Killing of Resistant Cancer Cells with Low Bak by a Combination of an Antimesothelin Immunotoxin and a TRAIL Receptor 2 Agonist Antibody

Xing Du1, Laiman Xiang1, Crystal Mackall2, and Ira Pastan1

Abstract

Purpose: Many solid tumors express cell surface mesothelin making them attractive targets for antibody-based therapies of cancer. SS1P [antimesothelin(Fv)PE38] is a recombinant immunotoxin (RIT) that has potent cytotoxic activity on several cancer cell lines and clinical activity in mesothelioma patients. Pancreatic cancers express mesothelin and are known to be resistant to most chemotherapeutic agents. The goal of this study is to treat pancreatic cancer with RIT by targeting mesothelin.

Experimental Design: We measured the cytotoxic activity of an antimesothelin immunotoxin on pancreatic cancer cells. We also measured the levels of several pro- and antiapoptotic proteins, as well as the ability of TNF-related apoptosis-inducing ligand (TRAIL) or the anti-TRAIL receptor 2 agonist antibody (HGS-ETR2) to kill pancreatic cells, and the cytotoxic activity of the two agents together in cell culture and against tumors in mice.

Results: In two pancreatic cancer cell lines, immunotoxin treatment inhibited protein synthesis but did not produce significant cell death. The resistant lines had low levels of the proapoptotic protein Bak. Increasing Bak expression enhanced the sensitivity to immunotoxins, whereas Bak knockdown diminished it. We also found that combining immunotoxin with TRAIL or HGS-ETR2 caused synergistic cell death, and together triggered caspase-8 recruitment and activation, Bid cleavage and Bax activation. Combining SS1P with HGS-ETR2 also acted synergistically to decrease tumor burden in a mouse model.

Conclusion: Our data show that low Bak can cause cancer cells to be resistant to immunotoxin treatment and that combining immunotoxin with TRAIL or a TRAIL agonist antibody can overcome resistance. Clin Cancer Res; 17(18); 5926–34. ©2011 AACR.

Introduction

Since the Food and Drug Administration approved the first monoclonal antibody (mAb) rituximab in 1997 for the treatment of B cell lymphomas, many mAbs have been evaluated for their ability to cause tumor regressions (1), but only a few have been effective. To improve the therapeutic usefulness of antibodies, they are now being used with a variety of cytotoxic substances such as small molecular drugs, radioisotopes, or protein toxins (2).

Our laboratory has focused on the development of recombinant immunotoxins (RIT) for cancer treatment (3). These proteins are composed of an Fv that binds to an antigen on a cancer cell fused to a 38-kD portion of Pseudomonas exotoxin A (PE38). They derive their potency from the toxin and their specificity from the antibody fragment to which they are attached. We now have several RITs in clinical trials; one of these is Moxetumomab pasudotox (also known as CAT-8015 or HA22), which targets CD22 on B cells malignancies. It has produced a very high complete remission rate in chemotherapy-resistant hairy cell leukemia and is now being evaluated in other B cell malignancies (4–6). Another is SS1P [antimesothelin(Fv) PE38] that targets mesothelin, a 40-kD cell surface glycoprotein that is present on mesotheliomas and pancreatic, ovarian, and lung cancers and cholangiocarcinomas (7–11). SS1P is composed of an antimesothelin Fv linked to PE38. It has shown significant cytotoxic activity in vitro against ovarian, mesothelioma, lung, and cholangiocarcinoma cancer cells (7–11). SS1P has been evaluated in 2 phase I clinical trials. It was well tolerated and showed some antitumor activity in patients with mesothelioma (12, 13). A new trial in which SS1P is being given in combination with cisplatin and pemetrexed is ongoing (14).

To kill cells, an immunotoxin must be internalized and the toxin portion delivered to the cytosol via the...
endoplasmic reticulum (Fig. 1A). In the cytosol, protein synthesis is inhibited by the ADP-ribosylation of elongation factor 2 and then the apoptosis cascade is activated and cell death ensues. The details of how the apoptosis pathway is activated after protein synthesis inhibition are still not completely understood. Our experiments with mouse embryonic fibroblast (MEF) cells showed that degradation of the antiapoptotic protein Mcl-1 is required, and that Bak, a mediator of the intrinsic pathway of apoptosis is critical for apoptosis induced by PE (15).

To expand the usefulness of antimesothelin immunotoxin therapy, and to further understand the mechanism of immunotoxin induced apoptosis, we evaluated the activity of SS1P or SS1P-KDEL, a mutant RIT with enhanced intracellular trafficking ability (16), on pancreatic cancer cells, which are known to be resistant to conventional treatments (17, 18). Recent findings indicate that constitutively activated autophagy may contribute to pancreatic ductal adenocarcinoma pathogenesis (19, 20). We show here that Mcl-1 and Bak also regulate apoptosis in human cells, and that pancreatic cancer cells with low Bak protein are resistant to immunotoxin treatment despite efficient protein synthesis inhibition. This resistance can be overcome by combining immunotoxin with TNF-related apoptosis-inducing ligand (TRAIL), or an anti-TRAIL receptor 2 agonist antibody that activates the extrinsic pathway. The cell killing produced by the combination treatment is highly synergistic and mitochondrial dependent and is initiated by caspase-8 recruitment and activation.

Translational Relevance

New treatments for pancreatic cancer are needed because they are resistant to most chemotherapeutic agents. Many solid tumors express cell surface mesothelin, which makes them attractive targets for antibody-based cancer therapies. We assessed the activity of an antimesothelin immunotoxin on pancreatic cancer cells and found that they are immunotoxin resistant and that low levels of the proapoptotic protein Bak contribute to the resistance. We show that combining TNF-related apoptosis-inducing ligand (TRAIL) or an anti-TRAIL receptor 2 agonist antibody, HGS-ETR2, with immunotoxin caused synergistic cell death and synergized in mice to cause a reduction in tumor size. Our results show that low Bak cancer cells are resistant to immunotoxin treatment and that combining immunotoxin with TRAIL or TRAIL agonist antibody is an effective way to overcome such resistance.
Synergistic antitumor activity was also observed in mice receiving a combination of immunotoxin and anti-TRAIL receptor 2 agonist antibody.

Materials and Methods

Reagents

Recombinant human and mouse TRAIL were purchased from R&D Systems. The anti-TRAIL receptor 2 agonist antibody HGS-ETR2 was provided by Human Genome Science. Immunotoxins SS1P, SS1P-KDEL, HB21(Fv)-PE40, TG-Fc-PE38, and LMB9 were prepared in our lab. Human insulin was obtained from the NIH pharmacy. Puromycin was purchased from Invitrogen. 3H-leucine was purchased from GE Healthcare.

Cells

A431/H9 (mesothelin stable cell line), KB31, Hela, MEF, and MEF (Bak−/−) were maintained in Dulbecco’s Modified Eagle Medium with 10% FBS. Pancreatic cell line Panc3.014 was obtained from Dr. Elizabeth Jaffee (Department of Oncology, Johns Hopkins University, Baltimore, MD) and maintained in RPMI-1640 with 20% FBS and 0.2 unit/mL human insulin. Pancreatic cell line HTB80 (Japan-2, American Type Culture Collection) was maintained in RPMI-1640 with 10% FBS.

Surface TRAIL receptor and mesothelin expression, protein synthesis, and apoptosis assay, immunoblot and immunoprecipitation

TRAIL receptor expression was detected with anti-human TRAIL R1 and R2 antibodies (R&D System). Mesothelin expression was detected with MORAB-009 (SS1 humanized mAb). Protein synthesis inhibition was measured with 3H-leucine incorporation. Detailed methods are described in the Supplementary Methods.

After treatment, both floating and adherent cells were collected, washed with cold DPBS twice, and solubilized in lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitors and subjected to Western blotting. For immunoprecipitation and Bax activation, cell lysates were prepared in 1% CHAPS lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and incubated with mouse anti-Bax (6A7). Detailed methods and antibodies for Western blotting are described in the Supplementary Methods.

Bak overexpression and siRNA knockdown of Bak, Bax, and Bid

A cDNA for human Bak was cloned into a lentiviral expression vector pLVX-PURO (Clontech). Lentivirus was produced by SAIC-Frederick (Frederick, MD) and transduced into KB31. Stable clones were selected with 1.5 μg/mL puromycin. Bak expression levels were confirmed by Western blot and cells were treated with 100 ng/mL SS1P for indicated times and subjected to apoptosis analysis.

siRNAs (control, Hs-Bak1_5, Hs_Bax_9, Hs_Bid_7; Qiagen) were transfected into cells at the indicated concentrations with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol.

Xenograft tumor model

KB31 (1.7 × 10⁶) with Matrigel (4.0 mg/mL, 200 μL per mouse) were implanted subcutaneously into the thigh area of the rear leg of 5- to 6-week-old athymic nude mice (18–20 g). Tumor dimensions were determined every other day with a caliper. Tumor volume (mm³) was calculated by the formula: (a) × (b²) × 0.4, in which (a) is tumor length and (b) is tumor width in millimeters.

When tumor size reached 110 mm³ (day 8), 10 mg/kg HGS-ETR2 was given intraperitoneally and 4 hours later, 3 doses of SS1P 0.3 mg/kg was given i.v. QOD (days 8, 10, and 12). The animal protocol was approved by the National Cancer Institute (NCI) Animal Care and Use Committee. All animal experiments were stopped when tumors reached 1,000 mm³ based on animal protocols.

Statistics

All data are presented as mean ± SD from at least 3 independent assays. Statistical analysis of synergy on tumor experiments was done by Drs. David Venzon and David Liewehr (Biostatistics and Data Management Section/Center for Cancer Research/NCI, Bethesda, MD). Tumor volumes were logarithmically transformed before analysis to normalize their distribution and to stabilize variances. Differences between treatment groups were assessed by repeated measures ANOVA, and 2-tailed P values from Dunnett’s test were reported for the multiple comparisons within days. Synergy was defined as an interaction effect significantly greater than the sum of the SS1P and HGS-ETR2 effect.

Results

Immunotoxin does not induce cell death despite efficient protein synthesis inhibition

Pancreatic cell lines HTB80 and Panc3.014 have strong cell surface mesothelin expression (Supplementary Fig. S1), and the expression levels are in the range of 2 sensitive cell lines often used in our lab, KB31 and A431/H9. We then treated the 4 cell lines with SS1P at 300 ng/mL and found that protein synthesis was completely arrested in A431/H9 and KB31 cells but not in HTB80 and Panc3.014 (data not shown). We have previously shown that in some cell lines changing the c-terminus of an immunotoxin to KDEL, which binds more tightly to the recycling receptor, can improve protein synthesis inhibition and indeed found SS1P-KDEL at 300 ng/mL profoundly inhibited protein synthesis in the 2 pancreatic cell lines (Fig. 1B) and employed SS1P-KDEL in all further experiments with pancreatic cells. When examined at 24 hours, more than 45% of the A431/H9 were undergoing apoptosis, which increased to more than 90% at 48 hours (Fig. 1C). Significant apoptosis became evident at 48 hours in the KB31 (Fig. 1C). No significant apoptosis was detected in HTB80 or Panc3.014 at 48 and even 72 hours (Fig. 1C).
and unpublished data). These results indicate that despite profound protein synthesis inhibition, SS1P-KDEL does not cause the death of HTB80 and Panc3.014 cells, indicating that these 2 cell lines are highly resistant to immunotoxin, as observed in a recent study that DLD1 cells do not undergo apoptosis when protein synthesis is inhibited by immunotoxins (21).

**Low expression of Bak protein in resistant cells and Bak-dependent apoptosis induced by immunotoxin**

Using MEFs, we have previously found that apoptosis induced by PE is Bak dependent (15). In this study, we examined Bak protein levels and found that expression levels were low in these 2 cell lines (Fig. 2A), compared with A431/H9 and KB31 cells. The levels of Bax, another proapoptotic multi-BH domain protein, were similar in all the cell lines. We found that treatment with SS1P-KDEL resulted in a large fall in Mcl-1 levels as previously shown in MEFs treated with native PE toxin (15), but this fall was not sufficient to induce apoptosis in HTB80 and Panc3.014 cells.

We reasoned that if Bak were important for apoptosis induced by immunotoxin, then increasing Bak expression would increase sensitivity to immunotoxin. We tried to make stable lines with high Bak by using HTB80 and Panc3.014 but failed because of low transfection efficiency. We were able to easily transfect KB31 cells that have lower BAK and are less sensitive to immunotoxins compared with A431/H9 cells.

We established several stable KB31 clones expressing Bak and found that each clone had about a 2-fold increase of Bak compared with parental KB31 cells. Bak protein levels were unchanged (Fig. 2B). All Bak clones showed increased sensitivity to SS1P, which induced 20% to 35% apoptotic cells after 24 hours and 34% to 60% after 48 hours. These values are significantly higher than the parental KB31 cells, or KB31 cells transduced with empty virus (Fig. 2B). Clone #2 had the lowest level of Bak and showed the lowest apoptosis. These data show that raising Bak levels increases immunotoxin killing.

To show that a decrease in Bak can cause resistance to immunotoxin, we used siRNA to knockdown Bak in A431/H9, because it has high Bak levels and is immunotoxin sensitive (Fig. 2C). We found that apoptotic cells induced by SS1P decreased from 39% to 8% at the 24-hour time point and from 65% to 14% at 36-hour time point.

To determine whether the apoptosis induced by other PE-based immunotoxins is also Bak dependent, we treated A431/H9 with HB21(Fv)-PE40 targeting the transferrin receptor and LMB9 targeting the Lewis Y antigen (3). Bak knockdown reduced the apoptotic cells induced by SS1P, HB21(Fv)-PE40, or LMB9 (Supplementary Fig. S2).
To determine whether Bak dependence exists in other cell types, we knocked down Bak in Hela cells and found substantial reduction of apoptotic cells induced by HB21 (Fv)-PE40 or TGFα-PE38 (transforming growth factor α fused to PE38) targeting epidermal growth factor receptor (3). Hela cells do not express Lewis Y and are not responsive to LMB9 (Supplementary Fig. S2).

**Immunotoxin combined with TRAIL synergistically induce cell death**

Because immunotoxins kill cells by lowering Mcl-1 levels in a Bak-dependent process, we hypothesized that activating the extrinsic apoptosis pathway would increase immunotoxin cell killing if Bak levels were low, because Bax is important for the extrinsic apoptosis pathway (22, 23). To do this, we needed to use cell lines expressing TRAIL receptors. We examined the pancreatic cancer lines and KB31 cells and found they all expressed TRAIL 1 and TRAIL 2 receptors and that the TRAIL 2 receptor was more highly expressed (Fig. 3A). Despite TRAIL receptor expression, these cells were all resistant to human TRAIL at 20 ng/mL 48 hours after treatment (Fig. 3B). SS1P-KDEL alone did not cause cell death of either HTB80 or Panc3.014 cells (Fig. 3B). When SS1P-KDEL and TRAIL were combined, there was considerable cell death: 51% apoptotic cells in HTB80 after 24 hours and 66% after 48 hours, 17% in Panc3.014 after 24 hours and 40% after 48 hours.

We examined the activation of cell death pathway proteins caspase-8, Bid and PARP in cells treated with immunotoxins and TRAIL. SS1P-KDEL or TRAIL alone did not induce apoptosis in HTB80 cells and did not activate caspase-8 or cause Bid cleavage (Fig. 4A). There was no PARP cleavage, which is consistent with the Annexin V staining assay data. Only the combination of SS1P-KDEL with TRAIL resulted in caspase-8, Bid, and PARP cleavage (Fig. 4A) and activation of Bax (Fig. 4B), indicating that the combination of immunotoxin and TRAIL successfully triggered the Bax-mediated apoptosis cascade by recruitment and activation of caspase-8.

To rule out the possible role of Bak in the Bax pathway, we utilized Bak knockout MEFs (Bak−/− MEF). We found that after 48 hours treatment with PE toxin alone or recombinant mouse TRAIL alone, there was no measurable apoptosis. However, PE combined with TRAIL induced massive apoptotic Bak−/− MEFs (Fig. 4C), suggesting that the combination of PE toxin and TRAIL synergistically activates Bax-mediated cell death, and that Bak is dispensable for this apoptosis pathway. Similar synergistic combination results were obtained when using Bak−/− HCT116 colorectal cancer cell line (Supplementary Fig. S3).

**Synergistic antitumor activity with a combination of TRAIL receptor 2 agonist antibody HGS-ETR2 and SS1P**

Lexatumumab (HGS-ETR2) is a fully human agonistic mAb targeting TRAIL receptor 2 that activates the extrinsic apoptosis pathway. It has preclinical antitumor activity and is being evaluated in clinical trials (24–27). To examine whether combining immunotoxin with HGS-ETR2 could overcome the resistance of Bak deficient cancer cells, we treated HTB80 cells with HGS-ETR2. As shown in Figure 5A, neither a very high concentration of HGS-ETR2 (50 μg/mL) nor SS1P-KDEL alone induced apoptosis. However, when SS1P-KDEL and HGS-ETR2 were combined, they synergistically triggered massive cell death, and the apoptosis was HGS-ETR2 dose dependent (Fig. 5A).

To examine the effect of the 2 agents in an animal model, we used the KB31 xenograft tumors because these cells grow well in immunodeficient mice and the pancreatic cancer cell lines do not (28, 29). Cell culture experiments showed that at 24 hours neither SS1P nor TRAIL nor HGS-ETR2 alone induced significant cell death. When SS1P was combined with either TRAIL or HGS-ETR2, substantial cell death was produced (Fig. 5B and C). For tumor
experiments, mice bearing KB31 tumors were treated on day 8 with a single dose of HGS-ETR2, or on days 8, 10, and 12 with SS1P, or with the 2 agents together. We found that each agent alone had a modest antitumor effect, and when combined the effect was much greater than with each agent alone (Fig. 5D). On day 12, we found the combination had substantially smaller tumors than the single treatments (P < 0.0001 for each). The calculated synergistic effect was significantly different from zero (P = 0.013). On day 14, the combination again had substantially smaller tumors than the single treatments (P < 0.0001 for each). The synergistic effect was slightly larger than on day 12 (P = 0.0028).

The mitochondrial dependent synergism between immunotoxin and HGS-ETR2 is not due to c-FLIP downregulation or mitochondrial feedback amplification loop

To understand the molecular mechanism behind such robust synergistic cell killing, we chose KB31 for further study because of its high transfection efficiency. We first determined whether the immunotoxin and HGS-ETR2 increases the mesothelin or TRAIL receptor 2 expression. As shown in Figure 6A, there was no change of mesothelin expression by HGS-ETR2 treatment or TRAIL receptor 2 expression by SS1P treatment. These findings rule out the possibility that increased surface receptors are the cause for the synergism of SS1P and HGS-ETR2 combination.

2 expression by SS1P treatment. These findings rule out the possibility that increased surface receptors are the cause for the synergism of SS1P and HGS-ETR2 combination.

Figure 4. Immunotoxin and TRAIL combination activates Bax-mediated apoptosis pathway, and Bak is dispensable for the synergism. A, HTB80 treated with 300 ng/mL SS1P-KDEL, 50 ng/mL TRAIL, or combination for 24 hours. Western blots were conducted with full-length caspase-8, cleaved caspase-8, Bid, Mcl-1, PARP, and actin antibodies. *, unknown protein. B, HTB80 treated with 300 ng/mL SS1P-KDEL, 50 ng/mL TRAIL, or combination for 24 hours. Cell lysates were prepared with CHAPS buffer, immunoprecipitated with mouse mAb 6A7, followed by Western blotting. WCL, whole-cell lysate. C, Bak−/− MEF’s treated with 30 ng/mL PE toxin, or 20, 100 ng/mL recombinant mouse TRAIL, or combination for 24 hours. Apoptotic cells are shown as Annexin V-positive population (n = 3).

Figure 5. Synergistic antitumor effect from the combination of immunotoxin and TRAIL receptor 2 agonist antibody HGS-ETR2. A, HTB80 treated with 300 ng/mL SS1P-KDEL, HGS-ETR2, or combination for 24 hours. B, KB31 treated with 100 ng/mL SS1P, 10 ng/mL rh-TRAIL, or combination for 24 hours. C, KB31 cells treated with 100 ng/mL SS1P, 0.5 mg/mL HGS-ETR2, or combination for 24 hours. Apoptosis was analyzed with Annexin V-PE and 7-AAD staining. D, KB31 cells (1.7 x 10^6) with Matrigel (4.0 mg/mL, 200 μL per mouse) implanted into nude mice, 5 mice per group. When tumor size reached 110 mm^3, 10 mg/kg HGS-ETR2 was given i.p, and 4 hours later, 3 doses of SS1P 0.3 mg/kg were given QOD i.v.
We found that caspase-8 and Bid cleavage in KB31 were synergistic cell killing, as suggested by the combination of SS1P and HGS-ETR2 (Fig. 6D), suggesting that cell death by combination treatment is still mitochondrial dependent. However, the production of the active form of caspase-8, p43/41, was not affected by either Bid knockdown, or by Bak/Bax double knockdown (Fig. 6E). Bid cleavage was also not affected by Bak/Bax double knockdown (Fig. 6E). These data suggest that a mitochondrial feedback amplification loop does not exist when cells are treated with the combination of SS1P and HGS-ETR2, because the downstream events did not affect the upstream events.

Discussion

In this study, we examined pancreatic cancer cell lines to see whether an immunotoxin against mesothelin that has previously been shown to kill other mesothelin expressing cancer cell lines (7–11) can kill them. We found that 2 pancreatic lines, HTB80 and Panc3.014, were resistant to immunotoxin killing despite complete inhibition of protein synthesis and showed that the resistance was associated with low expression of Bak. We then carried out experiments to determine whether Bak regulated the ability of immunotoxins to kill cells and found that increasing Bak makes cells more immunotoxin sensitive and that lowering Bak makes cells immunotoxin resistant. Thus, it is clear that after arrest of protein synthesis, Bak, which acts through the intrinsic mitochondrial apoptosis pathway, has an important role in the ability of immunotoxins to kill cells. To our knowledge, this is the first article showing that cancer cells with low levels of endogenous Bak are resistant to some anticancer drugs. We did not expect the level of Bak to be so crucial that even a 2-fold increase of Bak could sensitize cells to immunotoxin.

Certain cancer cells, such as DU145 and LoVo, carry frameshift mutations in the Bak gene and do not express Bak protein (31). To determine whether mutations in the Bak gene are a cause of low Bak expression in HTB80 and Panc3.014, we sequenced the genomic DNA and found no changes except silent mutations in the Bak gene (data not shown). Other cancer cells such as colon cancer cell line HCT116 also contain low Bak protein (unpublished data and ref. 32). HCT116 is resistant to HB21(Fv)-PE40 despite profound protein synthesis inhibition and growth arrest, and no significant apoptotic cells can be detected even after 48 hours of treatment (Supplementary Fig. S2 and unpublished data). Thus, there are cancer cells that contain much less Bak protein for unknown reasons and are resistant to immunotoxin.

To overcome immunotoxin resistance, we simultaneously targeted both the mitochondrial (intrinsic) and death receptor (extrinsic) apoptosis pathways; this has been shown to be an effective strategy with the drugs etoposide, bortezomib, and vorinostat (30, 33–35). We found that HTB80 and Panc3.014 express TRAIL receptors, yet are resistant to treatment with TRAIL. Remarkably, when SS1P-KDEL and TRAIL were combined on HTB80 and Panc3.014 cells, they synergized to produce massive cell death induced by the combination of SS1P and HGS-ETR2 (Fig. 6D), suggesting that cell death by combination treatment is still mitochondrial dependent. However, the production of the active form of caspase-8, p43/41, was not affected by either Bid knockdown, or by Bak/Bax double knockdown (Fig. 6E). Bid cleavage was also not affected by Bak/Bax double knockdown (Fig. 6E). These data suggest that a mitochondrial feedback amplification loop does not exist when cells are treated with the combination of SS1P and HGS-ETR2, because the downstream events did not affect the upstream events.

Discussion

In this study, we examined pancreatic cancer cell lines to see whether an immunotoxin against mesothelin that has previously been shown to kill other mesothelin expressing cancer cell lines (7–11) can kill them. We found that 2 pancreatic lines, HTB80 and Panc3.014, were resistant to immunotoxin killing despite complete inhibition of protein synthesis and showed that the resistance was associated with low expression of Bak. We then carried out experiments to determine whether Bak regulated the ability of immunotoxins to kill cells and found that increasing Bak makes cells more immunotoxin sensitive and that lowering Bak makes cells immunotoxin resistant. Thus, it is clear that after arrest of protein synthesis, Bak, which acts through the intrinsic mitochondrial apoptosis pathway, has an important role in the ability of immunotoxins to kill cells. To our knowledge, this is the first article showing that cancer cells with low levels of endogenous Bak are resistant to some anticancer drugs. We did not expect the level of Bak to be so crucial that even a 2-fold increase of Bak could sensitize cells to immunotoxin.

Certain cancer cells, such as DU145 and LoVo, carry frameshift mutations in the Bak gene and do not express Bak protein (31). To determine whether mutations in the Bak gene are a cause of low Bak expression in HTB80 and Panc3.014, we sequenced the genomic DNA and found no changes except silent mutations in the Bak gene (data not shown). Other cancer cells such as colon cancer cell line HCT116 also contain low Bak protein (unpublished data and ref. 32). HCT116 is resistant to HB21(Fv)-PE40 despite profound protein synthesis inhibition and growth arrest, and no significant apoptotic cells can be detected even after 48 hours of treatment (Supplementary Fig. S2 and unpublished data). Thus, there are cancer cells that contain much less Bak protein for unknown reasons and are resistant to immunotoxin.

To overcome immunotoxin resistance, we simultaneously targeted both the mitochondrial (intrinsic) and death receptor (extrinsic) apoptosis pathways; this has been shown to be an effective strategy with the drugs etoposide, bortezomib, and vorinostat (30, 33–35). We found that HTB80 and Panc3.014 express TRAIL receptors, yet are resistant to treatment with TRAIL. Remarkably, when SS1P-KDEL and TRAIL were combined on HTB80 and Panc3.014 cells, they synergized to produce massive cell death induced by the combination of SS1P and HGS-ETR2 (Fig. 6D), suggesting that cell death by combination treatment is still mitochondrial dependent. However, the production of the active form of caspase-8, p43/41, was not affected by either Bid knockdown, or by Bak/Bax double knockdown (Fig. 6E). Bid cleavage was also not affected by Bak/Bax double knockdown (Fig. 6E). These data suggest that a mitochondrial feedback amplification loop does not exist when cells are treated with the combination of SS1P and HGS-ETR2, because the downstream events did not affect the upstream events.
death accompanied by caspase-8 and Bid cleavage, and Bax activation.

Antibodies to TRAIL receptors have advantages over TRAIL itself as therapeutic agents because they do not bind to decoy receptors and have a long half-life. HGS-ETR2 is a human IgG1 agonist antibody to TRAIL receptor 2 that has been evaluated in several studies for its antitumor activity (24–27). We found that combining SS1P-KDEL with HGS-ETR2 induced synergistic cell killing in HTB80 and that combining SS1P with HGS-ETR2 or TRAIL induced synergistic cell killing of KB31. When given to mice with KB31 tumors, SS1P combined with HGS-ETR2 had greater antitumor activity than either agent alone on slowing tumor growth, although the tumor regressions were not observed. Optimization of the timing of administration of immunotoxin and HGS-ETR2 may enhance tumor penetration and improve their antitumor effect.

Recruitment and activation of caspase-8 seems to be the mechanism for the synergism observed with the combination of immunotoxin and TRAIL or HGS-ETR2 (Fig. 4A and Fig. 6B). To investigate how it was initiated, we analyzed the expression of mesothelin and TRAIL receptor 2 and found that they did not change. Downregulation of c-FLIP, a negative regulator of caspase-8 activation, has been reported to be important for the synergism of the combination of mitochondrial and death receptor apoptosis agents in some studies (33, 36, 37) but not in others (34, 38). In this study, we found no change of c-FLIP expression after treatment with SS1P, HGS-ETR2, or the combination.

A mitochondrial feedback amplification loop was suggested to account for the synergistic cell killing by the combination of TRAIL and etoposide (30). In that scenario, extensive caspase-8 cleavage seen during TRAIL–etoposide synergy is a consequence and not a cause of the apoptotic cascade activated downstream of Bid, and Bid knockdown was shown to disrupt the mitochondrial feedback loop, and inhibit the production of p43/41, the active form of caspase-8 (30). When we combined immunotoxin and HGS-ETR2, Bid knockdown or Bak/Bax double knockdown did not inhibit caspase-8-p43/41 formation (pro-domain cleavage, Ref. 39 and 40). Also, Bak/Bax double knockdown did not affect Bid cleavage. Collectively, these data indicate that the combination of immunotoxin and TRAIL or HGS-ETR2 does not induce mitochondrial feedback amplification. Although we found the combination of immunotoxin and TRAIL or HGS-ETR2 synergistically induces the cleavage and activation of caspase-8, and triggers downstream the apoptosis pathway, the mechanism of caspase-8 activation under combination treatment remains unclear. It is noteworthy that caspase-8 p18 formation was reduced by either Bid knockdown or Bak/Bax double knockdown, suggesting that the maturation of caspase-8 into p18 (inter-domain cleavage) may occur downstream of mitochondrial involvement (39, 40).

In summary, cancer cells with endogenous low Bak protein are resistant to immunotoxin treatment despite profound protein synthesis inhibition. Combining the immunotoxin with TRAIL or TRAIL agonist antibodies can induce synergistic mitochondrial-dependent cell killing by recruitment and activation of caspase-8, although the mechanism needs further study. Combining immunotoxin with activators of the death receptor pathway is an effective approach to overcome resistance due to Bak deficiency.

Disclosure of Potential Conflicts of Interest

Ira Pastan is an inventor on immunotoxin patents that are owned by the NIH.

Acknowledgments

We thank Dr. David FitzGerald for reading the manuscript and helpful comments. Dr. Raffit Hassan for providing the pancreatic cell lines and MORAB antibodies. Dawn A. Walker and other lab members for their helpful discussion, and Dr. Dan Soppet (Laboratory of Molecular Technology, NCI-SAIC) for genomic Bak sequencing. We also thank Drs. Richard Youle and Chuxin Wang (NINDS, NIH) for providing a human Bak gene cDNA and HCT116 cells and for their helpful comments.

Grant Support

This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, and Center for Cancer Research. 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 May 12, 2011; revised July 19, 2011; accepted July 24, 2011; published OnlineFirst August 3, 2011.

References

9. Li Q, Verschraegen CG, Mendoza J, Hassan R. Cytotoxic activity of the recombinant anti-mesothelin immunotoxin, SS1(dsFv)PE38, towards
tumor cell lines established from ascites of patients with peritoneal mesotheliomas. Anticancer Res 2004;24:1327–35.


Clinical Cancer Research

Killing of Resistant Cancer Cells with Low Bak by a Combination of an Antimesothelin Immunotoxin and a TRAIL Receptor 2 Agonist Antibody

Xing Du, Laiman Xiang, Crystal Mackall, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-1235

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/08/04/1078-0432.CCR-11-1235.DC1

Cited articles
This article cites 40 articles, 24 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/17/18/5926.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/17/18/5926.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.