The death ligand, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), has shown great promise for inducing apoptosis selectively in tumors. Although many tumor cells are resistant to TRAIL-induced apoptosis alone, they can often be sensitized by co-treatment with DNA-damaging agents such as etoposide. However, the molecular mechanism underlying this therapeutically important synergy is unknown. We explored the mechanism mediating TRAIL-DNA damage apoptotic synergy in human mesothelioma cells, a tumor type particularly refractory to existing therapies. We show that Bid, a cytoplasmic Bcl-2 homology domain 3-containing protein activated by caspase 8 in response to TRAIL ligation, is essential for TRAIL-etoposide apoptotic synergy and, furthermore, that exposure to DNA damage primes cells to induction of apoptosis by otherwise sublethal levels of activated Bid. Finally, we show that the extensive caspase 8 cleavage seen during TRAIL-etoposide synergy is a consequence and not a cause of the apoptotic cascade activated downstream of Bid. These data indicate that TRAIL-etoposide apoptotic synergy arises because DNA damage increases the inherent sensitivity of cells to levels of TRAIL-activated Bid that would otherwise be insufficient for apoptosis. Such studies indicate how the adroit combination of differing proapoptotic and sublethal signals can provide an attractive strategy for treating refractory tumors.

Resistance to apoptosis is critical to tumor development and maintenance (1, 2). In part, this is because activated oncogenes and deregulated proliferation are potent triggers of apoptosis that must be countermanded before tumors can arise (3). Moreover, because most cancer therapies act by inducing apoptosis in tumor cells, suppression of apoptosis is also likely a critical feature of chemo- and radioresistance (4). Restoring an effective capacity to execute apoptosis therefore represents an attractive general strategy for the selective therapeutic targeting of tumor cells.

Relatively little is known concerning the mechanisms by which apoptosis is suppressed in cancers. However, because all tumor cells appear to retain the capacity to undergo apoptosis when subjected to sufficiently strong insults, apoptosis suppression in cancer cells in the main seems to involve dysfunctions in apoptotic and survival signaling pathways rather than lesions in the highly redundant basal apoptotic machinery itself (5). A frequent example of this is the mutational inactivation of the p53 pathway observed in most tumor cells. p53 is a transcription factor that integrates disparate stress and damage responses and couples them to the basal apoptotic machinery in a great part through transcriptional modulation of effectors that impact on the mitochondrion (6). Because inactivation of p53 does not compromise the apoptotic machinery but instead severs it from upstream damage signals, such cells should, in principle, remain amenable to activation of apoptosis by p53-independent pathways.

Recently, the tumor necrosis factor-related apoptosis-inducing ligand TRAIL/Apo2L has excited much interest because of its potential selectivity in triggering apoptosis in tumor cells rather than in their normal counterparts (7, 8). This lack of general toxicity is reflected in the fact that TRAIL has been proven relatively safe in in vivo studies of rodents and primates compared with the other death receptor ligands, tumor necrosis factor and Fas ligand, both of which induce significant inflammation and tissue injury (9, 10). Unfortunately, many tumor cell types are resistant to killing by TRAIL alone. Nonetheless, such tumor cells can often be rendered TRAIL-sensitive by co-treatment with DNA-damaging agents (11–16), indicating that the TRAIL and DNA damage apoptotic signaling pathways converge at some level to cooperate in triggering the cell death machinery. However, although synergy between TRAIL and DNA damage is reported in many cancer cell types, both in vitro (11–16) and in vivo (9, 12, 13, 17), no clear mechanism has been elucidated for its striking effect.

TRAIL binds to its cognate receptors, DR4 and DR5, inducing their trimerization and intracellular recruitment of the adaptor protein Fas-associated death domain (18, 19). The Fas-associated death domain, in turn, recruits procaspase 8 into a death-inducing signaling complex that triggers autocatalytic cleavage and activation of caspase 8 (20, 21). In so-called “Type I” cells, such as T lymphocytes, death-inducing signaling complex-activated caspase 8 directly activates downstream effector caspases that mediate cell death (22). However, the molecular mechanism of TRAIL-mediated apoptosis in tumor cells is less clear, with much controversy over whether TRAIL triggers an independent caspase 8-independent pathway (23). While TRAIL can induce apoptosis in some tumor cells, most human tumor cell lines are resistant to TRAIL-induced apoptosis (24, 25). One attractive general strategy for the selective therapeutic targeting of tumor cells, therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The on-line version of this article (available at http://www.jbc.org) contains a supplemental movie.

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Cancer Center, Houston, TX), and primary human mesothelial cells were grown in standard media (31). Both cell lines have been shown to have inactive p53 (31, 32). Primary human mesothelial cells were cultured from ascites fluid from patients without infection or malignancy according to a protocol approved by the University of California San Francisco Committee on Human Research. Cells were γ-irradiated using a cesium irradiator.

Immunoblotting Analysis—Cell extracts were fractionated by SDS gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes, which were then blocked with 1% nonfat dried milk. Anti-caspase 8 (1C12, 1:250; Cell Signaling, Beverly, MA), anti-human Bid (clone 2002, 1:1000; Cell Signaling), and anti-human DR5 (AB16942, 1:500; Chemicon International) were used as primary antibodies, and horseradish peroxidase-linked rabbit anti-mouse (1:5000) or horseradish peroxidase-linked donkey anti-rabbit (1:2500, Amersham Biosciences) antibodies were used as appropriate secondary reagents. Actin was measured to confirm equivalent loading of cell extracts.

Apoptosis was quantitated using fluorescein isothiocyanate-annexin V (BD Biosciences) as described (34). 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 (34).

Microinjection and Fluorescence Time-lapse Microscopy—For microinjection, Bid was dissolved in phosphate-buffered saline (PBS) and combined with dextran-conjugated Oregon Green (0.5% final concentration, Molecular Probes, Eugene, OR) as a co-injection marker. Cells were plated on sterile glass-bottomed coverslip dishes in a phenol red-free medium and, when adherent, exposed to either etoposide (10 μg/ml) or the Me2SO carrier (1.6000 for 15 h. Etoposide- or Me2SO-exposed cells were microinjected with either tBid (5 nM) or phosphate-buffered saline as previously (35). At least 100 cells in two discrete areas in each of two separate experiments were microinjected in each case. The estimated volume injected into each cell was 2–5 pl (36), equivalent to ~6,000–15,000 molecules of tBid. Prior to time-lapse analysis, all cells received fresh media containing propidium iodide (2 μg/ml).

Microinjected cells were placed in a thermostatic chamber on an Axiovert S 100 TV microscope (Zeiss, Thornwood, NY). The chamber was continuously flushed at 37°C. Cells were imaged every 25 min with an Orca C4742–95 charged couple device camera (Hamamatsu Photonics, Hamamatsu City, Japan) at 8 bit using Openlab software (Improvision, Lexington, MA), which also controlled the XY-stage, shutters, and filter wheels. Phase contrast images were illuminated with a 100-watt halogen bulb while a mercury vapor lamp and automated excitation filter wheel were used to excite the fluorescent probes. Oregon Green was excited using a 480/25 bandpass filter, and propidium iodide was excited using a 525/40 bandpass filter. The emission from these two fluorophores were detected using a polychromatic beamsplitter 8600/78a (Spectra Services, Rochester, NY) followed by an automated emission filter wheel using 525/40 and 620/60 bandpass filters, respectively. Images for each time point were merged using Openlab software. The phase contrast signal, Oregon Green, and propidium iodide were imaged using a 488-nm laser and a 590-nm long-pass filter. Cells were controlled the XY-stage, shutters, and filter wheels. Phase contrast images were illuminated with a 100-watt halogen bulb while a mercury vapor lamp and automated excitation filter wheel were used to excite the fluorescent probes. Oregon Green was excited using a 480/25 bandpass filter, and propidium iodide was excited using a 525/40 bandpass filter. The emission from these two fluorophores were detected using a polychromatic beamsplitter 8600/78a (Spectra Services, Rochester, NY) followed by an automated emission filter wheel using 525/40 and 620/60 bandpass filters, respectively. Images for each time point were merged using Openlab software. The phase contrast signal, Oregon Green, and propidium iodide were imaged using a 488-nm laser and a 590-nm long-pass filter.

RESULTS

To validate apoptotic cooperation between TRAIL and DNA damage, M28 and REN mesothelioma cells were exposed to various doses of TRAIL, etoposide, or γ irradiation, and apoptosis was determined 18 h later. At commonly used therapeutic doses (25 ng/ml TRAIL, 10 μg/ml etoposide, 12 gray of γ irradiation), each insult individually induced negligible apo-

factor caspases and triggers apoptosis. However, in the majority of cells (“Type II”), TRAIL-induced activation of caspase 8 is insufficient to kill without recruiting the mitochondrial apoptotic program through cleavage and activation of the BH3 protein intermediary Bid (22).

In principle, DNA damage signals might interact with the TRAIL signaling pathway at any point to induce a synergistic apoptotic response. Some evidence exists that DNA damage can up-regulate expression of the TRAIL receptors DR4 and DR5 (12, 13, 23, 24) or modulate assembly or function of the death-inducing signaling complex (25, 26), either of which might augment TRAIL-induced caspase 8 cleavage. On the other hand, because TRAIL and DNA damage signals both impact on the mitochondrion, it is possible that DNA damage signals might instead act to lower the threshold for release of apoptotic effectors by Bid or vice versa. Unfortunately, dissecting how such pathways interact is confounded by the fact that, once the apoptotic program is initiated, widespread activation of effector caspases leads to promiscuous cleavage of the remaining initiator and effector procaspases as well as other caspase targets (27, 28). This widespread activation makes it difficult to distinguish cause from consequence.

Mesothelioma is a highly aggressive tumor conspicuous for its resistance to existing anticancer therapies (29). In previous work, we have shown that mesothelioma cells can be sensitized to TRAIL-induced apoptosis by various DNA-damaging signals (30, 31). Such sensitization occurs despite the absence of functional p53 in these cells (31, 32), making the apoptotic synergy between TRAIL and DNA damage a valuable model for combinatorial therapeutics of particular relevance to the majority of solid tumors, which lack a functional p53 pathway. Synergy also appears to be selective for malignant mesothelioma cells; despite earlier experiments in which primary mesothelial cells had variable responses suggesting synergy (30); currently, with more careful harvesting and culturing, the primary cells consistently show a lack of synergy between TRAIL and DNA-damaging agents (see “Results”). We thus sought to understand the mechanism underlying this striking synergy between TRAIL and DNA-damaging agents in malignant mesothelioma cells.

Using a combination of RNA interference and microinjection, we have perturbed the TRAIL pathway to dissect the molecular basis of the TRAIL-DNA damage synergy and show that it is mediated by the essential intermediary Bid, a BH3 protein that is a target for death receptor signaling pathways. We also show that TRAIL by itself causes low level cleavage of Bid that alone is insufficient to trigger apoptosis in mesothelioma cells. However, exposure of mesothelioma cells to sublethal DNA-damaging agents profoundly increases sensitivity to the pro-apoptotic effects of Bid. Hence, the combination of sublethal TRAIL and sublethal DNA damage efficiently triggers activation of the mitochondrial apoptotic program and death of mesothelioma cells.

EXPERIMENTAL PROCEDURES

Reagents—Human recombinant TRAIL (375-TL, histidine-tagged extracellular domain) was purchased from R&D Systems Inc. (Minneapolis, MN), and the topoisomerase II inhibitor etoposide was purchased from Bedford Laboratories (Bedford, OH), dissolved in Me2SO, and used at a final concentration of 10 μg/ml. The proteasome inhibitor MG132 was purchased from Calbiochem.

Recombinant tBid was generated from full-length human Bid cDNA with a thrombin cleavage sequence inserted at amino acids 57–62 and a His8 tag on the COOH terminus (33). After thrombin cleavage and purification, the tBid fragment generated is identical to that resulting from caspase 8 cleavage of wild-type full-length Bid and runs as a single band at the expected molecular mass of 18–20 kDa in SDS gels.

Cell Culture—Human malignant mesothelioma cell lines M28 (from Dr. Brenda Gerwin, NCI, National Institutes of Health, Bethesda, MD), REN (from Dr. Roy Smythe, University of Texas, M. D. Anderson...
ptosis (Fig. 1, A–C), even when assayed as late as 72 h after the insult. Indeed, even at far higher doses (250 ng/ml TRAIL, 100 μg/ml etoposide, 50 gray of γ irradiation), each insult alone induced negligible apoptosis (data not shown). In contrast, exposure of mesothelioma cells to the combination of sublethal TRAIL (25 ng/ml) together with either sublethal etoposide (10 μg/ml) or γ irradiation (12 gray) induced substantial apoptosis (Fig. 1, a–c). No such enhancement of apoptosis was observed when the two genotoxic insults, etoposide and γ irradiation, were combined (data not shown). Moreover, the apoptotic synergy between TRAIL and DNA damage that we observed in M28 and REN mesothelioma tumor cells was absent in normal primary human mesothelial cells (Fig. 1d).

It has been reported that DNA damage can sensitize cells to the effects of TRAIL through up-regulation of TRAIL receptor expression, specifically of DR5 (24). However, exposure of mesothelioma cells to etoposide had no measurable effect on DR5 expression (Fig. 2), consistent with our previous published observations indicating no induction of DR5 by doxorubicin (30). Thus, some other mechanism must underlie the apoptotic synergy between TRAIL and etoposide that we observe.

To explore the mechanism underlying the apoptotic cooperation between TRAIL signaling and DNA damage, we first assessed the impact of TRAIL and etoposide exposure, separately and together, on cleavage of the TRAIL death effectors caspase 8 and Bid. By 15 h, the earliest time at which synergistic apoptosis becomes evident, we observed substantial caspase 8 cleavage only in cells exposed to the combination of
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TRAIL and etoposide (Fig. 3). Likewise, Bid cleavage (as judged by the decrease in full-length Bid) was extensive only in cells exposed to the combined insults (Fig. 3). By contrast, cells treated with TRAIL alone exhibited little evident caspase 8 cleavage, which appeared inconsistently in immunoblots. Nonetheless, as noted elsewhere (39), such low level caspase 8 activation was sufficient to induce modest but measurable cleavage of Bid (Figs. 3 and 6). No caspase 8 or Bid cleavage was evident in cells exposed solely to etoposide (Fig. 3).

The Bid cleavage that we observe in cells exposed to TRAIL alone indicates that even though TRAIL alone is insufficient to trigger apoptosis, its cognate pathway is activated to some degree in such cells. This raises the possibility that DNA damage might cooperate with TRAIL to induce apoptosis by increasing the sensitivity of cells to low levels of tBid generated by TRAIL that would otherwise be insufficient to trigger cell death. To test this hypothesis directly, we asked whether etoposide exposure enhances sensitivity to induction of apoptosis from sublethal levels of tBid introduced directly into mesothelioma cells by microinjection. First, we microinjected mesothelioma cells with a fixed volume containing various concentrations of recombinant tBid (5–500 nM) and monitored the cells for apoptosis for the next 36 h to establish a sublethal tBid dose. Although 50–500 nM tBid induced significantly more apoptosis than the phosphate-buffered saline control (data not shown), 5 nM tBid proved non-toxic, and this sublethal dose was then used for further study.

To determine whether etoposide treatment sensitizes mesothelioma cells to sublethal levels of tBid, M28 cells were exposed either to etoposide (10 μg/ml) or the Me2SO carrier for 15 h and then microinjected with either 5 nM tBid or phosphate-buffered saline together with the neutral fluorescent marker Oregon Green. Significant apoptosis occurred only in cells exposed both to etoposide and microinjected tBid (Fig. 4; also see supplemental movie). These data unambiguously demonstrate that DNA damage increases sensitivity to induction of apoptosis by otherwise sublethal levels of tBid. Because tBid mediates its apoptotic actions through interactions with the Bcl-2/Bax family of proteins, the most plausible convergence for tBid and DNA damage signals is at the level of the mitochondrion.

To confirm the pivotal and unique role of Bid in mediating TRAIL-DNA damage apoptotic synergy, we used RNA interference to suppress the Bid protein in both M28 and REN cells. Bid protein expression was almost completely ablated after two successive rounds of transfection with siRNA oligonucleotides (Fig. 5a), whereas the siRNA had no discernible effect on expression of other proteins such as actin or key determinants of apoptosis such as caspase 8 (Fig. 5a) and Bcl-xL (not shown). Ablation of Bid rendered mesothelioma cells substantially resistant to induction of apoptosis by combinations of TRAIL and DNA damage (either etoposide or γ irradiation) that are normally lethal (Fig. 5, b and c). Thus, expression of Bid is essential for the apoptotic synergy between TRAIL and DNA damage.

Our data are consistent with the notion that DNA damage cooperates with TRAIL signaling in inducing apoptosis by decreasing the threshold at which tBid triggers the basal apoptotic machinery. However, such a model does not explain why the combination of TRAIL and DNA damage gives rise to greatly enhanced activation of ostensibly upstream apoptotic effectors like caspase 8 and Bid (see Fig. 3). One possibility is that, in addition to its downstream sensitizing role, DNA damage also acts upstream to augment caspase 8 and Bid activation by TRAIL. Alternatively, the massive cleavage of caspase 8 and Bid observed upon co-treatment with TRAIL and etoposide could be a consequence of the execution of the apoptotic program, which promiscuously activates downstream and upstream caspases. In this latter case, the extensive caspase 8 cleavage induced by co-treatment with TRAIL and etoposide should be completely dependent upon its downstream effector Bid. Therefore, to discriminate between these possibilities, we used siRNA to suppress Bid expression in M28 mesothelioma cells and then exposed the cells to TRAIL, etoposide, or both. After 15 h, extensive caspase 8 cleavage was evident in control mesothelioma cells exposed to both TRAIL and etoposide (Fig. 6). In contrast, caspase 8 cleavage in Bid-deficient cells co-treated with TRAIL and etoposide was dramatically reduced to the low level observed in Bid-competent cells treated with TRAIL alone (see Fig. 6). Because Bid is ostensibly a downstream cleavage target of caspase 8, the extensive caspase 8 cleavage we see in TRAIL-etoposide synergy cannot be due to cooperation upstream of caspase 8 but is most likely a secondary consequence of the promiscuous transactivation of caspases that mediates the execution phase of apoptosis.

DISCUSSION

Combinatorial therapies for cancer offer the tantalizing prospect of combining subtoxic treatments in a manner that induces death selectively in tumors. Much evidence indicates that tumor cells are inherently sensitized to apoptosis by virtue of the oncogenic and mitogenic lesions that drive their uncontrolled proliferation. Such innate sensitization suggests that disparate pro-apoptotic triggers, each individually subtoxic, might be combined in various ways to induce death selectively in tumor cells. Such combination therapies offer the promise of increasing the diversity of tractable candidates available for drug targeting, of ameliorating side effects of cancer therapies, and of finding rational strategies for bypassing specific apoptotic lesions responsible for tumor progression and drug resistance in particular tumor types. In this article, we have explored the mechanism underlying an efficacious combinatorial therapeutic approach in mesothelioma, an especially intractable neoplasm, and demonstrated how two disparate pro-apoptotic but individually subtoxic signals cooperate to trigger death. Understanding such apoptotic synergy may allow manipulation of molecular pathways in tumors to achieve potent therapeutic responses.

To investigate the mechanism of apoptotic synergy between TRAIL signaling and DNA damage in mesothelioma cells, we examined the impact of activating each pathway separately...
and in combination on the well characterized TRAIL-signaling apoptotic effectors caspase 8 and Bid. The combination of TRAIL and etoposide induced extensive caspase 8 and Bid cleavage and profound apoptosis. In contrast, neither TRAIL nor etoposide alone had any appreciable impact on apoptosis at the levels used. Nonetheless, TRAIL alone but not etoposide alone triggered a modest cleavage of Bid, indicating that even in the absence of a cooperating DNA damage signal, the downstream TRAIL effector pathway had been activated to a low, non-lethal extent by TRAIL alone. This raised the possibility that DNA damage might cooperate with TRAIL not by enhancing the TRAIL signaling pathway per se but rather by lowering the threshold at which TRAIL signaling triggers the apoptotic machinery. We confirmed this by directly demonstrating that exposure of mesothelioma cells to DNA damage rendered them sensitive to induction of apoptosis by levels of the well characterized apoptotic TRAIL pathway effector tBid that are otherwise completely sublethal. We also confirmed that Bid is the essential intermediary of the apoptotic synergy between TRAIL signaling and DNA damage by abrogating Bid with siRNA. In the absence of Bid, we observed no synergy between the two pro-apoptotic signals. Although extensive evidence exists for the key role of Bid as a mediator of TRAIL signaling to the mitochondrion in Type II cells, these data identify for the first time the key role of Bid in the synergy between TRAIL and DNA damage.

The extensive caspase 8 cleavage that we observed upon exposing cells to the combination of TRAIL and DNA damage suggested that DNA damage might also interact with the TRAIL pathway to enhance TRAIL signaling at a site proximal to Bid. In the absence of Bid, however, the extensive cleavage of caspase 8 was prevented even in cells exposed to both TRAIL and DNA damage, indicating that the two signals do not cooperate upstream of Bid. Rather, the extensive caspase 8 and Bid cleavage that we observed during TRAIL-DNA damage cooperation in the presence of Bid appears to be a consequence, not a

**FIG. 4. Etoposide induces sensitivity of M28 cells to microinjected tBid.**

- **a**, M28 cells were exposed either to etoposide or to the Me2SO carrier for 15 h and were then microinjected either with sub-lethal recombinant tBid or with saline solution. Microinjected cells were studied for their cell fate by time-lapse videomicroscopy. Survival is plotted for different groups compared with the control group (saline-injected Me2SO-exposed cells) (n = 4 in each group, mean ± S.E.; *, significantly different from survival after tBid or etoposide alone; p < 0.02).
- **b**, fluorescent image of microinjected cells immediately after microinjection and after 30 h. Microinjected live cells are green, and dead cells have a red nucleus because of staining with propidium iodide. Of note, with death microinjected cells lose their green color; thus their fate is best determined by time-lapse videomicroscopy (see supplemental movie). The supplemental movie shows 30 h of 4-frame time-lapse video of the M28 cells exposed either to etoposide or the carrier control and then microinjected either with tBid or with saline.
cause, of the promiscuous activation of caspases that occurs during the execution phase of apoptosis (27, 28).

Genotoxic agents have long been the mainstay of classical cancer therapy. The efficacy and therapeutic specificity of such ostensibly nonspecific agents is thought to arise from an innately heightened sensitivity of tumor cells to DNA damage and perturbations of the cell proliferative machinery. This, in turn, is in great part a consequence of the obligate growth deregulatory mutations that underpin tumorigenesis and act to sensitize cells to a wide range of pro-apoptotic insults (5, 40). Unfortunately, use of genotoxic therapeutics is circumscribed by their collateral toxicity to sensitive normal tissues as well as the inexorable evolution and selection of resistant neoplastic clones harboring mutations that compromise the signaling pathways mediating genotoxic responses. Nonetheless, it is now clear that DNA damage signals are but one of many classes of pro-apoptotic signaling pathways that converge on and are integrated at the common basal cell suicide machinery. From this has arisen the attractive notion that adroit activation of multiple different pro-apoptotic signaling pathways, each at an individually sublethal level, might cumulatively prove sufficient to trip the threshold for cell death activation. We have explored this possibility in mesothelioma cells, a particularly intractable tumor because of its refractoriness to existing modes of therapy. Our studies show that co-activation of the TRAIL and DNA damage pathways together at levels where each individually induces no measurable toxicity, is profoundly cytotoxic and that Bid mediates this apoptotic synergy.

Recently, Deng et al. (41) showed that c-Jun NH2-terminal kinase activation enhances the sensitivity of cells to tumor necrosis factor-induced apoptosis via generation of a novel cleavage fragment of Bid, jBid, that is required for cleavage of caspase 8. Indeed, we have previously noted that etoposide-

FIG. 5. RNAi knock-down of Bid expression abrogates TRAIL-etoposide apoptotic synergy. a, M28 mesothelioma cells were subjected to two sequential rounds of transfection with either random or Bid-specific siRNAs. Bid expression is almost completely abrogated by the Bid-specific RNAi; there is no effect on expression of actin or caspase 8. b, M28 cells subjected to two sequential rounds of Bid-specific RNAi were exposed for an additional 18 h either to TRAIL, etoposide, γ irradiation, or a combination. In Bid-deficient cells, all apoptotic synergy between TRAIL and etoposide or TRAIL and γ irradiation is abolished (n = 3, mean ± S.E.; *, significantly different from apoptosis due to TRAIL alone plus that due to etoposide or γ irradiation alone; p < 0.05). c, likewise, RNAi inhibition of Bid expression abrogates TRAIL-etoposide synergy in REN mesothelioma cells (n = 3, mean ± S.E.; *, significantly different from apoptosis due to TRAIL plus that due to etoposide; p < 0.05).
sothelioma cells were transfected with random (R) or Bid siRNA and then exposed to TRAIL alone, etoposide alone, or both together. At 15 h, cells were harvested and either prepared for immunoblotting for caspase 8 or assayed for apoptosis by annexin V binding. Low level caspase 8 cleavage is evident in cells treated with TRAIL alone, whereas extensive caspase 8 cleavage is evident in cells co-exposed to TRAIL and etoposide. With Bid suppression, extensive caspase 8 cleavage is evident in cells treated with TRAIL alone, etoposide alone, or both together. At 15 h, cells were harvested and either prepared for immunoblotting for caspase 8 or assayed for apoptosis by annexin V binding. Low level caspase 8 cleavage is evident in cells treated with TRAIL alone, whereas extensive caspase 8 cleavage is evident in cells co-exposed to TRAIL and etoposide. With Bid suppression, extensive caspase 8 cleavage is evident in cells treated with TRAIL alone, etoposide alone, or both together.

Fig. 6. Bid knock-down prevents the extensive caspase 8 cleavage that accompanies TRAIL-etoposide synergy. M28 mesothelioma cells were transfected with random (R) or Bid siRNA and then exposed to TRAIL alone, etoposide alone, or both together. At 15 h, cells were harvested and either prepared for immunoblotting for caspase 8 or assayed for apoptosis by annexin V binding. Low level caspase 8 cleavage is evident in cells treated with TRAIL alone, whereas extensive caspase 8 cleavage is evident in cells co-exposed to TRAIL and etoposide. With Bid suppression, extensive caspase 8 cleavage is evident in cells treated with TRAIL alone, etoposide alone, or both together.

Enhanced Image

Caspase 8

Bid

TRAIL

Etoposide

siRNA

R

R

Bid

R

Bid

Bid

-22 kDa

-56 kDa

-43 kDa

-40 kDa

-17 kDa

-17 kDa

Actin

Apoptosis (%) 4 3 8 6 54 12

Fig. 7. Model for proposed mechanism of apoptotic synergy between TRAIL and DNA damage in mesothelioma cells. TRAIL by itself induces cleavage of Bid, but this is insufficient to activate the mitochondrial apoptotic machinery in mesothelioma cells. However, a separate DNA damage signal lowers the threshold at which Bid can trigger the mitochondrial apoptotic program. Consequent activation of the apoptotic machinery then leads to promiscuous cleavage of caspases and their substrates, including caspase 8 and Bid. There is no requirement in this model for any interaction between DNA damage signals and the TRAIL-DR4/5-death-inducing signaling complex (DISC)-caspase 8 pathway upstream of the mitochondrion. Consequently, synergy is completely dependent on Bid, as evident in Figs. 5 and 6. In normal mesothelial cells, even these combined apoptotic signals are insufficient to exceed the threshold for apoptosis because their mitochondria are not sensitized by pro-apoptotic oncogenic lesions.

suggest that this type of manipulation of the mitochondrial cell death threshold would be a productive generic strategy for augmenting combinatorial anticancer therapies.

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