The present studies were performed to determine whether lysosomal permeabilization contributes to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) cytotoxicity and to reconcile a role for lysosomes with prior observations that Bcl-2 family members regulate TRAIL-induced apoptosis. In KMCH cholangiocarcinoma cells stably expressing Mcl-1 small interference RNA (siRNA), treatment with TRAIL induced a redistribution of the cathepsin B from lysosomes to the cytosol. Pharmacological and small hairpin RNA-targeted inhibition of cathepsin B attenuated TRAIL-mediated apoptosis as assessed by morphological, biochemical, and clonogenic assays. Neither Bid siRNA nor Bak siRNA prevented cathepsin B release. In contrast, treatment of the cells with Bim siRNA or the JNK inhibitor SP600125 attenuated lysosomal permeabilization and cell death. Moreover, Bim and active Bax co-localized to lysosomes in TRAIL-treated cells in a JNK-dependent manner, and Bax siRNA reduced TRAIL-induced lysosomal permeabilization and cell death. Finally, BH3 domain peptides permeabilized isolated lysosomes in the presence of Bax. Collectively, these data suggest that TRAIL can trigger an apoptotic pathway that involves JNK-dependent activation of Bim, which in turn induces Bax-mediated permeabilization of lysosomes.

Tumor necrosis factor (TNF)2-related apoptosis-inducing ligand (TRAIL), a death ligand that preferentially induces apoptosis in transformed and virally infected cells, is under evaluation as an anti-cancer agent in humans (1–3). TRAIL induces apoptosis by binding to death receptors 4 and 5 (DR4/

TRAIL-R1 and DR5/TRAIL-R2), type I transmembrane proteins that contain death domains in their cytosolic tails. TRAIL binding results in receptor oligomerization (4) followed by recruitment of Fas-associated protein with death domain protein (5). This polyprotein in turn recruits procaspases-8 and -10 to the receptor complex through homotypic interactions involving death effector domains in Fas-associated protein with death domain and these two apical caspases (4). The resulting activation of the bound caspases is essential for TRAIL-induced apoptosis (5).

The cytotoxicity of TRAIL is also regulated by members of the Bcl-2 family of polypeptides (6). In particular, the multidomain proapoptotic Bcl-2 family member Bax is essential for TRAIL-induced killing of a colon cancer cell line that has low level expression of Bak (7). Other Bcl-2 family members also affect TRAIL action. We and others have demonstrated that the multidomain antiapoptotic protein Mcl-1 blocks TRAIL-mediated apoptosis in various cell lines (8–10). The related proteins Bcl-2 and Bcl-XL have also been reported to inhibit TRAIL-induced killing (11, 12). Conversely, certain proapoptotic BH3-only family members, which function as sensors of various cellular stresses (13), participate in TRAIL-induced killing. Specifically, Bid and Bim have both been implicated in TRAIL cytotoxicity (10, 14). How these Bcl-2 family members integrate into a composite signaling pathway involving apical caspases and subsequent cellular killing remains incompletely understood.

To add further complexity, ligation of TRAIL receptors is also associated with activation of various mitogen-associated protein kinases, including p38, extracellular signal-regulated kinases 1 and 2 (ERK 1/2), and c-Jun N-terminal kinase (JNK) (15). Of these, JNK has been most strongly associated with apoptosis induction (16). JNK may induce apoptosis by catalyzing an activating phosphorylation of Bid (17, 18), which in turn may activate Bax, inactivate antiapoptotic Bcl-2 family members, or both (19). Thus, analysis of TRAIL signaling must also take into account kinase-activation events.

TRAIL has extensive homology with TNF-α, which can also trigger apoptosis, in part, by inducing lysosomal permeabilization (20–23). The release of lysosomal cathepsins, especially cathepsin B, into the cytosol is associated with apoptosis (20, 23, 24). Cytosolic cathepsin B triggers mitochondrial dysfunction, thereby inducing cytochrome c release followed by caspase 9 activation (20). The one or more mechanisms by which lysosomal permeabilization occurs remains incompletely understood. Although sphingosine, ceramide, and reactive oxygen species have all been implicated in this pheno-
non (25), it is also possible that Bcl-2 proteins participate in the permeabilization of this organelle. Consistent with this possibility, Bax translocation to lysosomes and lysosomal permeabilization has recently been reported in fatty liver (26, 27).

Despite the structural homology between TRAIL and TNF-α, the role of the lysosomal pathway in TRAIL-mediated cytotoxicity has remained unclear. In studies designed to address this question, we now identify a pathway that involves caspase-8-dependent JNK activation followed by a Bim- and Bax-dependent lysosomal permeabilization step that participates in TRAIL-induced cytotoxicity upstream of mitochondrial events.

**MATERIALS AND METHODS**

**Cell Lines and Culture**—KMCH and MZ-CHA-1 human cholangiocarcinoma cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 0.1% penicillin, 100 µg/ml streptomycin, and 100 µg/ml gentamycin. KMCH cells stably transfected with an Mcl-1 small hairpin RNA (shMcl-1 KMCH cells) (8) were cultured in DMEM supplemented with 10% fetal bovine serum, 10% calf serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, 100 µg/ml gentamycin, and 200 µg/ml G418. Huh-7 human hepatocellular carcinoma cells were also cultured in DMEM containing the supplements described above, whereas Huh-7 cells stably transduced with a commercially available shRNA (Ctsb shRNA-Huh-7) (28) were cultured in DMEM containing 200 µg/ml G418.

**Quantification of Apoptosis**—Apoptosis was quantified by assessing the characteristic nuclear changes of apoptosis (i.e., chromatin condensation and nuclear fragmentation) after staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) and fluorescence microscopy using excitation and emission wavelengths of 380 and 430 nm, respectively. Caspase-3/7 activity in cell cultures was assessed by measuring rhodamine release from the caspase-3/7 substrate rhodamine 110, bis(N-CBZ-l-aspartyl-l-glutamyl-l-valyl-l-aspartic acid amide), using the Apo-ONE™ homogeneous caspase-3/7 kit (Promega, Madison, WI) following the supplier’s instructions.

**Image Acquisition**—Cellular images were obtained using an inverted confocal microscope (LSM510, Carl Zeiss Microimaging, Inc., Thornwood, NJ) equipped with a 63× numerical aperture (NA) 1.4 or a 100× NA 1.4 lens using LSM510 imaging software (Carl Zeiss Microimaging, Inc.).

**Immunocytochemistry**—Cells for Bax and cytochrome c immunocytochemistry were grown on glass coverslips and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.1% w/v PIPES, 1 mM EGTA, and 3 mM MgSO4. For cathepsin B immunocytochemistry, cells were fixed in PBS containing 4% paraformaldehyde and 0.1% picric acid. Cells were permeabilized with 0.0125% (w/v) CHAPS in PBS at 37 °C for 10 min and blocked for 1 h at room temperature with PBS containing 5% goat serum albumin, 5% glycerol, and 0.04% sodium azide. After incubation with primary antibody in blocking buffer at 4 °C overnight, cells were washed three times with PBS, incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR), or Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:500 in blocking buffer for 1 h at 37 °C. Cells were then washed three times in PBS and three times in H2O, mounted onto slides using a ProLong Antifade Kit (Molecular Probes), and imaged by confocal microscopy, with excitation and emission wavelengths of 488 nm and 507 nm (Alexa Fluor 488), and 577 nm and 590 nm (Cy3), respectively. The following primary antibodies were used: monoclonal mouse anti-cathepsin B from Calbiochem (La Jolla, CA), monoclonal mouse anti-Bax (Clone 6A7) from Exalpha Biologicals (Watertown, MA), monoclonal mouse anti-Bax (B-9) from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal mouse anti-protein disulfide-isomerase (PDI, Affinity BioReagents, Golden, CO) and monoclonal mouse anti-cytochrome c (#556432) from BD Pharmingen (San Jose, CA).

**siRNA-targeted Protein Knockdown and Generation of the Mz-CHA-1-Bim shRNA Cell Line**—A specific double-stranded 21-nucleotide RNA sequence homologous to the target message was used to silence human Bim, Bid, Bax, or Bak expression. siRNAs were designed and synthesized using a Silencer siRNA Construction kit from Ambion Inc. (Austin, TX) and software provided by the company. The sequences targeted in the current experiments were as follows: for Bim, 5'-AAC CTT GCA TAG TAA GCG CCT GTC TC-3' (T7-promoter in bold); for Bid, 5'-AAG AAG ACA TCA TCC GGA ATA CCT GTC TC-3'; for Bak, 5'-AAG TAC GAA GAT TCT TCA AAT CCT GTC TC-3'; and for Bax, 5'-AAG ACG AAC TGC ACA GAA ATT TAT GAT ACC AGA-3'. As a control, cells were also transfected with a scrambled RNA duplex with the following sequence: 5'-AAC GTG ATT TAT GAC ACC AGA-3'. Inhibition of protein expression was assessed by immunoblot analysis after transfection with the siRNAs. Briefly, cells grown in 12-well dishes were transiently transfected with 20 nm siRNA using 6 µl/ml siPORT Lipid (Ambion Inc.) in a total transfection volume of 0.5 ml of DMEM containing 10% fetal bovine serum. After incubation at 37 °C in 5% CO2 for 4 h, 1.5 ml of normal growth medium was added. Cells were then incubated in medium for 40 h before being subjected to further studies. The Mz-CHA-1-Bim siRNA cell line was generated as follows. pSilencer 4.1-CMV neo (Ambion) plasmid containing the Bim targeting sequence (above) was transfected into Mz-CHA-1 cells using a standard Lipofectamine plus method. Stably transfected Mz-CHA-1 cells were selected in DMEM containing 100 µg/ml G418. Individual colonies were subcloned and screened for Bim protein expression by immunoblot analysis.

**Bim-GFP**—Green Fluorescent Protein (Bim-GFP)—Using primers designed to produce full-length Bim (forward primer, 5'-GTC CAT GAA TCC ACC ATG GCA AAG CAA CCT-3' ; reverse primer, 5'-ACC TCA TCC TAG GTC ATT GCA TTC A-3'), the 417-bp product was amplified by PCR, separated on a 1% agarose gel, and further purified by using the QIAquick gel extraction kit (Qiagen, Chatsworth, CA). The purified PCR product and pEGFP-N1 vector (Clontech, Mountain View, CA) were digested with EcoRI and BamHI (Invitrogen), ligated using standard techniques, and transformed into One Shot TOP10 competent cells (Invitrogen), which were plated on agar plates containing 30 µg/ml kanamycin. DNA for transfection was subsequently purified using a Quantum Prep™ Plasmid Midiprep Kit from Bio-Rad (Hercules, CA). Splice junctions
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and insert were sequenced to confirm fidelity of the PCR reaction and the orientation of the Bim insert.

Immunoblot Analysis—Whole cell lysates or lysosomal fractions were subjected to immunoblot analysis. Lysosomes were isolated from cell cultures using a commercially available Lysosome Isolation Kit (Sigma). Whole cell lysates were obtained by incubating cells for 30 min on ice with lysis buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfon fluoride; 1 μg/ml aprotinin, leupeptin, pepstatin; 1 mM Na3VO4; 1 mM NaF) followed by sedimentation at 14,000 × g for 15 min at 4 °C. Samples were resolved by 15% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with the indicated primary antibodies at a dilution of 1:1,000. Peroxidase-conjugated secondary antibodies (BIOSOURCE International, Camarillo, CA) were incubated at a dilution of 1:5,000. Bound antibodies were visualized using enhanced chemiluminescence reagents (ECL, Amersham Biosciences) and Kodak X-OMAT film. Primary antibodies used were the following: monoclonal mouse anti-lysosomal associated membrane protein 1 (LAMP1) (BD Pharmingen); monoclonal mouse anti-PDI (Affinity BioReagents); monoclonal rat anti-Bim (Chemicon, Victoria, Australia); polyclonal rabbit anti-caspase 9, polyclonal rabbit anti-Bak, anti-JNK, and anti-phospho-JNK (Upstate Cell Signaling, Charlottesville, VA); and monoclonal mouse anti-Bax (B-9), polyclonal goat anti-cytochrome c oxidase II (K-20), polyclonal goat anti-cathepsin B (C-19), polyclonal goat anti-actin (C-11), and polyclonal goat anti-Bid (C-20) (Santa Cruz Biotechnology).

Assessment and Quantitation of GFP-coupled Protein Compartmentalization by Confocal Microscopy—The Bax-GFP plasmid was described previously (29). MZ-CHA-1 cells were transfected with Bax-GFP or Bim-GFP (described above) using Lipofectamine Plus (Invitrogen). Briefly, transfections were performed using 1 ml of Opti-MEM-1 containing 6 μl of Plus reagent, 1 μg/ml plasmid DNA, and 6 μl/ml Lipofectamine reagent following the supplier’s instructions. LysoTracker Red DND-99 or MitoTracker Red CMXRs (Molecular Probes) was loaded into cells by incubation in probe-containing medium at a final concentration of 50 nM for 15 min 37 °C. Cells were imaged by confocal microscopy using excitation and emission wavelengths of 488 and 507 nm for GFP and 577 and 590 nm for red fluorescence, respectively. Quantitation of co-localization was accomplished using the LSM510 imaging software. Briefly, random lines one pixel in width were drawn through the cells, and fluorescence intensity for both the green and red channels was quantified. A ratio of the green to red fluorescence was calculated yielding a percent pixel overlap. The data are expressed as percent pixel overlay for each experimental condition.

Determination of Organelle-associated Cathepsin B and Cytochrome c Release into the Cytosol—Cells were either transfected with cathepsin B-RFP, or immunofluorescence for cathepsin B or cytochrome c was performed as described previously (22, 28). Cells were imaged by confocal microscope and scored as either displaying diffuse versus punctate fluorescent pattern.

Measurement of Lysosomal Permeabilization in Cell-free Systems—Highly purified lysosomes free of mitochondria were isolated from mouse liver as described (30). To examine lysosomal permeabilization by Bcl-2 family members, 20-μg aliquots of isolated lysosomes were treated with increasing concentrations (5–25 μM) of synthetic 25-mer peptides corresponding to the BH3 region of Bad, Bim, or Noxa (19) or with commercially available human truncated Bid (tBid, 25–50 ng/ml), in the presence or absence of mouse recombinant Bax (10 μg/ml), in a final volume of 50 μl of lysosomal buffer (10 mM HEPES, pH 7.4, 0.3 M sucrose). After incubation at 30 °C for 30 min, the reaction mixture was centrifuged at 15,000 × g for 30 min at 4 °C to pellet the lysosomes. A 20-μl aliquot of each supernatant was subjected to SDS-PAGE and analyzed by immunoblot for cathepsin B (C-19, Santa Cruz Biotechnology) and cathepsin D (C-20, Santa Cruz Biotechnology) as described above. The pellet was also subjected to immunoblot analysis for cathepsin B.

Clonogenic Assay—Cells were split at 100 cells per 35-mm dish in 1 ml of medium. 24 h later the cells were treated with TRAIL as indicated in the figure legend. After removal of TRAIL, cells were then allowed to grow until isolated colonies were visible (~7 days). The colonies were then washed once with PBS, stained with Coomassie Blue, and counted.

Materials—Reagents were obtained from the following suppliers: Recombinant human TRAIL from R&D Systems (Minneapolis, MN); shRNA cat B plasmid and staurosporine from Sigma; the cathepsin B inhibitor CA-074, calpain inhibitor III and p38 inhibitor SB203580 from Calbiochem; R3032 from Celera Genomics (South San Francisco, CA); LEHD-fmk from Enzyme Systems (Livermore, CA); SP600125 and 3-O-methylsphingomyelin from BIOMOL (Plymouth Meeting, PA); z-IETD-fmk from MP Biomedicals (Aurora, OH); human recombinant tBid from Alexis Biochemicals (San Diego, CA); mouse recombinant Bax from ProteinX Lab (San Diego, CA); monoclonal mouse anti-Fas (CH11) from Upstate (Charlottesville, VA); and pepstatin from Chemicon. Plasmid encoding CrmA was a kind gift from Charles Young (Mayo Clinic, Rochester, MN).

Statistical Analysis—All data represent at least three independent experiments using cells from a minimum of three separate isolations and are expressed as mean ± S.E. Differences between groups were compared using two-tailed Student’s t-tests.

RESULTS

Involvement of Cathepsin B in TRAIL-induced Cytochrome c Release, Caspase 9 Activation, and Cell Death—The present studies were initially performed in cholangiocarcinoma cells, a human epithelial malignancy that has been previously used for examining TRAIL signaling and resistance (8, 9, 31). Because cholangiocarcinoma cells are relatively resistant to TRAIL-mediated apoptosis unless they are depleted of Mcl-1 (shMcl-1 KMCH cells) or abundantly express Bim (Mz-CHA-1 cells) (8), we have previously established a cholangiocarcinoma cell line with a knockdown of Mcl-1 by stable transfection with a shRNA targeting Mcl-1 (shMcl-1 KMCH cells) (8). When these were incubated with recombinant human TRAIL (5 ng/ml) for 4–5
h, morphological evidence of apoptosis was present in ≥60% of the shMcl-1 KMCH cells but was minimal in the parental KMCH cells (Fig. 1A). Examination of caspase-3/7 activity yielded similar results (Fig. 1B). In further experiments, cathepsin B localization was examined during the course of TRAIL-induced cell death (Fig. 1C). Under basal conditions, both cell lines displayed punctate fluorescence consistent with lysosomal localization of cathepsin B. However, after treatment with TRAIL, shMcl-1 KMCH cells showed diffuse fluorescence, suggesting a redistribution of cathepsin B from lysosomes to the cytosol (Fig. 1C). In contrast, parental KMCH cells continued to display punctate fluorescence after TRAIL treatment.

To assess the importance of cathepsin B release, the effect of two cathepsin B inhibitors, CA-074 and R3032, as well as the cathepsin D inhibitor pepstatin and calpain inhibitor III on TRAIL-induced killing was examined. Inhibition of cathepsin B, but not cathepsin D nor calpain, significantly reduced TRAIL-induced apoptosis and effector caspase activity in shMcl-1 KMCH cells (Fig. 2A, caspase-3/7 data not shown). Furthermore, the cathepsin B inhibitors also prevented mitochondrial cytochrome c release (Fig. 2B), shRNA-targeted knockdown of cathepsin B also reduced TRAIL-mediated apoptosis as assessed morphologically and by quantitation of effector caspases-3/-7 activity (Fig. 2C, caspase-3/7 data not shown).

We further confirmed the role of lysosomal permeabilization in TRAIL-mediated apoptosis using the lysosomal stabilizing agent 3-O-methyl sphingomyelin (32). This agent attenuated cathepsin B release as anticipated, and more, importantly reduced apoptosis (Fig. 3). Taken together, these data suggest lysosomal release of cathepsin B is upstream of mitochondria dysfunction during TRAIL-mediated apoptosis.

To rule out the alternative possibility that lysosomal cathepsin B release was downstream of mitochondrial dysfunction, we examined lysosomal permeabilization in the presence of the caspase-9 inhibitor LEHD-fmk. LEHD-fmk did not attenuate cathepsin B release from lysosomes (Fig. 4A), although, as anticipated, it reduced the post-mitochondrial events of apoptosis, including the nuclear morphological features of apoptosis and caspase-3/7 activation (Fig. 4B, caspase-3/7 data not shown) as well as blocking TRAIL induced autocleavage of caspase 9 (Fig. 4C).

To generalize these findings to an additional cell type, we next performed complementary studies in Huh-7 cells, a human hepatocellular carcinoma cell line commonly employed in cell biology studies (33, 34). The parental cell line and Huh-7 cells stably transfected with an shRNA targeting cathepsin B...
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(cstB-shRNA Huh-7) were employed for these studies. TRAIL-induced cell death did occur in the Huh-7 cell line, and this cytotoxicity was significantly reduced in the cstB-shRNA Huh-7 cell line (Fig. 5A). The dependence of TRAIL-mediated cytotoxicity on cathepsin B was further delineated in a clongenic assay, which demonstrated enhanced long term survival of TRAIL-treated cells deficient in cathepsin B (Fig. 5B). These results cannot be attributed to differences in expression of other death effector proteins (Fig. 5C).

Collectively, the observations in Figs. 1–5 establish that TRAIL-induced killing in shMcl-1 KMCH, and Huh-7 cells requires activity of cathepsin B and caspase-9, that cathepsin B is released from lysosomes into the cytoplasm during TRAIL-induced killing, and that this permeabilization of lysosomes occurs upstream of mitochondrial perturbation and caspase-9 activation.

TRAIL-induced Lysosomal Cathepsin B Release Depends on JNK and Bim—We next examined the role of JNK and caspase 8 in TRAIL-mediated lysosomal cathepsin B release. To determine a role for caspase 8 in lysosomal permeabilization Mz-CHA-1 cells were transfected with CrmA, a smallpox protein that potently inhibits caspase 8 (35). Transfection with a CrmA expression plasmid inhibited cathepsin B-RFP release (Fig. 6A) indicating an upstream role for caspase 8 in lysosomal permeabilization by Bax.
and then treated with TRAIL or staurosporine (Fig. 6B). Importantly, pretreatment with the JNK inhibitor SP600125 also significantly reduced cathepsin B release from lysosomes (Fig. 6C) as well as TRAIL-mediated apoptosis (Fig. 6D). The observations were specific for SP600125 and TRAIL, because the p38 mitogen-associated protein kinase inhibitor SB203580 did not prevent TRAIL cytotoxicity nor did SP600125 significantly reduce staurosporine-induced apoptosis (Fig. 6D). These observations identify caspase-8-dependent JNK activation as a critical step in TRAIL-mediated apoptosis and place it upstream of lysosomal cathepsin B release.

One mechanism tying JNK activation to the initiation of apoptosis involves an activating phosphorylation of Bim (17). To determine whether Bim is involved in TRAIL-induced cell death, cells were depleted of Bim by siRNA. Mz-ChA-1 human cholangiocarcinoma cells were employed for these studies, because they abundantly express Bim, facilitating assessment of siRNA efficacy by immunoblotting (Fig. 7A). Transfection with Bim siRNA, but not scrambled siRNA, reduced TRAIL cytotoxicity (Fig. 7A). As recently suggested by others (10), these data implicate Bim in TRAIL-mediated apoptosis.

To reconcile these findings with participation of cathepsin B in TRAIL-induced apoptosis, Bim localization was examined. Initial experiments employed a Bim-GFP construct and confocal microscopy. Under basal conditions, Bim-GFP did not significantly localize with either mitochondria or lysosomes (Fig. 7, B and C) but instead was found for most part throughout the cytoplasm. Following treatment with TRAIL, however, Bim-GFP became punctate and co-localized with LysoTracker Red rather than the MitoTracker Red (Fig. 7, B and C). This redistribution of Bim-GFP was abrogated by the JNK inhibitor SP600125. Cell fractionation coupled with immunoblot analysis was employed to independently assess Bim localization. Consistent with the confocal microscopy findings, Bim translocation to lysosomes was also observed following TRAIL treatment (Fig. 7D).

Because Bid, another BH3-only protein, has also been implicated in TRAIL cytotoxicity (14), the action of TRAIL was assessed in cells depleted of Bid by siRNA. Targeted Bid knockdown neither attenuated TRAIL-mediated apoptosis (Fig. 7A) nor prevented cathepsin B release from lysosomes in these cells (data not shown). However, apoptosis by the Fas agonist CH-11 was reduced by Bid knockdown with siRNA, confirming that the magnitude of Bid reduction was sufficient to reduce cell death by another death receptor (Fig. 7A). Collectively, this series of experiments suggests lysosomal permeabilization is JNK- and Bim-dependent and that Bim is acting at the level of the lysosomes.

**Cathepsin B Release from Lysosomes Requires Bax**—Because BH3-only polypeptides require Bax or Bak to permeabilize lysosomes and mitochondria (19), we reasoned by analogy that either Bax or Bak might also be necessary for lysosomal destabilization. After treatment with siRNA targeting Bax or Bak (inset, Fig. 8), TRAIL-induced cathepsin B release was assessed. Bax siRNA blocked TRAIL-induced cathepsin B release, whereas Bak depletion did not (Fig. 8). These findings suggested that Bax, along with Bim, must translocate to lysosomes during TRAIL cytotoxic signaling. To test this prediction, we exam-
ined the cellular localization of Bax-GFP following exposure of Mz-ChA-1 cells to TRAIL. Under basal conditions, Bax-GFP fluorescence was predominantly diffuse, with minor co-localization to organelles staining with LysoTracker Red and MitoTracker Red (Fig. 9, A and B). Upon treatment with TRAIL, Bax-GFP fluorescence became punctate and displayed an intense co-localization with LysoTracker Red but not with MitoTracker Red. In contrast, when cells were treated with staurosporine, an agent that causes cell death exclusively through the mitochondrial pathway, Bax-GFP co-localized with MitoTracker Red rather than LysoTracker Red. Because Bax has also been reported to localize to the endoplasmic reticulum, double immunofluorescence for Bax and PDI (an endoplasmic reticulum marker) was performed. However, no co-localization of Bax with the endoplasmic reticulum was observed following TRAIL treatment (Fig. 9 C).

To determine if Bax is active when it translocates to lysosomes and to exclude the possibility that lysosomal localization of Bax after TRAIL treatment represented an effect of the GFP tag or forced overexpression, endogenous Bax was also examined by immunofluorescence with a monoclonal antibody (6A7) that recognizes only the active form of Bax. After TRAIL treatment, endogenous Bax activation was readily observed by immunofluorescence (Fig. 9 D). However, the TRAIL-induced Bax activation was prevented in the Mz-CHA-1 cells in which Bim was knocked down by siRNA (Fig. 9 D). The active Bax observed following TRAIL administration co-localized with lysosomes and not mitochondria (Fig. 9 E). Immunoblotting (Fig. 7 D) also demonstrated Bax translocation to the lysosomal fraction following TRAIL treatment. These data suggest active Bax can translocate to lysosomes following treatment with TRAIL by a Bim dependent process.

To determine whether Bax is capable of permeabilizing lysosomes, a cell-free system containing isolated lysosomes, purified Bax, recombinant tBid, and BH3-only peptides of Bim, Bid, and Bad was employed (Fig. 10). We verified that isolated lysosomes were free of the endoplasmic reticulum marker PDI and the mitochondria protein cytochrome c oxidase (Fig. 10 A). After
purified lysosomes were incubated in the presence of Bax with or without recombinant tBid or the BH3 peptides, lysosomal permeabilization was assessed by blotting for cathepsins B in the supernatant and the pellet (Fig 10B). Incubation with tBid, Bax, or the BH3 peptides alone was insufficient to induce significant lysosomal permeabilization. In the presence of the purified Bax and Bim BH3 peptide together lysosomal release of cathepsin B was greatly enhanced. Taken together, these data indicate that, during TRAIL-mediated apoptosis of a cholangiocarcinoma cell line, Bax translocates to lysosomes and induces cathepsin B release analogous to the release of cytochrome c from mitochondria.

**DISCUSSION**

The results of the present study indicate that TRAIL-induced cytotoxicity in cholangiocarcinoma and hepatoma cells (i) is associated with a redistribution of the lysosomal protease cathepsin B from lysosomes to the cytosol; (ii) is, in part, mediated by cathepsin B, as shRNA and pharmacologic inhibition of this lysosomal protease blocks apoptosis upstream of mitochondrial dysfunction; and (iii) involves JNK-dependent activation of Bim, which in turn allows activation of Bax to induce lysosomal permeabilization. These findings not only suggest that TRAIL can trigger the lysosomal pathway of cell death but also demonstrate that this pathway is regulated by Bcl-2 proteins. Collectively, these observations have potentially important implications for current understanding of TRAIL-induced cytotoxicity.

Several recent studies indicate that lysosomal permeabilization with release of lysosomal proteases into the cytosol participates in death receptor-mediated cell death (20, 23, 37). However, those studies were performed using TNF-α, often in the presence of the general transcription inhibitor actinomycin D, a confounding variable. Here we demonstrate that TRAIL-induced apoptosis can also be associated with early lysosomal permeabilization. This TRAIL-induced release of lysosomal enzymes to the cytosol occurred in multiple cholangiocarcinoma cell lines and was observed in the absence of a transcription or protein synthesis inhibitor. Importantly, pharmacological inhibition of cathepsin B attenuated TRAIL-induced cytochrome c from mitochondria and the attendant cytotoxicity of TRAIL. In contrast, inhibition of caspase-9, a caspase activated downstream of mitochondrial dysfunction, inhibited cell death but did not prevent lysosomal permeabilization. These observations are consistent with previous studies demonstrating that lysosomal permeabilization is upstream of and triggers mitochondrial dysfunction (20, 38, 39). Although multiple lysosomal proteases are released into the cytosol upon lysosomal permeabilization, most are inactive at neutral pH. Cathepsin B is not only one of the most abundant lysosomal proteases, it also retains endopeptidase activity at neutral pH and induces mitochondrial cytochrome c release in cell-free systems (20, 25). Combined with the results of the present work, these studies suggest that cathepsin B plays an instrumental role in TRAIL-induced cell death.

How cathepsin B is released during death receptor-mediated apoptosis has previously been unclear. It is well established that JNK is activated by death receptor-signaling cascades (40). JNK has been strongly implicated in TNF-α-mediated and toxin-induced cell death (41–43). The ability of the JNK inhibitor SP600125 to inhibit TRAIL-induced cathepsin B release and cytotoxicity suggests that JNK plays an important role in lysosomal permeabilization.

Previously reported roles for JNK in apoptotic pathways include phosphorylation of Bim, generation of an active form of BID referred to as tBid, enhancement of proteasome-mediated degradation of cFLIP, and activator protein-1 activation (17, 44–46). In the current studies, cFLIP levels were unaltered following TRAIL administration in cholangiocarcinoma cells (data not shown), apparently ruling out cFLIP down-regulation as a mode of JNK cytotoxic signaling (47). Moreover, we did not observe inhibition of lysosomal cathepsin B release or apoptosis in cells depleted of BID by siRNA, suggesting that tBid or tBid have limited roles in this cell type. In contrast, cellular depletion of Bim attenuated lysosomal cathepsin B release and apoptosis.

Further support for the view that JNK-mediated Bim activation plays a role in TRAIL-induced cytotoxicity comes from the observation that Mcl-1 modulates TRAIL sensitivity in cholangiocarcinoma cells (8). Mcl-1 binds Bim with a high affinity, allowing it to sequester this otherwise toxic molecule (48). In contrast, the ability of Mcl-1 to bind tBid remains controversial (48, 49). When combined with the present observations, these prior results suggest that JNK participates in TRAIL-induced cell death by activating Bim, an observation consistent with recent reports suggesting an integral role for Bim in TRAIL cytotoxicity (10).

Because Bim cannot permeabilize lysosomes directly, we next asked whether Bax and/or Bak, which are required for apoptosis induced by other stimuli (50), participate in lysosomal permeabilization as well. Bax activation downstream of JNK has been reported in a variety of models, supporting our model of TRAIL cytotoxicity (51–53). Results of our studies...
indicate that siRNA targeting Bax, but not Bak, inhibits lysosomal cathepsin B release following TRAIL administration. Consistent with these observations, experiments using Bax-GFP or immunocytochemistry for active Bax demonstrate Bax translocation to lysosomes in TRAIL-treated cholangiocarcinoma cells. These results are consistent with two recent studies that have also implicated Bax in lysosomal permeabilization (26, 54). Thus, in addition to its well described translocation to mitochondria (55, 56), Bax apparently can also translocate to lysosomes. Perhaps Bim promotes Bax translocation to lysosomes, whereas tBid facilitates its association with mitochondria? This would explain the Bim-dependent, tBid-independent observations of our current study.

The present studies also begin to provide insight into the process by which Bax permeabilizes lysosomes. In the presence of a BH3-only protein to disable anti-apoptotic proteins or directly ligate Bax, Bax has been reported to form channels in membranes (57). Our studies with isolated lysosomes demonstrate that Bax-mediated membrane permeabilization is not dependent upon mitochondrial specific lipids (e.g. cardiolipin) but does require a proapoptotic BH3-only domain peptide or protein. The ability of BH3 domains to cooperate with Bax in the permeabilization of lysosomes under cell-free conditions is consistent with the view that certain BH3 domains can directly bind to and activate Bax (19). Alternatively, immunoelectron microscopy has previously demonstrated localization of Bcl-2 to vesicular bodies that might be lysosomes in addition to the well known localization to the cytoplasmic surfaces of the mitochondria, nuclei, and endoplasmic reticulum (58, 59) consistent with the view that BH3 domains can facilitate Bax activation by neutralizing antiapoptotic Bcl-2 family members (60). Further study is required to determine the precise mechanism by which Bim induces

![Image](http://example.com/fig9.png)

**FIGURE 9.** Activated Bax selectively translocates to lysosomes in a JNK-dependent manner following TRAIL-treatment. **A** and **B**, Mz-ChA-1 cells were transfected with Bax-GFP and subsequently treated with TRAIL (5 ng/ml) for 3 h or staurosporine (1 μg/ml). The cells were then loaded with MitoTracker Red (A) or LysoTracker Red (B) imaged by confocal microscopy (upper panels), and quantitation of red/green fluorescence overlay was performed (lower panels). 100× lens. Bars, 10 μm. **C**, Mz-ChA-1 cells were treated with TRAIL (5 ng/ml) for 3 h. The cells were fixed and immunofluorescence for total Bax and PDI was performed. The cells were imaged by confocal microscopy (upper panel) and quantitation of red/green fluorescence overlay was performed (lower panel). 100× lens. Bars, 10 μm. **D**, Mz-ChA-1 and Mz-ChA-1Bim siRNA cells treated with TRAIL (5 ng/ml) for 3 h. The cells were fixed and immunofluorescence for active Bax was performed using the 6A7 monoclonal antibody. Cells were imaged by confocal microscope (upper panel), and total fluorescence was quantified (lower panel). The effectiveness of Bim protein knockdown by siRNA was assessed by immunoblotting (inset). Bars, 20 μm. **E**, Mz-ChA-1 cells were treated with TRAIL (5 ng/ml) for 3 h loaded with MitoTracker Red or LysoTracker Red. Immunofluorescence for active Bax was performed in fixed cells that were then imaged by confocal microscopy (upper panel). Red/green fluorescence overlay was quantified as described under “Materials and Methods” (lower panel). 100× lens. Bars, 10 μm.
Bax-mediated lysosomal permeabilization in cholangiocarcinoma and hepatoma cell lines.

Based on the present results, it appears that TRAIL can induce at least three different cell death pathways. One involves the direct activation of caspase 8, which in turn activates caspase 3. A second involves caspase 8-mediated cleavage of Bid, which then activates the mitochondrial pathway. Our data suggest a third pathway in which TRAIL activates JNK, which in turn activates Bim to permeabilize lysosomes and activate the mitochondrial pathway. Although we have observed the involvement of this third pathway during TRAIL-induced apoptosis in multiple cell lines, including neoplastic cells derived from hepatocytes as well as bile duct epithelium, it is unclear how widespread the involvement of the lysosomal pathway will be. Further study in cells derived from other embryological origins will be required to further assess which of these three pathways predominates in various cellular contexts and to determine the full extent to which the lysosomal pathway contributes to TRAIL-induced apoptosis.

In summary, these data support a model in which TRAIL induces lysosomal permeabilization as part of its cell death program in cholangiocarcinoma cells. Although TNF-α has been shown to induce liver injury via a cathepsin B-dependent process in vitro and in vivo, these studies are the first to delineate this pathway in TRAIL cytotoxicity. Specifically, the model implicates TRAIL-mediated JNK activation by a caspase-8-dependent mechanism. JNK then either directly or indirectly activates Bim, which likely engages Bax to cause lysosomal release of cathepsin B into the cytosol. Cytosolic cathepsin B then induces mitochondrial dysfunction and activation of the intrinsic apoptotic pathway (20). Although the processes that regulate Bim and Bax translocation to lysosomes require further investigation, the observation that the lysosomal cell death pathway may be “up-regulated” in transformed cells (12, 61) raises the possibility that the events described in this report may play an important role in TRAIL-mediated apoptosis of cancer cells.

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FIGURE 10. Bax-dependent lysosomal permeabilization in vitro requires Bim. A, the purity of isolated lysosomes was verified by immunoblot analysis for lysosomes (LAMP1), endoplasmic reticulum (PDI), and mitochondria (cytochrome c oxidase). B, isolated lysosomes (20 μg of protein) from mouse liver were incubated at 30 °C for 30 min with full-length recombinant Bax (10 μg/ml), human recombinant tBid (50 ng/ml), Bim BH3 peptide (25 μM), Bad BH3 peptide (25 μM), or Bid BH3 peptide (25 μM), alone or in combination as indicated. The lysosomes were pelleted by centrifugation at 15,000 × g for 30 min; the resulting supernatants were subjected to SDS-PAGE and Western blot analysis for cathepsin B. The depicted blots are representative of three separate experiments.
Lysosomal Permeabilization by Bax

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