cFLIP_L prevents TRAIL-induced apoptosis of hepatocellular carcinoma cells by inhibiting the lysosomal pathway of apoptosis

Maria Eugenia Guicciardi, Steven F. Bronk, Nathan W. Werneburg, and Gregory J. Gores
Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905
Submitted 24 October 2006; accepted in final form 30 January 2007

Guicciardi ME, Bronk SF, Werneburg NW, Gores GJ. cFLIP_L prevents TRAIL-induced apoptosis of hepatocellular carcinoma cells by inhibiting the lysosomal pathway of apoptosis. Am J Physiol Gastrointest Liver Physiol 292: G1337–G1346, 2007. First published February 1, 2007; doi:10.1152/ajpgi.00497.2006.—Sensitivity to TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis and the lysosomal pathway of cell death are features of cancer cells. However, it is unknown if TRAIL cytotoxic signaling engages the lysosomal pathway of cell death. Our aim, therefore, was to ascertain if TRAIL killing involves lysosomal permeabilization. TRAIL-induced apoptosis of hepatocellular carcinoma cells (HuH-7, Hep3B) was associated with lysosomal permeabilization, as demonstrated by redistribution of the lysosomal protease cathepsin B into the cytosol. Pharmacological and short hairpin RNA-targeted inhibition of cathepsin B reduced apoptosis. Because cellular FLICE-inhibitory protein (cFLIP) inhibits TRAIL-induced cell death and is frequently overexpressed by human cancers, the ability of cFLIP to prevent lysosomal permeabilization during TRAIL treatment was examined. Enforced long-form cFLIP (cFLIP_L) expression reduced release of cathepsin B from lysosomes and attenuated apoptosis. cFLIP_L overexpression was also associated with robust p42/44 MAPK activation following exposure to TRAIL. In contrast, cFLIP_S overexpression attenuated p38 MAPK activation and had no significant effect on JNK and NF-κB activation. Inhibition of p42/44 MAPK by PD98059 restored TRAIL-mediated lysosomal permeabilization and apoptosis in cFLIP-overexpressing cells. In conclusion, these results demonstrate that lysosomal permeabilization contributes to TRAIL-induced apoptosis of hepatocellular carcinoma cells and suggest that cFLIP_L cytoprotection is, in part, due to p42/44 MAPK-dependent inhibition of lysosomal breakdown.

caspase-8; cathepsin B; PD98059; p42/44 mitogen-activated protein kinase

RECOMBINANT TNF-RELATED apoptosis-inducing ligand (TRAIL) and agonistic monoclonal antibodies for TRAIL-cognate receptors are promising anticancer agents (30). Their ability to selectively trigger cell death in a wide variety of cancers while sparing normal cells renders them attractive therapeutic agents (1, 38). Although of significant interest, the selectivity of TRAIL cytotoxicity for transformed cells remains largely unexplained. Recent studies have described a pathway of apoptotic cell death involving lysosomal disruption with release of proteolytic enzymes into the cytosol (15). This pathway seems to be particularly relevant in transformed cells (6), which often have mutations in the classic caspase-dependent apoptotic machinery but enhanced expression of lysosomal enzymes (19, 21). Indeed, oncogene-driven transformation of immortalized cells results in significant increases in expression of lysosomal cysteine cathepsins and sensitization to lysosome-mediated cell death (6). Cathepsin B, an abundant lysosomal cysteine protease with active endopeptidase activity at physiological pH, is a key mediator of this cytotoxic pathway (14). Although the ability of TRAIL to trigger lysosomal disruption has been suggested, the signaling pathway(s) involved in this process remain to be defined (7, 27).

TRAIL interacts with at least four membrane receptors belonging to the TNF receptor family: TRAIL-receptor 1 (TRAIL-R1, or death receptor 4, DR4), TRAIL receptor 2 (TRAIL-R2, or death receptor 5, DR5), TRAIL receptor 3 (TRAIL-R3, or decoy receptor 1, DcR1), and TRAIL receptor 4 (TRAIL-R4, or decoy receptor 2, DcR2) (11). Only TRAIL-R1 and TRAIL-R2 have cytoplastic death domains that allow recruitment of the adaptor Fas-associated protein with death domain (FADD) to the receptor complex. FADD is essential for activation of apical caspases, such as procaspase-8 and procaspase-10, resulting in apoptosis (32). Active caspase-8 induces apoptosis by directly activating the effector caspases-3 and -7 and by triggering the mitochondrial pathway of cell death via proteolytic activation of the BH3-only protein Bid (22, 23). The antiapoptotic protein cFLIP (cellular FLICE/caspase-8 inhibitory protein) can interrupt cytotoxic signaling by the TRAIL receptor complex (20, 36). cFLIP is frequently overexpressed in human tumors (24), including hepatocellular carcinoma (28). Two widely expressed isoforms of FLIP are generated by alternative splicing, the long isoform cFLIP_L and the short isoform cFLIP_S, both containing two amino-terminal death effector domains capable of interacting with the death effector domain of FADD. A third isoform, cFLIP_K, has recently been described (10), but its expression pattern, especially in malignancy, is unknown. Whereas cFLIP_L and cFLIP_S only have the amino-terminal death effector domains, cFLIP_K has an additional carboxy-terminal caspase-like domain and structurally resembles procaspase-8 and -10 but has no proteolytic activity. Because cFLIP_K also binds to FADD via a homotypic death effector domain interaction, it was originally thought to antagonize the recruitment and activation of procaspase-8 at the receptor complex. However, recent data suggest that cFLIP_K can heterodimerize with procaspase-8, thereby enhancing caspase-8 activation at the receptor complex (25). These data suggest that cFLIP_K may exert other molecular mechanisms that inhibit TRAIL-mediated apoptosis. Consistent with this concept, cFLIP_L can modulate a variety of kinase signaling cascades, many of which have prosurvival functions (9, 18).

TRAIL-R1 and TRAIL-R2 are also able to initiate kinase cascades resulting in activation of NF-κB, and members of the...
MAPK family, including JNK, p38, and p42/44 MAPK (ERK1/2) (16, 26, 32, 33). Given the ability of both TRAIL and cFLIP to initiate kinase signaling cascades, it is plausible that enhanced expression of cFLIP serves as a molecular switch, converting TRAIL signaling from cytotoxic caspase cascades to prosurvival kinase pathways. In particular, activation of the p42/44 MAPK and NF-κB pathways are associated with cytoprotection in numerous models of cytotoxicity (17, 35). These concepts remain unexplored, as does the potential effect of kinase pathways on lysosomal disruption.

The current study explores the above concepts in human hepatocellular carcinoma (HCC) cells. The results indicate that TRAIL triggers lysosomal permeabilization and cathepsin B-dependent apoptosis of HCC cells. cFLIP effectively reduces TRAIL-induced apoptosis, at least in part, by p42/44 MAPK-mediated lysosome stabilization.

MATERIALS AND METHODS

Cell lines. Human hepatocarcinoma HuH-7 cells were stably transduced to generate cFLIPl-overexpressing cell lines (HuH-cFLIP clone 1 and clone 5), as previously described by us (12). HuH-7, HuH-cFLIP clones, and human hepatocarcinoma Hep3B cell lines were cultured in DMEM supplemented with 10% FBS.

Quantitation of apoptosis. Apoptosis was quantitated by assessing nuclear changes that are indicative of apoptosis by using the DNA-binding dye DAPI (10 μg/ml). Cells were viewed by fluorescence microscopy (Nikon Eclipse TE200; Nikon, Tokyo, Japan) using excitation and emission filters of 380 and 430 nm, respectively. Caspase-3 and -7 activity in cell cultures was quantitated using Apo-ONE homogeneous caspase-3/7 kit (Promega, Madison, WI) following the manufacturer’s instructions.

Cathepsin B immunofluorescence. Cells were grown on 35-mm glass coverslips. After treatment, cells were washed once in PBS, fixed in ice-cold methanol for 6 min at 4°C, and permeabilized with 0.3% Tween 20 in PBS for 3 min at room temperature. After being washed with PBS, cells were incubated in blocking buffer (20% goat serum, 0.05% Tween 20 in PBS for 1 h at 37°C, then incubated overnight at room temperature with mouse anti-human cathepsin B (dilution 1:500 in 1% BSA, 0.05% Tween 20 in PBS) for 1 h at 37°C, mounted by using Prolong Antifade kit (Molecular Probes), and visualized by using an inverted laser-scanning confocal microscope with excitation and emission wavelengths of 488 and 507 nm, respectively.

Generation of cathepsin B short hairpin RNA HuH-7 cell line. HuH-7 cells were transduced using OptiMEM I (GIBCO-Invitrogen, Carlsbad, CA) containing 6 μl/ml Lipofectamine (Invitrogen), 1 μg/ml plasmid DNA [cathepsin B, MISSION short hairpin RNA (shRNA) lentiviral plasmid; Sigma Aldrich, St. Louis, MO] and 6 μl/ml Plus reagent (Invitrogen). Forty-eight hours after transfection, fresh DMEM containing 6 μl/ml puromycin was added to induce selective pressure on the culture. Surviving clones were separated and individually cultured. Cathepsin B expression in the clones was assessed by immunoblot analysis.

Preparation of cytosolic fractions and immunoblot analysis. Purified cytosolic fractions (S-100) from cell cultures were obtained by N2 cavitation as previously described (13). Immunoblot analysis of cytosolic extracts and whole cell lysates was performed as previously described by us (13).

NF-κB p65 activation. NF-κB DNA-binding assay was performed on purified nuclear extracts (10 μg protein) obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) according to the manufacturer’s instructions. Nuclear protein extracts were screened for NF-κB DNA-binding activity by using the TransAM assay (Active Motif, Carlsbad, CA) employing the NF-κB consensus binding motif 5’-GGGACTTTCC-3′ nucleotide sequence and p65 antisera in a quantitative ELISA-like assay. Degradation of NF-κB regulatory subunit IκB-α was assessed by immunoblot analysis as described above.

Cathepsin B-red fluorescent protein transfection. The rat cathepsin B-red fluorescent protein (RFP) expression vector previously described by us (40) was transiently transfected into Hep3B cells using OptiMEM containing 6 μl/ml Plus reagent, 2 μg/ml cathepsin B-RFP cDNA, and 6 μl/ml Lipofectamine reagent, following the manufacturer’s instructions. Cathepsin B-RFP was visualized by using an inverted laser-scanning confocal microscope with excitation and emission wavelengths of 577 and 590 nm, respectively.

Statistical analysis. All data represent at least three independent experiments and are expressed as means ± SE unless otherwise indicated.

Fig. 1. Recombinant TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of HuH-7 cells is time- and concentration-dependent, and is inhibited by cellular FLICE-inhibitory protein, long isoform (cFLIP). A: HuH-7 cells were incubated with increasing concentrations of human TRAIL for up to 5 h. Apoptosis was quantitated by morphological criteria after DAPI staining. B: HuH-7 and HuH-cFLIP cells were incubated with TRAIL (4 ng/ml) for 8 h or with etoposide (75 μM) for 48 h. Apoptosis was quantitated by morphological criteria after DAPI staining. *P < 0.05, HuH-7 vs. HuH-cFLIP. C: Activation of effector caspases-3 and -7, expressed as fold increase over control (untreated) value of relative fluorescence units (RFLU), was measured by a fluorogenic assay. *P < 0.01, HuH-7 vs. HuH-cFLIP.
indicated. Differences between groups were compared by using an unpaired two-tailed t-test, and P values <0.05 were considered statistically significant.

**Antibodies.** Anti-cathepsin B was from Oncogene Research Products (Boston, MA). Anti-cathepsin D (C-20; dilution 1:500), anti-β-actin (C-11; 1:500), and anti-IκB-α (C-21; 1:500) were from Santa Cruz Biochemicals (Santa Cruz, CA). Anti-SAPK/JNK (1:1,000), anti-phospho-SAPK/JNK (Thr183/Tyr185; 1:1,000), anti-p42/44 MAPK (1:1,000), anti-phospho-p42/44 MAPK (Thr202/Tyr204; 1:1,000), and anti-p38 MAPK (1:1,000) were from Cell Signaling Technology (Beverly, MA). Anti-active p38 (1:2,000) was from Promega. Anti-cFLIP (NT; 1:500) was from ProSci (Poway, CA). Peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, and swine anti-goat IgG were from Biosource International (Camarillo, CA). Alexa Fluor 488-conjugated goat anti-mouse IgG (heavy plus light) was from Molecular Probes. Cy3-conjugated goat anti-rabbit IgG was from Jackson Immunoresearch Laboratories (West Grove, PA).

**Reagents.** Human recombinant TRAIL was from R&D Systems (catalog no. 375-TEC). R-3032 was from Celera Genomics (South San Francisco, CA) and was used at a concentration of 20 μM in DMSO. SB203580, PD98059, and U0126 were from Calbiochem (San Diego, CA) and were used at concentrations of 0.5–1 μM, 50 μM, and 20 μM in DMSO, respectively. z-IETD-fmk was from Enzyme Systems Products (Irvine, CA) and was used at a concentration of 10 μM in DMSO. Etoposide, BSA, DAPI, Bradford reagent, and all other chemicals used were from Sigma Aldrich.

**RESULTS**

**TRAIL-induced apoptosis of HuH-7 cells is regulated by cFLIP L.** Time- and concentration-dependent apoptosis was readily observed in HuH-7 cells treated with TRAIL (Fig. 1A). Because concentrations of 2–5 ng/ml were effective in inducing apoptosis in 36–75% of the cells, respectively, after 5 h, this range of concentrations was used for the remainder of the study. To confirm inhibition of cFLIP L in TRAIL-mediated apoptosis, the cFLIP L-overexpressing cell line HuH-cFLIP was treated with TRAIL (4 ng/ml) for up to 8 h. As expected, apoptosis and activation of effector caspase-3 and -7, measured as DEVDase activity, were significantly reduced in the HuH-cFLIP cells (Fig. 1, B and C). Conversely, HuH-cFLIP cells were not resistant to etoposide-induced apoptosis, confirming that the cFLIP cytoprotective effect is limited to death receptor-mediated apoptosis (Fig. 1B). Thus, HuH-7 cells are sensitive to TRAIL-induced cell death, which is effectively diminished by overexpression of cFLIP L.

**TRAIL-induced apoptosis of HuH-7 cells involves lysosomal cathepsin B release.** To ascertain whether lysosomal permeabilization occurs in HuH-7 cells following TRAIL treatment, analysis of subcellular distribution of cathepsin B was performed by immunofluorescence. Cathepsin B fluorescence was...
punctate and mainly perinuclear in untreated cells, consistent with lysosomal localization. However, following incubation with TRAIL, cathepsin B became progressively diffuse as it was released from lysosomes into the cytosol (Fig. 2A). In contrast to the parent cell line, cathepsin B fluorescence remained punctate in TRAIL-treated HuH-cFLIP cells (Fig. 2A). Consistent with previous observations suggesting that cathepsin B activity is required for its own release from the lysosomes (40), inhibition of cathepsin B by the reversible inhibitor R-3032 significantly reduced lysosomal permeabilization (Fig. 2A). This inhibitor is highly selective for cathepsin B, as the $K_i$ for cathepsin B is at least two logs lower than it is for other lysosomal cathepsins, such as cathepsin L, cathepsin K, and cathepsin S, and does not inhibit caspases (Robert M. Rydzewski, Celera Genomics; personal communication).

These data demonstrated that TRAIL-induced lysosomal permeabilization that occurs in HCC cells is, in part, cathepsin B dependent and is inhibited by cFLIP_L.

Although lysosomal permeabilization occurs in TRAIL-treated cells, the above observations do not demonstrate an essential role for this process in cell death. Therefore, we generated HuH-7 cells stably expressing shRNA complementary to cathepsin B (CtsB shRNA-HuH-7), in which the protein level of cathepsin B was strongly reduced (Fig. 2B, inset). These cells were resistant to TRAIL-mediated lysosomal permeabilization (data not shown) and apoptosis (Fig. 2, B and C). These results were also confirmed by using the cathepsin B inhibitor R-3032 (Fig. 2C). Thus our observations suggest that cathepsin B contributes to TRAIL-induced apoptosis in HuH-7 cells.

cFLIP_L enhances p42/44 MAPK activation by TRAIL. cFLIP is increasingly recognized as an important adaptor molecule in kinase signaling cascades. Therefore, we next compared TRAIL-mediated activation of MAPK pathways in HuH-7 and HuH-cFLIP cells. TRAIL triggered p38 phosphorylation after 4–6 h in HuH-7 cells but not in HuH-cFLIP cells (Fig. 3A).

The ability of cFLIP_L to inhibit p38 MAPK phosphorylation has been previously described in bile acid-treated HuH-7 cells (12). In contrast, overexpression of cFLIP_L resulted in constitutive and sustained phosphorylation of p42/44 MAPK, which was further augmented by TRAIL treatment, whereas only transient p42/44 MAPK activation was detected in the wild-type HuH-7 cells (Fig. 3A). Although transient JNK phosphorylation was observed in HuH-cFLIP cells, sustained JNK activation, which is required for cell death (4), was not observed in either HuH-7 or HuH-cFLIP cells after TRAIL treatment (Fig. 3A). Thus, cFLIP_L appears to switch TRAIL signaling from a p38 activation process to a cascade activating p42/44 MAPK.

Next, we determined whether TRAIL differentially activates NF-κB in HuH-7 and HuH-cFLIP cells and whether this may also contribute to the resistance of HuH-cFLIP cells to TRAIL-induced lysosomal permeabilization and apoptosis. Indeed, the enhanced chemoresistance of cFLIP_L overexpressing cells has been associated with activation of NF-κB-regulated survival pathways (18). Nonetheless, untreated HuH-cFLIP cells showed comparable NF-κB DNA-binding activity compared with HuH-7 cells (OD450 0.48 ± 0.04 vs. 0.26 ± 0.01), suggesting that high levels of cFLIP_L are not sufficient to induce constitutive activation of NF-κB in these cells. Following TRAIL treatment, only a delayed and modest increase in NF-κB DNA-binding activity was observed in nuclear extracts from both HuH-7 and HuH-cFLIP cells (Fig. 3B). In contrast, DNA-binding activity was significantly increased in HuH-7 and HuH-cFLIP cells following TNF-α treatment, demonstrating that the cells have retained their
ability to activate this transcription factor (Fig. 3B). Consistent with these observations, no degradation of IκB-α was detected by immunoblot analysis in either cell line after treatment with TRAIL for up to 8 h (Fig. 3C). Thus NF-κB is only slightly activated and with similar kinetics in the two cell lines, and therefore it cannot be responsible for their differential sensitivity to TRAIL.

Inhibition of p42/44 MAPK restores TRAIL-induced lysosomal permeabilization and apoptosis in HuH-cFLIP cells. Based on our observations on differential activation of MAPK cascades in HuH-7 and HuH-cFLIP cells following TRAIL treatment, we proceeded to determine if these kinases modulate TRAIL-induced lysosomal permeabilization and/or apoptosis. Because p38 MAPK is only activated in HuH-7 cells, we speculated that activation of this kinase may be required for the execution of cell death. If this were the case, inhibition of p38 MAPK should render HuH-7 cells resistant to TRAIL-induced apoptosis. However, the selective p38 inhibitor SB203580 did not attenuate TRAIL cytotoxicity (Fig. 4A), suggesting that activation of p38 MAPK does not contribute to cell death. In contrast, apoptosis was strongly increased in HuH-cFLIP cells treated with TRAIL in the presence of the p42/44 MAPK inhibitors PD98059 (Fig. 4, B and C) and U0126 (Fig. 4, D and E). Inhibition of p42/44 MAPK also significantly increased TRAIL-induced lysosomal permeabilization in HuH-cFLIP cells, as demonstrated by immunofluorescence analysis (Fig. 5A) and immunoblot analysis (Fig. 5B) of cathepsin B and cathepsin D redistribution from lysosomes to cytosol.

**Fig. 4.** Inhibition of p38 MAPK does not protect from TRAIL-induced apoptosis, whereas inhibition of p42/44 MAPK enhances TRAIL toxicity. A: HuH-7 cells were treated with TRAIL (4 ng/ml) for up to 8 h in the presence or absence of the p38 MAPK inhibitor SB203580 (0.5–1 μM). Apoptosis was quantitated by morphological criteria after DAPI staining. B: HuH-7 and HuH-cFLIP cells were incubated with TRAIL (4 ng/ml) for up to 8 h in the presence or absence of the p42/44 MAPK inhibitor PD98059 (50 μM). Apoptosis was quantitated by morphological criteria after DAPI staining. The results are representative of 3 separate experiments performed in triplicate and are expressed as means ± SE. *P < 0.05, TRAIL vs. TRAIL + PD98059. C: activation of effector caspases-3 and -7 was measured by a fluorogenic assay. Data represent means ± SE of RFLU from 3 separate experiments performed in triplicate and are expressed as increments over control value (arbitrarily set at 1). *P < 0.05, TRAIL vs. TRAIL + PD98059. D: HuH-7 and HuH-cFLIP cells were incubated with TRAIL (4 ng/ml) in the presence or absence of the p42/44 MAPK inhibitor U0126 (20 μM). Apoptosis was quantitated by morphological criteria after DAPI staining. *P < 0.05. E: activation of effector caspases-3 and -7 was measured by a fluorogenic assay. Data represent means ± SE of RFLU from 3 separate experiments performed in triplicate and are expressed as increments over control value. *P < 0.05.
Fig. 5. Inhibition of p42/44 MAPK increases TRAIL-induced lysosomal permeabilization in cFLIP-overexpressing cells, whereas inhibition of caspase-8 partially prevents it. A: HuH-7 and HuH-cFLIP cells were incubated with TRAIL (4 ng/ml) in the presence or absence of PD98059 (50 μM) or z-IETD-fmk (10 μM) for 4 h. Localization of cathepsin B was analyzed by immunofluorescence and confocal microscopy (×100). B: cytosolic cathepsin D was analyzed by immunoblot on S-100 fractions obtained at the indicated time points. Immunoblot analysis for actin was performed to ensure equal protein loading. The depicted blot is representative of 2 separate experiments. C: HuH-cFLIP cells were incubated with 4 ng/ml TRAIL in the presence or absence of z-IETD-fmk (10 μM). At the indicated time points, cell lysates were analyzed by immunoblot for total and phosphorylated p42/44 MAPK. D: HuH-7 and HuH-cFLIP cells were incubated with TRAIL (4 ng/ml) for 8 h in the presence or absence of z-IETD-fmk (10 μM) and/or PD98059 (50 μM). Apoptosis was quantitated by morphological criteria after DAPI staining.
tively, these results suggest that cFLIP_L may exert its cytoprotective effect against TRAIL-induced apoptosis by p42/44 MAPK-mediated lysosome stabilization.

Caspase-8 catalytic activity is not essential for TRAIL-mediated p42/44 MAPK activation. Although caspase-8 activity is necessary for TRAIL-mediated p38 MAPK, JNK, and NF-κB activation (37), a role for caspase-8 activity in p42/44 MAPK activation has not been examined. Interestingly, p42/44 MAPK phosphorylation in HuH-cFLIP cells was not prevented by the addition of the caspase-8 inhibitor z-IETD-fmk (Fig. 5C), suggesting that cFLIP_L-modulated TRAIL signaling activates p42/44 MAPK through a molecular pathway independent of caspase-8 catalytic activity. Although caspase-8 was not necessary for p42/44 MAPK activation, inhibition of this protease did abrogate the cytotoxic effects of PD98059 plus TRAIL in both HuH-7 and HuH-cFLIP cells (Fig. 5D). Together, these data suggest that a caspase-8-independent pathway and a p42/44 MAPK-mediated pathway are independently activated in cFLIP_L-overexpressing cells following TRAIL treatment.

cFLIP_L and p42/44 MAPK-dependent inhibition of TRAIL-mediated lysosomal permeabilization and apoptosis in cFLIP_L-overexpressing cells is not due to clonal differences. To verify that TRAIL-induced lysosomal permeabilization and its regulation by p42/44 MAPK cascade observed in HuH-cFLIP cells was not due to clonal differences, we proceeded to verify our findings in another stable clone of HuH-cFLIP cells obtained during the same screening (clone 5). Like the first clone used in this study (clone 1), HuH-cFLIP clone 5 also displayed increased resistance to TRAIL-mediated apoptosis, although the cell death rate was slightly higher than that observed in HuH-cFLIP clone 1 (Fig. 6A). Importantly, inhibition of p42/44 MAPK also significantly increased TRAIL-induced cell death (Fig. 6A) and lysosomal permeabilization (Fig. 6B) in this clone, demonstrating that these findings are likely due to the overexpression of cFLIP_L rather than clonal differences.

TRAIL-induced apoptosis of Hep3B cells is mediated by lysosomal release of cathepsin B. Finally, to establish that our observations were not unique to HuH-7 cells, we extended our study to include another hepatocarcinoma cell line, Hep3B, which constitutively expresses high levels of cFLIP_L (Fig. 7A). Hep3B cells were treated for 4 and 8 h with TRAIL in the presence or absence of different inhibitors, and apoptosis was measured by morphological criteria and by activation of effector caspases-3 and -7. Moderate apoptosis was observed after 8 h of TRAIL treatment (Fig. 7, B and C). These cells were less sensitive to TRAIL toxicity than HuH-7 cells and HuH-cFLIP cells (Fig. 1, B and C), consistent with the higher expression of cFLIP_L (Fig. 7A). Importantly, cathepsin B inhibition prevented TRAIL-induced apoptosis, whereas inhibition of p42/44 MAPK greatly sensitized cells to TRAIL killing (Fig. 7, B and C). To investigate whether TRAIL induced lysosomal permeabilization in these cells, Hep3B cells were transiently transfected with an expression plasmid encoding cathepsin

Fig. 6. cFLIP_L and p42/44 MAPK inhibition of TRAIL-mediated lysosomal permeabilization and apoptosis in cFLIP_L-overexpressing HuH-7 cells is not due to clonal differences. A: HuH-7 cells and two clones of cFLIP_L-overexpressing HuH-7 cells (HuH-cFLIP clone 1, used in the rest of the study, and HuH-cFLIP clone 5) were treated with TRAIL (4 ng/ml) for 8 h. Apoptosis was quantitated by morphological criteria after DAPI staining. B: HuH-7 and HuH-cFLIP cells (clone 5) were incubated with TRAIL (4 ng/ml) in the presence or absence of PD98059 (50 μM) for 4 h. Localization of cathepsin B was analyzed by immunofluorescence and confocal microscopy (×100).
B-RFP, which localizes within the lysosomes (40). Redistribution of cathepsin B-RFP from lysosomes to the cytosol was minimal in cells treated with TRAIL alone (Fig. 7D). In contrast, cathepsin B-RFP release from lysosomes was readily apparent in cells treated with TRAIL plus PD98059 (Fig. 7D). Thus, a similar pathway of TRAIL cytotoxicity occurred in Hep3B cells as observed in HuH-7 cells.

**DISCUSSION**

The principal findings of this study relate to the mechanisms of TRAIL-induced apoptosis of HCC cells and, in particular, to the activation of the lysosomal pathway of apoptosis. Our data demonstrate that 1) TRAIL induces HCC cell apoptosis by triggering lysosomal permeabilization with release of cathepsin B into the cytosol; 2) cathepsin B inhibition attenuates TRAIL cytotoxicity; and 3) enhanced cFLIP<sub>L</sub> expression inhibits TRAIL-induced apoptosis by blocking lysosomal permeabilization via activation of p42/44 MAPK. These results demonstrate that lysosomal permeabilization with release of cathepsin B contributes to TRAIL-induced apoptosis of tumor cells and suggest that cFLIP<sub>L</sub> mediates TRAIL resistance by multiple mechanisms.

Several recent studies indicate that lysosomal destabilization with release of lysosomal proteases into the cytosol can play an important role in death receptor-mediated cell death (5, 7, 14). However, those studies were generally performed using the death-inducing ligands TNF-α or FasL, whereas data regarding the role of lysosomes in TRAIL-mediated apoptosis are scarce. Herein, we demonstrated that TRAIL-mediated apoptosis in HCC cells is associated with early lysosomal dysfunction and release of lysosomal enzymes in the cytosol. For example, lysosomal release of cathepsin B and D into the cytosol was observed prior to the morphological changes of apoptosis. Both pharmacological and shRNA-targeted inhibition of cathepsin B also reduced TRAIL-induced apoptosis as measured morphologically and by activation of effector caspases. The observation that lysosomal cathepsin B release is required for caspase-3 and -7 activation is consistent with prior studies demonstrating that lysosomal breakdown is proximal to mitochondrial dysfunction and effector caspase activation (2, 3,
Thus, like TNF-α, TRAIL can also trigger the lysosomal pathway of apoptosis.

Enhanced expression of cFLIPL in HuH-7 cells conferred resistance to TRAIL-induced apoptosis. These results were not unexpected, as cFLIP is a potent inhibitor of death receptor-mediated apoptosis, and cFLIP expression has been shown to positively correlate with resistance to death receptor apoptosis in HCC cells (8, 28). Consistent with its antiapoptotic effects, cFLIP overexpression also reduced lysosomal permeabilization. Because cFLIP is increasingly recognized as an important mediator of kinase cascades (9, 18), we explored differences in MAP kinase and NF-κB activation between HuH-7 cells with and without enforced cFLIP expression. Consistent with previous observations (12), p38 MAPK was actually inhibited by cFLIP-enforced expression, whereas only delayed and slight NF-κB activation was observed in HuH-7 cells independent of cFLIP transfection. Although transient JNK activation occurred in HuH-cFLIP cells, sustained JNK activation is necessary for cell death (4). In contrast, p42/44 MAPK was differentially activated in cFLIP-overexpressing cells, and pharmacological inhibition of p42/44 MAPK potentiated lysosomal permeabilization and apoptosis. These data are consistent with several prior observations that p42/44 MAPK protects against TRAIL-induced apoptosis (29, 31, 34, 41). In particular, these prior studies demonstrated that, although caspase-8-mediated cleavage of Bid occurred following TRAIL treatment, cell death was still prevented by p42/44 MAPK activation upstream of mitochondria. Based on our previous observations that TNF-α-induced lysosomal permeabilization requires truncated Bid (tBid) (13, 39), it is conceivable that p42/44 MAPK exerts its cytoprotective effect by directly or indirectly inhibiting tBid activity and/or function.

In summary, we have shown that TRAIL induces apoptosis of HCC cells, a process associated with lysosomal permeabilization and release of cathepsin B into the cytosol. cFLIPL inhibits TRAIL-induced apoptosis by reducing lysosomal permeabilization through the activation of p42/44 MAPK. The precise mechanism by which p42/44 MAPK contributes to lysosomal stability is unclear and requires further investigation. However, we propose the following model where TRAIL-stimulated caspase-8 and p42/44 MAPK activation occur as independent signaling events (Fig. 8). In this model, caspase-8 is activated at the TRAIL-induced signaling complex in the presence or absence of cFLIPL, whereas p42/44 MAPK activation is amplified by cFLIPL. This model would explain the observations that caspase-8 inhibition does not block p42/44 MAPK activation but still prevents cell death despite p42/44 MAPK blockade. The ability of p42/44 MAPK to inhibit cell death despite caspase-8 activation could be explained by either a direct or an indirect effect on tBid function. Finally, the data suggesting that p42/44 MAPK inhibition augments TRAIL cytotoxicity in HCC cells by promoting lysosomal permeabilization have therapeutic implications; inhibition of p42/44 MAPK may be a therapeutic strategy to circumvent TRAIL resistance in cFLIP-overexpressing cancers.

ACKNOWLEDGMENTS

We thank Erin Bungum for excellent secretarial assistance.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-63947 (G. J. Gores), the Palumbo Foundation, and the Mayo Foundation.

REFERENCES

36. Tschopp J, Irmler M, Thome M. Inhibition of Fas death signals by FLIPs. Curr Opin Immunol 10: 552–558, 1998.