabine-induced apoptosis can be similarly potentiated by wortmannin in the orthotopic model. Fluorescence image analysis of TUNEL-labeled tumor sections revealed that gemcitabine or wortmannin alone did not produce significant increases in apoptotic cells compared with the vehicle control. However, gemcitabine but not wortmannin monotherapy demonstrated some antitumor activity. Contrary to our data, other groups have reported inhibition of tumor growth by PI3K inhibitors (22, 30). Such discrepancies might be attributable to the use of differential doses, schedules, and cell lines. Tumors treated with gemcitabine and wortmannin in combination showed a significant 5-fold increase in apoptosis and decrease in weight versus the vehicle control. Our TUNEL measurements demonstrated high intratumoral reproducibility; hence, any observed differences were attributable to intertumor variations consequent to treatments. Taken together, these results suggest a place for PI3K inhibitors as adjuncts in the treatment of pancreatic cancer. Although wortmannin demonstrated efficacy in potentiating apoptosis and tumor growth inhibition in pancreatic cancer in situ, its ability to inhibit PKB/Akt phosphorylation in normal tissues remains a concern. Wortmannin administered daily at twice the MTD for 10 days has been reported to cause 100% mortality (22), indicating its narrow therapeutic index. Nonetheless, wortmannin can serve as a lead for the development of PI3K inhibitors with better pharmacokinetic and pharmacodynamic properties.

In summary, we conclude that wortmannin inhibits PKB/Akt phosphorylation and enhances apoptosis as well as tumor growth inhibition induced by gemcitabine in orthotopic human pancreatic cancer xenografts in vivo. Our findings further support the preclinical development of treatment protocols that target the PI3K-PKB/Akt cell survival pathway to be used in pancreatic cancer patients.
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