The Bile Acid Glycochenodeoxycholate Induces TRAIL-Receptor 2/DR5 Expression and Apoptosis*

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Toxic bile salts induce hepatocyte apoptosis by both Fas-dependent and -independent mechanisms. In this study, we examined the cellular mechanisms responsible for Fas-independent, bile acid-mediated apoptosis. HuH-7 cells, which are known to be Fas deficient, were stably transfected with the sodium-dependent bile acid transporting polypeptide. The toxic bile acid glycochenodeoxycholate (GCDC)-induced apoptosis in these cells in a time- and concentration-dependent manner. Apoptosis and mitochondrial cytochrome c release were inhibited by transfection with dominant negative FADD, CrmA transfection, or treatment with the selective caspase 8 inhibitor IETD-CHO. These observations suggested the Fas-independent apoptosis was also death receptor mediated. Reverse transcriptase-polymerase chain reaction demonstrated tumor necrosis factor-R1, tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-R1/DR4, -R2/DR5, and TRAIL, but not tumor necrosis factor-α expression by these cells. GCDC treatment increased expression of TRAIL-R2/DR5 mRNA and protein 10-fold while expression of TRAIL-R1 was unchanged. Furthermore, aggregation of TRAIL-R2/DR5, but not TRAIL-R1/DR4 was observed following GCDC treatment of the cells. Induction of TRAIL-R2/DR5 expression and apoptosis by bile acids provides new insights into the mechanisms of hepatocyte apoptosis and the regulation of TRAIL-R2/DR5 expression.

In cholestasis (a pathophysiologic condition of the liver defined as an impairment in bile formation), the enterohepatic circulation is interrupted and bile acids accumulate within the liver because they cannot be secreted into bile (1). Elevated bile acid concentrations within the liver promote liver injury and the development of liver cirrhosis and liver failure. For example, children lacking the canalicular transport protein for bile acid secretion develop a progressive liver disease due to the inability to excrete bile acids from the hepatocyte (2). Numerous studies indicate that toxic bile acids induce hepatocyte injury in vitro by inducing apoptosis (3–5). Moreover, hepatocyte apoptosis has also been shown in animal models of cholestasis demonstrating congruence between the in vitro and in vivo observations (6). Thus, the mechanisms of bile acid-induced hepatocyte apoptosis are of clinical and scientific importance.

Apoptosis may occur by two fundamental pathways: (i) the death receptor or extrinsic pathway; and (ii) the mitochondrial or intrinsic pathway. Bile acids induce hepatocyte apoptosis in a short term in vitro paradigm by a Fas death receptor-dependent mechanism (3). Hepatocyte apoptosis was also decreased in the lpr bile duct-ligated mouse, which has minimal Fas expression (6). Although hepatocyte apoptosis was diminished in this model of extrahepatic cholestasis, the rate of apoptosis increased over time suggesting a Fas-independent mechanism of apoptosis was also occurring in these animals (6). The mechanism responsible for this delayed, Fas-independent pathway of bile salt-induced apoptosis was not elucidated in this study. This delayed apoptosis may also be death receptor mediated as hepatocytes express tumor necrosis factor receptor R1, tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptor 1 (TRAIL-R1, also referred to as death receptor-4 (DR4)), and TRAIL receptor 2 (TRAIL-R2, also called death receptor 5 (DR5)/killer/TRICK2). All these death receptors signal apoptosis by recruiting the cytoplasmic adapter protein FADD (Fas-associated death domain) to an oligomerized receptor complex (7–11). FADD in turn facilitates the binding and activation of procaspase 8, an initiator caspase (10), which then catalyzes a series of proteolytic events that contribute to the process recognized biochemically and morphologically as apoptosis.

Bile acids have also been postulated to cause direct mitochondrial cytoxicity, and the Fas-independent apoptosis could also occur via the mitochondrial pathway (12, 13). Indeed, bile acids will induce mitochondrial generation of reactive oxygen species, mitochondrial membrane potential depolarization, and the mitochondrial permeability transition (4, 14). The biochemical hallmark of the mitochondrial pathway of apoptosis is cytochrome c release from the intermembrane space into the cytosol (13, 15). Cytosolic cytochrome c allosterically facilitates binding of apoptosis activating factor-1 with procaspase 9. This multimeric protein complex facilitates activation of caspase 9 which then initiates a caspase cascade causing cell death by apoptosis. Mitochondrial dysfunction can also occur in

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¶ The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis inducing ligand; AFC, 7-amino-4-trifluoromethylcoumarin; RT-PCR, reverse transcriptase-polymerase chain reaction; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; TNF, tumor necrosis factor; CDC, chenodeoxycholate; GFP, green fluorescent protein; TCDC, taurochenodeoxycholate.

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death receptor-mediated apoptosis, especially so called “Type II cells” such as hepatocytes (12). Mitochondrial cytochrome c release is FADD/caspase 8-dependent during death receptor-mediated apoptosis of type II cells, whereas in the classic mitochondrial pathway of apoptosis, mitochondrial dysfunction is independent of FADD/caspase-8 activation (13). Thus, inhibition of FADD/caspase 8 signaling can help differentiate death receptor-associated versus direct mitochondrial dysfunction in type II cells such as hepatocytes.

The overall objective of this study was to examine the mechanisms contributing to Fas-independent apoptosis during bile acid cytotoxicity. To address this objective, we formulated the following questions. (i) Do toxic bile acids induce apoptosis in Fas-deficient cells? (ii) Does cytochrome c release occur in this model of apoptosis? (iii) Is mitochondrial cytochrome c release-dependent or -independent of the FADD/caspase 8 activation process and (iv) does bile acid modulate expression of death receptors or their cognate ligands? To address these questions, we generated a human cell line expressing the human bile acid transporter to induce bile acid-mediated apoptosis. We choose the hydrophobic bile acid glycinechenodeoxycholate (GCDC) as the toxic bile salt for these studies because it is a primary bile salt that accumulates intrahepatically during cholestasis and is a poten inducer of hepatocyte apoptosis (1, 16).

EXPERIMENTAL PROCEDURES

Generation of HuH-BAT Cell Lines and Culture

HuH-7 cells, a human hepatocellular carcinoma cell line, were stably transfected with the sodium-dependent taurocholate co-transporting polypeptide (NTcp). An expression vector for the human NTcp (pcDNA3-NTcp) was a generous gift from Peter Maier, Zurich, Switzerland. HuH-7 cells were cultured until 30% subconfluent in Eagle’s minimum essential medium containing 10% fetal bovine serum. Transfection with pcDNA3-NTcp was performed using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD). Stably transfected clones were selected in medium containing 1200 mg/ml G418. Individual clones were subcloned and screened for bile acid transport measurement as previously described (17). Established clones (HuH-BAT for HuH-Bile Acid Transporting) were grown in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum, 10% bovine calf serum, 100,000 units/liter penicillin, 100 mg/liter streptomycin, 100 mg/liter gentamycin, and 200 mg/liter G418.

Quantitation of Apoptosis

Apoptosis was quantitated by assessing 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 (16).

Plasmids and Transfection

Plasmids for dominant-negative (DN-FADD-green fluorescent protein (GFP) (pcDNA3-GFP-ΔFADD) (3) and CrmA (pc-CrmA) (3) have been previously described. The expression vector pc-DNA3-GFP-ΔFADD encodes a polypeptide that contains GFP fused to the COOH-terminal death domain. This construct acts as a dominant-negative inhibitor because it lacks the NH2-terminal death effector domain of FADD (11). The pcG2-full, pgl2 SV-half, pgl2-SV-Smal, and pgl2 gene reporter constructs for TRAIL-R2/DR5 have been previously described (11). The TK-Renilla-CMV plasmid was purchased from Promega (Madison, WI) and used to normalize for transfection efficiency in luciferase assays. HuH-BAT cells were transiently transfected using LipofectAMINE as previously described by us (19). In brief, cells grown in 3.5-cm dishes were transfected by adding 1 ml of Opti-MEM containing 6 μg of LipofectAMINE (Life Technologies, Inc.), 6 μl of LipofectAMINE Plus reagent (Life Technologies, Inc.), and each plasmid: pcDNA3-GFP-ΔFADD (0.5 μg) and pc-CrmA (0.45 μg). The cells were then incubated in the above mixture for 24 h at 37°C in 5% CO2, 95% air incubator. After this incubation, 1 ml of Eagle’s minimum essential medium containing 20% fetal bovine serum was added to the transfection medium in each culture dish. Twenty-four hours later, the medium was aspirated and replaced with 2 ml of Eagle’s minimum essential medium containing 10% fetal bovine serum. The transfection of CrmA was performed by co-transfection with the GFP expression plasmid (PE-GFP-N1, 0.15 μg/dish) to identify the transfected cells under fluorescent microscopy. The transfection efficiency was ~60–70% for all plasmids as estimated by the percentage of cells expressing GFP was visualized by fluorescence microscopy.

Subcellular Fractionation

Cytoplasmic extracts for the immunoblot assay of cytochrome c were obtained as described by Leist et al. (20). Briefly, at the desired time points, the culture medium was exchanged with premeabilization buffer (210 mM t-mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM sucinate, 0.2 mM EGTA, 0.15% bovine serum albumin, 80 μg/ml digitonin, pH 7.2). The permeabilization buffer was removed and centrifuged for 10 min at 13,000 × g. Supernatants representing the cytosolic extract were employed for the immunoblot analysis.

Measurement of Caspase-8-like Activity

Cytoplasmic extracts for the enzyme assay were prepared by the method as described (17) with minor modification. In brief, cells were homogenized in hypotonic buffer (25 mM HEPES, 5 mM MgCl2, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml peptatin, 2 μg/ml leupeptin, pH 7.5), and centrifuged for 10 min at 1,000 × g. Caspase-8-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 phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, and 20 μM IETD-AFC. Fluorescence was measured using a fluorometer (model LS50, PerkinElmer Life Sciences, Norwalk, CT) as described previously (3, 17).

Reverse Transcriptase (RT)-PCR

Death Receptors—Expressions of Fas, TNF receptor 1, DR4, DR5, and DR6 in HuH-BAT cell were evaluated using RT-PCR. Total RNA was extracted from the cells (3). cDNA was prepared using an oligo(dT) primer and Moloney leukemia virus reverse transcriptase as previously described in detail (3). Primers used in this experiment were: Fas, 5′-GCAAGAGCCTATTTTCTCC-3′ and 5′-ATTATTGCCAGCTTGTGACCG-3′; TNF-receptor 1, 5′-TGCTCTGCGATTCAC TG-3′ and 5′-ACGAATTCTTTCCTCCAGCCCA-3′; DR4, 5′-CAGAAGGCTCTGGAGGCGTCTGAAC-3′ and 5′-ATGTCATGGTGATCTTGTGG-3′; DR5, 5′-GGGGAAAGATCTTGCATGAGTGG-3′ and 5′-CACTTGGTCTCACCAGG-3′; TRAIL, 5′-AGACCTCGGGTATCATCAGG-3′ and 5′-TATTTGTGGCCGCAGGACC-3′. Total cellular RNA was isolated using Trizol reagent (Life Technologies, Inc.). After reverse transcription as previously described (3), the cDNA product was amplified by PCR with Taq DNA polymerase using standard protocols (3). The amplified products (8 μl) were separated on 1% agarose gels, stained with ethidium bromide, and photographed using ultraviolet illumination.

For the quantitation of TRAIL-R2/DR5 mRNA, real time PCR was performed with the Roche LightCycler (Roche Molecular Biochemicals, Branchburg, NJ, USA) using SYBR green as the fluorophore (Molecular Probes, Eugene, OR). After electrophoresis in 1% low melting temperature agarose gel, gels containing the expected base pairs PCR product bands were cut and the products were eluted into Tris-HCl using DNA elution kit (Qiagen, Valencia, CA). The TRAIL-R2/DR5 standards were prepared at the concentrations of 100, 105, 109, 107, 104, and 103 copies/μl. The linear relationship between copy number and cycle number was then determined. The standard curve was used to calculate the copy number in the experimental sample.

Reporter Gene Assay

HuH-BAT cells cultured in 6-well plates were co-transfected with 20 ng of TK-Renilla-CMV and 1 μg of pcG2-full, pcG2 SV-half, pcG2-SV-Smal, or pgl2. Twelve hours after the transfection, cells were incubated with GCDC or media (control) for 8 h and then cell lysates were prepared as previously described (22). Both firefly and Renilla luciferase activities were quantitated using the dual luciferase reporter assay.
TRAIL-mediated Apoptosis

**Fig. 1.** GCDC-induced apoptosis in HuH-BAT cells. A, the Fas-deficient human hepatoma cell line, HuH7, was stably transfected with the sodium-dependent bile salt transporter (Ntcp) generating the HuHBAT cell line. Ntcp expression was verified by immunoblot analysis (inset). Kinetic analysis demonstrated linear, sodium-dependent bile acid uptake in the transfected but not the parent cell line. Bile acid uptake was assessed using radiolabeled taurocholate. B, apoptosis was observed in the bile acid transporting HuH-BAT cell line but not in the parent HuH7 cells after 12 h of incubation with GCDC (50–200 μM). Apoptosis was quantitated using DAPI and fluorescent microscopy. C, HuH-BAT cells were incubated in the presence of GCDC (200 μM) or diluent (media) for 12 h and apoptosis quantitated over time. All data were expressed as mean ± S.D. from three individual experiments.

**Immuno blot Analysis**

Samples were resolved by 14% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies at concentrations of 1:1,000. Peroxidase-conjugated secondary antibodies (BIOSOURCE International, Camarillo, CA) were incubated at a dilution of 1:2,000. Bound antibody was visualized using chemiluminescent-substrate (ECL; Amersham Pharmacia Biotech, Arlington Heights, IL) and exposed to Kodak X-Omat film. Primary antibodies: rabbit anti-DR4, goat anti-DR5, rabbit anti-DR6, and rabbit anti-FLIP were obtained from Alexis. Goat anti-TNF-receptor 1, goat anti-TRAIL, and goat anti-actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit anti-DR5 was obtained from R&D systems Co. Mouse anti-cytochrome c was obtained from Phrmin-gen, San Diego, CA.

**Cross-linking and Immunoprecipitation**

Cells were treated with 2 mM 3,3′-dithiobis(succinimidylpropionate) (Pierce Chemical Co., Rockford, IL), a cleavable cross-linker, for 20 min at 4 °C. After the reaction was quenched with 50 mM Tris-HCl for 10 min at 4 °C, cells were washed with PBS. Cells were lysed for 30 min on ice with lysis buffer (1% Triton X-100, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 15,000 g for 15 min at 4 °C. Immunoprecipitation was performed by incubating 2 mg of supernatant protein in 1 ml of lysis buffer containing goat anti-DR5 antibody (Alexis Co., Lausen, Switzerland) and 40 ml of protein-Sepharose 4B (Zymed Laboratories Inc. Laboratories, San Francisco, CA) at 4 °C overnight with agitation. Polypeptides were eluted by boiling for 5 min in 2 × Laemmli sample buffer. For an analysis of DR5 oligomerization, immunoprecipitation was performed with excess and limited amounts of antibody as described previously (3).

**Immunocytochemistry**

Cells were cultured in 35-mm dishes on collagen-coated glass coverslips. For immunocytochemistry, the media was aspirated and the cells were rinsed with phosphate-buffered saline (pH 7.2). Next, the cells were fixed with freshly prepared 3% paraformaldehyde in PBS (pH 6.9) for 20 min at 37 °C. After the fixation, the cells were washed three times with PBS (pH 7.2), and then incubated in PBS containing 0.1% Triton X-100 for 2 min. The dishes were rinsed in PBS, and the cells next incubated with the primary antisera for 2 h at 37 °C. Primary antisera were rabbit anti-DR4 (Alexis, 3,000 dilution) and goat anti-DR5 (Alexis, 1,300 dilution). After three washes with PBS, the cells were incubated with tetramethylrhodamine methyl ester-conjugated secondary antibodies (Molecular Probes Inc., Eugene OR), 10 μg/ml, for 45 min at 37°C. Cells were washed again, and the coverslips mounted on glass slides and viewed by laser scanning confocal microscopy (Axiovert 100 M-LSM 510, Carl Zeiss Inc., Thornwood, NY) using excitation and emission wavelengths of 555 and 580 nm, respectively.

**Materials and Reagents**

DAPI was from Molecular Probes Inc. (Eugene, OR). IETD-CHO and IETD-AFC was obtained from Enzyme Systems Products (Livermore, CA). GCDC was obtained from Sigma.

**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 ANOVA for repeated measures and a post-hoc Bonferroni test to correct for multiple comparisons.
TRAIL-mediated Apoptosis

GCDC Induces Apoptosis in the Fas-deficient HuH-BAT Cell Line—Because bile acid-mediated apoptosis requires transport of bile acids into cells (3), we first developed a bile salt transporting cell line. The Fas-deficient p53 mutant, human hepatoma cell line, HuH-7, was stably transfected with the sodium-dependent bile acid transporter (HuH-BAT). Expression of the transport protein was verified by immunoblot analysis, and efficient sodium-dependent bile acid uptake was confirmed by performing uptake studies with radiolabeled taurocholate (Fig. 1A). GCDC-induced HuH-BAT cell apoptosis was concentration- and time-dependent (Fig. 1, Band C). Maximal apoptosis was observed at 12 h at a GCDC concentration of 200 μM. GCDC did not induce apoptosis in the parent non-bile salt transporting HuH-7 cells. Chenodeoxycholate (CDC) and taurochenodeoxycholate (TCDC) were also examined for their ability to induce apoptosis in this cell line. Following incubation with 200 μM CDC or TCDC for 12 h, HuH-BAT cell apoptosis was 43.1 ± 7.9 and 23.9 ± 3.7%, respectively (data not shown). As has been reported in other studies (23), ursodeoxycholate (100 μM) did not induce apoptosis but did inhibit GCDC-mediated apoptosis by 38% (data not shown). This latter observation helps establish the validity of this model for studying bile acid apoptosis. Taken together, these results suggest GCDC and other bile acids can induce a delayed apoptosis in the absence of Fas by a mechanism requiring bile acid transport into the cell.

GCDC-mediated Cytochrome c Release and Apoptosis Is FADD and Caspase-8 Dependent—Mitochondrial release of cytochrome c into the cytosol was observed in this model of Fas-independent, bile acid-mediated apoptosis (Fig. 2). The mitochondrial release of cytochrome c was time dependent and maximal at 4 h; the occurrence of cytochrome c release preceded the morphologic changes of apoptosis. Next, the effect of inhibiting the FADD/caspase-8-activation process on mitochondrial cytochrome c release was examined to determine if a known death receptor was involved. Transfection of HuH-BAT cells with a dominant negative FADD-GFP construct attenuated GCDC-mediated cytochrome c release (Fig. 2A). GCDC associated cytochrome c release was also prevented by transfection with CrmA (a small pox protein which potently inhibits caspase 8 and caspase 1) or using the selective tetrapeptide caspase 8 inhibitor IETD-CHO (Fig. 2B). All three approaches of inhibiting the FADD/caspase-8 activation pathway also markedly attenuated GCDC cytotoxicity (Fig. 3, A-C). Transfection with dominant negative FADD-GFP also inhibited GCDC-associated increases in caspase 8-like activity demonstrating caspase 8 activation was FADD-dependent (Fig. 3D). Collectively, these observations support a death receptor pathway in GCDC-mediated apoptosis despite the absence of Fas expression.

RESULTS

FIG. 2. FADD and caspase 8 mediate GCDC-induced apoptosis in HuH-BAT cells. A, HuH-BAT cells in 3.5-cm dishes were transfected with DN-FADD-GFP expressing plasmid DNA (0.5 μg of DNA/dish). Equivalent amounts of GFP expressing plasmid was used as a control for the DN-FADD-GFP transfection. Cells were used for experiments 48 h after transfection. Apoptosis was assessed after treatment with GCDC (200 μM, 12 h) or media using DAPI loading and fluorescent microscopy. Apoptotic cells are expressed as a percentage of total apoptosis. Taken together, these results suggest GCDC and other bile acids can induce a delayed apoptosis in the absence of Fas by a mechanism requiring bile acid transport into the cell.

FIG. 3. A-FADD plus GCDC by ANOVA. Data were expressed as mean ± S.D. from three (A, B, and C) or four (D) individual experiments.

FIG. 4. HuH-BAT cells express multiple death receptors and ligands. Reverse transcriptase-PCR analysis of death receptors and their ligands. HuH-BAT cells were cultured for 4 h in the presence or absence of 200 μM GCDC. Total RNA was isolated from HuH-7 cells (lane 2), HuH-BAT cells (lane 3), and GCDC-treated HuH-BAT cells (lane 4), and subsequent RT-PCR was performed using Fas, TNF receptor 1, TRAIL receptor 1, TRAIL receptor 2, DR6, Fas ligand, TRAIL (ligand), TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lane 1 is a positive control for each PCR. SW480 cells, a human colon cancer cell line, were used as a positive control for Fas, TNF receptor 1, TRAIL receptor 1, TRAIL receptor 2, and DR6. LNCaP cells, a human prostate cancer cell line, were used as a positive control for Fas ligand and TNF-α. Col6(7) cells, a human melanoma cells, were used as a positive control for TRAIL.
HuH-BAT Cells Express Multiple Death Receptors, but Only the Death Ligand TRAIL—Because GCDC-induced apoptosis of HuH-BAT cells appeared to be death receptor-mediated, we profiled the mRNA expression of known human death receptors and ligands in this cell line by RT-PCR (Fig. 4). As reported by others these cells do not express Fas (24). However, mRNA expression for TNF-R1, TRAIL-R1/DR4, -R2/DR5, and death receptor-6 (DR6) was observed. These cells also express TRAIL but not TNF-α or Fas ligand. To confirm this mRNA expression profile at the protein level, immunoblot analysis was performed (Fig. 5). Protein expression for all mRNA transcripts was observed. Although multiple death receptors are expressed in these cells, the only death ligand detected was TRAIL. Thus, these observations suggest TRAIL and one of its cognate receptors contributes to GCDC cytotoxicity in the absence of Fas.

**GCDC Enhances TRAIL-R2/DR5 Expression**—Because there was constitutive expression of TRAIL and TRAIL-R1/DR4 and -R2/DR5 without apoptosis, we reasoned that GCDC induced apoptosis by increasing expression of the ligand or its receptors. Indeed, TRAIL-R2/DR5 but not TRAIL-R1/DR4 or TRAIL increased following GCDC treatment of HuH-BAT cells (Fig. 5). TRAIL-R2/DR5 was observed as two bands of 49 and 43 kDa by polyacrylamide gel electrophoresis. This observation is consistent with published data demonstrating two splice variants for TRAIL-R2/DR5 (21, 25). Both TRAIL-R2/DR5 isoforms increased following GCDC treatment of the cells suggesting enhanced transcription was occurring as opposed to alternations in mRNA splicing. To further examine this concept, we evaluated TRAIL-R2/DR5 mRNA levels by quantitative real time PCR (Fig. 6). GCDC treatment of HuH-BAT cells increased TRAIL-R2/DR5 mRNA expression 10-fold. The increased expression of TRAIL-R2/DR5 mRNA and protein by GCDC suggested this death receptor may contribute to GCDC-mediated apoptosis.

To determine if GCDC increased TRAIL-R2/DR5 expression by enhancing transcription, we next performed luciferase reporter gene assays (Fig. 7). The full genomic fragment, pGL2-full, demonstrated basal transactivation of TRAIL-R2/DR5 in this p53 mutated cell line. Moreover, GCDC enhanced this p53-independent transactivation of pGL2-full. The pGL2-full construct contains three high homology lesions to the p53 consensus DNA-binding sequence (18) as indicated in Fig. 7 (BS1, BS2, and BS3). A previous study demonstrated that BS2 is the most responsive to p53-dependent transactivation of pGL2-SmaI, which contains the BS2 site, demonstrated transactivation following transfection of wild-type p53 (18). Interestingly, the transcriptional activation by GCDC was observed in both pGL2 and pGL2-Sv-half but not in pGL2-Sv-SmaI. Indeed GCDC increased expression of the pGL2-Sv-half ~3-fold. These results suggest GCDC increases TRAIL-R2/DR5 gene expression, in part, by enhancing transcription.

**GCDC Sensitizes HuH-BAT Cells to TRAIL**—We reasoned that if GCDC increases TRAIL-R2/DR5 expression, it should sensitize the cells to exogenous TRAIL. HuH-BAT cells were treated with GCDC (200 μM), FLAG-tagged TRAIL plus an anti-FLAG antibody to induce ligand aggregation, or both
Indeed, apoptosis in the presence of GCDC plus TRAIL was greater than the sum of apoptosis in GCDC-treated and TRAIL-exposed cells. These data demonstrate that GCDC sensitizes cells to TRAIL presumably by up-regulating the receptor for TRAIL-R2/DR5. Recently, the expression of FLICE inhibitory protein (FLIP) has been implicated as an inhibitor of TRAIL-induced apoptosis (26–29). Therefore, we next evaluated cellular expression levels of FLIP in GCDC-treated cells (Fig. 8B). However, the expression of FLIP was unchanged during GCDC exposure suggesting that the sensitization to TRAIL-induced apoptosis by GCDC is not mediated by down-regulation of FLIP, but rather by increased expression of TRAIL-R2/DR5.

**GCDC-induced Apoptosis Is Associated with TRAIL-R2/DR5, but Not TRAIL-R1 Oligomerization**—As assessed by crystal structure analysis, TRAIL and its cognate receptors have been shown to oligomerize, a process necessary for transducing the death signal (8, 30, 31). Therefore, we next evaluated cellular expression levels of FLIP in GCDC-treated cells (Fig. 8B). However, the expression of FLIP was unchanged during GCDC exposure suggesting that the sensitization to TRAIL-induced apoptosis by GCDC is not mediated by down-regulation of FLIP, but rather by increased expression of TRAIL-R2/DR5.

Treatment of HuH-BAT cells for 60 min, we observed more efficient immunoprecipitation of TRAIL-R2/DR5 than in untreated cells under limiting antibody conditions (Fig. 9A). In contrast, TRAIL-R1 did not appear to be oligomerized by GCDC treatment as assessed by this assay (Fig. 9A). In addition, aggregation of TRAIL-R2/DR5 receptors was also observed using immunocytochemistry (Fig. 9B). The cellular distribution of TRAIL-R2/DR5 receptors was diffuse in untreated cells but became punctate in GCDC-treated cells consistent with aggregation or capping of the receptor. In contrast, TRAIL-R1/DR5 immunofluorescence remained diffuse following exposure to GCDC (Fig. 9B). These observations are consistent with activation of TRAIL-R2/DR5 by cytotoxic bile acids.

**DISCUSSION**

The principal findings of this study relate to the cellular mechanisms of Fas-independent bile salt-mediated apoptosis. The results demonstrate that GCDC-mediated apoptosis in a Fas-deficient cell line: (i) is FADD and caspase 8 dependent; (ii) is associated with increased TRAIL-R2/DR5 mRNA and protein expression without a change in FLIP protein levels; and (iii) involves oligomerization of TRAIL-R2/DR5 but not TRAIL-R1. The results provide new information suggesting bile acid cytotoxicity, in the absence of Fas expression, is mediated by TRAIL and its cognate receptor TRAIL-R2/DR5. Each of these findings will be discussed below.

Deoxycholate (100 μM) has previously been shown to induce apoptosis of HuH-7 cells (23). In contrast, we did not observe apoptosis by GCDC in this cell line in the absence of Ntcp. It is widely accepted that unconjugated bile acids such as deoxy-
cholate either diffuse across plasma membranes or are transported by a family of transport polypeptides referred to as organic anion transporters (34). In contrast, conjugated bile acids such as GCDC are predominantly transported into cells by the Ntcp (34). In our experience, bile acid transport into the cell is required to render GCDC apoptotic (35). Likely, the differences between the previous study and our current results relate to the bile acid employed, GCDC versus DCA, and the

**FIG. 9.** GCDC treatment of HuH-BAT cells results in DR5, but not DR4 aggregation. A, untreated (Control) and GCDC (200 μM, 4 h)-treated HuH-BAT cells were incubated with the cleavable cross-linking agent 3,3′-dithiobis(succinimidyldipropionate) and lysed for immunoprecipitation using limiting (1 μg/ml) or excess (10 μg/ml) amounts of anti-DR5 or DR4 antisera. Western blot analysis of the immunoprecipitate was performed as described under “Experimental Procedures.” B, control and GCDC (200 μM, 4 h)-treated cells were fixed and immunocytochemistry performed with anti-DR4/TRAIL-R1 and anti-DR5/TRAIL-R2 antisera. Immunofluorescence was granular and cytoplasmic for DR4 in both treated and untreated cell. Although DR5 immunofluorescence was granular in untreated cells it became punctate consistent with receptor aggregation in treated cells.
different modes of transport into the cell for these two bile acids.

Previous in vitro experiments demonstrated delayed hepatocyte apoptosis in the bile duct-ligated lpr mouse. This observation suggested that, in addition to activating a Fas (6), bile acids may also activate additional apoptotic pathways. However, lpr mice are not completely Fas deficient and do express minute amounts of Fas making the above interpretation of the in vivo data problematic. Therefore, to determine if bile acids may also activate other apoptotic pathways, we determined if bile acid apoptosis occurred in a Fas-deficient human hepatoma cell line stably transfected with a sodium-dependent bile acid transporter, HuH-BAT cells. This cell line underwent apoptosis despite the absence of Fas. These data suggest cytotoxic bile acids can activate multiple apoptotic processes.

Several observations implicate a death receptor pathway and not direct mitochondrial cytotoxicity for bile acid-mediated apoptosis despite the absence of Fas expression. Apoptosis by GCDC was inhibited by transfection with a dominant negative FADD implying a necessary role for FADD in the apoptotic cascade. Likewise, inhibition of caspase 8 by transfection with FADD implying a necessary role for FADD in the apoptotic cascade. Likewise, inhibition of caspase 8 by transfection with a dominant negative FADD transfection and caspase 8 inhibition (Fig. 2). These data are also consistent with a recent report showing that a caspase 8 inhibitor also blocked mitochondrial generation of reactive oxygen species in bile acid-treated hepatocytes (36). Thus, death receptor-mediated signaling appears to be responsible for mitochondrial dysfunction during bile acid cytotoxicity.

Death receptor-mediated bile acid cytotoxicity in the Fas-deficient cell line appears to be TRAIL-mediated. Although TRAIL and TRAIL-R1/DR4 and -R2/DR5 are constitutively expressed by the cells, the cytotoxic bile acid GCDC sensitized the cells to TRAIL cytotoxicity. This sensitization was associated with an increased expression of TRAIL-R2/DR5 mRNA and protein. Sensitization to TRAIL cytotoxicity by up-regulating TRAIL-R2/DR5 has also been observed in p53 overexpression-induced apoptosis (28). These data suggest that enhanced expression of TRAIL-R2/DR5 is a common mechanism for apoptosis induction by injurious agents. In addition to increasing TRAIL-R2/DR5 expression, bile acids may also sensitize the cells to TRAIL killing by altering affinity of binding proteins for the receptor complex or by enhancing the density of death receptors in plasma membrane microdomains. Both inhibitor and enhancing proteins for death receptor signaling have been identified (37, 38), and it is possible that bile acids bind to these cytoplasmic proteins influencing their activities. Aggregation of plasma membrane receptors and their signaling in cholesterol-rich lipid rafts has also been described (39), and bile acids which are known to insert into lipid bilayers may increase the density of the death receptors in lipid rafts promoting their aggregation. The mechanisms by which bile acids sensitize cells TRAIL-R2/DR5 apoptosis may be multifactorial and will require further experimental definition.

The potential hepatotoxicity of TRAIL has been controversial (40). Recently, data have emerged suggesting that TRAIL in a reduced state with high zinc binding is not hepatotoxic while more oxidized and less zinc containing ligands may be hepatotoxic (40). Our data suggest the context of the hepatocyte may also be important in determining if TRAIL will be hepatotoxic. In cholestasis with impairment of bile acid secretion, the retention of bile acids within the hepatocyte may sensitize the cell to TRAIL-mediated apoptosis by enhancing TRAIL-R2/DR5 expression. This information will be important in selecting patients for anti-tumor therapy with this ligand as is currently being considered.

We identified enhanced transcription for TRAIL-R2/DR5 in HuH-BAT cells following GCDC exposure. Both p53-dependent and -independent mechanisms for TRAIL-R2/DR5 induction have been identified (41, 42). HuH-BAT cells are derived from the HuH-7 cell line which is known to have a cytokine for tyrosine mutation at codon 220 (A:T → G:C) (43). Mutations in this region of the gene are known to disrupt p53 function (43). Because the HuH-BAT cells have a mutant p53, bile acid induction of TRAIL-R2/DR5 would appear to be p53 independent.

Bile acids have been shown to modulate gene expression by binding to the nuclear hormone receptor FXR (44). Thus, death receptor-mediated signaling appears to be responsible for transcriptionally active AP1 complexes (47). The role of these different pathways in regulating TRAIL-R2/DR5 induction by bile acids will require further study. These studies may, however, be important for many fields of biology. For example, induction of TRAIL-R2/DR5 may promote apoptosis of cancer cells by chemotherapeutic agents. If bile acid activation of the FXR nuclear hormone receptor is shown to induce TRAIL-R2/DR5, agonists for this receptor (e.g., GW4064) could be employed in the treatment of cancer (48).

Collectively, our previous and current studies demonstrate the importance of death receptor-mediated apoptosis in bile salt cytotoxicity. These studies suggest that death receptor-mediated apoptosis should also be important in liver injury during cholestasis. However, further in vivo studies are required to evaluate this concept. Appropriate studies employing cholestatic models of liver injury in the Bid knockout mouse or CrmA (a potent inhibitor of caspase 8) expressing transgenic animals would be useful to determine the in vivo relevance of our in vitro observations. Such further studies would not only provide a more complete picture of the role of death receptors in cholestatic liver injury, but also help evaluate Bid and caspase 8 as respective therapeutic targets for reducing liver injury during cholestasis.

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