Malignant mesothelioma cells are rapidly sensitized to TRAIL-induced apoptosis by low-dose anisomycin via Bim

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Abstract

Tumor necrosis factor–related apoptosis inducing ligand (TRAIL) holds promise for the treatment of tumors; however, many tumors are resistant to TRAIL alone. We previously showed that resistant malignant mesothelioma cells are sensitized to TRAIL-induced apoptosis by diverse toxic insults including chemotherapy, irradiation, or protein translation inhibitors such as cycloheximide. In seeking nontoxic sensitizers for TRAIL, we tested the protein translation inhibitor anisomycin at subtoxic concentrations 10- to 100-fold below those reported to inhibit protein translation. At these low concentrations (25 ng/mL), anisomycin potently and rapidly sensitized mesothelioma cells to TRAIL-induced apoptosis. Moreover, such sensitization occurred in malignant but not in nonmalignant mesothelial cells. Sensitization by anisomycin was dependent on Bid, indicating a role for mitochondrial amplification in the apoptotic synergy with TRAIL signaling. Consistent with this, we found that anisomycin induces rapid accumulation of the BH3-only protein Bim; moreover, small interfering RNA knockdown of Bim inhibits anisomycin-induced sensitization. Bim accumulation seems not to be transcriptional; instead, it is associated with Bim phosphorylation and increased stability, both consistent with the activation of c-Jun NH2-terminal kinase signals by anisomycin. Overall, our data indicate that the rapid and selective sensitization by anisomycin in mesothelioma cells is mediated by posttranslational potentiation of Bim, which primes the cells for apoptosis via the death receptor pathway. Such subtoxic approaches to sensitization may enhance the value of TRAIL in cancer therapy.

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Introduction

Acquisition of resistance to apoptosis is a defining feature of tumor cells (1), which are otherwise primed for apoptosis by virtue of their oncogenic programs (2–5). Thus, strategies that bypass nodes of apoptotic resistance may be used to trigger cell death selectively in tumor cells. One potential strategy for activating selective apoptosis is to activate death receptors, which can directly engage the apoptotic caspases of the cell. The death receptor ligand, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), shows selectivity toward transformed or malignant cells (6); nonetheless, many tumor cells including those derived from malignant mesothelioma, a particularly aggressive and chemoresistant tumor, are resistant to apoptosis induced by TRAIL alone (7–9). Thus, a key strategy for making TRAIL clinically useful becomes finding means to sensitize to TRAIL-induced apoptosis without losing the selectivity of TRAIL for tumor cells and without inducing toxicity.

The apoptotic response to TRAIL can often be enhanced by a concurrent toxic signal such as from chemotherapeutic agents, γ irradiation, or protein translation inhibitors such as cycloheximide (7). We have shown that human malignant mesothelioma cell lines can be sensitized to TRAIL-induced apoptosis by these approaches (7–9). Using etoposide or irradiation, we found that DNA damage sensitized the malignant cells by lowering the threshold of response in mitochondria to the TRAIL-induced cleavage product of Bid, tBid (9). This suggested that other, perhaps less toxic, agents might similarly sensitize mitochondria to TRAIL signals. To explore this possibility, together with the mechanism by which such sensitization occurs, we explored the efficacy of anisomycin, a protein translation inhibitor known to induce a ribotoxic stress response at subtoxic low concentrations (10–100 nmol/L; refs. 10, 11) and, at high concentrations (1–50 μmol/L), to induce apoptosis by itself (12, 13) or together with either TRAIL (14) or Fas ligand (15).

We report for the first time that anisomycin, when used at low subtoxic concentrations (90 nmol/L or 25 ng/mL), is nonetheless a potent sensitizer to TRAIL-induced apoptosis in mesothelioma cells. This sensitization is associated with stabilization and intracellular accumulation of the proapoptotic BH3-only protein Bim. Bim stabilization correlates with its phosphorylation and both...
Bim accumulation and phosphorylation are inhibited by selective inhibition of c-jun NH2-terminal kinase (JNK), a kinase activated by low levels of anisomycin. Thus, we show that tumor-specific therapeutic efficacy of TRAIL can be significantly potentiated by coexposure to subtoxic concentrations of a sensitizer that, via the intermediary Bim, lowers the threshold for triggering the mitochondrial apoptotic program.

Materials and Methods

Reagents and Antibodies

Human recombinant TRAIL (375-TEC) was purchased from R&D Systems, Inc. Anisomycin (Sigma-Aldrich) was dissolved in Me2SO at 5 mg/mL (18.8 mmol/L) and used at 25 ng/mL (0.09 μmol/L). The topoisomerase II inhibitor etoposide (Bedford Laboratories) was dissolved in Me2SO at 1 mg/mL (1.7 mmol/L) and used at 10 μg/mL (17 μmol/L). The proteasome inhibitor MG-132 (Calbiochem) was purchased in Me2SO solution at 4.8 mg/mL (10 mmol/L) and used at 100 μg/mL, and the translation inhibitor cycloheximide (Sigma-Aldrich) was dissolved in ethanol at 10 mg/mL (35.5 mmol/L) and used at 1 μg/mL (35.5 μmol/L). SP600125, a chemical inhibitor of JNK kinase activity, was a gift from the Signal Research Division of Celgene. SP600125 was dissolved in Me2SO at 4.4 mg/mL (17 mmol/L). At this concentration, SP600125 effectively blocked phosphorylation of JNK, with significantly less effect on other mitogen-activated protein kinases. Matching concentrations of Me2SO or ethanol were used as vehicle controls.

Antihuman Bax, Bid, Mcl-1, JNK, and phospho-JNK (Cell Signaling), antihuman Bim (Axxora), and anti–Bcl-2 and Bcl-x (BD Biosciences) were used as primary antibodies for immunoblotting. Anti-Bim (clone 14A8; Chemicon) was used for immunoprecipitation in pulse-chase experiments. Horseradish peroxidase–linked donkey anti-rabbit antibody (1:2,000; Amersham Biosciences) was used as the secondary antibody.

Cell Culture

The human mesothelioma cell lines M28 (from Dr. Brenda Gerwin, National Cancer Institute, NIH, Bethesda, MD) and REN (from Dr. Roy Smythe, University of Texas M. D. Anderson Cancer Center, Houston, TX) and primary human mesothelial cells were all cultured in standard media (8) in a 37°C incubator at 100% humidity and 5% CO2. Primary human mesothelial cells were cultured from ascites fluid from patients without infection or malignancy according to a protocol approved by the UCSF Committee on Human Research. All cell lines were tested and found to be negative for Mycoplasma every 2 months by PCR analysis as previously described (16).

Annexin V Assay for Apoptosis

Apoptosis was quantitated by flow cytometry with FITC-Annexin V (BD Biosciences), as described (17). The extent of apoptosis and the lack of necrosis (<5%) were confirmed in selected experiments by direct morphologic analysis of cells stained with acridine orange and ethidium bromide, as described (17).

Immunoblot Analysis

Protein analysis was done on whole-cell lysates prepared on ice from 10-cm-diameter plates of M28 or REN cells or primary human mesothelial cells at 70% confluence after the appropriate treatment. The lysis buffer consisted of 20 mmol/L Tris-HCl (pH 8), 137 mmol/L NaCl, 50 mmol/L sodium fluoride, 10% glycerol, 1% Triton X-100, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L sodium orthovanadate (pH 8), and a 1× working solution of Protease Inhibitor Cocktail Set I (Calbiochem). The protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad).

Samples were boiled for 5 min with sample buffer [0.2 mol/L Tris (pH 6.8), 5% SDS, 3% glycerol, and 0.01% bromophenol blue]; 40 μg of sample were loaded into each well and fractionated with 7.5% or 12.5% SDS-PAGE. The separated proteins were electroblotted onto nitrocellulose membranes, which were then blocked with 5% bovine serum albumin (Sigma). Primary antibodies were used at a dilution of 1:1,000 in 5% bovine serum albumin overnight at 4°C. The secondary antibody was used between 1:2,000 and 1:5,000 for 1 h at room temperature. Actin was measured to confirm the equal loading of protein. The blot was developed using SuperSignal chemiluminescent substrate for detection of horseradish peroxidase (Pierce Biotechnology).

RNA Interference: Lipid Transfection

BimEL, BimL, and BimS knockdown was achieved by transfection with small interfering RNA (siRNA) as previously described (ref. 18; antisense strand, ACUUAACUACAGGUGUC dTT; Xeragon, Inc.). A second nonoverlapping siRNA to Bim was used to confirm the observations with Bim knockdown (antisense strand, CUCCGAUACCCGCAACCUC dTT). An irrelevant nonsilencing siRNA (antisense strand, ACGUGACGUCCGAGAA dTT) was used as a control. siRNA duplexes were transfected into target cells using Lipofectamine 2000 (Invitrogen) as described (9). Transfected cells were studied 48 h after transfection at the time of maximal protein knockdown.

RNA Interference: Cell Nucleofection

To confirm a functional role for Bim and to minimize toxicity, nucleofection was used as an additional approach to introduce siRNA duplexes into both M28 and REN cells. M28 or REN cells (5 × 10⁴) were pelleted and resuspended in 100 μL of buffer (solution V, Amaza Biosystems) with 3 μg of the appropriate siRNA duplex. This suspension was transferred to a sterile cuvette and nucleofected using program T-20 on a Nucleofector II device (Amaza Biosystems). Cells were recovered for 30 min in serum-free RPMI medium before being plated.

Additionally, to confirm that anisomycin sensitization to TRAIL was occurring at the level of the mitochondrion, nucleofection as described above was used to introduce siRNA duplexes, previously determined to be specific for the knockdown of Bid (9), into M28 cells.

Fluorescence Time-Lapse Microscopy

Before time-lapse analysis, all Bim knockdown cells received fresh medium containing propidium iodide.
Anisomycin Sensitizes to TRAIL via Bim

(2 μg/mL). Time-lapse was done as previously described (9) and cells were imaged every 10 min. Phase and fluorescent images for each time point were merged to generate a color time-lapse movie; dying cells rounded up and developed a red nucleus as they became permeable to propidium iodide. All cells in the field were followed for their fate (survival or death) and death as a percentage of the total number of cells was plotted versus time (hours after treatment).

**Quantitative Real-time Reverse Transcription-PCR**

Reverse transcription-PCR primers to human BimEL/ BimL and glyceraldehyde-3-phosphate dehydrogenase were synthesized according to the previously published sequences (18, 19). Total RNA was extracted from M28 and REN monolayers using the RNeasy mini kit (Qiagen, Inc.). One microgram of total RNA was reverse transcribed to cDNA and reverse transcription-PCR reactions were done and analyzed with SYBR Green using the ABI PRISM 7700 sequence detection system (PE Applied Biosystems) as previously described (19).

**Two-Dimensional Electrophoresis**

Cellular proteins were solubilized in 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, and 10 mmol/L DTT, and isoelectric focusing was done on 17-cm pH 3–10 immobilized gradient strips using the Bio-Rad Protean IEF system. After focusing, IEF strips were equilibrated in 0.375 mol/L Tris (pH 8.8), 2% SDS supplemented with 2% DTT and then with 2.5% iodoacetamide. Second-dimension SDS-PAGE was done on 12.5% acrylamide gels using the Bio-Rad Protean II system.

**Analysis of BimEL Phosphorylation**

Protein lysates were prepared for immunoblot analysis as described above, except that phosphatase inhibitors (sodium fluoride and sodium orthovanadate) and the Protease Inhibitor Cocktail Set I were not included. To inactivate endogenous proteases, a final 1× concentration of Complete Mini EDTA-Free Protease Inhibitor Cocktail (Roche) was added to the lysis buffer. Lysates from anisomycin-treated and untreated cells were each divided into four 40-μg aliquots of protein and were left untreated at 37°C or 4°C for 1 h; incubated at 37°C for 1 h with 25 units of calf intestinal alkaline phosphatase (New England Biolabs) to induce widespread dephosphorylation; or incubated with calf intestinal alkaline phosphatase and 50 mmol/L sodium orthovanadate (Sigma-Aldrich) at 37°C for 1 h to inactivate the calf intestinal alkaline phosphatase and confirm the role of phosphorylation in any change in gel migration. Samples were boiled in sample buffer, fractionated by 12.5% SDS gel electrophoresis, blotted onto a nitrocellulose membrane, and probed for Bim expression, as described above.

**Analysis of BimEL Persistence by Pulse-Chase**

M28 cells were washed twice with methionine and cysteine-free DMEM and then pulsed with 0.71 μCi/10⁶ cells of ³⁵S-labeled methionine/cysteine (NEG-072, Perkin-Elmer) for 40 min. Following this incubation, the cells were washed again and cold methionine (100 μg/mL) and cysteine (500 μg/mL) were added (chase). At this time, a zero time point was taken and SP600125 (20 μmol/L) or Me₂SO control was added for 1 h, where appropriate, followed by anisomycin (25 ng/mL) for 6 h. Cells were then lysed with ice-cold radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS] containing protease inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mmol/L Na₃VO₄, 2 mmol/L NaF). Protein G sepharose beads were incubated with anti-Bim monoclonal antibody (40 μL beads/1 μg antibody ratio, clone 14A8, Chemicon) for 1 h at 4°C under gentle agitation. Armed beads were then washed thrice with ice-cold radioimmunoprecipitation assay buffer to remove unbound antibodies. One hundred micromilligrams of protein extract in 500 μL of radioimmunoprecipitation assay buffer from each time point were then incubated with 40 μL of antibody-bead slurry for 3 h at 4°C. Precipitated proteins were then separated on a 10% acrylamide gel. The gel was then fixed for 30 min (50% methanol, 10% acetic acid, and 40% water), rehydrated in 30 volumes of water for 1 h, and soaked in 1 mol/L sodium salicylate for 1 h before being dried and autoradiographed with intensifying screens for 2 days at −80°C.

**Statistics**

Differences among groups were determined using ANOVA with post hoc analysis by Tukey’s test (GraphPad Prism version 4.0, GraphPad Software, Inc.). P < 0.05 was considered significant. All data are shown as mean ± SE for at least three separate experiments.

**Results**

**Anisomycin Rapidly Sensitizes Malignant Mesothelioma Cells to TRAIL-Induced Apoptosis**

We examined whether low-dose anisomycin can sensitize malignant or nonmalignant human mesothelial cells to TRAIL-induced apoptosis at 6 to 8 h and 20 h (Fig. 1A). Given alone, TRAIL, anisomycin, or cycloheximide each induced minimal apoptosis. However, anisomycin sensitized malignant M28 and REN cells to TRAIL-induced apoptosis in a dose-dependent manner. The effect of anisomycin was rapid; after anisomycin priming, TRAIL induced significant apoptosis within 6 to 8 h, which is significantly earlier than in our previous study where, after etoposide exposure, TRAIL-induced apoptosis was evident only after 16 to 18 h (9). In contrast, anisomycin

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3 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
did not sensitize nonmalignant human mesothelial cells to TRAIL-induced apoptosis. Such lack of response was not due to an inability to respond to TRAIL because nonmalignant human mesothelial cells undergo TRAIL-induced apoptosis when sensitized with cycloheximide (Fig. 1A). In separate experiments, anisomycin also failed to sensitize primary murine hepatocytes to TRAIL-induced apoptosis, although cycloheximide was able to do so (Supplementary Fig. 1).3

**Anisomycin Sensitizes at Doses That Are Subtoxic**

To confirm that anisomycin is subtoxic at the low concentrations and times of exposure that elicit sensitivity to TRAIL, M28, REN, and nonmalignant human mesothelial cells were all exposed to anisomycin (10 and 25 ng/mL) or to other agents at concentrations previously shown to sensitize to TRAIL: the topoisomerase II inhibitor etoposide (10 μg/mL) and the proteasome inhibitor MG-132 (100 μmol/L) for 6 h. A 6-h exposure to anisomycin did not interfere with cell growth in either the malignant or nonmalignant human mesothelial cells (Fig. 1B). In contrast, a 6-h exposure to either etoposide or MG-132 was toxic, suppressing cell proliferation over the next 3 days. In addition, a 6-h exposure to anisomycin (25 ng/mL) had no effect on mitochondrial potential difference as shown by TMRM fluorescence (see Supplementary Fig. 2). All subsequent experiments were done using anisomycin at the subtoxic dose of 25 ng/mL.

**Anisomycin Sensitization Requires Bid and Thus Amplification at the Mitochondria**

Previously, we had shown that etoposide sensitization to TRAIL signals requires Bid, thereby identifying the need for amplification at the mitochondria (9). To confirm that anisomycin is also acting via the mitochondria, we ablated Bid expression using siRNA as previously described (9). Bid knockdown significantly reduced TRAIL plus anisomycin–induced apoptosis at 8 and 20 h (Fig. 2A), showing that anisomycin effectively acts to lower the threshold at which TRAIL-induced tBid triggers the mitochondrial apoptosis program. In addition, anisomycin did not decrease FLIP levels (see Supplementary Fig. 3),3 further supporting the findings that anisomycin sensitizes to TRAIL-induced apoptosis at the level of the mitochondria.

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**Figure 1.** Anisomycin sensitizes malignant cells, but not nonmalignant cells, to TRAIL-induced apoptosis at concentrations that are nontoxic. A, anisomycin sensitizes mesothelioma cells to TRAIL-induced apoptosis at 8 and 20 h. M28 and REN mesothelioma cells and nonmalignant human mesothelial cells were exposed to TRAIL (1 ng/mL) and anisomycin (1–25 ng/mL) alone or in combination. TRAIL plus cycloheximide (10 μg/mL) was used as a positive control. Anisomycin potentiated TRAIL-induced apoptosis in the malignant but not the nonmalignant cells at 8 and 20 h. *, P < 0.05, significantly greater than the sum of apoptosis due to agents alone (n = 3). Baseline levels of apoptosis at 8 h were M28, 6 ± 1%; REN, 9 ± 3%; and nonmalignant cells, 14 ± 6%. Baseline levels of apoptosis at 20 h were M28, 8 ± 2%; REN, 5 ± 2%; and nonmalignant cells, 20 ± 6%. B, cells were exposed to various agents at concentrations that we have shown to induce sensitization to TRAIL, including etoposide (10 μg/mL), MG-132 (100 μmol/L), cycloheximide (10 μg/mL), and anisomycin (10, 25 ng/mL), for 6 h. Exposure to anisomycin had no effect on cellular proliferation whereas exposure to etoposide or MG-132 significantly reduced cell proliferation. *, P < 0.05, compared with control (n = 3).
Anisomycin Up-regulates BimEL Selectively in Malignant Cells

The most plausible mediators of anisomycin sensitization to TRAIL are one or more members of the Bcl-2/Bax/BH3-only family of proteins because these are the principal determinants regulating the apoptotic threshold within cells. However, we observed no decrease in levels of antiapoptotic molecules such as Bcl-2, Bcl-x, or Mcl-1 (a major binding partner of Bim) within the time frame of anisomycin sensitization, nor any increase in proapoptotic molecules Bax or Bid (Fig. 2B for Bid; others in Supplementary Fig. 2B-S2).3 Alone of all the BH3-only molecules examined, BimEL levels rapidly increased within 1 to 2 h of anisomycin exposure (Fig. 2B). The other major Bim isoforms, BimL and BimS, were undetectable in the cell lines used in this study. Bim was undetectable in nonmalignant mesothelial cells and did not increase following anisomycin exposure (Fig. 2B).

Bim Knockdown Inhibits the Ability of Anisomycin to Sensitize to TRAIL-Induced Apoptosis

To confirm the role for Bim in mediating apoptotic synergy between TRAIL and anisomycin in malignant cells, we used RNA interference to knockdown Bim protein. After lipid transfection or nucleofection with two nonoverlapping siRNA duplexes, but not with the random control duplexes, BimEL protein expression was completely ablated by 48 h and remained suppressed at 72 h in the malignant cell lines (Fig. 3A). Bim knockdown did not affect expression of Bid (data not shown). Knockdown of Bim inhibited apoptosis induced by TRAIL plus anisomycin for at least 10 h after treatment, as shown by time-lapse videomicroscopic analysis (Fig. 3B and C; also see Supplementary movie Bim.avi). Such inhibition was confirmed in separate experiments using flow cytometric analysis: Bim knockdown inhibited the sensitization by anisomycin to TRAIL-induced apoptosis in both M28 and REN at 8 h (Fig. 3D).

Anisomycin-Induced Up-regulation of Bim Protein Is Consistent with Bim Phosphorylation via JNK

To determine the mechanism by which anisomycin induces rapid accumulation of Bim protein, we first examined the effect of anisomycin on transcription of bim mRNA by reverse transcription-PCR (Fig. 4A). However, exposure of mesothelioma cells to anisomycin did not increase bim mRNA, although etoposide at 12 h (used as a positive control) did (20). Thus, increased transcription of Bim message does not account for the increase in Bim protein we observed 1 to 2 h after anisomycin treatment.

Modulation of Bim stability through phosphorylation is a major mechanism regulating steady-state Bim protein

Figure 2. Sensitization by anisomycin requires Bid, indicating amplification at the mitochondria, and is associated with elevation of BimEL. A, Bid knockdown inhibits TRAIL plus anisomycin–induced apoptosis of M28 cells at 8 and 20 h. Such dependence on Bid indicates the involvement of the mitochondria in the sensitization by anisomycin. The effect of Bid knockdown on apoptosis due to TRAIL alone indicates some sensitization of cells to TRAIL by the transfection process, as previously seen (9). *, P < 0.05, significantly different from apoptosis following random siRNA duplex (n = 3). Inset, Bid protein 48 h after transfection of RNAi duplexes. Blot is representative of three experiments (mean densitometry is shown in Supplementary Fig. 2A-S). B, M28 and REN mesothelioma cells and nonmalignant human mesothelial cells were stimulated with anisomycin (25 ng/mL) for 0 to 6 h and lysed for immunoblot analysis of proapoptotic and antiapoptotic Bcl-2 family members. In mesothelioma cells, there is a basal level of BimEL protein that increases 1 to 2 h after anisomycin treatment. In nonmalignant mesothelial cells, BimEL was at undetectable levels at baseline, with no apparent increase following anisomycin. Bid, another BH3-only molecule, showed no change. Blot is representative of three separate experiments (mean densitometry of changes in Bim relative to Bid is shown in Supplementary Fig. 2B-S1). There were no evident proapoptotic changes of other BH3-containing molecules including Bcl-2, Bcl-xL, Mcl-1, and Bax (see Supplementary Fig. 2B-S2).
levels (21, 22). We therefore asked whether anisomycin treatment elicits posttranslational modification of Bim protein in mesothelioma cells. Indeed, two-dimensional electrophoresis indicated that anisomycin treatment causes a shift of Bim species to a more acidic isoelectric point (Fig. 4B). This is consistent with an increase in Bim phosphorylation, which we confirmed by showing that the observed shift in BimEL migration due to anisomycin is abrogated by pretreatment of cell lysates with alkaline phosphatase (Fig. 4C).

The ribotoxic stress response is a potent activator of the JNK pathway (10), a pathway known to phosphorylate and activate Bim protein (23). Indeed, anisomycin induced significant increases in phospho-JNK at 1 and 2 h in M28 and REN malignant cells (Fig. 5A). The level of total JNK remained constant throughout in all cell lines, indicating that the increase in phospho-JNK is due to an increase in the phosphorylation of the existing pool of JNK. Anisomycin also increased phospho-extracellular signal–regulated kinase (ERK) in the malignant cells although levels of phospho-p38 remained unchanged (data not shown). Consistent with its lack of sensitization of the nonmalignant cells, anisomycin did not lead to phosphorylation of JNK in nonmalignant human mesothelial cells (Fig. 5A).
To test whether anisomycin-induced JNK phosphorylation of Bim is responsible for stabilizing Bim protein, anisomycin was added to mesothelioma cells and the molecular weight and level of Bim were monitored. Anisomycin induced the expected increase in apparent Bim molecular weight due to phosphorylation, together with significant accumulation of Bim protein (Fig. 5B). Both phosphorylation and accumulation of Bim were inhibited by treatment of cells with the JNK inhibitor SP600125 (Fig. 5B).

To confirm that anisomycin altered Bim protein persistence, anisomycin was added to 35S-labeled mesothelioma cells. In some conditions, SP600125 was added 1 h before anisomycin. Anisomycin increased the persistence of the labeled Bim protein. Pretreatment with SP600125 inhibited the ability of anisomycin to enhance Bim persistence (Fig. 5C). These studies indicate that subtoxic anisomycin exposure enhances Bim stability and supports a role for anisomycin-induced JNK activation in the rapid accumulation of Bim.

Discussion

An attractive strategy for cancer therapy is to combine agents at doses that are individually nontoxic or subtoxic but that together selectively kill tumor cells. An effective combinatorial strategy would exploit the obligate attributes of cancer cells that distinguish them from their normal counterparts. TRAIL, the death receptor ligand, has been of intense interest due to its ability to induce apoptosis selectively in some malignant cells (6, 24) although the mechanism underlying this selectivity is unclear.

Figure 4. Anisomycin fails to increase Bim message but does induce Bim phosphorylation. A, anisomycin does not induce bim mRNA levels. M28 and REN mesothelioma cells were exposed to anisomycin (25 ng/mL) for 1, 2, or 4 h; to Me2SO as a vehicle control for 4 h; or to etoposide (10 μg/mL) as a positive control for 12 h. RNA was extracted, reverse transcribed to cDNA, and Bim message quantitated by reverse transcription-PCR. Data from each sample were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase message. Data are expressed as the ratio of Bim expression (normalized to glyceraldehyde-3-phosphate dehydrogenase expression) in the experimental samples compared with control. *, P < 0.05, significantly different from Bim message in control conditions (n = 3). B, anisomycin induces a shift in Bim migration consistent with phosphorylation. M28 cells treated for 2 h with anisomycin (25 ng/mL) or control (Me2SO) were lysed and Bim protein migration was analyzed by two-dimensional electrophoresis. Bim is shifted toward a more acidic isoelectric point in anisomycin-treated M28 cells compared with control cells (arrow). Consistent with an increase in Bim phosphorylation (representative of two blots). C, anisomycin-induced changes in BimEL migration are due to phosphorylation. Anisomycin treatment elicits a shift in BimEL to a higher apparent molecular weight, most markedly in samples maintained at 4°C to inhibit endogenous phosphatases. This shift in molecular weight is abrogated on dephosphorylation with calf intestinal alkaline phosphatase (CIP) but restored if the phosphatase is inhibited with sodium orthovanadate (CIP + Na3VO4). Representative of three blots (densitometry of upper band as percentage of total BimEL band intensity is shown in Supplementary Fig. 4C-S).
Unfortunately, despite its potential antitumor selectivity, many tumor cells remain resistant to TRAIL-induced apoptosis. Here we show that a second stimulus, itself subtoxic, can nonetheless effectively sensitize previously refractory mesothelioma cells to TRAIL-induced apoptosis. Understanding the mechanisms of such combinatorial approaches holds promise for optimizing cancer treatments, even in recalcitrant solid tumors such as mesothelioma.

Anisomycin is known to induce a ribotoxic stress response by binding to the actively translating ribosomal 28S subunit (10). This ribotoxic stress response involves a strong and early JNK activation, together with variable extents of p38 and/or ERK activation depending on cell type. The notion that anisomycin activation of the ribotoxic stress response may be used selectively to potentiate killing of tumor cells (25) is an attractive one; however, almost all studies have used anisomycin at concentrations high enough also to interfere with protein translation (10–100-fold greater than used in this study; refs. 11, 13–15), a cause of significant collateral toxicity. Our study is important because it shows that levels of anisomycin sufficient to engage only the ribotoxic stress response are nonetheless competent to sensitize cells to killing via the TRAIL death receptor pathway and that this sensitization operates, in large part, through the BH3-only molecule BimEL.

Bim is a member of the diverse BH3-only proapoptotic family of proteins that act as the terminal cell death effectors of a wide range of signaling and damage response pathways; together with tBid and Puma, Bim directly activates the obligate proapoptotic multimeric molecules Bax and Bak at the mitochondrion outer membrane (26). As expected for such a potentially toxic molecule, Bim function is highly regulated by both transcriptional and posttranslational mechanisms. Of the 18 Bim isoforms thus far described (27), the major species are formed by alternative splicing into short (BimS), long (BimL), and extra long (BimEL) variants. The BimL and BimEL isoforms contain a domain mediating binding to the dynein light chain of microtubules, where some studies suggest the proteins reside (28). Bim is a key mediator of apoptosis initiated by anoikis (29), by trophic factor withdrawal (30), and by chemotherapeutic agents such as paclitaxel (31). Here we show for the first time that Bim also plays a key role in the ability of a stress response to sensitize to TRAIL-induced apoptosis. Whereas Bim and tBid have each been reported to activate Bax/Bak directly (26), we presume that the level of Bim induced by subtoxic anisomycin and the level of activated tBid induced by TRAIL were each alone insufficient to trigger apoptosis of mesothelioma cells. This is consistent with contemporary models of cell death control in which the cell threshold for firing the mitochondrial apoptotic program is established by the mutually
antagonistic activities of the proapoptotic Bax/Bak proteins buffered by the prosurvival Bcl-2/Bcl-xL proteins. By promoting Bax/Bak activity and/or inhibiting Bcl-2/Bcl-xL protection, multiple BH3 proteins can act cumulatively to breach the threshold for apoptotic firing. Indeed, the knockdown of Bim did not completely abrogate the sensitization of anisomycin, which may indicate the action either of a small residual amount of Bim or of other BH3-only molecules at the mitochondria.

Prior studies had identified JNK as mediating the effects of anisomycin, although the means by which JNK activation might trigger apoptosis were not identified (12, 14, 15, 32). In our study, the activation of Bim and sensitization of the cells by anisomycin is consistent with the action of JNK. Moreover, JNK has previously been implicated in sensitization to TRAIL of mesothelioma cells (which incidentally lack functional p53) by chemotherapeutic agents (8). JNK has previously been shown to augment the proapoptotic functions of Bim by at least two distinct mechanisms: increased bim transcription (33) and phosphorylation leading to translocation of BimL from dynein light chains to mitochondria (23, 34). However, despite activation of JNK by anisomycin exposure, we observed no effect on bim transcription; we did not examine Bim localization and cannot exclude that translocation also plays a role. We did observe an increase in Bim stability and an accumulation of Bim protein associated with JNK-dependent phosphorylation. One possibility is that Bim phosphorylation by JNK leads to Bim protein stabilization by counteracting the phosphorylation by ERK, which is known to target Bim for degradation via the proteasome (refs. 22, 35; see Fig. 6). Interestingly, we observed that anisomycin treatment elicited an increase in both phospho-JNK and ERK, so the predilection for apoptosis may reflect subtleties in the relative timing and persistence of the JNK and ERK signals within the cell (34). Intrinsic asymmetries in signal strength and decay have recently emerged as important determinants of tumor cell death during withdrawal from oncogenic signaling (36).

Enhancement of TRAIL-induced apoptosis could have great potential value in treating mesothelioma, a disease with a particularly poor prognosis. Mesothelioma is an aggressive tumor of the mesothelial lining of serosal surfaces, with median survival from diagnosis ranging from 8 to 14 months (37). Mesothelioma is highly resistant to radiotherapy and chemotherapy (38), most likely due to disruption of the core apoptosis machinery (39). The exact cause of this apoptotic defect is not known but mesothelioma often exhibits defects in the p53 pathway, most notably frequent deletions of p14ARF (40) and overexpression of Bcl-xL (41). Despite these obstacles, however, mesothelioma cell lines undergo cell death in response to combinatorial approaches involving stimuli that activate members of the BH3-only apoptotic family. Thus, activation of death receptor pathways promotes apoptosis in these cells via tBid (9), and we now show that the intrinsic apoptotic pathway activated by a ribotoxic stimulus operates via Bim. Intriguingly, one of the targets of Bim, the proapoptotic Bax, is typically expressed at high levels in mesothelioma (42, 43), which may explain the effectiveness of signaling pathways that activate Bim in mesotheliomas. Importantly, activation of the JNK stress pathway may obviate the need for a functional p53 pathway by engaging BH3-only molecules targeted by JNK such as Bim instead of those targeted by p53 such as Puma or Noxa (44, 45). This proapoptotic combinatorial approach may have practical clinical relevance because TRAIL and anisomycin have

Figure 6. Schematic model for the mechanism of anisomycin sensitization of TRAIL-induced apoptosis in malignant cells. TRAIL alone is insufficient to induce significant apoptosis (Fig. 2A) due to insensitivity of the mitochondrion to the TRAIL effector, tBid (9). Anisomycin acts to reduce the threshold at which the mitochondrion responds to tBid (Fig. 2A). Anisomycin stimulates the JNK signaling pathway (Fig. 5A), which leads to phosphorylation and stability of BimEL (Fig. 5B), causing elevation of BimEL protein. BimEL, by virtue of its elevated level, its phosphorylation, or both, sensitizes the mitochondria to TRAIL-induced tBid. Thus, the two BH3-only molecules, BimEL and Bim, each generated by stimuli that are individually subtoxic, cooperate to induce apoptosis. (Schema was modified from Fig. 7 of reference 9 to reflect the contribution of anisomycin and BimEL in sensitizing to TRAIL-induced apoptosis.)
each been administered in vivo without apparent toxicity (6, 25), whereas mesothelioma offers the opportunity for intraperitoneal or i.p. drug delivery, thereby offering localized delivery strategies.

The selectivity of our combinatorial approach for killing tumor cells over normal cells is striking although the mechanism underlying such selectivity remains unclear. It may be that mesothelioma cells, like many tumor cells, have an innately lowered threshold for apoptosis by virtue of their proapoptotic oncogenic lesions (2), and that the combination of TRAIL and anisomycin is sufficient to breach the lowered threshold specific to tumor cells. Tumor cell selectivity may also arise from selective activation of JNK in tumor cells: the ribotoxic response requires actively translating ribosomes (10), which are likely to be a more prominent feature in rapidly proliferating malignant cells compared with their slower growing normal counterparts (see also Fig. 1B). Indeed, in one study, anisomycin (at high concentrations) induced both JNK activation and apoptosis only in cells transformed with Ha-Ras but not in their nontransformed counterparts (12). We also note that basal levels of Bim are higher in tumor cells than in normal mesothelial cells. Hence, in the normal cells, stabilization of Bim may lead to insufficient accumulation of the protein to breach the threshold for apoptotic triggering. Clearly, defining the precise mechanisms underlying tumor cell sensitivity may indicate chinks in the malignant arm that treatment strategies can be designed to exploit.

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References


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