Pancreatic Cancer–Specific Cell Death Induced In Vivo by Cytoplasmic-Delivered Polyinosine–Polycytidylic Acid

Praveen Bhoopathi1, Bridget A. Quinn1, Qin Gui1, Xue-Ning Shen1, Steven R. Grossman1,2,3, Swadesh K. Das1,3, Devanand Sarkar1,3,4, Paul B. Fisher1,3,4, and Luni Emdad1,3,4

Abstract

Polyinosine–polycytidylic acid [pIC] is a synthetic dsRNA that acts as an immune agonist of TLR3 and RLR to activate dendritic and natural killer cells that can kill tumor cells. pIC can also trigger apoptosis in pancreatic ductal adenocarcinoma cells (PDAC) but its mechanism of action is obscure. In this study, we investigated the potential therapeutic activity of a formulation of pIC with polyethylenimine ([pIC]PEI) in PDAC and investigated its mechanism of action. [pIC]PEI stimulated apoptosis in PDAC cells without affecting normal pancreatic epithelial cells. Mechanistically, [pIC]PEI repressed XIAP and survivin expression and activated an immune response by inducing MDA-5, RIG-I, and NOXA. Phosphorylation of AKT was inhibited by [pIC]PEI in PDAC, and this event was critical for stimulating apoptosis through XIAP and survivin degradation. In vivo administration of [pIC]PEI inhibited tumor growth via AKT-mediated XIAP degradation in both subcutaneous and quasi-orthotopic models of PDAC. Taken together, these results offer a preclinical proof-of-concept for the evaluation of [pIC]PEI as an immunochemotherapy to treat pancreatic cancer. Cancer Res; 74(21); 6224–35. ©2014 AACR.

Introduction

Pancreatic cancer is one of the deadliest cancers and ranks fourth in cancer-related deaths in the United States (1–4). Pancreatic cancer death rates have been increasing in the United States over the past several years, as compared with the downward trend in death rates for most other major cancers (5, 6). The overall 5-year survival rate of patients with pancreatic cancer is less than 6%, and this dismal prognosis has not improved in recent years, resulting in an increasing number of deaths (7). The high fatality of pancreatic cancer is attributed to failure to diagnose the disease early (before it has metastasized to other organs) and resistance to current therapies (8).

Surgical removal of pancreatic cancer can cure a small percentage of patients, but survival rates after pancreatectomy are extremely low (9). Chemotherapy or radiation used alone or in combination, is only palliative and provides little or no benefit for patients suffering from advanced pancreatic cancer (10). The current FDA-approved cytotoxic treatment for advanced pancreatic cancer remains gemcitabine (11). Even though gemcitabine is well tolerated, its efficacy is marginal with a median survival of 6 months. The combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) showed superiority over single-agent gemcitabine in patients with metastatic pancreatic cancer (from 6.8 months with gemcitabine to 11.1 months with FOLFIRINOX; ref. 11). However, FOLFIRINOX had greater toxicity, including grade 3 or 4 neutropenia, febrile neutropenia, thrombocytopenia, diarrhea, neuropathy, vomiting, and fatigue (12). Considering its aggressiveness and lack of currently effective therapies, a dire need exists for newer strategies to treat pancreatic cancer. Characterization of new activated oncogenic signaling pathways, defining molecular pathways involved in resistance and/or targeting those genes involved in these pathways in pancreatic cancer cells, may lead to more effective therapeutic strategies for pancreatic cancer treatment (13–15).

The immune system plays a pivotal role in pathophysiology of pancreatitis and pancreatic ductal adenocarcinoma (PDAC) development (16). Eukaryotic cells contain pattern recognition receptors that detect viral/bacterial nucleic acids activating antiviral immune responses (17, 18). Toll-like receptors (TLR) function as pattern recognition receptors and primary sensors of bacteria and viruses (19). Activation of TLRs decreases expression of costimulatory molecules on tumor cells, immunosuppressive functions of regulatory T cells, and production of antiproliferative cytokines (20). TLR agonists activate immune responses...
promoting tolerance to tumor antigens and serve as adjuvants in cancer immunotherapy clinical trials (21).

Polynosine–polycytidylic acid [pIC] is a synthetic dsRNA directly activating dendritic cells and triggering natural killer (NK) cells to kill tumor cells (22). [pIC] mimics viral RNA serving as an agonist of TLR3 and RLRs, and has been extensively used as an immune adjuvant in clinical trials (23). Although [pIC] initiates apoptosis in several cancers, its mechanism of action in human PDAC is not clear. [pIC] induces high levels of type I IFNs and activates several nuclear and cytoplasmic enzyme systems, including OAS (oligoadenylate synthetase), the dsRNA-dependent protein kinase (PKR), RIG-I Helicase, and MDA-5 (melanoma differentiation associated gene-5) that are involved in antiviral and antitumor host defenses (19, 23). Polyethylenimine (PEI) is a promising reagent increasing transfection efficiency of nucleic acids, including DNA, siRNA, or RNA, when administered in vivo (24). Complexing Jet-PEI with several DNA or other vectors leads to a significant increase in transfection efficiency (25). When [pIC] is coadministered with PEI as a carrier, [pIC]PEI, it profoundly affects cancer cell growth, induces apoptosis and toxic autophagy, and promotes potent immune modulating capacities (25–27). [pIC]PEI induces toxic autophagy by recruitment of Atg-5 in melanoma cells linking toxic autophagy to apoptotic caspases (25). In addition, [pIC]PEI decreases viability through apoptosis in breast cancer cells in vitro and in vivo in tumor xenograft models through activation of mda-5 (26). The precise role of cellular intermediates and signaling pathways involved in induction of apoptosis and toxic autophagy by [pIC]PEI remains to be determined.

Our main objectives were to assess the biologic and molecular effects of [pIC]PEI on human PDAC cells and to decipher the mechanism(s) involved in its antitumor activity. Cytoplasmic delivery of [pIC] using jetPEI induced pancreatic cancer–specific cell death without affecting normal pancreatic epithelial cells. We linked for the first time inhibition of AKT activation followed by XIAP degradation as prime mediators in cancer-specific cell killing by [pIC]PEI. Considering the absence of toxicity in vivo of [pIC]PEI and profound cytotoxic activity on PDAC cells, use of this reagent, alone and in combination with other therapeutic agents, could culminate in a novel, safe, and effective approach for treating pancreatic cancer.

Materials and Methods

Cells and reagents

Human PDAC cell lines (MIA PaCa-2, PANC-1, BxPC-3, and AsPC-1) and the hTERT-HPNE cell line were purchased from ATCC. LT-2 cell line was obtained from Millipore life sciences. ATCC authenticates these cell lines using short tandem repeat analysis. All the cell lines were expanded and frozen immediately after receipt. The cumulative culture length of the cells was less than 6 months after resuscitation. Early passage cells were used for all experiments and they were not reauthenticated. All the cell lines were frequently tested for mycoplasma contamination using a mycoplasma detection kit from Sigma. Cell culture conditions and other reagents are described in Supplementary Materials and Methods.

Transfections with [pIC] using jetPEI

All treatments were performed using in vitro jetPEI (Polyplus transfection) transfection reagent using the manufacturer’s protocol. Briefly, [pIC] was mixed with jetPEI (1:2 ratio) in 500 μL of 150 mmol/L sodium chloride and left for 20 minutes to allow complex formation, which was then added to cells in fresh medium.

Plasmid transfection

Plasmid transfection experiments used FuGene HD transfection reagent using the manufacturer’s protocol (Roche) and are described in Supplementary Materials and Methods.

Cell proliferation assays (MTT assay)

Cell growth rate was determined using a modified MTT assay as described (28).

 Colony formation assays

Cells were either mock treated or exposed to [pIC], PEI, or [pIC]PEI for 48 hours. Cells were trypsinized and seeded (100 cells) in 6-well plates in triplicate. On Day 14 of incubation, cells were fixed in methanol, stained with Giemsa, and colonies (>50 cells) counted. Survival fraction was defined as number of colonies divided by number of plated cells.

LC3 assay

We used a previous protocol with minor changes (29) as described in Supplementary Materials and Methods in detail.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Induction of apoptosis in PDAC cells treated with [pIC]PEI as well as in xenograft tumor tissue sections of [pIC]PEI-treated mice was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) enzyme reagent (Roche) following the manufacturer’s instructions and as described (30). Apoptotic index (%) = 100 × (apoptotic cells/total cells).

Annexin V assay

PDAC cells were mock treated or exposed to [pIC], or PEI or [pIC]PEI for 48 hours. Cells were harvested through trypsinization, and washed twice with cold PBS, resuspended in 1 × binding buffer (100 μL) at a density of 1 × 10^6 cells per mL. Cells incubated with 5 μL of FITC-conjugated Annexin V and 5 μL of propidium iodide (PI) for 15 minutes at room temperature in the dark. The 1 × binding buffer (400 μL) was added, and the samples were analyzed by flow cytometry.

Real-time PCR

Cells cultured in 100-mm plates were mock treated or treated with [pIC], PEI, or [pIC]PEI for 48 hours. Total RNA was extracted
using RNAeasy Kit (Qiagen) and equal amounts of RNA were used for cDNA synthesis according to the manufacturer’s protocols using SuperScript VILO cDNA Synthesis Kit (Applied Biosystems). TaqMan probes for XIAP and survivin were from Applied Biosystems and qRT-PCR was performed (28).

Western blotting
Western blotting analysis was performed as described (28, 30).

In vivo studies
To directly evaluate the effect of [pIC]PEI on tumor growth in vivo, we subcutaneously implanted \(5 \times 10^6\) MIA PaCa-2 cells and tumors that developed received peri-tumoral injections of either [pIC], PEI, or pIC complexed with in vivo JetPEI on 7, 11, 15, and 21 days posttumor cell implantation. Tumor growth was monitored in mice by measuring tumor size every alternate day until completion of the experiment [sacrificed according to our Institutional Animal Care and Use Committee (IACUC) protocol]. After termination of the experiment, tumors were fixed and sections were used for IHC analysis.

Quasi-orthotopic tumor studies
MIA PaCa-2-luc cells \((5 \times 10^6)\) were injected intraperitoneally into nude mice. Bioluminescence imaging (BLI) was performed every week after tumor cell implantation. After 2 weeks of cell implantation, mice were divided into two groups with 10 mice per group. One group was used as tumor growth control, without any treatment, and the other group was injected twice weekly with [pIC]PEI \((1 \text{ mg/kg})\) i.p. (total of four doses). Control and treated mice were observed for tumor progression using BLI. All mice were sacrificed once mice either lost body weight or developed impaired mobility.

Statistical analysis
All data are presented as mean ± SD of at least three independent experiments, each performed at least in triplicate. One-way ANOVA combined with the Tukey posthoc test of means was used for multiple comparisons. Statistical differences are presented at probability levels of \(P < 0.05\), \(P < 0.01\), and \(P < 0.001\).

Results
Cytosolic delivery of [pIC] using PEI selectively decreases PDAC cell growth
Cytosolic delivery of [pIC] in human melanoma, breast carcinoma, and hepatoma cells in vitro and in vivo inhibits growth and induces apoptosis and toxic autophagy (25–27, 31). However, the precise mechanisms underlying these effects are not fully understood. The effect of [pIC]PEI on normal and PDAC cell growth was evaluated using MTT assays (Fig. 1). Treatment of PDAC cells with [pIC]PEI decreased proliferation in a dose- and time-dependent manner reaching 70% to 80% growth inhibition with 2 µg/mL concentration at 72 hours as compared with either control, pIC-, or PEI-treated cells. In contrast, normal immortal pancreatic epithelial cells (LT-2 and hTERT-HPNE) showed minimal growth inhibition (~8% maximum reduction at 72 hours) without any change in proliferation (Figs. 1A and B). Clonogenic assays evaluated [pIC]PEI treatment on long-term survival and growth of pancreatic cells. [pIC]PEI treatment decreased PDAC colony formation (both size and number) in a dose-dependent manner reaching 87%, 86%, and 79% inhibition in MIA PaCa-2, AsPC-1, and PANC-1, respectively, when compared with [pIC] (~3% reduction), PEI alone (~4% reduction), or mock controls, without altering LT-2 clonogenic ability (~9% reduction; Fig. 1C).

[pIC]PEI induces autophagy in PDAC cells
Previous studies demonstrated that treatment of human melanoma [25] or breast cancer [26] cells with [pIC]PEI induces autophagy. Accordingly, we initially determined whether [pIC]PEI transfection promoted autophagy in PDAC cells. PDAC and HPNE cells were treated with [pIC], PEI, or [pIC]PEI and cultured for 48 hours (Fig. 2). Cells were then collected and cell lysates were assessed for autophagy markers LC3 and Atg-5. [pIC]PEI transfection increased LC3B and Atg-5 levels in PDAC cells in a dose-dependent manner (Fig. 2A and B), whereas HPNE cells did not show any appreciable changes in either LC3B or Atg-5 level. To further confirm induction of autophagy, we assessed GFP-LC3 lipidation and foci formation in AsPC-1 cells following [pIC]PEI treatment. GFP-LC3 lipidation and foci formation were evident in [pIC]PEI-treated AsPC-1 cells versus nontreated cells (Fig. 2C).

[pIC]PEI selectively induces apoptosis in PDAC cells
Flow-cytometric analysis was used to determine whether growth inhibition in [pIC]PEI-treated cells was a consequence of cell-cycle–related events and/or apoptosis. PI staining indicated that [pIC]PEI treatment increased Sub-G1, apoptotic cells by 56%, 42%, and 52% in MIA PaCa-2, AsPC-1, and PANC-1, respectively, versus mock controls (Supplementary Fig. S1). Annexin V and TUNEL assays corroborated these findings (Fig. 3). Flow-cytometric analyses demonstrated increased Annexin V-PI-positive PDAC cells (~60%–70%) following [pIC]PEI treatment, indicating increased cell death (Fig. 3A). In addition, TUNEL positivity was increased in PDAC following [pIC]PEI transfection, but not in normal LT-2 and HPNE cells (Fig. 3B). Western blotting analysis revealed that [pIC]PEI treatment increased cleavage of caspase-3 and PARP in PDAC but not in normal HPNE cells (Fig. 3C). To confirm the involvement of caspases in [pIC]PEI-induced PDAC cell death, we used the pan-caspase inhibitor z-VAD, which rescued PDAC from [pIC]PEI-induced cell death (Supplementary Fig. S2). These studies confirm that [pIC]PEI treatment inhibits PDAC cell growth by inducing apoptosis, without inhibiting cell proliferation or inducing cell death in normal pancreatic cells.

[pIC]PEI induces MDA-5, RIG-I, and NOXA in PDAC cells, but not in normal pancreatic epithelial cells
[pIC]PEI induces MDA-5 and TLR-3 in melanoma cells, which was critical for induction of toxic autophagy (25). In breast
cancer cells, knockdown of MDA-5 significantly restored viability after transfection with [pIC], whereas knockdown of TLR-3 did not protect cells (26). [pIC]PEI transfection in PDAC increased RIG-I, MDA-5, and NOXA as compared with mock controls (Fig. 4) with IFNβ treatment as a positive control. [pIC] or PEI did not significantly change RIG-I, MDA-5, or NOXA levels (data not shown). To confirm further that [pIC]PEI induces autophagy-mediated apoptosis in PDAC, we used the autophagy inhibitor 3MA (25). Inhibition of autophagy with 3MA rescued PDAC from [pIC]PEI-induced apoptosis (Supplementary Fig. S2). The experiments with 3MA and z-VAD indicate that [pIC]PEI treatment in PDAC, but not in normal cells, induces autophagy followed by apoptotic cell death (Supplementary Fig. S2).

[pIC]PEI triggers X-linked inhibitor of apoptosis protein and survivin degradation in PDAC cells

Overexpression of the Bcl-2 family of antiapoptotic proteins is a frequent occurrence in PDAC and serves as a barrier to effective treatment (3, 4). X-linked inhibitor of apoptosis protein (XIAP) overexpression is frequently observed in PDAC and XIAP degradation or inhibition can sensitize PDAC to apoptosis (12). On the basis of these considerations, the effect of [pIC]PEI on the expressions of Bcl-2 family and other relevant antiapoptotic proteins commonly overexpressed in PDAC, including XIAP and survivin, were determined by Western blotting. Treatment of PDAC with [pIC]PEI decreased MCL-1, survivin, and XIAP levels (Fig. 5A and 5B), but did not alter either XIAP or survivin in HPNE cells. Interestingly, [pIC]PEI also increased the amount of the proapoptotic protein, Bax (Fig. 5B).

Blocking XIAP degradation does not block apoptosis, whereas overexpressing XIAP rescues [pIC]PEI-mediated apoptosis in PDAC cells

[pIC]PEI transfection in PDAC decreased XIAP protein levels, whereas RNA levels were not altered significantly, suggesting that XIAP degradation may be translationally or posttranslationally regulated (Supplementary Fig. S3). To determine whether XIAP degradation plays a key role in [pIC]PEI-induced
apoptosis, we overexpressed XIAP in PDAC and then treated cells with different doses of [pIC]PEI. Overexpression of XIAP partially rescued PDAC from [pIC]PEI-induced cell death (Fig. 3B). However, when a proteasome inhibitor, MG132, was used to block [pIC]PEI-induced degradation of XIAP (Supplementary Fig. S4), PDACs were not rescued from [pIC]PEI-mediated apoptosis.

Figure 2. [pIC]PEI induces autophagy in PDAC cells. A and B, HPNE and PDAC cells were treated with either [pIC], PEI, or indicated doses of [pIC]PEI as in Fig. 1 for 48 hours and cell lysates were subjected to Western blotting to detect LC3 (A) and Atg5 (B). β-Actin served as loading control. C, AsPC-1 cells were treated with 1 μg/mL of [pIC]PEI for 48 hours and stained for LC3 localization. Results are representative of three independent experiments.

Figure 3. [pIC]PEI induces apoptosis in PDAC cells. A, PDACs were treated with 0.5 μg/mL of [pIC]PEI for 16, 24, or 48 hours and stained with FITC-Annexin V and analyzed by FACS. Columns, mean of triplicate experiments; bars, SD. *P < 0.01 versus 0 hours. B, pancreatic cancer cells were cultured in an 8-well chamber slide and treated as above for 48 hours. Cells were fixed and TUNEL assays performed. TUNEL-positive cells were counted per microscopic field and data presented as percent positive cells versus untreated control cells. *P < 0.01 versus pIC alone. C, PDAC and HPNE cells were treated as above for 48 hours and cell lysates were subjected to Western blotting for PARP and active caspase-3. β-Actin served as loading control.
apoptosis. This phenomenon was confirmed using TUNEL analysis, which shows that MG132 did not rescue PDAC cells from [pIC]PEI-induced death (Supplementary Fig. 5B). These results suggest that an additional pathway(s) may contribute to [pIC]PEI-induced cell death.

**Overexpression of survivin partially rescues PDAC cells from [pIC]PEI-mediated cell death**

Survivin is overexpressed in PDAC (32) and provides a biomarker for monitoring prognosis of patients with pancreatic cancer. Our results demonstrate that [pIC]PEI dose dependently inhibits survivin expression in PDAC but not in HPNE cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A). To explore the relevance of this molecule in [pIC]PEI-induced cell death, we transiently transfected PDAC cells (Fig. 5A).

Overexpression of survivin in PDAC partially rescued cells from [pIC]PEI-induced apoptosis (Supplementary Fig. 5A), without substantially altering pAKT levels (Fig. 6B). Similarly, when MG132 blocked [pIC]PEI-induced XIAP degradation, pAKT levels remained unchanged (Fig. 6A). To check the specificity of the [pIC]PEI effect on PDAC, we also determined levels of pAKT and total AKT in HPNE cells. [pIC]PEI did not alter the levels of pAKT or total AKT in HPNE cells as compared with [pIC], PEI, or mock controls (Fig. 6A).

To confirm that AKT is an upstream modulator of XIAP, PDAC cells were transiently transfected with an AKT expression plasmid for 24 hours and then treated with [pIC], PEI, or [pIC]PEI for an additional 48 hours. Cell lysates were prepared and subjected to Western blotting analysis for XIAP and pAKT. XIAP overexpression in PDAC partially rescued cells from [pIC]PEI-induced apoptosis (Supplementary Fig. 5A), without substantially altering pAKT levels (Fig. 6A). Similarly, when MG132 blocked [pIC]PEI-induced XIAP degradation, pAKT levels remained unchanged (Fig. 6A). To check the specificity of the [pIC]PEI effect on PDAC, we also determined levels of pAKT and total AKT in HPNE cells. [pIC]PEI did not alter the levels of pAKT or total AKT in HPNE cells as compared with [pIC], PEI, or mock controls (Fig. 6A).

Enhanced the rescue of PDAC following [pIC]PEI treatment (Supplemental Fig. 5C). Taken together, these results confirm that both XIAP and survivin contribute to [pIC]PEI-induced PDAC death.

[pIC]PEI induces AKT-mediated XIAP degradation and overexpression of AKT rescues PDAC cells from [pIC]PEI-mediated cell death

Overexpression of XIAP plus survivin increases survival in [pIC]PEI-treated PDAC. Treatment with MG132 suggests that blocking XIAP degradation alone cannot rescue cells from apoptosis. On the basis of these observations, we hypothesized that upstream regulators of XIAP may play an important role in [pIC]PEI-induced apoptosis. Previous reports suggest that phosphorylation of XIAP by AKT protects XIAP from ubiquitination and degradation (33). We therefore determined the levels of total AKT and its phosphorylation upon [pIC]PEI treatment in PDAC. [pIC]PEI decreased the levels of pAKT in three pancreatic cancer cell lines (AsPC-1, MIA PaCa-2, and PANC-1) as compared with [pIC], PEI, or mock controls, whereas total AKT levels were unchanged (Fig. 6A). To check the specificity of the [pIC]PEI effect on PDAC, we also determined levels of pAKT and total AKT in HPNE cells. [pIC]PEI did not alter the levels of pAKT or total AKT in HPNE cells as compared with [pIC], PEI, or mock controls (Fig. 6A).

To confirm that AKT is an upstream modulator of XIAP, PDAC cells were transiently transfected with an AKT expression plasmid for 24 hours and then treated with [pIC], PEI, or [pIC]PEI for an additional 48 hours. Cell lysates were prepared and subjected to Western blotting analysis for XIAP and pAKT. XIAP overexpression in PDAC partially rescued cells from [pIC]PEI-induced apoptosis (Supplementary Fig. 5A), without substantially altering pAKT levels (Fig. 6B). Similarly, when MG132 blocked [pIC]PEI-induced XIAP degradation, pAKT levels remained unchanged (Fig. 6A). To check the specificity of the [pIC]PEI effect on PDAC, we also determined levels of pAKT and total AKT in HPNE cells. [pIC]PEI did not alter the levels of pAKT or total AKT in HPNE cells as compared with [pIC], PEI, or mock controls (Fig. 6A).

Enhanced the rescue of PDAC following [pIC]PEI treatment (Supplemental Fig. 5C). Taken together, these results confirm that both XIAP and survivin contribute to [pIC]PEI-induced PDAC death.

[pIC]PEI mediates mTOR-PKC-ε-mediated AKT regulation in PDAC cells

Previous studies show that mTOR can regulate AKT in several cancers (34). AKT has also been reported to be a downstream effector of PKC-ε in ethanol-induced cardioprotection, and
inhibition of PKC-ε prevented the increase in AKT activity (35). To define the effects of [pIC]PEI on mTOR-PKC-ε link, we performed Western blot analysis for mTOR and PKC-ε. [pIC]PEI transfection in PDAC decreased mTOR protein levels and mTOR activation in a dose-dependent manner (Supplementary Fig. S7). We also showed that [pIC]PEI treatment of PDAC decreased PKC-ε in a dose-dependent manner (Fig. 6 and Supplementary Fig. S7). Our results emphasize that PKC-ε and AKT act in a positive feedback loop, since AKT overexpression led to an increase in PKC-ε in PDAC cells (Fig. 6D). These results suggest a potential role of mTOR-PKC-ε in regulating AKT following [pIC]PEI treatment, which warrant further future investigation.

**In vivo delivery of [pIC] using in vivo jetPEI decreases human pancreatic tumor growth in subcutaneous and quasi-orthotopic xenograft mouse models**

MIA PaCa-2 (5 × 10^6) cells were subcutaneously injected into male nude mice and when the tumor reached approximately 75 mm³, they were mock treated (PBS, control) or treated with [pIC], PEI, or [pIC]PEI peri-tumorally and tumor growth was monitored (Fig. 7A). A significant decrease in tumor volume and tumor mass was evident in mice receiving [pIC]PEI as compared with mock-treated (PBS) control mice or mice treated with [pIC] or PEI. Analysis of tumor mass and volume showed approximately 90% inhibition in tumor growth in [pIC]PEI-treated animals (Fig. 7B and C). To determine
whether [pIC]PEI also induced AKT- and XIAP-mediated apoptosis in vivo, tumor sections were prepared and IHC was performed. IHC confirmed downregulation of XIAP and survivin in [pIC]PEI-treated tumor sections when compared with [pIC] or PEI alone or in control PBS-treated tumor sections (Supplementary Fig. S8). TUNEL analysis for apoptosis in tumors confirmed enhanced TUNEL positivity in the [pIC]PEI-treated tumor sections (Supplementary Fig. S8). Taken together, these results show that [pIC]PEI treatment in vivo inhibits PDAC tumor growth through increased apoptosis as evidenced by increased TUNEL and cleaved caspase-3 staining and this effect is mediated through the AKT-XIAP pathway.

To confirm further that [pIC]PEI could inhibit growth of human PDAC in vivo, we used a quasi-orthotopic model in which 5 × 10⁶ MIA PaCa-2-luc cells were injected intraperitoneally into nude mice (Fig. 7D). After 2 weeks, mice received [pIC]PEI IP and BLI monitored tumor growth. Mice were sacrificed once control tumors reached the termination point, as indicated by compromised health. The total pancreatic region with tumors for each mouse was collected and weighted (Fig. 7D). The pancreas in control group mice with tumors weighted approximately 0.5 g, whereas the [pIC]PEI-treated animals’ pancreas weighted approximately 0.3 g (Fig. 7D). BLI also showed high luminescence in control mice compared with [pIC]PEI-treated animals (Fig. 7D). Taken together, these results demonstrate that [pIC]PEI treatment significantly reduced tumor burden in a quasi-orthotopic pancreatic tumor model.
Discussion

Pancreatic cancer remains one of the most difficult cancers to treat effectively. Rapid progression of the disease and diagnosis only after the primary tumor has metastasized are factors contributing to the failure of current approaches to mitigate this disease. Moreover, the lack of noteworthy benefits of current treatment modalities highlights the need for innovative approaches to combat this invariably lethal neoplasm. We presently demonstrate that delivery of [pIC] into the cytosol using PEI selectively promotes PDAC apoptosis without affecting normal pancreatic epithelial cells. This effect is observed both in cells in culture and in two in vivo pancreatic cancer xenograft models, involving subcutaneous tumor injection and quasi-orthotopic intraperitoneal delivery of human PDAC. In addition, through a series of hypothesis-based experiments, we define the mechanism of this selective activity of [pIC]<sup>PEI</sup>, which involves AKT-mediated XIAP degradation, resulting in apoptosis and toxic autophagy uniquely in PDAC.

 Ideally, optimum anticancer therapies should not alter the immunologic status of cancer patients, but instead should boost immune system responses because immunologic status significantly impacts on therapeutic outcome of patients with cancer (26). [pIC] has been used for decades as a synthetic dsRNA mimic to enhance the immune system in an IFN-dependent manner (25) and was tested in multiple clinical cancer therapy trials. However, clinical trials with naked [pIC] were not promising in melanoma because of poor stability of [pIC] and IFN induction (25). [pIC] complexed with low-molecular weight poly-L-lysine, carboxymethylcellulose, liposomes, or PEI increased therapeutic activity via IFN-dependent immune responses (25, 27). [pIC] activates professional antigen-presenting cells, such as dendritic cells that secrete type-I IFN, resulting in effective...
activation of NK cells (25). Interestingly, [pIC][PEI] also displayed efficient antitumor activity in animals with defective NK, T, or B-cell signaling (25, 26). [pIC] induces death in a panel of human cancer cell lines; however, it is only in the last few years that receptors for pIC recognition (MDA-5, TLR3, and PKR) have been discovered (27). Transfection of [pIC] into human melanoma cells induced cell death through the cytosolic [pIC] receptor, MDA-5 (25). When [pIC] was delivered into the cytosol by PEI, endogenous MDA-5 was induced, which facilitated killing of melanoma cells by activation of NOXA. However, when MDA-5 or NOXA was depleted, it did not abrogate cell killing of melanoma cells by activation of NOXA. [pIC] induces an IFN-independent proapoptotic signaling pathway that was activated by RIG-I and MDA-5 in normal cells as well as in melanoma cells, which led to apoptosis only in melanoma cells (27). Interestingly, primary cells were less susceptible due to an intact Bcl-XL pathway, resulting in preferential tumor cell death in vitro and in vivo in immune deficient mice. In hepatoma cells, [pIC]-liposomes induced caspase-dependent apoptosis and upregulated RIG-I and MDA-5 (31). Transfection of [pIC] into breast cancer cells resulted in growth inhibition and apoptosis by inducing MDA-5 and TLR3 and selective knockdown of MDA-5, but not TLR3, partially protected cells from the [pIC]. We show that [pIC][PEI] transfection selectively inhibits cell growth and induces apoptosis in PDAC without affecting normal pancreatic epithelial cells. Moreover, [pIC][PEI] transfection in PDAC increased RIG-I, MDA-5, and NOXA both at the mRNA and protein level, which is consistent with recent reports (25, 27, 31). Apoptosis induction in PDAC is caspase dependent since using a pan-caspase inhibitor z-VAD, rescued PDAC from [pIC][PEI]-induced cell death. In line with the previous reports, we also found that [pIC][PEI] induced autophagy in PDAC as evidenced by LC3 cleavage and Atg5 induction. Inhibition of autophagy by treatment with 3-MA in PDAC reduced [pIC][PEI]-mediated apoptosis. IAPs are deregulated in PDAC and contribute to therapy resistance (36, 37). XIAP and survivin are two essential members of the IAP family, which are overexpressed in numerous human malignancies, including PDAC, and predict poor prognosis (38–43). Interestingly, [pIC][PEI] treatment in PDAC degraded survivin and XIAP in a dose-dependent manner. Overexpression of survivin or XIAP individually in PDAC resulted in partial resistance to [pIC][PEI] treatment, whereas overexpression in combination provided greater resistance to [pIC][PEI]. Although XIAP degradation contributes to [pIC][PEI]-induced PDAC death, rescue of XIAP degradation by MG132 did not provide resistance to apoptosis. AKT can phosphorylate XIAP at serine 87 in vitro and in vivo and can also interact with XIAP (33). The XIAP phosphorylation at serine 87 by AKT inhibits its autoubiquitination and ubiquitination functions promoting resistance to cisplatin-induced XIAP degradation, caspase-3 activation, and apoptosis (33, 44, 45). AKT, also known as protein kinase B, is a serine/threonine kinase involved in regulation of cell proliferation, survival/apoptosis, angiogenesis, metabolism, and protein synthesis (46). AKT plays a seminal role in various cancers (47–53), [pIC][PEI] treatment of PDAC decreased activation of AKT in a dose-dependent manner. A myristoylated, constitutively active form of Akt (myr-Akt) rescued PDAC from XIAP degradation and [pIC][PEI]-induced apoptosis, whereas XIAP overexpression failed to rescue inhibition of AKT phosphorylation by [pIC][PEI]. Taken together, these studies suggest that AKT functions upstream of XIAP and regulates degradation of XIAP, resulting in cell death. Interestingly, we found that [pIC][PEI] treatment dramatically decreases mTOR phosphorylation in PDAC in a dose-dependent manner. Moreover, we found that [pIC][PEI] treatment of PDAC decreased PKC-ε, which was shown previously to act in a positive feedback loop with AKT (35). We plan to extend this interesting observation in future studies.

In summary, we demonstrate that cytosolic delivery of [pIC] using PEI in PDAC results in AKT-XIAP-mediated apoptosis in vitro and in vivo. [pIC][PEI] displayed strong antitumor activity in immunodeficient mice, using both human pancreatic tumor xenograft and quasi-orthotopic models, demonstrating that an intact immune system is not mandatory for anticancer activity, supporting previous studies in melanoma (25, 27) and breast cancer (26). Apoptosis is sequentially triggered by inhibition of AKT activation followed by XIAP degradation in [pIC][PEI]-treated PDAC. This conclusion is supported by the observations that AKT modulation regulated XIAP expression, but XIAP alteration did not affect AKT expression. Our in vitro and in vivo experimental data suggest that [pIC][PEI] may provide a clinically relevant treatment strategy for aggressive and invariably fatal pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Development of methodology: P. Bhoopathi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.A. Quinn
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.K. Das, P.B. Fisher, L. Emdad
Writing, review, and/or revision of the manuscript: P. Bhoopathi, B.A. Quinn, S.R. Grossman, D. Sarkar, P.B. Fisher, L. Emdad
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Q. Gui, X.-N. Shen, L. Emdad
Study supervision: P.B. Fisher, L. Emdad

Acknowledgments

The authors thank Dr. Richard A. Roth (Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA) for providing plasmid myr-AKT delta4–129 and Dr. Paola M. Barral for assistance with Western blotting.

Grant Support

This work was supported in part by the NIH, NCI Grant 1H01 CA127641, VCU MCC Developmental Funds (P.B. Fisher), and a VCU MCC Pilot Project (L. Emdad, S.R. Grossman, and P.B. Fisher).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 24, 2014; revised July 8, 2014; accepted July 19, 2014; published OnlineFirst September 9, 2014.

www.aacajournals.org Cancer Res; 74(21) November 1, 2014 6233
References


Pancreatic Cancer–Specific Cell Death Induced In Vivo by Cytoplasmic-Delivered Polyinosine–Polycytidylic Acid

Praveen Bhoopathi, Bridget A. Quinn, Qin Gui, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-0819

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/09/09/0008-5472.CAN-14-0819.DC1

Cited articles
This article cites 53 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/21/6224.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/74/21/6224.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.