Predominant Bcl-XL Knockdown Disables Antiapoptotic Mechanisms: Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Based Triple Chemotherapy Overcomes Chemoresistance in Pancreatic Cancer Cells In vitro

Jirong Bai, Jianhua Sui, Aram Demirjian, Charles M. Vollmer Jr., Wayne Marasco, and Mark P. Callery

Abstract

Pancreatic cancer is lethal because of its invasiveness, rapid progression, and profound resistance to chemotherapy and radiation therapy. To identify the molecular mechanisms underlying this, we have examined the expression and potency of three major death receptors: tumor necrosis factor receptor (TNFR), TNF-related apoptosis-inducing ligand receptor (TRAIL-R), and Fas in mediating cytotoxicity in four invasive pancreatic cancer cell lines. We have analyzed the expression of major antiapoptotic factors, cell cycle regulators and death receptor decoys (DcR) in comparison with normal pancreas tissues and five other human malignant tumor cell lines. We have found that different pancreatic cancer cell lines coexpress high-level TRAIL-R, Fas, and TNF-R1 but are strongly resistant to apoptosis triggered by the death receptors. DeR2 and DcR3 overexpression may partly contribute to the resistance of pancreatic cancer cells to TRAIL-R– and Fas-mediated cytotoxicity. Bcl-XL and Bcl-2 are predominantly overexpressed in pancreatic cancer cell lines, respectively. Bcl-XL is also predominantly overexpressed in prostate, colorectal, and intestinal cancer cells. The knockdown of the predominant Bcl-XL overexpression significantly reduces the viability of pancreatic cancer cells to TNFα- and TRAIL-mediated apoptosis by sublethal-dose single and combined antitumor drugs, including geldanamycin, PS-341, Trichostatin A, and doxorubicin. Geldanamycin and PS-341 synergistically block NFκB activation, suppress Akt/PKB pathway, and down-regulate Bcl-XL, Bcl-2, cIAP-1, and cyclin D1 expression. This combined regimen dramatically enhances TRAIL cytotoxic effects and breaks through chemoresistance. Bcl-XL plays a vital role in pancreatic cancer chemoresistance. Geldanamycin, PS-341, and TRAIL triple combination may be a novel therapeutic strategy for pancreatic cancer. (Cancer Res 2005; 65(6): 2344-52)

Introduction

Pancreatic cancer is extraordinarily lethal because of its invasive growth, rapid progression, and profound resistance to any therapy now offered (1). The biological mechanisms underlying this phenomenon remain elusive (2, 3). Apoptosis is a normal cell suicide program that is highly conserved among all species. This regulated cell death process is primarily mediated by three different types of death receptors: tumor necrosis factor receptor (TNFR), TNF-related apoptosis-inducing ligand receptor (TRAIL-R), and Fas and plays a critical role in removing unwanted cells during embryogenesis, immune responses, and tissue homeostasis (4). These natural surveillance mechanisms against tumorigenesis are severely compromised in pancreatic cancer.

RelA/p50, a heterodimeric component of NFκB transcription factors (5, 6), is constitutively activated in the majority of pancreatic cancer cell lines and tumors but not in normal pancreatic tissues (7). The activation of NFκB deregulates the biological functions of its downstream antiapoptotic factors (8), including Bcl-2 and Bcl-XL (9–12), inhibitors of apoptotic proteins (IAP; refs. 13–15), Fas-associated phosphatase-1 (Fap-1; ref. 16), and cFLIP (8, 13), as well as death receptor decoys (DcR; refs. 17, 18). These factors cooperatively and negatively regulate apoptotic pathways in tumor cells by blocking proapoptotic functions and suppressing activation of caspase cascades. Bcl-XL overexpression is detected in pancreatic cancer tissues (19) and is linked to abnormal NFκB signaling (11). However, its role in pancreatic cancer chemoresistance has not been established.

In this study, we hypothesize that down-regulation of a predominantly overexpressed antiapoptotic factor could disable antiapoptotic mechanisms in pancreatic cancer cells. We have asked whether death receptor–based apoptotic pathways remain potent in pancreatic cancer cells. Which antiapoptotic factors are predominantly overexpressed? Do the predominantly overexpressed antiapoptotic factors play a vital role in chemoresistance? What roles may DcRs play in pancreatic cancer cell survival? To answer these questions, we have examined the surface expression of TNF-R1 and TRAIL-R1/2 and Fas in four pancreatic cancer cell lines with diverse biological characteristics and have analyzed their potency in mediating apoptosis. Pancreatic cancer has a much poorer prognosis than any other type of tumor (1). At the molecular level, we have investigated the cellular coexpression of antiapoptotic factors Bcl-2, Bcl-XL, cIAP-1, Survivin, cFLIP and Fap-1, and DcRs, as well as cell cycle regulators cyclin D1 and p21Cip1 in pancreatic cancer cells and compared the results with those from five other human malignant tumor cell lines to identify if pancreatic cancer cells express some unique factors.

Clinical investigations show that fluorouracil-based standard chemoradiation or gemcitabine chemotherapy protocols do not prolong overall survival rates in patients with pancreatic cancer (3, 20–22). New biological antitumor therapies based on the following drugs are being evaluated today (23, 24). Geldanamycin, a heat shock protein 90 inhibitor, disrupts heat shock protein

Requests for reprints: Jirong Bai, Research West 857, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. Phone: 617-667-0877; Fax: 617-975-5562; E-mail: jbaire@bidmc.harvard.edu. ©2005 American Association for Cancer Research.
90 chaperoning functions and enhances TNF apoptotic effects (25). PS-341 is a specific proteasome inhibitor that blocks NFκB activation and suppresses tumor growth (26, 27). Trichostatin A, a histone deacetylase inhibitor, modifies chromatin structure and alters the biological functions of transcription factors involved in oncogenesis (26). But the efficacy of these drugs in pancreatic cancer is unknown. In this study, we have determined the cytotoxic effects of geldanamycin, PS-341, Trichostatin A, and doxorubicin on pancreatic cancer cells in the presence or absence of death receptor ligands. We have analyzed whether these drugs influence NFκB activation, Akt/PKB pathway, and the expression of Bcl-2, Bcl-XL and cIAP-1, as well as cell cycle regulators (cyclin D1 and p21CIP1) in pancreatic cancer cells. Ultimately, we have investigated the direct effects of loss of predominant Bcl-XL function on chemoresistance in human pancreatic cancer by developing retroviral-based RNA interference (RNAi) expression vectors and Bcl-XL knockdown pancreatic cancer cell lines. We show for the first time that the genetic depletion of the predominant endogenous Bcl-XL overexpression drives pancreatic cancer cells to death receptor-triggered apoptosis by using various drug treatments.

Materials and Methods

Cell Culture. Human pancreatic cancer (AsPC-1, BxPC-3, Mia PaCa-2, and Panc-1) and other human malignant tumor cell lines, including T-cell lymphoma (Jurkat), cervical carcinoma (HeLa), colon (SW480), prostate (LNCap), and intestinal cancer (HT29) were maintained according to American Type Culture Collection (Manassas, VA) instructions.

Establishment of Bcl-XL Knockdown Pancreatic Cancer Cells. Plasmid DNA for Bcl-XL knockdown and control retroviral vectors were transfected into a murine retroviral packaging cell line to produce VSV-G pseudotyped retroviral particles as described previously (28, 29). Then pancreatic cancer cells were infected with VSV-G pseudotyped retroviral particles at 50 MOI in the presence of 4 μg/mL polybrene (29). Subsequently, GFP+ cells were isolated by fluorescence-activated cell sorting (FACS).

Antibodies, Cytokines, Drugs, and Protein Lysates. Rabbit polyclonal antibodies against the human Bcl-XL, cIAP-1 and cIAP-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal and monoclonal antibodies (mAb) to human caspase-3, caspase-7, and caspase-8 were purchased from BD Pharmingen (San Diego, CA). Antibodies to human caspase-9, Akt, Bcl-2, cyclin D1, and p21CIP1 were obtained from Cell Signaling Technology (Beverly, MA). mAb to human β-actin (AC-15) and human poly(ADP-ribose) polymerase (PARP) were obtained from Sigma (St. Louis, MO) and BIOMOL (Plymouth Meeting, PA), respectively. Recombinant human TRAIL, recombinant human TNFα, mAb to cFLIP, and an agonistic anti-human CD95 mAb (clones DX2; ref. 30) were purchased from Alexis Biochemicals (San Diego, CA). Geldanamycin and doxorubicin were purchased from Sigma. Fas mAb DX2 (0.1-1,000 ng/mL; Fig. 1A), respectively. TNFα exposure caused concentration dependent apoptosis (0.25-25 ng/mL) in Mia PaCa-2 culture (Fig. 1C). Higher TNFα concentration did not enhance its killing of Mia PaCa-2 cells. TNFα showed no toxic effects on AsPC-1, BxPC-3, and Panc-1.

Pancreatic Cancer Cells Predominantly Overexpress Bcl-XL or Bcl-2. Pancreatic cancer and other five human malignant tumor cell lines, including T-cell lymphoma (Jurkat), cervical (HeLa), prostate (LNCap), colon (SW480), and small bowel cancer (HT29) cell lines, simultaneously overexpressed Bcl-2, Bcl-XL, and cIAP-1 (Fig. 2A) but not cFLIP (data not shown). Normal pancreas tissues did not express these proteins. cIAP-2 and Fap-1 expressions were not detected in pancreatic cancer cell lines. Survivin was detected in all four pancreatic cancer cell lines (data not shown). Bcl-XL or Bcl-2 is apparently the predominant endogenous antiapoptotic factors in these pancreatic cancer cell lines. Bcl-XL protein levels were significantly higher than Bcl-2 in BxPC-3 and Panc-1. AsPC-1 and Mia PaCa-2, however, produced more Bcl-2 than Bcl-XL. AsPC-1 produced the largest amounts of Bcl-2 and Bcl-XL among the pancreatic cancer cell lines. LNCap, SW480, and HT29 predominantly overexpressed Bcl-XL.

Pancreatic cancer cell lines overexpressed cyclin D1 compared with normal pancreas tissues (Fig. 2B). This protein was expressed at much higher levels in prostate (LNCap), colorectal (SW480), and small bowel (HT29) cancer cells. p21CIP1 overexpression was identified in BxPC-3, Panc-1, and four other epithelial cancer cell lines but was not detected in normal pancreas tissues and Jurkat cells.

Apoptosis Assay. Cell sensitivity to treatment of TNFα, TRAIL, or agonistic anti-CD95 mAb or drugs was measured by nonradioactive cell proliferation assays ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenoxy)-2(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assays] from Promega (Madison, WI) following the manufacturer's instructions. Vector-transduced and nontransduced pancreatic cancer cells were seeded in 96-well plates overnight. The cells of duplicate wells were treated with diluted cytokines, mAb, or drugs at 37°C for 48 hours. Subsequently, MTS reagent was added to each well. The results were measured four hours later. Readings from control wells of each set of tests (incubated with growth medium alone) served as positive controls, representing 100% cell survival. Based on this, the percentage of cell viability of drug-treated cells was calculated. Statistical significances in cell viability between Bcl-XL knockdown and control cells following drug treatments were evaluated by two-tailed Student's t test. A probability level of <0.05 is considered significant.

Results

Pancreatic Cancer Cell Lines Highly Express Death Receptors But Are Strongly Resistant to Receptor-Triggered Apoptosis. We determined the surface expression of three major death receptors in four pancreatic cancer cell lines (AsPC-1, BxPC-3, Mia PaCa-2, and Panc-1) by antibody staining and fluorescence-activated cell sorting analysis. These cell lines express variable levels of TRAIL-R2 and Fas (S1). AsPC-1 cells also express TRAIL-R1 (data not shown). According to fluorescence density, TRAIL-R2 surface levels could be ranked as Mia PaCa-2 > AsPC-1 > BxPC-3 > Panc-1. Panc-1 (S1a) and Mia PaCa-2 (S1b) also stained positive for TNF-R1. TNF-R1 expression on AsPC-1 and BxPC-3 cells was very low according to fluorescence-activated cell sorting analysis (data not shown). Ligand induced activation assays suggest that Panc-1, Mia PaCa-2, and BxPC-3 have functioning TNF and TRAIL receptors, but AsPC-1 do not (data not shown).

All pancreatic cancer cell lines showed no apoptosis upon stimulation by TRAIL (1-100 ng/mL; Fig. 1A) and an agonistic anti-Fas mAb DX2 (0.1-1,000 ng/mL; Fig. 1B), respectively. TNFα exposure caused concentration dependent apoptosis (0.25-25 ng/mL) in Mia PaCa-2 culture (Fig. 1C). Higher TNFα concentration did not enhance its killing of Mia PaCa-2 cells. TNFα showed no toxic effects on AsPC-1, BxPC-3, and Panc-1.

www.aacrjournals.org 2345 Cancer Res 2005; 65: (6). March 15, 2005
Pancreatic cancer cells also overexpress DcRs (Fig. 2C). Eight tumor cell lines overexpressed DcR3, a Fas decoy, compared with normal pancreas tissues. DcR2, a TRAIL-R decoy, was only identified in three of four pancreatic cancer cell lines, but not in normal pancreas tissues, Panc-1 and other nonpancreatic tumor cells. DcR1, also a TRAIL-R decoy, was not detected in these cancer cell lines. Compared with human epithelial cancer cell lines, Jurkat cells only expressed low-level Bcl-XL and cleaved Bcl-2 and DcR3 but not other factors. DcR2 overexpression in pancreatic cancer cell lines may be associated with increased resistance to TRAIL induced apoptosis (S2a and S2b).

**Genetic Inactivation of Bcl-XL in Pancreatic Cancer Cells.** We established three different Bcl-XL RNAi expression vectors (Fig. 3A and B) to determine the role of Bcl-XL in pancreatic cancer chemoresistance. These RNAi vectors were introduced into Panc-1 cells by retroviral infection. Panc-1 cells predominantly overexpress Bcl-XL. Compared with nontransduced and SINeG-XRi117 transduced cells, Bcl-XL expression was dramatically down-regulated by SINeG-XRi117 transduction (Fig. 3C). SINeG-XRi458 was more effective (Fig. 3C). SINeG-XRi, containing both XRi117 and XRi458 cassettes, completely abolished the Bcl-XL expression (Fig. 3D). Bcl-XL knockdown did not affect the expression of Bcl-2, cIAP-1 and cyclin D1 in these cells (data not shown).

Sorted GFP+ Bcl-XL knockdown and control RNAi-transduced cells were morphologically normal like their parental Panc-1 cells. They were expanded for functional analysis for TNFα- and TRAIL-mediated cytotoxicity. Bcl-XL expression in Mia PaCa-2 was not predominant. Bcl-XL knockdown Mia PaCa-2 cells were also established (data not shown). Its effects on Mia PaCa-2 apoptosis will be addressed in the Discussion.

**Bcl-XL Depletion Drives Pancreatic Cancer Cells to Apoptosis by Geldanamycin and PS-341 in the Presence of TNFα.** Bcl-XL depletion dramatically increased the susceptibility of Bcl-XL knockdown cells (SINeG-XRi) to sublethal-dose geldanamycin and PS-341 in the presence of TNFα (Fig. 4A and B). In the absence of...
geldanamycin and PS-341, TNFα alone did not significantly reduce the viability of Bcl-XL knockdown, control vector–transduced (SINeG-XRi), and nontransduced Panc-1 cells (Fig. 4A, TNFα alone). When cells were exposed to drugs alone (0 μg/mL TNFα) for 48 hours, the viability of Bcl-XL knockdown cells declined by 23% for proteasome inhibitor PS-341 (Fig. 4A) and 30% for heat shock protein 90 inhibitor geldanamycin (Fig. 4B) whereas the viability of control cells (Panc-1 and SINeG-XRi transducers) only declined by 7% to 9% (PS-341) and 13% to 14% (geldanamycin) under the same conditions. This suggests that Bcl-XL depletion sensitizes pancreatic cancer cells to geldanamycin and PS-341. In the presence of 1 to 50 ng/mL TNFα and sublethal-dose PS-341 (10 nmol/L) or geldanamycin (500 nmol/L), the viability of Bcl-XL knockdown cells decreased by 45% to 64% for PS-341 treatment and 47% to 53% for geldanamycin treatment. However, under the identical conditions, the viability of control cells (Panc-1 and SINeG-XRi transducers) decreased by 15% to 30% (PS-341) and 15% to 22% (geldanamycin), indicating that Bcl-XL depletion significantly decreased pancreatic cancer cell viability by an average of 30% (geldanamycin treatment) or 30% to 34% (PS-341 treatment) over the background of the control cells (P < 0.01) treated with TNFα/PS-341 or TNFα/geldanamycin.

TNFα alone (25 ng/mL) did not activate caspase-3 and caused no cleavage of pro-PARP in both Bcl-XL knockdown and control cells (data not shown). However, combining PS-341 with TNFα activated caspase cascades and prompted the effective cleavage of the majority of pro-caspase-3 and degradation of almost the entire pro-PARP in SINeG-XRi-transduced cells (Fig. 4D, lane 2). For Panc-1 cells, the cleavage of pro-caspase-3 was not as effective as that occurred in SINeG-XRi cells treated by TNFα/PS-341. Under this condition, about 50% of pro-PARP remained uncleaved in Panc-1 cells.

Geldanamycin combined with TNFα displayed similar effects on caspase-3 activation and pro-PARP cleavage during 48-hour treatment (Fig. 4D, lane 4). These were consistent with the results from apoptosis assays described in Fig. 4A and B.

**Heat Shock Protein 90 and Proteasome Inhibitors Synergistically Potentiate TNFα Killing of Pancreatic Cancer Cells.** Combining geldanamycin (500 nmol/L) with PS-341 (10 nmol/L) without TNFα slightly improved the cytotoxic effects of geldanamycin and PS-341 on Bcl-XL knockdown but not on control pancreatic cancer cells compared with relevant single drug regimens (Fig. 4C). However, the combination of TNFα (1–50 ng/mL) with sublethal-dose geldanamycin/PS-341 dramatically enhanced the tumoricidal effects of geldanamycin/PS-341 and TNFα on both Bcl-XL knockdown and control cells. Furthermore, the geldanamycin/PS-341/TNFα triple combination significantly decreased control cell viability (Panc-1 and SINeG-XRi–transduced cells) by 45% to 57% (P < 0.01) in comparison with geldanamycin/TNFα and PS-341/TNFα treatments. The latter two regimens killed only 15% to 22% (Fig. 4B) and 15% to 30% (Fig. 4A) of control cells, respectively. Even a low dose of TNFα (1 ng/mL) caused 45% of Panc-1 and 43% of SINeG-XRi–transduced cells to proceed to apoptosis in the presence of geldanamycin/PS-341 combination therapy. As to Bcl-XL–knockdown cells, geldanamycin/PS-341 combination further increased TNFα–induced cytotoxicity by an average of 15%, representing 60% to 70% cell death (P < 0.05) compared with control cells under the same conditions (Fig. 4C).

Geldanamycin and PS-341 combinatorial therapy more effectively activated caspase cascades than either drug in the presence of TNFα (25 ng/mL) in both Bcl-XL knockdown and control pancreatic cancer cells (Fig. 4D). Combined treatment caused the cleavage of most pro-PARP in control cells and complete...
degradation of pro-PARP in Bcl-XL knockdown counterparts (Fig. 4D, lane 3).

**Predominant Bcl-XL Overexpression Blocks both Intrinsic and Extrinsic Apoptosis Pathways.** The cleavage of pro-caspase-9 and pro-caspase-8 activates intrinsic and extrinsic apoptosis pathways, respectively. In the presence of 10 nmol/L PS-341 alone, SINeG-Ri– and SINeG-XRi–transduced counterparts showed no cleaved caspase-8 (Fig. 5A, lane 1) and caspase-9 (Fig. 5B, lane 1). Combining PS-341 with TNFα did not cause the cleavage of pro-caspase-8 and pro-caspase-9 in SINeG-Ri–transduced cells. However, SINeG-XRi cells produced abundant cleaved caspase-8 (Fig. 5A, lanes 2-3) and caspase-9 products (Fig. 5B, lanes 2-3) under the same conditions. The cleavage of executioner caspase-3 and caspase-7 could be seen in SINeG-XRi cells treated with PS-341 and TNFα but not in control cells (Fig. 5C and D). PS-341 and TNFα combination caused the degradation of most full-length pro-PARP proteins in SINeG-XRi cells but not in control cells (Fig. 5E). Taken together, these results strongly support that Bcl-XL predominant overexpression protects Panc-1 cells from TNFα-mediated killing by blocking both intrinsic and extrinsic apoptotic pathways.

**Bcl-XL Knockdown Sensitizes Pancreatic Cancer Cells to TRAIL-Induced Apoptosis in the Presence of Combinatorial Antitumor Drugs.** Bcl-XL knockdown and control cells (Panc-1 and SINeG-Ri” transducers) were similarly insensitive to TRAIL-triggered cytotoxicity at 1 to 50 ng/mL (data not shown). Coadministration of low-dose TRAIL (1-10 ng/mL) and sublethal-dose geldanamycin (500 nmol/L), PS-341 (10 nmol/L), or Trichostatin A (250 nmol/L) showed no significant cytotoxicity to Bcl-XL knockdown and control cells, with one exception that TRAIL, and doxorubicin (250 nmol/L) combination killed 50% to 68% Bcl-XL knockdown cells versus 30% to 48% control cells (P < 0.05) depending on TRAIL concentrations (1-25 ng/mL; data not shown).

Geldanamycin/PS-341, Trichostatin A/PS-341 (Fig. 6A and B), and Trichostatin A/geldanamycin (data not shown) combinations with low-dose TRAIL were all effective in initiating apoptosis in pancreatic cancer cells compared with single drug-based treatments. Bcl-XL knockdown cells were much more sensitive to TRAIL-induced apoptosis than control counterparts that overexpress Bcl-XL under identical conditions (Fig. 6A-B). For geldanamycin/PS-341 regimen, TRAIL induced 35%, 59%, and 77% apoptosis for Bcl-XL knockdown cells at 5, 10, and 25 ng/mL. TRAIL versus 13%, 26%, and 56% apoptosis for control cells under the same conditions (Fig. 6A). This indicates that Bcl-XL depletion significantly reduced the viability on these cells ~2-fold (P < 0.01) compared with control cells.

When Bcl-XL-depleted cells and control counterparts were exposed to Trichostatin A/PS-341, TRAIL induced 20%, 35%, and 75% cell death in Bcl-XL knockdown cells at 5, 10, and 25 ng/mL TRAIL, respectively (Fig. 6B). However, the same experimental conditions only killed 5%, 13%, and 53% of control cells, indicating

---

**Figure 4.** Bcl-XL depletion drives pancreatic cancer cells to TNFα-induced apoptosis by sublethal-dose GA and PS-341. Panc-1 and vector-transduced derivatives were incubated with (A) PS-341, (B) geldanamycin (GA), and (C) GA/PS-341 in the presence of diluted TNFα for 48 hours followed by MTS assays described in Materials and Methods. SDs were <2%. TNFα alone: SINeG-XRi–transduced cells were incubated with TNFα in the absence of drugs and the subsequent results were presented for comparison. D, caspase and pro-PARP cleavage assays. Panc-1, SINeG-Ri”– and SINeG-XRi–transduced cells were incubated with TNFα (25 ng/mL), GA (500 nmol/L), and PS-341 (10 nmol/L) for 48 hours. Subsequently, 30 μg protein samples from different treatments were analyzed in a reducing condition by immunoblot with specific antibodies to human caspase-3 and PARP. We omitted the results for SINeG-Ri”-transduced cells as they were similar to those presented for Panc-1 cells. Cells, not treated or treated with TNFα alone, showed no cleavage for caspase-3 and PARP. Filters were stripped and reprobed with anti-β-actin mAb to show equivalent protein loading.
that Bcl-XL depletion significantly increased Trichostatin A/PS-341 killing effects on pancreatic cancer cells in the presence of 10 and 25 ng/mL TRAIL ($P < 0.01$). Compared with Trichostatin A/PS-341 treatment (Fig. 6b), cell viability for geldanamycin/PS-341 treatment decreased proportionally with increasing TRAIL concentrations in a linear fashion (Fig. 6a). Trichostatin A and geldanamycin combination killed 19%, 66%, and 74% of Bcl-XL knockdown cells upon exposure to 5, 25, and 50 ng/mL TRAIL (data not shown). The same treatments caused 4%, 31%, and 51% cell death in control counterparts, indicating that Bcl-XL depletion dramatically increased the sensitivity of the pancreatic cancer cells to Trichostatin A/geldanamycin–mediated killing in the presence of 25 ($P < 0.01$) and 50 ng/mL TRAIL ($P < 0.05$).

Geldanamycin/PS-341 Combination Is More Effective in Inducing Cytotoxicity than Trichostatin A/Geldanamycin and Trichostatin A/PS-341 in Pancreatic Cancer Cells. In the presence of 5, 10, 25, and 50 ng/mL TRAIL (Fig. 6c), geldanamycin/PS-341 reduced the viability of Panc-1 cells by 18%, 35%, 67%, and 78%, respectively. Trichostatin A/geldanamycin treatment reduced cell viability by 14%, 18%, 31%, and 56% and Trichostatin A/PS-341 by 11%, 18%, 54%, and 77% under the same conditions. It seems that geldanamycin/PS-341 was more effective than Trichostatin A/geldanamycin in killing pancreatic cancer cells in the presence of 10, 25, and 50 ng/mL TRAIL ($P < 0.01$).

Forty-eight hours after drug treatment, TRAIL-treated pancreatic cancer cells (TRAIL alone, 25 ng/mL) and nontreated Bcl-XL knockdown (Fig. 6d, lane 1, SINeG-XRi) and control cells (Fig. 6d, lane 1, Panc-1) did not produce 85-kDa cleaved PARP fragments. In the absence of TRAIL, geldanamycin/PS-341 did not cause PARP cleavage in Panc-1 cells (Fig. 6d, lane 3, Panc-1) but yielded visible 85-kDa fragments in Bcl-XL depleted cells (lane 3, SINeG-XRi). Trichostatin A/PS-341 caused no PARP cleavage in both Bcl-XL knockdown and control counterparts (Fig. 6d, lane 5). Bcl-XL knockdown and control cells treated with geldanamycin/Trichostatin A, however, showed similar levels of cleaved 85-kDa products (Fig. 6d, lane 4). Compared with Panc-1 cells in the presence of TRAIL, PARP proteins were nearly completely degraded in Bcl-XL depleted cells following combinatorial drug treatments. Triple therapy with geldanamycin, PS-341, and TRAIL, caused pro-PARP to disappear altogether in Bcl-XL depleted cells (Fig. 6d, lane 6). For Bcl-XL knockdown cells treated with Trichostatin A/geldanamycin and Trichostatin A/PS-341, little pro-PARP remained following TRAIL stimulation (Fig. 6d, lanes 7 and 8, SINeG-XRi) compared with control Panc-1 cells under the same conditions (Fig. 6d, lanes 7 and 8, Panc-1).

When the extent of PARP cleavage in Panc-1 cells was compared between geldanamycin/PS-341/TRAIL (Fig. 6d, lane 6, Panc-1), Trichostatin A/geldanamycin/TRAIL (lane 7), and Trichostatin A/PS-341/TRAIL (lane 8), the degradation of PARP was significant for geldanamycin/PS-341/TRAIL compared with two other regimens. There were no significant differences between Trichostatin A/PS-341/TRAIL and Trichostatin A/geldanamycin/TRAIL treatments as to PARP cleavage (Fig. 6d, lanes 7 and 8, Panc-1).

NFκB-driven reporter gene assays show that geldanamycin and PS-341 combination is much more effective in blocking constitutive (20.9-fold, S3a) and TNFα-induced (29-fold, S3b) NFκB activation than either drug. Geldanamycin and PS-341 combination nearly completely blocked IκBα degradation (S3c) and caused Akt/PKB depletion (S3d). In addition, this combination more effectively suppressed the expression of Bcl-XL, cIAP-1, and cyclin D1 than either drug (S3d). Akt/PKB degradation and the suppression of the antiapoptotic factors were more profound in Bcl-XL knockdown cells (S3d).

Discussion

Pancreatic cancer is a major unsolved health problem worldwide. Currently used fluorouracil-based chemoradiation and gemcitabine-based chemotherapies do not improve the survival rates for patients (3, 20–22). To reveal the molecular basis of chemoresistance in pancreatic cancer, we have determined the molecular profiles of four pancreatic cancer cell lines in comparison with normal pancreas and five other human malignant tumors. Pancreatic cancer cells seem to differ from cervical, prostate, colorectal, and small bowel cancer cells in the expression of Bcl-XL, Bcl-2, cIAP-1, and DcR3. Although they simultaneously express TRAIL receptors and Fas, pancreatic cancer cells are strongly resistant to relevant ligand-triggered apoptosis. Three of four pancreatic cancer cell lines tested express high levels of FasL than Fas and the expression of DcR3 is much lower than other human malignant tumors. Chemoresistance in pancreatic cancer, we have determined the molecular profiles of four pancreatic cancer cell lines in comparison with normal pancreas and five other primary tumors. Pancreatic cancer cells seem to differ from cervical, prostate, colorectal, and small bowel cancer cells in the expression of Bcl-XL, Bcl-2, cIAP-1, and cyclin D1. Although they simultaneously express TRAIL receptors and Fas, pancreatic cancer cells are strongly resistant to relevant ligand-triggered apoptosis. Three of four pancreatic cancer cell lines tested express high levels of FasL than Fas and the expression of DcR3 is much lower than other human malignant tumors.
overexpression coincides with resistance to Fas-triggered apoptosis in pancreatic cancer cell lines; however, its exact role in pancreatic tumorigenesis remains to be established. We have shown that DcR2 is overexpressed in three of four pancreatic cancer cell lines but not in normal pancreas tissues and five other human tumor counterparts. DcR2 negative Panc-1 cells express a lower level of TRAIL receptors than DcR2-positive BxPC-3 and Mia PaCa-2. However, Panc-1 cells are more sensitive to TRAIL-induced apoptosis than BxPC-3 and Mia PaCa-2 in the presence of geldanamycin and PS-341 combinatorial therapy (S1). This suggests that DcR2 overexpression may be associated with the increased resistance of BxPC-3 and Mia PaCa-2 to TRAIL-induced apoptosis.

Our experiments reveal that Bcl-XL or Bcl-2 is predominantly overexpressed in pancreatic cancer cell lines tested. Bcl-XL is not only predominantly overexpressed in pancreatic cancer cells, but also in three other epithelial cancer cell lines: prostate (LNCap), colorectal (SW480), and small bowel (HT29). Previous studies have used overexpression systems with a foreign promoter to define Bcl-XL functions (32, 33). It is not clear, however, such systems can accurately simulate the exact role that endogenous Bcl-XL plays in a naturally occurring tumor. As such, we have used RNA interference to knock down Bcl-XL overexpression, and for the first time, have examined the effects of Bcl-XL depletion on apoptosis in pancreatic cancer cells.

We show that the predominant Bcl-XL overexpression in pancreatic cancer cells effectively blocks the activation of intrinsic and extrinsic apoptosis pathways. RNAi-mediated knockdown of the predominant Bcl-XL significantly enhances antitumor drug–induced caspase activation as well as TNFα- or TRAIL-triggered apoptosis. In contrast, RNAi-mediated knockdown of non-predominant Bcl-XL in Mia PaCa-2 cells did not increase the sensitivity of Mia PaCa-2 to the similar treatments used in this study (data not shown). Bcl-XL knockdown shows

**Figure 6.** Predominant Bcl-XL knockdown sensitizes pancreatic cancer cells to TRAIL-induced apoptosis in the presence of sublethal-dose antitumor drugs. Apoptosis assays were carried out as described in Materials and Methods. Panc-1 and vector-transduced derivatives were incubated respectively with TRAIL alone (25 ng/mL) or TRAIL plus combined drugs at defined concentrations: 10 nmol/L PS-341, 500 nmol/L geldanamycin (GA), 250 nmol/L Trichostatin A (TSA). In the absence of drugs, TRAIL showed no toxicity to control and Bcl-XL-knockdown cells. Therefore, only results from SINeG-XRi cells treated with TRAIL alone were presented in each graph for comparison. A, GA/PS-341/TRAIL. B, TSA/PS-341/TRAIL. C, comparison of cytotoxicity of combined drug regimens in Panc-1 cells. Panc-1 treated with TRAIL alone served as control. D, comparison of PARP cleavage between Panc-1 and SINeG-XRi derivatives following treatments with TRAIL, drugs, and drugs/TRAIL. Cells in 12-well plates were not treated or treated with TRAIL (25 ng/mL), alone or combined drugs or drugs/TRAIL for 48 hours. The extents of PARP cleavage were determined by Western blot. Pro-PARP: 116 kDa, not cleaved; 85-kDa fragments are enzyme-cleaved products.
that a predominantly overexpressed antiapoptotic protein is a critical factor for the survival of pancreatic cancer cells. These findings emphasize that Bcl-XL is a critical antiapoptotic factor that may contribute to chemoresistance in various cancers, including pancreatic cancer, and suggests Bcl-XL as an important molecular target for future drug discovery.

We have found that the majority of pancreatic cancer cell lines are strongly resistant to PS-341, gledanycinamycin, Trichostatin A, and doxorubicin. Chemotherapy regimens based on these drugs at sublethal doses effectively kill leukemia (34), breast, and lung cancer cells in vitro (35, 36) but only have marginal cytotoxic effects on pancreatic cancer cells. Our study reveals that it is necessary to combine a death ligand, such as TNFα, with two optimal anti-tumor drugs to overcome the anti-apoptotic mechanisms in pancreatic cancer cells in vitro. TNFα is extremely toxic in vivo, ruling out its usefulness in pancreatic cancer chemotherapy. TRAIL receptors, however, are widely expressed in various types of human tumor cells (37–39), including the pancreatic cancer cell lines tested in this study. Recombinant TRAIL is harmless to many normal human cells and causes no adverse effects on non-human primates. It effectively induces apoptosis in a variety of human tumor cell lines and tumor xenografts (38–41).

Unfortunately, TRAIL alone does not cause cytotoxicity in pancreatic cancer cells. However, we now show that combining TRAIL with gledanycinamycin/PS-341, and gledanycinamycin/Trichostatin A or Trichostatin A/PS-341 effectively kills control pancreatic cancer cells. This killing effect is maximized when Bcl-XL is depleted.

Gledanycinamycin and PS-341 synergistically enhance TNFα- and TRAIL-triggered apoptosis by increasing the activation of caspase cascades through down-regulation of antiapoptotic factors and cell cycle regulators, a phenomenon not previously described. Gledanycinamycin and PS-341 induce these effects by both blocking NF-κB activation and disrupting the Akt/PKB signaling pathway as well as cell cycle progression. This study suggests that gledanycinamycin, PS-341, and TRAIL combination may be a novel therapeutic strategy in the future treatment of pancreatic cancer.

Acknowledgments

Received 10/1/2004; revised 12/8/2004; accepted 1/4/2005.

Grant support: National Pancreas Foundation and Beth Israel Deaconess Medical Center.

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.

We thank Ben Enos and Akikazu Murakami of Dana-Farber Cancer Institute for technical support.

References


Predominant Bcl-XL Knockdown Disables Antiapoptotic Mechanisms: Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Based Triple Chemotherapy Overcomes Chemoresistance in Pancreatic Cancer Cells In vitro

Jirong Bai, Jianhua Sui, Aram Demirjian, et al.


Updated version
Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/65/6/2344

Supplementary Material
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2005/03/30/65.6.2344.DC1

Cited articles
This article cites 44 articles, 17 of which you can access for free at: http://cancerres.aacrjournals.org/content/65/6/2344.full#ref-list-1

Citing articles
This article has been cited by 22 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/65/6/2344.full#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.