Nuclear factor-κB maintains TRAIL resistance in human pancreatic cancer cells

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Abstract

Although it displays promising activity in other tumor models, the effects of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) on human pancreatic cancer cells have not been comprehensively explored. We report that a majority of human pancreatic cancer cell lines (seven of nine) underwent apoptosis when they were exposed to recombinant human TRAIL in vitro. Characterization of surface TRAIL receptors by fluorescence-activated cell sorting showed that TRAIL-resistant cells (Panc-1 and HS766T) expressed lower levels of DR4 and DR5 than did TRAIL-sensitive cells. The proteasome inhibitor bortezomib (PS-341, Velcade) further increased TRAIL responsiveness in the TRAIL-sensitive cells and synergized with TRAIL to reverse resistance in Panc-1 and HS776T cells. The effects of bortezomib were mimicked by transfection with a small interfering RNA construct specific for the p65 subunit of nuclear factor-κB (NF-κB) or exposure to a selective chemical inhibitor of IKK (PS-1145). Silencing IκBα prevented TRAIL sensitization by PS-1145, confirming that IκBα mediated the effects of PS-1145. NF-κB inhibition resulted in down-regulation of BCL-XL and XIAP, and silencing either restored TRAIL sensitivity in TRAIL-resistant cells. Finally, therapy with TRAIL plus PS-1145 reversed TRAIL resistance in vivo to produce synergistic growth inhibition in orthotopic Panc-1 tumors. Together, our results show that NF-κB inhibits TRAIL-induced apoptosis in human pancreatic cancer cells and suggest that combination therapy with TRAIL and NF-κB inhibitors, such as bortezomib, PS-1145, or curcumin, should be considered as a possible treatment strategy in patients with pancreatic cancer. [Mol Cancer Ther 2006;5(9):2251–60]

Introduction

Pancreatic cancer is the fourth leading cause of cancer deaths in both men and women (1). Despite advances in surgical techniques and adjuvant therapy, survival has changed little in the last 20 years (2), with a 5-year survival of 4% according to the American Cancer Society (2). More than 50% of patients will have metastatic disease at presentation, 10% to 15% will be resectable, and the remainder will have locally advanced unresectable disease as well as an incidence of unrecognized metastases (3). Adjuvant chemotheraphy and radiation therapy are not very effective, extending survival by an average of 3 to 6 months. The nucleoside analogue, gemcitabine, is currently considered to be the most active agent in the disease, but its effects are mostly palliative, and the majority of patients succumb to their disease within 6 months (4). Ongoing studies are evaluating the therapeutic activity of gemcitabine-based combination therapy, but the preliminary results of these trials do not suggest that a major increase in overall or 6-month survival will be obtained (4). Therefore, there is a great need to develop more active regimens in this disease, and relevant biological targets are being evaluated aggressively in preclinical models.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a cytokine that plays important roles in inflammation and immunity (5). There is considerable enthusiasm for developing TRAIL for cancer therapy because preclinical studies showed that it is a very potent stimulus for apoptosis in a variety of different human cancer cell lines but not in normal cells (6). Furthermore, unlike its structural and functional homologues, tumor necrosis factor and Fas ligand, systemic exposure to TRAIL does not lead to appreciable toxicity in rodents and primates (6). The mechanisms underlying the TRAIL resistance of normal cells are not clear but may be related to their lower expression levels of functional TRAIL receptors (DR4, DR5) relative to cancer cells (7), to expression of nonfunctional (“decoy”) TRAIL receptors (5, 8–10), or to differences in cell cycle control (11). Phase I clinical trials using recombinant Apo-2L/TRAIL were recently opened at our institution and others.

Studies conducted over the past 10 years have implicated the inflammation-associated transcription factor, nuclear factor-κB (NF-κB), in cancer progression and resistance to therapy (12–14). With respect to pancreatic cancer, studies
showed that NF-κB is maximally activated in over 70% of human pancreatic cancer cell lines and primary tumors (15). Other work established that NF-κB functions as an inhibitor of apoptosis via its ability to regulate the expression of a variety of different antiapoptotic proteins (BCL-2, BCL-XL, IAPs, c-FLIP; refs. 16, 17). Many different small molecule and molecular inhibitors of NF-κB have been identified, and preclinical studies have shown that they can sensitize cells to apoptosis induced by a variety of different stimuli, including conventional chemotherapy (16, 18, 19). One of these agents is the proteasome inhibitor bortezomib (20), which recently received Food and Drug Administration approval for the treatment of multiple myeloma (21, 22).

Given that conventional chemotherapy is almost inactive in the disease, we initiated the present study to determine whether human pancreatic cancer cells are sensitive to the proapoptotic activity of TRAIL. Our results show that a majority of human cell lines are TRAIL sensitive at baseline and that bortezomib and other NF-κB inhibitors promote further increases in TRAIL-induced cell death.

Materials and Methods

Cell Culture and Reagents

The human pancreatic cancer cell lines Aspc1, BxPC3, Capan2, ClPacl, HPAF2, HS766T, MiaPaca2, and Panc1 were obtained from the American Type Culture Collection (Manassas, VA). The L.3.6pl pancreatic adenocarcinoma cell line was derived from COLO-357 following selection for metastases from the pancreas to the liver in nude mice (23). All of the cell lines, with the exception of BxPC3, were maintained in MEM supplemented with 10% fetal bovine serum, MEM vitamin solution, 1-glutamine, nonessential amino acids, sodium pyruvate, and penicillin/streptomycin solutions in a 37°C incubator under an atmosphere of 5% CO2 in the air. BxPC3 was maintained under the same conditions in RPMI medium.

Bortezomib and PS1145 were provided by Millenium Pharmaceuticals (Cambridge, MA). Tumor necrosis factor-α was purchased from Promega (Madison, WI). The recombinant human TRAIL (rhTRAIL) used in vitro was purchased from R&D Systems, Inc. (Minneapolis, MN). For in vivo studies, recombinant soluble TRAIL (amino acids 98–281) was expressed in Escherichia coli by transforming BL-21 cells with a pET15b plasmid containing a partial TRAIL cDNA. Clones were isolated and grown to log phase, followed by addition of 1 mmol/L isopropyl-1-thio-β-D-galactopyranoside to induce protein expression, and incubated for 2 hours. The bacteria were lysed, and the His-tagged protein was purified under native conditions using the QIAexpress system from Qiagen (Valencia, CA). Bacteria were then suspended in lysis buffer [50 mmol/L NaH2PO4, 300 mmol/L NaCl, and 10 mmol/L imidazole (pH 8.0)] with 100 μg/mL lysozyme. The lysates were sonicated and centrifuged at 10,000 × g for 20 minutes at 4°C. Lysates were incubated with nickel-nitrilotriacetic (NiNta) agarose mixture (Qiagen) for 90 minutes at 4°C while rotating. The lysate-NiNta mixture was washed onto a column and washed with lysis buffer, and His-tagged TRAIL was eluted in 250 mmol/L imidazole. Post elution, fractions containing high concentrations of TRAIL were collected and stored in aliquots containing 10% glycerol at 80°C.

Analysis of Viability by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Reduction

Cells were harvested in their exponential growth phase by a 2-minute treatment with 0.25% trypsin/0.02% EDTA (w/v). (When this procedure was followed, the viability of harvested cells always exceeded 95%). Cells were seeded into 96-well microculture plates at 5,000 per well and allowed to attach for 24 hours. The medium was removed and replaced with normal medium containing TRAIL with or without increasing concentrations of bortezomib, and cells were incubated for 48 hours. Cells survival/cytostasis was then quantified using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (24). Each experimental data point represented average values obtained from eight replicates, and each experiment was done in triplicate.

Quantification of DNA Fragmentation

DNA fragmentation was measured by propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis as described previously (24, 25). After incubation in vitro, cells were harvested by trypsinization, pelleted by centrifugation, and resuspended in PBS containing 50 μg/mL propidium iodide, 0.1% Triton-X 100, and 0.1% sodium citrate. Samples were stored at 4°C and analyzed by flow cytometry (FL3 channel).

Cell Surface Staining and FACS Analysis

For detection of cell surface expression of death receptors, cells were harvested by trypsinization and pelleted by centrifugation. Cells were incubated with 1 μg anti-TRAIL-R1 antibody (e-Biosciences, San Diego, CA) or anti-TRAIL-R2 antibody (e-Biosciences) or an irrelevant isotype control antibody in 50 μL PBS buffer containing 1% fetal bovine serum and 10 μg/mL sodium azide for 30 minutes on ice. Cells were washed twice with 0.5 mL cold PBS FACS buffer, and cells were stained with an IgG1-specific, FITC-conjugated secondary antibody (e-Biosciences) for an additional 30 minutes on ice in the dark. After two further washes with FACS buffer, cells were resuspended in 400 μL FACS buffer and analyzed by flow cytometry.

Small Interfering RNA

Cells in six-well plates were grown to confluence and transfected with small, double-stranded, interfering RNA molecules (siRNA) for IκBα and p65 (Upstate Cell Signaling Solutions; Lake Placid, NY) or with siRNA Nonspecific Control IV (Dharmacon RNA Technologies, Lafayette, CO) in serum-free MEM without antibiotic supplements using Oligofectamine (Invitrogen Life Technologies, Carlsbad, CA). Cells were incubated under these conditions for 48 hours, and silencing was then confirmed by immunoblotting.
**Western Blotting**

Cells were lysed directly in six-well plates by incubation for 1 hour at 4°C in 100 μL Triton lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 25 mmol/L Tris (pH 7.5), 1 mmol/L glycerol phosphate, 1 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 1 Complete Mini Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, IN)]. Lysates were centrifuged for 10 minutes at 14,000 × g at 4°C. Supernatants were saved and 30 μg from each sample was mixed with SDS-PAGE sample buffer (50 mmol/L Tris-HCL, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol). Samples were boiled for 5 minutes and then resolved by 12% SDS-PAGE (100 V). Polypeptides were electrophoretically transferred (90 minutes, 100 V) to nitrocellulose membranes (0.2 μm pore size, Schleicher & Schuell, Keene, NH). Membranes were blocked for 1 hour in 5% milk diluted with TBS-T (25 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween 20) or 3% bovine serum albumin diluted in PBS containing 0.1% Tween 20. Membranes were incubated overnight at 4°C with primary antibodies specific for IKK (Cell Signaling, Beverly, MA; 1:1,000 dilution), phosphorylated IκBα (Cell Signaling; 1:1,000 dilution), p65 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000 dilution), BclX(L) (BD Biosciences, PharMingen, San Diego, CA; 1:1,000 dilution), XIAP (R&D Systems; 1:1,000 dilution), or Bcl-2 (Santa Cruz Biotechnology; 1:1,000 dilution). Membranes were washed thrice, for 5 minutes each, and incubated with species-specific secondary antibodies [donkey anti-rabbit IgG (Amersham; 1:2,000 dilution) or donkey anti-mouse IgG (Amersham, Piscataway, NJ; 1:2,000 dilution)]. Membranes were incubated with horseradish sheep antimouse (Amersham; 1:1,000 dilution)] for 1 hour at 4°C. Blots were washed thrice, for 5 minutes each, and developed by chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA).

**Immunochemistry and Immunofluorescence**

Paraffin-embedded sections were used for analysis of BclXL. Approximately 5-μm-thick sections of formalin-fixed, paraffin-embedded tissue were deparaffinized in xylene, treated serially with dilutions of alcohol (100%, 95%, and 80% ethanol), and subsequently rehydrated in PBS (pH 7.5). The protocol for detection of BclX(L) used citrate/PBS-T antigen retrieval with incubation for 5 minutes in a microwave set on high power. Endogenous peroxidase activity was blocked by incubating tissue sections in 3% hydrogen peroxide diluted in PBS for 15 minutes. Tissues were washed in PBS thrice for 3 minutes each followed by incubation with the protein blocking solution for 20 minutes at room temperature. The protein blocking solution contained 5% normal horse serum and 1% normal goat serum in PBS (pH 7.5). Excess protein block was drained and tissues were incubated at 4°C overnight with mouse monoclonal anti-BclXL antibody (Santa Cruz Biotechnology; 1:50). The tissues were then washed thrice for 3 minutes each and incubated with peroxidase-conjugated goat anti-mouse IgG and IgM (Jackson Immunoresearch, West Grove, PA; 1:50) for 1 hour at room temperature. Positive staining was visualized by incubating tissue in 3,3′-diaminobenzidine (Research Genetics, Huntsville, AL) for 10 to 15 minutes. Following three washes in PBS, nuclei were counterstained with Gill’s no. 3 hematoxylin (Sigma, St. Louis, MO) for 10 seconds. Tissue sections were finally washed with distilled H2O thrice and treated with PBS for 1 minute. Slides were mounted with Universal Mount (Research Genetics) before imaging. Negative control samples incubated in secondary antibodies alone exhibited no staining.

Images were captured from tumor areas under a light microscope at fields of ×100 and absorbance was quantified using the Optimas software program (Bioscan, Edmonds, WA). Five different tumors were analyzed from each group, and a minimum of four images was captured from each tumor to yield an average measurement of staining intensity from 20 independent fields. Results were graphed as percentage of positively stained nuclei/cells versus total number of nuclei/cells quantified using the Optimas software (Bioscan).
Quantification of Apoptosis by Fluorescent Terminal Deoxyribonucleotide Transferase–Mediated Nick-End Labeling

DNA fragmentation was analyzed in frozen sections by FITC-labeled terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL; Promega). Percentages of positive cells were determined using the Optimas software program (Bioscan). Data were acquired from four fields in at least three tumors derived from each treatment group, and results are reported as means ± SE.

Results

Recent work from our laboratory and others has shown that the proteasome inhibitor bortezomib (PS-341, also known as Velcade) is a potent TRAIL-sensitizing agent in a variety of different TRAIL-resistant human cancer cell lines (10, 26–35). We therefore explored the effects of TRAIL with or without bortezomib on cell viability in two bortezomib-sensitive (L3.6pl, BxPC-3) and two bortezomib-resistant (Panc-1, HS766T) human pancreatic cancer cell lines (24) by MTT reduction analysis. Consistent with our previous work (24), L3.6pl and BxPC-3 cells were both strongly growth-inhibited by bortezomib, and both were also growth inhibited by exogenous TRAIL (1 ng/mL; Fig. 1A and B). Incubation with both agents resulted further growth inhibition (Fig. 1A and B). To more directly determine their relative sensitivities to TRAIL-induced apoptosis, we exposed nine human pancreatic cancer cell lines (including the four described above) to increasing concentrations of the cytokine for 24 hours and measured DNA fragmentation by propidium iodide staining and FACS analysis. Seven of the nine cell lines displayed significant, concentration-dependent increases in DNA fragmentation (Fig. 2A). The only two cell lines that did not undergo apoptosis in response to exogenous TRAIL were the Panc-1 and HS766T cells, consistent with the growth inhibition observed in the MTT analyses (Fig. 1C and D). Because surface receptor levels could play a critical role in determining cellular responsiveness to TRAIL, we measured, by FACS, the surface expression of the two active receptors of TRAIL (DR4 and DR5) on the nine cell lines within our panel. Interestingly, all of the TRAIL-sensitive lines displayed relatively uniform, high-level expression of both DR4 and DR5, whereas the TRAIL-resistant Panc-1 and HS-766T cells expressed lower levels of both receptors (Fig. 2B and C). Nonetheless, bortezomib synergized with TRAIL to promote apoptosis in both TRAIL-resistant cell lines (Fig. 2D), as we and others have observed in pancreatic cancer and other models previously (10, 29, 32–35). The effects of bortezomib were associated with up-regulation of surface DR5 (data not shown), also consistent with previous observations (10, 33).

Although bortezomib is a potent inhibitor of NF-κB by virtue of its ability to stabilize the endogenous inhibitor of NF-κB (IκB), it also has a variety of other effects on cell cycle progression and cell death that are not mediated by NF-κB inhibition (20, 36). To more directly evaluate the role of NF-κB inhibition in the restoration of TRAIL sensitivity in the Panc-1 and HS-766T cells, we...
characterized the effects of a more selective chemical inhibitor of NF-κB (the IKK inhibitor PS-1145; refs. 37–39) on TRAIL-induced apoptosis by propidium iodide/FACS. Control experiments confirmed that PS-1145 blocked NF-κB DNA-binding activity, and immunofluorescence staining and confocal microscopy showed that PS-1145 prevented tumor necrosis factor–induced nuclear localization of the p65 subunit of NF-κB (data not shown). PS-1145 also blocked both basal and tumor necrosis factor–induced phosphorylation of IκBα (data not shown). Concentrations of the drug up to 100 μmol/L did not increase DNA fragmentation in the Panc-1 or HS766T cells or in any of the other cell lines within our panel (Fig. 3A; data not shown). Furthermore, and in contrast to bortezomib, exposure to PS-1145 had no effect on DNA synthesis as measured by [3H]thymidine incorporation (data not shown). In addition, PS-1145 did not appreciably augment apoptosis induced by gemcitabine in either the Panc-1 or HS766T cells (Fig. 3B) and it had no effect on surface DR4 or DR5 expression (data not shown). However, like bortezomib, PS-1145 interacted with TRAIL to promote DNA fragmentation in both TRAIL-refractory cell lines in a concentration-dependent fashion (Fig. 3A).

To more rigorously define the effects of NF-κB inhibition on TRAIL sensitivity, we compared the levels of TRAIL-induced apoptosis in cells transiently transfected with a p65-specific siRNA construct or an off-target control. Like PS-1145, p65 knockdown synergized with TRAIL to promote DNA fragmentation in the Panc-1 cells (Fig. 3C), and under these conditions no additional effect of exposure to PS-1145 was observed. Immunoblotting confirmed that the p65-specific siRNA construct efficiently reduced p65 protein levels in the cells (Fig. 3C). Conversely, we reasoned that if the effects of PS-1145 were truly due to
accumulation of IκBα downstream of IKK inhibition, then siRNA-mediated silencing of IκBα would render cells resistant to PS-1145–mediated TRAIL sensitization. Consistent with this idea, an siRNA construct specific for IκBα (but not a control construct) completely blocked the DNA fragmentation observed in cells treated with PS-1145 plus TRAIL (Fig. 3D). Again, immunoblotting confirmed that the IκBα-specific siRNA construct strongly inhibited IκBα protein expression, whereas the off-target control construct had no effect (Fig. 3D).

Previous studies have established that the antiapoptotic proteins, BCL-XL and XIAP, are transcriptional targets of NF-κB in pancreatic cancer and other tumor types (16, 17, 40). Consistent with these observations, PS-1145 (Fig. 4A) and PS-341 (data not shown) down-regulated expression of both proteins in Panc-1 cells, whereas Bcl-2

![Figure 3. Effects of PS-1145 on TRAIL-induced apoptosis.](image-url)

**A**

**B**

**C**

**D**

Figure 3. Effects of PS-1145 on TRAIL-induced apoptosis. **A**, effects on DNA fragmentation. Cells were incubated in the presence of the indicated concentrations of PS-1145, rhTRAIL, or both agents for 24 h, and DNA fragmentation was measured by propidium iodide staining and FACS analysis as described in Materials and Methods. **Columns**, mean (n = 3); **bars**, SD. **B**, effects of PS-1145 on gemcitabine-induced apoptosis. Panc-1 or HS766T cells were incubated with the indicated concentrations of PS-1145, gemcitabine, or both agents for 24 h and DNA fragmentation was measured by propidium iodide staining and FACS analysis as described in Materials and Methods. **Columns**, mean (n = 3); **bars**, SD. **C**, effects of p65 silencing on apoptosis induced by TRAIL. Panc-1 cells were transfected with an siRNA construct specific for p65 or a control construct for 48 h. Cells were then incubated with 30 μmol/L PS-1145, 25 ng/mL rhTRAIL, or both agents for 24 h, and DNA fragmentation was measured by propidium iodide staining and FACS analysis. **Columns**, mean (n = 3); **bars**, SD. **D**, effects of silencing IκBα on PS-1145–mediated sensitization to TRAIL. Cells were transfected with an siRNA construct specific for IκBα or a control construct for 48 h. Cells were then incubated with 25 ng/mL rhTRAIL, 30 μmol/L PS-1145, or both agents for 8 h, and DNA fragmentation was measured by propidium iodide staining and FACS analysis. **Columns**, mean (n = 3); **bars**, SD. IκBα protein expression was determined by immunoblotting. Actin served as an internal control for protein loading. Similar results were obtained in three independent experiments.
levels remained unaffected. To determine whether these effects were functionally significant, we tested the effects of gene silencing on TRAIL-induced cell death. Knockdown of either XIAP (Fig. 4B) or BCL-XL (Fig. 4C) resulted in substantial TRAIL sensitization in the Panc-1 and HS766T cells.

Finally, we examined the effects of combination therapy with PS-1145 plus TRAIL on tumor growth inhibition and toxicity in mice bearing established orthotopic Panc-1 xenografts. Therapy consisted of 50 mg/kg PS-1145, 10 mg/kg rhTRAIL, or a combination of the two, delivered daily 5 d/wk via i.p. injection. Tumor growth was monitored noninvasively by bioluminescence imaging throughout the course of therapy; representative images obtained from animals at the experimental end point are shown in Fig. 5A. Animals were sacrificed after 4 weeks of therapy. All of the animals survived therapy and displayed similar body weights at the end of the study (Fig. 5B). Therapy with either PS-1145 or rhTRAIL alone had little effect on tumor growth (Fig. 5C). However, combination therapy with PS-1145 plus TRAIL produced frank tumor regressions (Fig. 5C), consistent with the strong proapoptotic activity of the drug combination in vitro. The effects of combination therapy in vivo were associated with decreases in BclXL expression as measured by immunohistochemistry (Fig. 5D). Single-agent therapy with PS1145 alone resulted in very few apoptotic cells, whereas higher levels of TUNEL staining were observed in tumors isolated from the animals treated with either TRAIL alone or the combination of PS1145 and TRAIL (Fig. 5E).

Discussion

Conventional chemotherapy has little effect on the course of disease progression in patients with pancreatic cancer, prompting a search for more effective agents that exploit the biology of disease progression. Here, we report that rhTRAIL induced some degree of apoptosis in a majority (seven of nine) of human pancreatic cancer cell lines. Chemical inhibitors of NF-κB (bortezomib and PS-1145) enhanced TRAIL-induced apoptosis in the TRAIL-sensitive cells and overcame TRAIL resistance in the other two cell lines (Panc-1 and HS766T). Molecular inhibition of NF-κB (via silencing of p65) also sensitized cells to TRAIL. Although the effects of NF-κB inhibition on cell survival are complex, our results confirm that the survival proteins, XIAP and BCL-XL, are down-regulated by PS-1145 in pancreatic cancer cells, and gene silencing experiments confirmed that the proteins both contribute to TRAIL resistance. Together, our data strongly suggest that NF-κB plays a central role in maintaining TRAIL resistance in human pancreatic cancer cells.

To determine whether combination therapy with TRAIL plus PS-1145 exerted antitumoral activity and/or toxicity, we evaluated the effects of combination therapy on the growth of xenografts derived from the TRAIL-refractory Panc-1 cells. Tumors were established in the pancreas glands of nude mice, and therapy was delivered daily 5 d/wk for 4 weeks. We observed some tumor growth inhibition in animals treated with PS-1145 or rhTRAIL alone, although the Panc-1 cells were resistant to either agent in vitro, suggesting that these agents might inhibit tumor growth via indirect effects on the tumor microenvironment. The increase in TUNEL observed with single-agent TRAIL therapy in the otherwise TRAIL-resistant Panc-1 tumors may have been due to sensitization of the tumors to TRAIL by the tumor microenvironment and/or to the antiangiogenic effects of TRAIL therapy.4 However, combination therapy was much more effective, resulting in

Figure 4. Roles of BCL-XL and XIAP in TRAIL sensitization. A, effects of PS-1145 on BCL-XL and XIAP expression. Cells were incubated with 40 μM PS-1145, 50 ng/mL TRAIL, or both for 24 h and target protein expression was measured by immunoblotting. Numbers below each lane, protein expression relative to control after normalization against actin (loading control). Similar results were obtained in three independent experiments. B, effects of XIAP silencing on TRAIL-induced apoptosis. TRAIL-refractory HS766T or Panc-1 cells were transiently transfected with an XIAP-specific siRNA construct or an off-target control for 48 h. Cells were then exposed to 50 ng/mL rhTRAIL for 24 h and DNA fragmentation was quantitated by propidium iodide/FACS. Bottom, effects of gene silencing on XIAP protein levels as measured by immunoblotting. Columns, mean (n = 3); bars, SD. C, effects of BCL-XL silencing on TRAIL-induced apoptosis. TRAIL-refractory HS766T or Panc-1 cells were transiently transfected with a BCL-XL-specific siRNA construct or an off-target control for 48 h. Cells were then exposed to 50 ng/mL rhTRAIL for 24 h and DNA fragmentation was measured by propidium iodide/FACS. Bottom, effects of gene silencing on BCL-XL protein levels as measured by immunoblotting. Columns, mean (n = 3); bars, SD.

4 L. Lashinger, unpublished observations.
regression of established tumors, and it produced no detectable toxicity. In parallel studies, we are evaluating the effects of proteasome inhibitors (bortezomib and NPI-0052) plus TRAIL in xenografts, and we have noted substantial systemic toxicity when the dose of proteasome inhibitor is delivered simultaneously with TRAIL at the maximum tolerated dose of the proteasome inhibitor.  

Figure 5. Effects of combination therapy with PS-1145 plus TRAIL in orthotopic Panc-1 tumors. Animals bearing established tumors were treated daily (5 d/wk) with 50 mg/kg PS-1145, 10 mg/kg rhTRAIL, or a combination of the two for 4 wks. Tumor volumes were assessed noninvasively as outlined in Materials and Methods. Animals were sacrificed, tumors were harvested, tumor sections were stained for detection of BclXL, or DNA fragmentation (using TUNEL), and expression levels were quantified as described in Materials and Methods. A, representative images of luciferase activity observed in representative orthotopic Panc-1 tumors at the experimental end point. Numerical values for the tumors displayed in the figure are indicated. B, effects of combination therapy with PS-1145 plus TRAIL on body weights. Data are body weights measured at the end of the experiment. Columns, mean (n = 8); bars, SE. C, tumor growth curves. Columns, mean; bars, SE (did not exceed 10% of mean values). D, effects of therapy on BclXL expression in vivo. Columns, mean; bars, SE, (n = 5). E, effects of therapy on apoptosis in vivo as measured by TUNEL. Columns, mean; bars, SE, (n = 5).
Thus, combining TRAIL with more selective NF-κB inhibitors (rather than proteasome inhibitors) may be a more attractive approach to the therapy of this malignancy.

On the other hand, in other recent studies, we showed that bortezomib sensitizes human prostate and bladder cancer cells to TRAIL (29, 35), but the effects of the drug as an NF-κB inhibitor may have been less important in these other models. Specifically, in the previous study, we showed that PS-1145 did not affect TRAIL sensitivity in the urological tumor cells, and our analyses of the molecular mechanisms involved showed that the effects of bortezomib in these models were dependent on accumulation of the cyclin-dependent kinase inhibitor p21 (35). Although these effects may cooperate with NF-κB inhibition to promote TRAIL sensitivity in pancreatic cancer cells exposed to bortezomib as well, we have found, in control experiments, that PS-1145 does not cause accumulation of p21 or p27, inhibit cyclin-dependent kinase 2 activation, or inhibit DNA synthesis (as measured by [3H]thymidine incorporation) in pancreatic cancer cells (data not shown). Furthermore, bortezomib (but not PS-1145) up-regulated surface DR5 expression on the Panc-1 and HS766T cells (data not shown), providing additional evidence that the effects of bortezomib are broader than those exerted by the more selective NF-κB antagonist. Thus, proteasome inhibitors seem to be more global TRAIL-sensitizing agents than NF-κB antagonists, and head-to-head comparisons of their efficacy and toxicity in appropriate preclinical models would seem warranted.

Conventional chemotherapy and radiotherapy can stimulate NF-κB in cancer cells, and NF-κB inhibitors have been reported to promote sensitivity to these agents in vitro and in vivo (18, 19). However, in our hands, the effects of bortezomib and PS-1145 on TRAIL-induced apoptosis were much greater than their effects on apoptosis induced by gemcitabine (Fig. 3B; ref. 36). As discussed above, bortezomib is a very strong inhibitor of cell cycle progression, and we suspect that cell cycle inhibition sometimes undermines the potential benefit of bortezomib-based combination chemotherapy by preventing cells from entering the phase of the cell cycle at which the agents are most active (36). On the other hand, TRAIL sensitivity may be enhanced when cancer cells are arrested in Gi (41), which distinguishes TRAIL from most other clinically relevant proapoptotic stimuli. In addition, our observation that PS-1145 has greater effects on apoptosis induced by TRAIL as opposed to other agents strongly suggests that TRAIL resistance is more tightly coupled to NF-κB activity than is resistance to other death stimuli.

Identifying the subset of patients who will benefit most from biological agents is a major challenge for clinical trial design. Our analyses of surface TRAIL receptor expression revealed that TRAIL-resistant cells expressed low levels of DR4 and DR5. Although results obtained with cell lines must be interpreted with caution and previous studies have concluded that cellular TRAIL receptor levels do not always correlate with TRAIL responsiveness (10), the data suggest that the possible relationship between basal death receptor expression and sensitivity to TRAIL should be explored further, particularly in primary tumors. Normal tissues also tend to express lower levels of DR4 and DR5 than tumor cells (7), and it is possible that this is one of the reasons why TRAIL is not toxic to normal tissues in vivo. In future studies, we plan to more directly evaluate the contributions of DR4 and DR5 to TRAIL-induced apoptosis in our panel of cell lines and characterize receptor expression in primary human pancreatic tumors displayed on tissue microarrays.
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