

Mcl-1 Mediates Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Resistance in Human Cholangiocarcinoma Cells

Makiko Taniai, Annette Grabhildner, Hajime Higuchi, Nate Verneburg, Steve F. Bronk, Daniel J. Fargugia, Scott H. Kaufmann, and Gregory J. Gores

Mayo Clinic School of Medicine, Rochester, Minnesota

ABSTRACT

Cholangiocarcinomas are usually fatal neoplasms originating from bile duct epithelia. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising agent for cancer therapy, including cholangiocarcinoma. However, many cholangiocarcinoma cells are resistant to TRAIL-mediated apoptosis. Thus, our aim was to examine the intracellular mechanisms responsible for TRAIL resistance in human cholangiocarcinoma cell lines. Three TRAIL-resistant human cholangiocarcinoma cell lines were identified. All of the cell lines expressed TRAIL receptor 1/death receptor 4 (TRAIL-R1/DR4) and TRAIL-R2/DR5. Expression of TRAIL decoy receptors and the antiapoptotic cellular FLICE-inhibitory protein (cFLIP) was inconsistent across the cell lines. Of the antiapoptotic Bcl-2 family of proteins profiled (Bcl-2, Bcl-xL, and Mcl-1), Mcl-1 was uniquely overexpressed by the cell lines. When small-interfering-RNA (siRNA) technology was used to knock down expression of Bcl-2, Bcl-xL, and Mcl-1, only the Mcl-1-siRNA sensitized the cells to TRAIL-mediated apoptosis. In a cell line stably transfected with Mcl-1-small-hairpin-RNA (Mcl-1-shRNA), Mcl-1 depletion sensitized cells to TRAIL-mediated apoptosis despite Bcl-2 expression. TRAIL-mediated apoptosis in the stably transfected cells was associated with mitochondrial depolarization, Bax activation, cytochrome c release from mitochondria, and caspase activation. Finally, flavopiridol, an anticancer drug that rapidly down-regulates Mcl-1, also sensitized cells to TRAIL cytotoxicity. In conclusion, these studies not only demonstrate that Mcl-1 mediates TRAIL resistance in cholangiocarcinoma cells by blocking the mitochondrial pathway of cell death but also identify two strategies for circumventing this resistance.

INTRODUCTION

Cholangiocarcinoma is a highly malignant, generally fatal adenocarcinoma arising from bile duct epithelial cells (cholangiocytes) of the intrahepatic or extrahepatic biliary system. Approximately 4000–5000 cholangiocarcinomas are diagnosed annually in the United States, representing ~20% of all hepatobiliary malignancies (1, 2). The incidence of this deadly neoplasm is increasing in Western countries (1–5). Surgical resection and liver transplantation are potentially curative therapies. Unfortunately, not more than two-thirds of cholangiocarcinomas are too advanced at the time of presentation to benefit from these surgical approaches. Moreover, recurrence after surgery is common and problematic. Currently available chemotherapeutic agents and radiation therapy are ineffective at improving survival (2). Accordingly, there is a need for improved therapies for this neoplasm.

When drugs or cytokines are used for the treatment of advanced cancers, selective induction of cancer cell apoptosis is a desirable outcome (6–8). The cytokine tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an attractive agent for achieving this goal (9). This death ligand selectively induces apoptosis in many transformed and malignant cells (10–12) without apparent toxicity to normal tissues in the rodent, baboon, and chimpanzee (13–15). Although one study has indicated that TRAIL might be toxic to human hepatocytes, this toxicity appears to reflect the formulation of cytokine used and to be circumvented with different TRAIL preparations (14, 16–19). The potential importance of the TRAIL pathway for the treatment of cancers is highlighted by the recent introduction of TRAIL receptor (TRAIL-R) agonistic antibodies into human Phase I trials (20).

One previous study has suggested that cholangiocarcinomas might be susceptible to TRAIL-induced apoptosis (12). In that report, however, several cholangiocarcinoma cell lines were resistant to TRAIL (12). TRAIL resistance is common in other cancers and has been attributed to the loss of TRAIL-Rs, up-regulation of TRAIL decoy receptors (DcRs), enhanced expression of cellular FLICE-inhibitory protein [cFLIP; an endogenous inhibitor of death receptor (DR) signaling], or alterations in expression of the Bcl-2 family of proteins (15, 16). For example, absence of the proapoptotic proteins Bak and Bax as well as enhanced expression of the antiapoptotic proteins Bcl-2 and Bcl-xL has been shown to convey TRAIL resistance (21–25). Cholangiocarcinomas, however, do not manifest changes in Bax, Bak, Bcl-2, or Bcl-xL but, instead, frequently overexpress the antiapoptotic Bcl-2 family member Mcl-1 (26, 27). The potential ability of Mcl-1 to inhibit TRAIL-mediated apoptosis has not been previously assessed but may be critical if TRAIL is to be useful for the treatment of cholangiocarcinoma.

The objective of this study was to determine whether Mcl-1 mediates TRAIL resistance in cholangiocarcinoma cells. To address this question, we used TRAIL-resistant cholangiocarcinoma cell lines and small-interfering-RNA (siRNA) technology to attenuate Mcl-1 expression (28–30). Results of this analysis not only suggest that selective Mcl-1 down-regulation sensitizes cholangiocarcinoma cell lines to TRAIL-mediated apoptosis, but also identify two approaches for overcoming this Mcl-1-mediated TRAIL resistance.

MATERIALS AND METHODS

Materials. Reagents were purchased from the following suppliers: anti-FLAG M2 mouse monoclonal antibody (IgG1) and Sepharose-CL 4B from Sigma (St. Louis, MO); 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and tetramethylrhodamine ethyl ester-conjugated goat-anti-mouse IgG (H + L) from Molecular Probes (Eugene, OR); mouse anti-Mcl-1 and mouse anti-Bcl-xL from Pharmingen (San Diego, CA); mouse (IgG2a) anti-cytochrome c from PharMingen (San Diego, CA); mouse (IgG2a) anti-BAX from Sigma (St. Louis, MO); mouse (IgG1) anti-active Bax from Exalphi Biologicals (Boston, MA); mouse (IgG1) anti-Bcl-2, mouse anti-Bax, goat anti-Bak, and goat anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA); goat anti-TRAIL-R2 (DR5), rabbit anti-TRAIL-R1 (DR4), goat anti-TRAIL-R3 (DcR1), and rat anti-cFLIP from Alexis (San Diego, CA); rabbit anti-TRAIL-R4 (DcR2) from Axxora (San Diego, CA), horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (H + L) from BioSource (Camarillo, CA); and Bcl-2- and Mcl-1-duplex-siRNA from Dharmacon (Lafayette, CO). Flavopiridol was a kind gift from Ed Sausville, National Cancer Institute (Bethesda, MD).

Cell Culture. The human cholangiocarcinoma cell lines KMCH, KMBC, and Witt were grown in DMEM supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin. The human cholangiocarcinoma cell lines KMCH, KMBC, and Witt were grown in DMEM supplemented with 10% fetal bovine serum.
10% bovine calf serum, 100,000 units/liter penicillin, 100 mg/liter streptomycin, 100 mg/liter gentamicin as described previously (31, 32). The nonmalignant human cholangiocyte cell line H69 (33) was cultured in the same medium.

**Cell Treatment and Quantitation of Apoptosis.** FLAG epitope-tagged human recombinant TRAIL (amino acids 95–281) was prepared as described previously (34, 35). Cells were incubated with the FLAG-TRAIL in the presence of anti-FLAG M2 monoclonal antibody (2 μg/ml). Flavopiridol was dissolved in DMSO and was diluted more than 1000-fold into culture medium before treatment. Apoptosis was quantitated by measuring the characteristic nuclear changes of apoptosis (i.e., chromatin condensation and nuclear fragmentation) using the nuclear binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and fluorescence microscopy (36).

**siRNA Treatment.** A specific double-stranded 21-nucleotide RNA sequence homologous to the target message was used to silence Mcl-1, Bcl-2, and Bcl-xL expression. siRNAs for human Bcl-2 and Mcl-1 were purchased from Ambion (Austin, TX) according to the manufacturer’s instructions. The sequence of the double-stranded RNA used to block Bcl-xL expression in the current experiments is 5′-AAC TGA CTC CAG CTG TAT CCC-3′ (T7-promoter bold).

Inhibition of protein expression and TRAIL-induced apoptosis was assessed after transient transfection of cholangiocarcinoma cells with siRNA. Briefly, cells grown in 12-well dishes were transiently transfection with 20 μl siRNA using 6 μl/mi 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/5% CO₂ for 15 min at 4°C, the supernatants were collected. Samples were then prepared and analyzed for apoptosis or by immunoblot as described below (see “Immunoblot Analysis”).

**Stable Transfection of Mcl-1 Hairpin RNA Expression Plasmid.** The pSSH plasmid containing the human H1 RNA promoter for the expression of small hairpin RNA (shRNA) was a gift from Dr. D. Billeaud (Mayo Clinic, Rochester, MN). Double-stranded DNA template (5′-CCC GCG GAC TGG CTA GTT AAA CTT CAA GAG AGT TTA ACT AGC CAG TCC GTG TTT GAG AAA-3′) was inserted into the pSSH plasmid after the H1 RNA promoter (pSSH1-Mcl-1). The DNA insert contains sense and antisense sequences (bold type) of the Mcl-1 mRNA and a 9-nucleotide linker sequence, yielding transcription of siRNA against Mcl-1. The plasmid was subjected to nucleotide sequencing for confirmation.

Transfection with the pSSH1-Mcl-1 plasmid was performed using a standard lipofection method. Stably transfected KMCH clones were selected in medium containing 1200 μg/ml G418. Individual colonies were subcloned and screened for Mcl-1 protein expression by immunoblot analysis. Established clones were grown in DMEM supplemented with 10% fetal bovine serum, 10% bovine calf serum, 100,000 units/liter penicillin, 100 mg/liter streptomycin, 100 mg/liter gentamicin, and 200 mg/liter G418.

**Immunoblot Analysis.** Cells were lysed by incubation on ice for 30 min in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na3VO4, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Complete Protease Inhibitor Cocktail; Boehringer-Mannheim Biochemica, Mannheim, Germany). After insoluble material was pelleted by centrifugation at 14,000 × g for 15 min at 4°C, the supernatants were collected. Samples were resolved by 12.5% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with primary antibodies at a dilution of 1:1000. Peroxidase-conjugated secondary antibodies (BioSource International, Camarillo, CA) were incubated at a dilution of 1:2000 to 1:10000. Bound antibody was visualized using chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL) and was exposed to Kodak X-OMAT film.

**Mitochondrial Membrane Potential.** The mitochondrial membrane potential was monitored in single-cultured KMCH cells using the mitochondrial membrane potential-sensitive dye tetramethylrhodamine ethyl ester as described previously (37). Briefly, cells were preincubated in Krebs-Ringer-HEPES buffer containing tetramethylrhodamine ethyl ester (1 μM) for 15 min at 37°C and then were incubated in the presence of 50 nM tetramethylrhodamine ethyl ester throughout the experiment. Cellular fluorescence was visualized using multiparameter digitized video microscope system (excitation 530 nm; emission 580 nm) and was quantitated using the Metafluor quantitative fluorescence software (Universal Imaging Corp., West Chester, PA) as described previously (38, 39).

**Immunocytochemistry.** KMCH cells were grown on coverslips and were fixed with PBS containing 3% paraformaldehyde for 20 min at 37°C. After three washes with PBS, the cells were permeabilized in PBS containing 0.0125% 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfate (CHAPS), washed again in PBS three times for 3 min, and then were blocked for 60 min at 37°C in blocking buffer containing 5% goat serum, 5% glycerc, 0.04% sodium azide in PBS (pH 7.2). The cells were then incubated with anti-active Bax antibody diluted 1:200 in blocking buffer at room temperature for 16 h, washed three times in PBS for 10 min, incubated with tetramethylrhodamine-conjugated goat-antimouse IgG at a concentration of 4 μg/ml for 45 min at 37°C, washed again in PBS three times for 3 min, rinsed in H₂O, and mounted using Prolong Antifade kit (Molecular Probes) before examination by fluorescence microscopy.

**Subcellular Fractionation.** Cytosolic extracts for cytochrome c immuno blot assay were obtained as described by Leist et al. (40). Briefly, at the desired time points, the culture medium was exchanged with ice-cold permeabilization buffer [210 mM n-mannitol, 70 mM sucrose, 10 mM HEPES, 0.2 mM EGTA, 0.15% BSA, 80 μM digoxin (pH 7.2)]. After a 5-min incubation at 4°C, cells were centrifuged for 10 min at 13,000 × g. Supernatants representing the cytosolic extracts were used for immunoblot analysis.

**Caspase Activation.** Cells were cultured on 35-mm glass-bottomed microwell dishes (MatTek Corp., Ashland, MA) at a density of 1000 cells/dish. Total cellular caspase activation was assessed in single cells using the fluorophore-tagged pancaspase inhibitor N-(6-carboxyfluoresceinvinylalanylnyl)aspartic acid fluoromethylketone ([FAM-VAD-fmk] CaspTag; Intergen Co., Purchase, NY). Briefly, the commercially supplied lyophilized FAM-VAD-FMK was reconstituted into a 150× stock with DMSO and was further diluted into a 30× working solution with PBS. Before the experiment, 10 μl of this solution was added to 290 μl of PBS for each dish. The DMEM was gently aspirated and was replaced with 300 μl of the fluorophore-containing buffer. Cells were incubated at 37°C for 1 h, washed twice with the supplied working solution wash buffer, and mounted on the microscope stage of an inverted fluorescent microscope (Axiovert 535; Carl Zeiss, Inc., Thornwood, NY). Excitation light provided by a 100-W mercury vapor lamp was passed through an interference and neutral-density filter wheel assembly (Eastern Microscope, Research Triangle Park, NC) to select wavelength and intensity under computer control. Fluorescence was visualized using excitation and emission wavelengths of 490 nm and 520 nm, respectively. Digital images were captured using a cooled charged coupled device camera (CCD camera, KAF-1400; Photometrometers, Tucson, AZ) and were analyzed and quantitated using fluorescence imaging software (Metafluor Imaging System; Universal Imaging Corp.). Fluorescence intensity of individual cells was quantitated; and the average fluorescence intensity of 100 cells per experimental group was determined.

**Statistical Analysis.** All of the data represent at least three independent experiments and are expressed as the means ± SD unless otherwise indicated. Differences between groups were compared using ANOVA and a post hoc Bonferroni test was used to correct for multiple comparisons.

**RESULTS**

Cholangiocarcinoma Cells Are Resistant to TRAIL-Induced Apoptosis. In initial experiments, the sensitivity of cholangiocarcinoma cells to the death ligand TRAIL was examined. The human cholangiocarcinoma cell lines KMCH, KMBC, and Witt, as well as the nonmalignant cholangiocyte cell line H69, were used for these studies. The cells were incubated with human recombinant FLAG-TRAIL at a concentration of 400 ng/ml for 16 h in the presence of 2 μg of anti-FLAG-M2 (Table 1). TRAIL induced minimal apoptosis, 2.5-fold above control values, in the transformed but nonmalignant cell line H69 (9 ± 2% versus 4 ± 1%; P < 0.05). In contrast, apoptosis above control rates was not observed for any of the three human cholangiocarcinoma cell lines. Thus, these three cholangiocarcinoma cell lines are resistant to TRAIL-mediated apoptosis.
Cholangiocarcinoma Cells Express Both Cytotoxic TRAIL-Rs and DcRs. Next, we sought to ascertain whether the resistance of the three human cholangiocarcinoma cell lines to TRAIL-induced apoptosis was due to failure to express the TRAIL DR4 and DR5, or to enhanced expression of the TRAIL DcRs. Immunoblotting demonstrated that all of the cell lines expressed the TRAIL DRs DR4 and DR5 (Fig. 1A). The DcRs DcR1 and DcR2 were also detected in nonmalignant cells and cholangiocarcinoma cells, albeit with a decreased expression of DcR1 in Witt cells and an increased expression in KMBC and KMCH cells. Thus, as has been reported in other cell lines (15, 16, 41), there was no consistent correlation between DcR expression and resistance to TRAIL cytotoxicity. Furthermore, TRAIL insensitivity could not be explained by a failure to express cognate TRAIL DRs.

Expression of cFLIP Is Not Enhanced in Cholangiocarcinoma Cells. cFLIP is a frequently expressed endogenous protein that inhibits DR-mediated apoptosis by interfering with the function of the death-inducing signaling complex. FLIP overexpression has been implicated in TRAIL resistance in some model systems (15, 23, 42). As assessed by immunoblot analysis, cFLIP protein was detectable in both nonmalignant H69 cells and in the cholangiocarcinoma cell lines (Fig. 1B). There was a slight elevation in expression of cFLIP_long in Witt cells and of cFLIP_short in KMBC cells, but in the third cholangiocarcinoma cell line KMCH, both forms of cFLIP were only weakly expressed in KMCH cells.

Table 1 Cholangiocarcinoma cells are resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis

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Fig. 1. Expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors, cellular FLICE-inhibitory protein (cFLIP), and Bcl-2 family members in cholangiocarcinoma cells. Whole cell lysates containing 30 μg of protein were subjected to SDS-PAGE and immunoblot analysis using antibodies against TRAIL death receptors DR4 and DR5, TRAIL decoy receptors DcR1 and DcR2 (A), as well as cFLIP_long (cFLIP-L) and cFLIP_short (cFLIP-S; B), and antibodies against the Bcl-2 family members Mcl-1, Bcl-2, Bcl-xL, Bak, and Bax (C). Representative results of at least three different experiments are depicted.

Fig. 2. Sensitivity to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis can be restored by down-regulation of Mcl-1. KMBC (A), KMCH (B), and Witt cells (C) were transiently transfected with specific small-interfering-RNA (siRNA) constructs targeting Mcl-1, Bcl-2, Bcl-xL, or a control siRNA and were incubated with 400 ng/ml TRAIL in the presence of anti-FLAG-M2 antibody for 10 h. Apoptosis was quantitated using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining and fluorescence microscopy. All of the data were expressed as mean ± SD from three individual experiments.

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expressed. Therefore, TRAIL resistance across cell lines also could not be explained by a uniform elevation of cFLIP.

**Mcl-1 Expression Is Enhanced in Cholangiocarcinoma Cell Lines.** In some model systems, TRAIL resistance has been ascribed to loss of proapoptotic and/or enhanced expression of antiapoptotic members of the Bcl-2 family. Therefore, expression of these proteins was analyzed by immunoblot analysis (Fig. 1C). The cell lines appeared to express comparable amounts of the antiapoptotic proteins Bax and Bak. The antiapoptotic protein Bcl-2 was expressed in the nonmalignant cell line H69 and in KMCH cells; however, it could not be identified in the two other cholangiocarcinoma cell lines Witt and KMBC. Bcl-xL was present in H69, Witt, and KMBC cells; but almost no Bcl-xL was found in KMCH cells. Mcl-1 was expressed in H69 cells; and its expression was even higher in all three cholangiocarcinoma cell lines. These data raised the possibility that Mcl-1 may render cholangiocarcinoma cell lines resistant to TRAIL-induced cell death.

**Mcl-1-siRNA, but Not Bcl-2- or Bcl-xL-siRNA, Sensitizes Cholangiocarcinoma Cells to TRAIL-Induced Apoptosis.** To further evaluate the ability of the three antiapoptotic Bcl-2 family members to inhibit TRAIL-induced apoptosis, siRNA technology was used. Transient transfection with specific siRNAs for Bcl-2, Bcl-xL, and Mcl-1 demonstrated their ability to reduce expression of the corresponding polypeptides (data not shown). In all three cholangiocarcinoma cell lines, the Bcl-2 and Bcl-xL siRNAs failed to significantly increase the sensitivity of the cells to TRAIL (Fig. 2). In contrast, Mcl-1 down-regulation significantly enhanced TRAIL-induced apoptosis, resulting in apoptosis of 30–50% of KMBC and KMCH cells (Fig. 2, A and B) and almost 70% of Witt cells (Fig. 2C). Taken together, these data strongly suggest that Mcl-1, but not other members of the Bcl-2-family, protect cholangiocarcinoma cells from TRAIL-induced apoptosis.

**Mcl-1 Inhibits TRAIL-Mediated Apoptosis by Blocking the Mitochondrial Pathway of Cell Death.** To minimize the variables associated with transient transfection, we developed a stably transfected cell line in which Mcl-1 expression was reduced by shRNA. The KMCH cells were chosen for these studies because they also express Bcl-2. If Mcl-1 were truly more potent than Bcl-2 in blocking TRAIL cytotoxicity, as suggested by the preceding studies, it would be most apparent in this cell line. The shRNA specifically reduced Mcl-1 expression in the stably transfected KMCH cells, but had no influence on expression of Bcl-2, Bcl-xL, Bax, or Bak (Fig. 3A). In addition, the expression of TRAIL death and DcRs as well as cFLIP-L and cFLIP-S was unaltered (Fig. 3, B and C). Subsequent studies were performed to compare the time- and dose-dependence of TRAIL-induced apoptosis in the stably transfected cell line and parental KMCH cells. At all of the concentrations examined, TRAIL-mediated apoptosis was significantly greater in the cells transfected with Mcl-1 shRNA, reaching 50% apoptosis at 50 ng/ml TRAIL and a maximum of more than 80% apoptosis at 400–800 ng/ml TRAIL (Fig. 4A). In contrast, the parental line barely exhibited 50% apoptosis at 1600 ng/ml TRAIL, indicating a 32-fold lower sensitivity to TRAIL. Thus, like the transient transfections, cells stably transfected with Mcl-1-siRNA were sensitized to TRAIL even over time (Fig. 4B).

Mcl-1 degradation was recently found to be necessary for activation...
of Bax and induction of mitochondrial dysfunction after genotoxic damage (43). To assess whether Mcl-1 also participates in a hierarchical pathway upstream of Bax (44) during TRAIL-induced apoptosis, Bax activation was examined by immunocytochemistry. These experiments used a monoclonal antibody that recognizes the NH2-terminus of activated Bax but not of inactive Bax. In untreated cells, minimal activated Bax was detected in either cell line (Fig. 5, B and C). After incubation with TRAIL for 6 h, a marked increase in activated Bax immunoreactivity was observed in the Mcl-1-shRNA-transfected cells (Fig. 5, B and C) but not in the TRAIL-treated parent cell line. Thus, inhibiting Mcl-1 expression appears to facilitate mitochondrial depolarization by permitting the activation of the pro-apoptotic Bcl-protein Bax at mitochondria.

Bax promotes apoptosis by inducing mitochondrial release of cytochrome c, which in turn facilitates caspase activation via the apoptosome (44). To ensure that the observed Bax activation resulted in the postulated downstream changes, cytochrome c release into the cytosol and caspase activation was examined. After incubation with TRAIL, cytochrome c was more readily detectable in cytosol from the cell line stably transfected with Mcl-1 shRNA than in cytosol from parental cells (Fig. 6A). TRAIL-induced activation of caspases was likewise facilitated by stable transfection with Mcl-1 shRNA (Fig. 6B). Taken together, these data suggest that Mcl-1 protects cholangiocarcinoma cells against TRAIL by inhibiting the mitochondrial pathway of apoptosis.

Flavopiridol Reduces Mcl-1 Expression and Sensitizes Cholangiocarcinoma Cells to TRAIL Cytotoxicity. Flavopiridol, a synthetic flavone currently in Phase I and Phase II trials as an anticancer agent, down-regulates a variety of proteins, including Mcl-1 (45–47).
Consistent with these earlier results, incubation of KMCH cells with 250 nM flavopiridol for 24 h caused a marked decrease in Mcl-1 protein expression (Fig. 7A). In contrast, other Bcl-2 family proteins, TRAIL-Rs, and cFLIP are unaltered (Fig. 7). As was seen with Mcl-1 siRNA (Fig. 3) and shRNA (Figs. 4), pretreatment with flavopiridol sensitizes the KMCH cells to TRAIL-mediated killing (Fig. 8).

DISCUSSION

The principal findings of this paper relate to the cellular mechanisms by which cholangiocarcinoma cells are resistant to TRAIL-mediated apoptosis. In human cholangiocarcinoma cell lines, we observed the following: (a) TRAIL resistance could not readily be explained by alterations in expression of DcRs or cFLIP; (b) Mcl-1 was the only antiapoptotic Bcl-2 family member consistently overexpressed; (c) down-regulation of Mcl-1, but not Bcl-2 or Bcl-xL, by siRNA sensitized cholangiocarcinoma cells to TRAIL-mediated apoptosis; and (d) loss of Mcl-1 potentiated mitochondrial dysfunction, Bax translocation, and caspase activation during TRAIL-induced apoptosis.

The present results demonstrate that cholangiocarcinoma cells express TRAIL-Rs but are nonetheless resistant to TRAIL. These cells also express TRAIL DcRs, which bind TRAIL but are incapable of initiating apoptotic signaling cascades because they lack complete cytoplasmic death domains and do not bind Fas-associated death domain protein (FADD; Refs. 15, 48). When first identified, the TRAIL DcRs were thought to induce TRAIL resistance by sequestering TRAIL and preventing it from binding to its legitimate DRs, TRAIL-R1/DR4 and TRAIL-R2/DR5 (48–53). Several subsequent studies, however, failed to find any correlation between DcR expression and TRAIL resistance in various cancer types (15, 16, 41). These results were similar to our present studies in human cholangiocarcinoma cell lines, in which expression of the DcRs was variable among the cell lines. Witt cells, for example, minimally expressed DcR1 as compared with the other cell lines but were equally resistant to TRAIL cytotoxicity.

Additional experiments were performed to assess other potential mechanisms of TRAIL resistance. cFLIP is an antiapoptotic protein that interferes with TRAIL signaling at the level of the death-inducing signaling complex (23, 54–58). Although cFLIP has been implicated in TRAIL resistance, expression of this polypeptide was also variable among the cholangiocarcinoma cell lines. cFLIPshort, which is universally regarded as an antiapoptotic polypeptide, was expressed abundantly only by KMBC cells. These data suggested that resistance to TRAIL was likely mediated downstream of the death-inducing signaling complex.

TRAIL-mediated apoptosis, like Fas-induced death, requires downstream mitochondrial dysfunction to fully engage the death program in many cell types (59, 60). This mitochondrial dysfunction, which is characterized by mitochondrial permeabilization, loss of mitochondrial transmembrane potential, and release of cytochrome c into the cytosol (61–64), is regulated by pro- and antiapoptotic members of the Bcl-2 family. For example, TRAIL-induced apoptosis in a colon carcinoma-derived cell line is dependent on the proapoptotic protein Bax (21, 22). Conversely, antiapoptotic Bcl-2 proteins have been shown to inhibit TRAIL-mediated cell killing (24, 65). Results of the present study indicate that the inhibition of TRAIL-mediated apoptosis in cholangiocarcinoma cells occurs at the level of mitochondria and is mediated by the antiapoptotic protein Mcl-1. Several observa-
tions support this interpretation. First, of the antiapoptotic Bcl-2 family of proteins, only Mcl-1 is consistently overexpressed in all three cholangiocarcinoma cell lines relative to the nonmalignant human cholangiocyte H69 cell line. These data are consistent with immunohistochemical studies suggesting that Mcl-1 is overexpressed in human cholangiocarcinoma (27). Second, when siRNA technology is used to diminish expression of the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1, only Mcl-1 siRNA sensitizes all of the cholangiocarcinoma cell lines to TRAIL-mediated apoptosis. Finally, in a cell line stably transfected with Mcl-1 shRNA, mitochondrial dysfunction with membrane depolarization and cytochrome c release is readily observed after TRAIL treatment. Taken together, these data strongly implicate Mcl-1-mediated inhibition of mitochondrial cytochrome c release as a mechanism of TRAIL resistance in human cholangiocarcinoma cells.

The present results indicate that Mcl-1 plays a dominant role in TRAIL resistance despite the expression of Bcl-2 in the KMCH cell line. This unexpected observation suggests that Mcl-1 and Bcl-2 may have different functions. Indeed, these observations are reminiscent of studies with Bcl-2 and Bcl-xL transgenic mice, in which differential protective effects are observed depending on the model of injury (65, 66). More recently, elimination of Mcl-1 has been shown to be required for the initiation of apoptosis after UV light irradiation despite the expression of Bcl-xL (43). The elegant studies by Nijhawan et al. (43) suggest that Mcl-1 is upstream of Bax translocation to mitochondria, an essential mechanism for TRAIL-mediated apoptosis in colon carcinoma cell lines (22). Our studies, demonstrating more efficient Bax activation and translocation to mitochondria in cells transfected with Mcl-1 shRNA, are consistent with and help integrate these observations. In human cholangiocarcinoma cells, Mcl-1 would appear to be upstream of Bax in mediating its antiapoptotic effects. Moreover, once Mcl-1 is eliminated, Bax is more efficiently activated by cytotoxic TRAIL-initiated signaling pathways.

Consistent with these results, we observed that flavopiridol, which reduces Mcl-1 expression, also sensitizes cholangiocarcinoma cells to TRAIL. These results confirm and extend the previous suggestion that flavopiridol plus a TRAIL agonist might be a rational combination for treating other malignancies (67).

The present study focused on the mechanisms of TRAIL resistance in cholangiocarcinoma cells. TRAIL resistance is common in malignant cell lines; and strategies to overcome this resistance will be necessary to augment the therapeutic efficacy of TRAIL for treatment of human solid tumors. Mechanisms of TRAIL resistance are complex and vary among different cell types. The current observations suggest that Mcl-1 is instrumental in mediating TRAIL resistance in human cholangiocarcinomas, as has been recently observed in epidermal growth factor-stimulated fibroblasts (68). On the basis of the present results, approaches to inhibit Mcl-1 expression will likely be necessary for successful treatment of human cholangiocarcinomas with TRAIL. Mcl-1 may be down-regulated by siRNA approaches or flavopiridol, as shown in this study, or by inhibiting Mcl-1 induction using selective inhibitors of receptor tyrosine kinases (46, 69, 70).

Given the uniformly fatal nature of this cancer, additional preclinical studies with Bcl-2 and Bcl-xL transgenic mice, in which differential protective effects are observed depending on the model of injury (65, 66). More recently, elimination of Mcl-1 has been shown to be required for the initiation of apoptosis after UV light irradiation despite the expression of Bcl-xL (43). The elegant studies by Nijhawan et al. (43) suggest that Mcl-1 is upstream of Bax translocation to mitochondria, an essential mechanism for TRAIL-mediated apoptosis in colon carcinoma cell lines (22). Our studies, demonstrating more efficient Bax activation and translocation to mitochondria in cells transfected with Mcl-1 shRNA, are consistent with and help integrate these observations. In human cholangiocarcinoma cells, Mcl-1 would appear to be upstream of Bax in mediating its antiapoptotic effects. Moreover, once Mcl-1 is eliminated, Bax is more efficiently activated by cytotoxic TRAIL-initiated signaling pathways.

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Given the uniformly fatal nature of this cancer, additional preclinical studies with Bcl-2 and Bcl-xL transgenic mice, in which differential protective effects are observed depending on the model of injury (65, 66). More recently, elimination of Mcl-1 has been shown to be required for the initiation of apoptosis after UV light irradiation despite the expression of Bcl-xL (43). The elegant studies by Nijhawan et al. (43) suggest that Mcl-1 is upstream of Bax translocation to mitochondria, an essential mechanism for TRAIL-mediated apoptosis in colon carcinoma cell lines (22). Our studies, demonstrating more efficient Bax activation and translocation to mitochondria in cells transfected with Mcl-1 shRNA, are consistent with and help integrate these observations. In human cholangiocarcinoma cells, Mcl-1 would appear to be upstream of Bax in mediating its antiapoptotic effects. Moreover, once Mcl-1 is eliminated, Bax is more efficiently activated by cytotoxic TRAIL-initiated signaling pathways.

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