Cholestasis Increases Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-R2/DR5 Expression and Sensitizes the Liver to TRAIL-Mediated Cytotoxicity

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ABSTRACT
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a potential chemotherapeutic agent for cancer, is not thought to be hepatotoxic. We have recently demonstrated, however, that bile acids increase TRAIL-R2/DR5 expression in a human liver cell line and render these cells susceptible to TRAIL-mediated apoptosis. These data suggest TRAIL may be hepatotoxic in cholestasis. The aim of this study was to directly assess TRAIL hepatotoxicity in bile duct-ligated mice, a model of extrahepatic cholestasis. Bile duct-ligated mice (3 days) were used for these studies. TRAIL-R2/DR5 expression was assessed by real-time and immunoblot analysis. The TRAIL death-inducing signaling complex (DISC) was evaluated by immunoprecipitation and immunoblot techniques. Bile duct ligation increased both liver TRAIL-R2/DR5 mRNA and protein expression (>10-fold). Following TRAIL administration (60 μg/mouse, i.v.) to bile duct ligation (BDL) mice, terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive hepatocytes, liver tissue caspase 3-like activity, and serum alanine aminotransferase values increased significantly compared with vehicle-treated BDL mice. The effect of TRAIL on the liver was direct, as the TRAIL DISC (Fas-associated death domain and procaspase 8 protein) was detected in liver tissue. TRAIL-mediated hepatocyte apoptosis in bile duct-ligated mice was associated with significant hepatotoxicity, as assessed by histopathology, although there was no animal mortality. In conclusion, these data define conditions under which TRAIL is hepatotoxic.

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also called as Apo2 ligand) is a proapoptotic ligand and is a member of tumor necrosis factor (TNF) super family (Wiley et al., 1995; Pitti et al., 1996). It is a type II membrane protein that is widely expressed (Pitti et al., 1996). TRAIL induces apoptosis by binding to TRAIL-receptors 1 and 2, also referred to as death receptor 4/Apo 2A and death receptor 5/killer/TRICK2, respectively (Griffith and Lynch, 1998). TRAIL binding to these cognate receptors induces their aggregation, resulting in the recruitment of the adaptor protein FADD (Fas-associated death domain) and procaspase 8, a cysteine protease, to the receptor complex. This death-inducing signaling complex (DISC) results in procaspase 8 self-activation initiated via the limited catalytic activity possessed by the zymogen proform (Thornberry and Lazebnik, 1998). Active caspase 8, an initiator caspase, activates a proapoptotic-signaling cascade culminating in cell death by apoptosis (Thornberry and Lazebnik, 1998).

Unlike TNF-α and Fas ligand, TRAIL seems to have a unique selectivity for triggering apoptosis in tumor cells while leaving normal tissues intact. Indeed, TRAIL has cytotoxic effects against a wide range of tumor cell types; however, most normal cells are resistant to TRAIL-mediated apoptosis (Griffith et al., 1998; Ashkenazi et al., 1999; Walczak et al., 1999). Recent studies have shown that administration of soluble recombinant TRAIL in experimental animals induces significant tumor regression without systemic toxicity (Ashkenazi et al., 1999; Walczak et al., 1999). Thus, TRAIL seems to be a potential chemotherapeutic agent for the treatment of human cancer.

Controversy regarding the use of human TRAIL as a chemotherapeutic agent has centered on its potential hepatotoxicity. Although not toxic to rodent or primate hepatocytes, different forms of soluble TRAIL seem to induce human hepatocyte apoptosis (Gores and Kaufmann, 2001). This potential problem may be circumvented by the use of specific
TRAIL-receptor 2/death receptor-5 agonists as this receptor, in contrast to TRAIL-receptor 1/death receptor-4, which is not expressed by hepatocytes (Ichikawa et al., 2001). TRAIL-R2/DR5, however, can be induced by both p53-dependent and -independent mechanisms (Takimoto and El-Deiry, 2000; Higuchi et al., 2001; Meng and El-Deiry, 2001), potentially rendering the involved tissues sensitive to TRAIL cytotoxicity. For example, bile acids will induce expression of TRAIL-R2/DR5 in liver cells, rendering them susceptible to TRAIL-induced apoptosis (Higuchi et al., 2001). These data suggest cholestasis, a common liver disease syndrome characterized by elevated tissue and serum bile acid concentrations, could render the liver susceptible to TRAIL-induced hepatotoxicity. It is difficult, however, to extrapolate these limited in vivo data to in vivo cholestasis. Thus, direct information on the effect of cholestasis on TRAIL-R2/DR5 expression and potential TRAIL hepatotoxicity is needed. Such information may prove important in selecting cancer patients for TRAIL chemotherapy and in understanding the mechanisms of cholestatic liver injury.

The overall objective of this study was to test the hypothesis that cholestasis renders the liver susceptible to TRAIL-mediated apoptosis. To address this objective, we formulated the following specific questions: 1) does cholestasis increase TRAIL-R2/DR5 mRNA and/or protein expression in the mouse liver; 2) is the increased TRAIL-R2/DR5 capable of signaling in this disease model; and 3) are hepatocytes in the cholestatic liver sensitive to TRAIL-induced apoptosis and liver injury? To address these questions, we used the common bile duct ligation (BDL) mice as a model of extrahepatic cholestasis.

Materials and Methods

Animal Studies. The use and the care of the animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Mayo Clinic. Common BDL was performed in 6- to 8-week-old male C57/BL mice (Jackson Laboratories, Bar Harbor, ME), as previously described by us in detail (Miyoshi et al., 1999). In brief, mice were anesthetized (ketamine 60 mg/kg and xylazine 10 mg/kg body weight by intraperitoneal injection), and the peritoneal cavity was opened. The common bile duct was double ligated and cut. Controls underwent a sham operation that consisted of common bile duct exposure but not ligation. After 3 days, the mice were sacrificed and the liver tissue was fixed in 4% formaldehyde and embedded in Tissue Path (Fisher Scientific, Pittsburgh, PA). Tissue sections (4 μm) were prepared, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays were performed following the manufacturer's instructions (in situ cell death detection kit; Roche Molecular Biochemicals, Mannheim, Germany). Hepatocyte apoptosis in liver sections was quantitated by counting the number of TUNEL-positive cells in 30 random microscopic fields (200×), as described (Miyoshi et al., 1999). Hematoxylin- and eosin-stained liver specimens were evaluated by light microscopy. Serum ALT determinations were performed using a commercially available assay kit (Sigma Diagnostics Kit no. 505; Sigma-Aldrich).

Quantitative Real-Time Polymerase Chain Reaction. Total RNA was isolated from liver tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was prepared using an oligo dT primer and mink leukemia virus reverse transcriptase, as previously described in detail (Higuchi et al., 2001). The cDNA product was amplified by PCR with Taq DNA polymerase using standard protocols (Higuchi et al., 2001). PCR primers were designed as follows: TRAIL-R2/ DR5, 5′-TGA CCG AGA AGA ACT GA-3′ and 5′-GCC TTG GAT CAT TG GATT A-3′; TRAIL, 5′-TCA GCA CTT CAG GAT GG-3′ and 5′-CAC CAT CTG TTT GGT TCT CA-3′. For an internal control, primers for 18S ribosomal RNA were purchased from Ambion, Inc. (Austin, TX). After electrophoresis in 1% low-melting-temperature agarose gel, the expected base pair PCR products were identified and the bands cut from the gel. Next, the PCR products were eluted into Tris-HCl using a DNA elution kit (QIAGEN, Valencia, CA). Extracted PCR products were prepared as standards at the concentrations of 100, 10, 10−1, 10−2, 10−3, and 10−4 copies/μL. The identity of the PCR products was verified using dye terminator real-time polymerase chain reaction. Real-time PCR was performed with the Roche LightCycler (Roche Molecular Biochemicals) using SYBR green as the fluorophore (Molecular Probes, Eugene, OR) (Higuchi et al., 2001). The linear relationship between copy number and cycle number was then determined. For the quantitation of each mRNA, real-time PCR was performed using the cDNA samples obtained from the mouse liver. The standard curve was used to calculate the copy number in the experimental sample. We note that for distinguishing specific from nonspecific products and primer dimers, melting curve analysis of all final PCR products were analyzed. Because different DNA products melt at different temperatures, it was possible to distinguish between genuine products and nonspecific products and primer dimers. All PCR conditions and primers were optimized to produce a single product of the correct base pair size.

Measurement of Caspase-3-Like Activity. Cytosolic extracts were prepared from liver samples by homogenization and centrifugation procedures, as previously described by us (Jones et al., 1998) with minor modification (Miyoshi et al., 1999). In brief, diced liver tissue was homogenized in hypotonic buffer (25 mM HEPES, 5 mM MgCl2, 1 mM EGTA, 0.5 mM PMFS, 2 μg/ml pepstatin, 2 μg/ml leupeptin, pH 7.5) and centrifuged for 10 min at 1,000g. Caspase-3-like activity was measured by adding 50 μl of cytosol to 450 μl of assay buffer containing 25 mM HEPES (pH 7.5), 10 mM dithiothreitol, 0.1% CHAPS, 0.5 mM PMFS, 100 U/ml aprotinin, and 20 μM DEVD-amino methyl coumarin (Enzyme Systems Products, Livermore, CA). Fluorescence was monitored using a fluorometer (model LS50; PerkinElmer, Norwalk, CT), as described previously (Jones et al., 1998; Faubion et al., 1999).

DISC Analysis by Immunoprecipitation. Liver tissue from either TRAIL or vehicle-injected mice was homogenized in DISC buffer (1% Triton X-100, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM PMFS), and insoluble debris were removed by centrifugation for 15 min at 14,000g for 4°C. The protein concentration of the supernatant was determined by the Bradford assay, and the protein concentration adjusted to a concentration of 5 mg of protein in 1 ml of DISC buffer. As a control, samples from vehicle-injected mice were supplemented with 0.5 μM of FLAG-TRAIL plus 1 μg of anti-FLAG antibody M2. After samples were precleared by Sepharose-CL 4B (Sigma-Aldrich), aliquots of protein...
G-Sepharose (40 μl) were added for immunoprecipitation, and samples were incubated over night in DISC buffer at 4°C. Immune complexes were then pelleted by centrifugation for 5 min at 14,000g, washed five times with DISC buffer, and released from the beads by boiling for 5 min in SDS sample buffer. Samples were then resolved by immunoblot analysis (see below).

**Immunoblot Analysis.** Samples were resolved by 12.5% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies. The membrane was incubated with peroxidase-conjugated secondary antibodies (1:10,000 dilution; Bio-source International, Camarillo, CA), and the bound antibody was visualized using a chemiluminescent substrate (ECL, Amersham, Arlington Heights, IL) and Kodak X-OMAT film (Eastman Kodak, Rochester, NY). Primary antibodies were goat anti-mouse DR5 (1:1000 dilution; R&D systems Co., Minneapolis, MN), rabbit anti-caspase 8 (1:1000 dilution; Pharmingen, San Diego, CA), goat anti-FADD (1:400 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and goat anti-actin (1:500 dilution, Santa Cruz Biotechnology, Inc.).

**Statistical Analysis.** All data represent at least three independent experiments and are expressed as the mean ± S.D. unless otherwise indicated. Differences between groups were compared using analysis of variance for repeated measures and a post hoc Bonferroni test to correct for multiple comparisons.

**Results**

**TRAIL R2/DR5 Liver Expression Is Increased in the BDL Mouse.** TRAIL-R2/DR5 mRNA expression was quantitated using real-time PCR and protein expression by immunoblot analysis. Similar to reports in human hepatocytes (Chaudhary et al., 1997; Ichikawa et al., 2001), despite the presence of TRAIL-R2/DR5 mRNA in murine liver, minimal protein expression was detected by immunoblot analysis (Fig. 1). In 3-day BDL mice, the copy number for liver TRAIL-R2/DR5 mRNA increased more than 10-fold compared with sham-operated animals, and in contrast to normal liver, TRAIL-R2/DR5 protein expression was readily identified by immunoblot analysis (Fig. 1). TRAIL mRNA was not detected by PCR in either liver tissue sham or BDL animals (data not shown). Unfortunately, because the TRAIL-R1/DR4 murine receptor has not been cloned and human antibodies do not cross-react with the mouse receptor, the effect of BDL on liver expression of this receptor could not be ascertained. These data demonstrate that like, in vitro bile acid treated cells (Higuchi et al., 2001), in vivo cholestasis also increases TRAIL-R2/DR5 expression.

**TRAIL Induces Hepatocyte Apoptosis in BDL Mice.** Because enhanced TRAIL-R2/DR5 expression may render cells more susceptible to TRAIL-mediated apoptosis (Higuchi et al., 2001; Kim et al., 2001), the sensitivity of the BDL mouse to potential TRAIL-mediated hepatotoxicity was next evaluated. Hepatocyte apoptosis was quantitated using the TUNEL assay (Fig. 2). In sham-operated mice, hepatocyte apoptosis was not observed in either TRAIL-injected (60 μg/mouse, i.v.) or vehicle-injected mice, which is consistent with previous reports that normal liver is not susceptible to TRAIL-induced apoptosis (Walczak et al., 1999). As previously reported by us (Miyoshi et al., 1999), hepatocyte apoptosis was readily apparent in 3-day BDL mice (Fig. 2). The number of TUNEL positive cells was 29 ± 5/field at 6 h and 33 ± 6 cells/field at 12 h following intravenous vehicle administration. More importantly, the number of TUNEL positive cells increased 3- and 4-fold at 6 and 12 h after admin-
the livers from TRAIL-treated sham-operated animals, TRAIL-R2/DR5 was coprecipitated with FLAG-TRAIL; however, the amount of immunoprecipitated DR5 was much greater in livers from BDL mice. In normal mouse liver, addition of FLAG-TRAIL plus anti-FLAG M2 antibody to the lysate resulted in the detection of equivalent amounts of TRAIL-R2/DR5 immunoprecipitated compared with liver lysates obtained from mice treated with TRAIL in vivo. These observations suggest similar binding affinities for recombinant human FLAG-TRAIL and mouse TRAIL-R2/DR5 both in vivo and in vitro and are consistent with the increased expression of DR5 protein level in the BDL liver described above. More importantly, both procaspase 8 and FADD are coprecipitated with TRAIL and TRAIL-R2/DR5, and the amounts of these immunoprecipitated proteins is greater in livers from BDL compared with sham-operated mice. In vehicle-treated mice, neither procaspase 8 nor FADD was coprecipitated with TRAIL-R2/DR5 from liver tissue. Thus, systemic TRAIL administration directly initiates TRAIL-R2/DR5-mediated DISC formation in liver of the BDL mouse. These observations strongly suggest the hepatocytes apoptosis observed in TRAIL-treated BDL animals is due to a direct effect of TRAIL on hepatocytes.

Fig. 2. Systemic trail administration induces hepatocyte apoptosis in BDL but not sham-operated mice. Mice were exposed to either BDL or a sham operation. Three days after the surgery, either FLAG-TRAIL (60 μg/mouse) or vehicle (anti-FLAG M2 antibodies 20 μg/mouse) was injected intravenously via the tail vein. Six and 12 h later, the mouse was anesthetized, and the liver was procured. The TUNEL assay was performed on liver sections, as described under Materials and Methods. A, representative fluorescent photomicrographs of TUNEL staining in mouse liver are displayed. In sham-operated mice, significant TUNEL-positive cells was not observed in either TRAIL or vehicle-injected group. In BDL mice livers, TUNEL-positive cells were significantly increased in TRAIL versus vehicle-treated mice. B, the number of TUNEL-positive cells was quantitated and expressed as cells/microscopic field (200×). Ten different fields were counted for each sample. Data from five independent animals were obtained and expressed as the mean ± S.D. *P < 0.01 for BDL-TRAIL versus BDL-vehicle, Sham-vehicle, or Sham-TRAIL. P < 0.01 for BDL-vehicle versus Sham-vehicle, or sham-TRAIL. P = not significant for sham-vehicle versus sham-TRAIL.

Fig. 3. TRAIL enhances caspase activation in BDL mouse liver. Mice were subjected to either BDL or a sham operation. Three days after the surgical procedure, either FLAG-TRAIL (60 μg/mouse) or vehicle (anti-FLAG M2 antibodies 20 μg/mouse) was injected via the tail vein. Six hours later, the mouse was anesthetized, the liver procured, and cytosolic extracts obtained, as described under Materials and Methods. Caspase 3-like activity was assayed in liver extract samples using the fluorogenic substrate DEVD-7-amino-4-methylcoumarin. Data points represent experiments from three independent animals and are expressed as the mean ± S.D. *P < 0.01 for BDL-TRAIL versus BDL-vehicle, sham-vehicle, or sham-TRAIL. P < 0.01 for BDL-vehicle versus sham-vehicle, or sham-TRAIL. P = not significant for sham-vehicle versus sham-TRAIL.

Fig. 4. BDL enhances TRAIL-induced DR5 activation with DISC formation. Mice were subjected to either BDL or a sham operation. Three days after the surgical procedure, either FLAG-TRAIL (60 μg/mouse) or vehicle (anti-FLAG M2 antibodies 20 μg/mouse) was injected via the tail vein. Six hours later, the mouse was anesthetized, the liver procured, and the liver homogenized as described under the Materials and Methods. The FLAG-TRAIL-anti FLAG M2 complex was immunoprecipitated, and precipitated samples were immunoblotted for TRAIL-R2/DR5, caspase 8, and FADD. In vehicle-treated groups, samples were supplemented with 0.5 μg of FLAG-TRAIL plus 1 μg of anti-FLAG antibody M2 following the immunoprecipitation.

IP: FLAG-TRAIL
TRAIL Is Hepatotoxic in the BDL Mouse. To ascertain if TRAIL-mediated hepatocyte apoptosis translated into significant hepatotoxicity, histopathologic examination of liver specimens was performed along with serum ALT determinations (Fig. 5). As reported by others (Walczak et al., 1999), when TRAIL was administered to sham-operated mice, no significant liver injury was observed as assessed by histopathology or serum ALT values. Histological examination of liver specimens from 3-day bile duct-ligated mice were compatible with extrahepatic cholestasis demonstrating bile infarctions, bile ductular proliferation along with minimal hepatocellular damage. In livers from BDL-mouse receiving TRAIL (60 μg/mouse), however, significant hepatocyte damage along with hepatocytes apoptosis was readily observed (Fig. 5A). Serum ALT values in TRAIL-treated BDL-mice were also significantly greater than those in vehicle-treated BDL mice (Fig. 5B). Indeed, serum ALT values were 698 ± 222 U/l at 6 h and 1184 ± 106 U/l at 12 h following TRAIL treatment compared with 396 ± 129 U/l at 6 h and 508 ± 67 U/l at 12 h after vehicle administration (p < 0.01). Thus, TRAIL seems to be nontoxic to normal liver; however, this death ligand is hepatotoxic in cholestatic animals.

Discussion

The principal findings of this study relate to TRAIL hepatotoxicity in cholestatic liver injury. The following observations were made in the bile duct-ligated mouse: 1) TRAIL-R2/DR5 mRNA and protein expression is markedly increased; 2) systemic TRAIL administration results in direct activation of the TRAIL-R2/DR5 within the liver causing hepatocyte apoptosis; and 3) the TRAIL-induced apoptosis is associated with significant hepatotoxicity. Each of these observations and their biological and clinical implications is discussed in greater detail.

TRAIL-R2/DR5 is only minimally expressed at the protein
level in human liver and, as shown in this study, in murine liver. TRAIL-R2/DR5, however, is inducible, and the current study demonstrated a significant induction of this death receptor in the liver of bile duct-ligated mice. Based on previous studies demonstrating that bile acids directly induce expression of TRAIL-R2/DR5 in a human hepatoma cell line, the elevated bile acid concentrations occurring in serum and liver tissue during extrahepatic cholestasis probably also contributed to the observed increase in hepatic TRAIL-R2/2 DR5 protein expression in this study. Inflammatory cytokines such as interferon-γ also induce TRAIL-R2/DR5 expression (Meng and El-Deiry, 2001). Like human cholestatic liver diseases, bile duct ligation in the mouse is associated with inflammation; therefore, a role for interferon-γ in inducing TRAIL-R2/DR5 expression cannot be excluded. Given the parallels between expression of this death receptor in human and murine liver, it is likely that cholestasis will also increase expression of TRAIL-R2/DR5 in human hepatocytes.

Systemic administration of TRAIL directly induced hepatocyte apoptosis in bile duct-ligated mice, but not control animals. The lack of TRAIL hepatotoxicity in control animals is consistent with previous observations (Ichikawa et al., 2001). The TRAIL-mediated hepatotoxicity in bile duct-ligated animals was associated with enhanced TRAIL-R2/DR5 expression, DISC formation, increased caspase activation, and hepatocyte apoptosis. Bax, a proapoptotic member of the Bcl-2 family, is necessary for TRAIL-mediated apoptosis in some cell types. We have previously reported, however, that Bax expression does not change in bile duct-ligated animals (Miyoshi et al., 1999). Changes in Bax expression is, therefore, an unlikely cause for enhanced TRAIL-induced liver cell apoptosis. Hepatic concentrations of bile acids are increased in cholestasis and negatively influence mitochondrial function (Krahenbuhl et al., 1992). It is possible that this altered mitochondrial state also renders the hepatocytes more susceptible to apoptosis by TRAIL in addition to the increased expression of TRAIL-R2/DR5.

The occurrence of hepatocyte apoptosis in TRAIL-treated BDL animals was associated with hepatotoxicity. This relationship between hepatocyte apoptosis and hepatotoxicity has been previously established (Miyoshi et al., 1999) but is further supported by these studies. Moreover, recent publications suggest significant hepatocyte apoptosis is proinflammatory in the liver, providing further evidence implicating apoptosis as an initial event in liver injury (Faouzi et al., 2001). The magnitude of the liver injury in TRAIL-treated BDL animals was less severe than that following treatment of mice with Fas agonists or TNF-α plus an inhibitor of NF-κB (Ogasawara et al., 1993; Guicciardi et al., 2001). Lethal hepatotoxicity with hemorrhagic necrosis of the liver is observed with the latter death receptor agonists (Ogasawara et al., 1993; Guicciardi et al., 2001). In contrast, no mortality was observed in TRAIL-treated BDL animals. In this regard, even when TRAIL is hepatotoxic, the toxicity is less than that observed with other death ligands.

Although the recruitment of procaspase 8 and FADD to the DISC was of a greater magnitude in the TRAIL-treated BDL mice, formation of the DISC was observed in TRAIL-treated control animals. Caspase 3-like activity, hepatocyte apoptosis, and hepatotoxicity, however, were not observed in these animals despite DISC formation. These results suggest the existence of anti-apoptotic mechanisms protecting hepatocytes from minor degrees of TRAIL-R2/DR5 activation. The existence of survival pathways blocking death receptor-mediated apoptosis is well documented and includes phosphatidylinositol-3 kinase/AKT kinase pathways, NF-κB-regulated transcription of survival proteins such as cFLIP, Bel-XL, c-IAP 1,2, GADD 45 β, A1, and XIAP, and mitogen-activated protein kinase pathways (Griffith et al., 1999; Burns and El-Deiry, 2001). Thus, in addition to increasing agonist-mediated TRAIL-R2/DR5 activation, cholestasis may also inhibit survival signals/pathways.

Previous studies suggesting that TRAIL is not hepatotoxic evaluated TRAIL hepatotoxicity only in experimental animals having normal liver function. The present data suggest cholestasis sensitizes the liver to TRAIL hepatotoxicity. Because cholestasis is a common feature of human liver injury or dysfunction (Popper, 1968; Patel et al., 1998) and may be subtle, the administration of TRAIL in the setting of abnormal liver function should be performed cautiously.

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References


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