Bile salts mediate hepatocyte apoptosis by increasing cell surface trafficking of Fas

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Sodeman, Thomas, Steven F. Bronk, Patricia J. Roberts, Hideyuki Miyoshi, and Gregory J. Gores. Bile salts mediate hepatocyte apoptosis by increasing cell surface trafficking of Fas. Am J Physiol Gastrointest Liver Physiol 278: G992–G999, 2000.—Toxic bile salts induce hepatocyte apoptosis by a Fas-dependent, Fas ligand-independent mechanism. To account for this observation, we formulated the hypothesis that toxic bile salts induce apoptosis by effecting translocation of cytoplasmic Fas to the cell surface, resulting in transduction of Fas death signals. In McNtcp.24 cells the majority of Fas was cytoplasmic, as assessed by cell fractionation and immunofluorescence studies. However, cell surface Fas increased sixfold after treatment with the toxic bile salt glycochenodeoxycholate (GCDC) in the absence of increased Fas protein expression. Moreover, in cells transfected with Fas-green fluorescence protein, cell surface fluorescence also increased in GCDC-treated cells, directly demonstrating Fas translocation to the plasma membrane. Both brefeldin A, a Golgi-disrupting agent, and nocodazole, a microtubule inhibitor, prevented the GCDC-induced increase in cell surface Fas and apoptosis. In conclusion, toxic bile salts appear to induce apoptosis by promoting cytoplasmic transport of Fas to the cell surface by a Golgi- and microtubule-dependent pathway.

brefeldin A; Fas-green fluorescence protein; J O-2 antisera; nocodazole; vesicle trafficking

CHOLESTASIS, DEFINED AS AN impairment in bile formation, is a feature of many chronic human liver diseases (28). The impairment in bile formation results in the retention of normal bile constituents within the liver, including high concentrations of bile salts (11). The retention and accumulation of bile salts within the liver during cholestasis is thought to exacerbate and promote liver injury (17, 23). Indeed, the pathophysiologic importance of bile salt-induced liver injury is demonstrated in children with subtype 2 of the progressive familial intrahepatic cholestasis syndromes (26). These children have mutations in the canalicular transport protein for bile salt secretion into bile and develop progressive liver disease because of the inability to excrete bile salts from the hepatocyte (26). The cellular and molecular mechanisms of bile salt-induced hepatocyte injury are, therefore, of clinical and scientific importance.

We have recently demonstrated that toxic bile salts induce hepatocyte apoptosis by a mechanism dependent on the Fas death receptor (10). The Fas receptor is a member of a growing family of death receptors that, when aggregated or oligomerized, signal a caspase protease-mediated death cascade culminating in apoptosis (9). Although Fas receptor activation is usually triggered by engaging the Fas ligand, in our studies the toxic bile salt glycochenodeoxycholate (GCDC) induced Fas oligomerization in the absence of Fas ligand. Furthermore, hepatocyte apoptosis was also observed in the bile duct-ligated, Fas ligand-deficient gld mouse (20). These observations suggest that a ligand-independent mechanism of Fas-mediated liver injury occurs during cholestasis. This is a potential new mechanism for cholestatic and toxin-induced liver injury.

Fas receptor oligomerization independent of ligand has been observed when Fas is overexpressed, when keratinocytes are exposed to ultraviolet light, and during drug-induced apoptosis by ganciclovir (1, 4, 21). Ganciclovir-induced apoptosis is mediated by a redistribution of cytoplasmic Fas to the cell surface (4). These observations suggest that one mechanism for the posttranscriptional regulation of Fas receptors is to sequester this death receptor within intracellular pools. The receptors can then be shuttled to the plasma membrane, presumably by a vesicular transport pathway, to initiate cell death signals. An increase in the cell surface density of Fas receptors likely promotes their aggregation, causing apoptosis. On the basis of this information, we formulated the hypothesis that bile salt-mediated, Fas-dependent apoptosis is caused by trafficking of preexisting cytoplasmic Fas receptors to the plasma membrane. To test this hypothesis, we sought to answer the following specific questions: 1) What is the cell surface vs. cytoplasmic distribution of Fas? 2) Does an increase in cell surface Fas occur during exposure to toxic bile salts? 3) Does inhibition of Fas translocation attenuate bile salt-mediated apoptosis? and 4) Are bile salt-treated hepatocytes more sensitive to Fas-stimulated apoptosis? We chose GCDC as the toxic bile salt for these studies because it is a primary bile salt whose concentrations increase during cholestasis.

EXPERIMENTAL PROCEDURES

Cell culture. McNtcp.24 cells, a hepatocyte-derived cell line that stably expresses the sodium-dependent taurocholate-cotransporting polypeptide and efficiently transports bile
salts, were cultured in DMEM containing 10% fetal bovine serum and 10% calf serum (22). We have previously shown that this cell line undergoes bile salt-mediated apoptosis with the same kinetics and at the same concentrations as primary rat hepatocytes (22). We have also demonstrated that this cell line does not express Fas ligand as assessed by PCR (10). For the cell fractionation experiments, mouse hepatocytes were employed. Mouse hepatocytes were isolated and cultured as previously described (10). p53 knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Quantitative immunofluorescence was performed using a digitized fluorescence microscopy system employing a Zeiss Axiosvert 35M microscope (Carl Zeiss, Thornwood, NY) and the Metafluor imaging system (Universal Imaging, West Chester PA), which has been previously described in detail (2). Briefly, MCTtcp.24 cells were cultured on collagen-coated glass coverslips in 30-mm dishes. The cells were fixed in formaldehyde (3% formaldehyde, 0.1 M PIPES, pH 7.2) for 30 min, were incubated with the polyclonal rabbit anti-Fas antisera. Membranes were washed three times in TTBS for 10 min each at room temperature and then incubated for 60 min at room temperature with a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. After three washes in TTBS for 10 min each at room temperature followed by a single wash in PBS for 10 min at room temperature, the blots were developed with the enhanced chemiluminescent substrate (Amersham, Arlington Heights, IL) and exposed to Kodak Biomax film.

Fas-GFP and confocal microscopy. Primers containing additional restriction site sequences (Bgl II in the sense and Sac II in the antisense) were made from the murine Fas sequence obtained from GenBank (accession no. E05335). The sense primer for Fas was 5’-AGATCTATGCTGTGATGCGCT-3’, and the reverse primer was 5’-CCGCGGTACGAGCTCC-3’. Mouse liver cDNA was amplified using PCR buffer (20 mM Tris, pH 8.4, and 50 mM KCl), 1.5 mM MgCl2, 200 μM dNTPs, 0.4 μM sense primer, 0.4 μM reverse primer, and 2.5 U Taq polymerase. Samples were heated to 94°C for 2 min and then amplified using the MJ Research (Wertwater, MA) PTC 200 Peltier thermal cycler for 30 cycles with the following times: 94°C for 1 min, 59.8°C for 1 min, and 72°C for 1 min. The samples were then placed at 72°C for 10 min. The PCR product was then cloned into Promega’s pGEM T-vector following the manufacturer’s instructions. The PCR product was cut out of the PCR vector with Bgl II and Sac II restriction enzymes and subcloned into Clontech’s pGFP-N1 vector that had been cut with the same enzymes. All PCR products were confirmed as the appropriate product by sequencing using dye terminator technology. MCTtcp.24 cells were transfected as previously described with the Fas-green fluorescence protein (GFP) construct (16). GFP fluorescence was imaged by confocal microscopy as previously described in detail (10, 22).

Quantification of apoptosis. We quantitated apoptosis as described in detail (10) by analyzing the nuclear changes of apoptosis using the nuclear binding dye 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) and fluorescence microscopy.

Bile acid uptake. Steady-state bile acid uptake by MCTtcp.24 cells was quantitated as we have previously described in detail (31). Tritiated taurocholate (New England Nuclear, Boston, MA) was employed for these studies.

Quantitative analysis. All data represent at least three experiments using cells, tissue, or extract from a minimum of three separate isolations and are expressed as means ± SE unless otherwise indicated. Differences between groups were compared using an ANOVA for repeated measures and a post hoc Bonferroni test for multiple comparisons. All statistical analyses were performed with the statistical software package Instat from GraphPAD (San Diego, CA).

Materials. DMEM media was purchased from Bio Whitaker (Walkersville, MD). Anti-Fas polyclonal antibody M-20 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit horseradish peroxidase was obtained from BioSource International (Camarillo, CA). Goat anti-rabbit Alexa 488 was obtained from Molecular Probes (Eugene, OR). Bovine serum and fetal calf serum were purchased from Summit Biotechnology (Ft. Collins, CO). Enhanced chemiluminescence Western blot detection kit was obtained from Amersham Pharmacia (Piscataway, NJ). J-02 antiserum
was purchased from PharMingen (San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO). Brefeldin A was in a stock solution of 5 mg/ml in DMSO and nocodazole 4 mM in dH2O.

RESULTS

What is the cytoplasmic vs. plasma membrane distribution of Fas? The cellular distribution of Fas, cytoplasmic vs. plasma membrane, was assessed using cell fractionation studies in mouse hepatocytes and quantitative fluorescence microscopy in Mkntcp.24 cells (Fig. 1). Cell fractionation studies (Fig. 1A) demonstrated that the majority of total cellular Fas in mouse hepatocytes was cytoplasmic and not associated with the plasma membrane (~71% of Fas was cytoplasmic vs. 29% in the plasma membrane). Because of the limitations of cell fractionation studies, including contamination of fractions, redistribution of proteins during the fractionation procedure, and proteolysis, we confirmed these results using quantitative fluorescence microscopy (Fig. 1B). Fas-associated immunofluorescence was measured in permeabilized cells (total cell Fas) and nonpermeabilized cells (cell surface Fas). These studies also demonstrated that Fas was predominantly cytoplasmic (54% in the cytoplasm). Thus in Mkntcp.24 cells and mouse hepatocytes, the majority of Fas is sequestered within intracellular compartments.

Does an increase in cell surface Fas occur during GCDC treatment? We next determined whether GCDC-mediated apoptosis was associated with an increase in cell surface Fas. The increase in cell surface Fas was initially determined by cell fractionation studies (Fig. 1). An approximately sixfold increase in plasma membrane Fas was identified after treatment of Mkntcp.24 cells with 50 µM GCDC for 1 h. We chose to confirm this observation by combining cell immunofluorescence with quantitative fluorescence microscopy in which we could visualize the cells' surface fluorescence directly (Fig. 2). Indeed, a sixfold increase in cell surface fluorescence was also observed using this approach. The increase in cell surface Fas could not be explained by an increase in Fas protein expression; immunoblot analysis of whole cell lysates following treatment with GCDC for 4 h identified no significant increase in total cellular Fas compared with untreated cells (Fig. 3). Furthermore, actinomycin D (200 µM), an inhibitor of transcription,
did not block the increase in cell surface Fas (data not shown).

To confirm independently the apparent translocation of Fas from the cell interior to the plasma membrane, cells were transfected with Fas-GFP and examined by confocal microscopy (Fig. 4). In control cells, Fas-GFP has an intracellular punctate fluorescence consistent with its compartmentation within organelles. In contrast, treatment with GCDC increased plasma membrane fluorescence, demonstrating increased Fas-GFP on the cell surface (Fig. 4). Thus GCDC cytotoxicity appears to be accompanied by translocation of Fas to the cell surface.

Does inhibition of Fas translocation attenuate bile salt-mediated apoptosis? Fas has been reported to be shuttled to the cell surface in fibroblasts by a protein secretory pathway involving the Golgi apparatus (5). Brefeldin A blocks Golgi-dependent protein secretion (7). Therefore, we postulated that brefeldin A would block the GCDC-mediated increase in cell surface Fas and apoptosis. Incubation of McNtcp.24 cells with brefeldin A (10 µg/ml) plus GCDC (50 µM) prevented the increase in cell surface Fas (Fig. 5). Moreover, brefeldin A also blocked GCDC-induced apoptosis (Fig. 5).

Protein translocation from the Golgi to the plasma membrane occurs along microtubules (19). Therefore, we postulated that nocodazole, a microtubule-disrupting agent (24), would attenuate the increase in both cell surface Fas and apoptosis during treatment of McNtcp.24 cells with GCDC. Like brefeldin A, nocodazole also blocked the increase in cell surface Fas and apoptosis by GCDC (Fig. 6). Neither brefeldin A nor nocodazole affected steady-state bile acid uptake by McNtcp.24 cells, demonstrating that alterations in bile acid uptake were not responsible for their effects (30-32).

Fig. 3. Total cellular Fas protein expression is not increased by GCDC. McNtcp.24 cells were treated with 50 µM GCDC or diluent for 4 h. The cells were then lysed, and immunobLOTS were performed for Fas and β-actin as a control for protein loading (top). Quantitative densitometry was performed and averaged for 3 experiments (bottom). No significant difference was observed for Fas protein expression between treated and untreated cells. OD, optical density. P = not significant.

Fig. 4. GCDC increases plasma membrane Fas-green fluorescence protein (GFP) in McNtcp.24 cells. Forty-eight hours after transfection with Fas-GFP, McNtcp.24 cells either were left untreated or were treated with 50 µM GCDC for 1 h. Cells were then treated with 100 µM digitonin for 15 min to release autofluorescent soluble compounds from the cell. GFP fluorescence was imaged by laser scanning confocal microscopy. Arrowheads indicate cell surface clustering of Fas-GFP after GCDC treatment.

Fig. 5. Brefeldin A inhibits the increase in cell surface Fas and attenuates apoptosis during treatment of McNtcp.24 cells with GCDC. A: McNtcp.24 cells were treated with 50 µM GCDC or diluent in the absence or presence of 10 µg/ml of brefeldin A for 60 min. After fixation, cell surface Fas was quantitated as described in Fig. 1 using immunocytofluorescence and quantitative fluorescence microscopy. B: McNtcp.24 cells were incubated as described for A for 4 h. Nuclear changes indicative of apoptosis were quantitated using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining and fluorescence microscopy.
min bile salt uptake: control = 2.3 ± 0.1, brefeldin A = 2.3 ± 0.1, and nocodazole = 2.2 ± 0.1 pmol·mg protein⁻¹·min⁻¹; P = not significant). These data bolster the conclusion that Fas shuttling to the cell surface by a classic protein secretory route is necessary for GCDC-induced apoptosis.

What is the mechanism for bile acid-associated increase in cell surface trafficking of Fas? Although p53 has been suggested to mediate Fas trafficking (5), bile-acid-associated Fas trafficking pathway was not p53 dependent (Fig. 7). Indeed, rates of GCDC-mediated apoptosis and increases in Fas-associated immunofluorescence were virtually identical in hepatocytes obtained from wild-type and p53 knockout mice.

Protein kinase C (PKC) has been implicated in secretory activity (25, 30). Moreover, we have previously demonstrated that PKC inhibitors attenuate bile salt-mediated hepatocyte apoptosis (15). Therefore, we determined whether the PKC inhibitor chelerythrine blocks Fas redistribution from the cell interior to the cell surface (Fig. 8). Incubation of MnTCP.24 cells with chelerythrine (1 µM) plus GCDC (50 µM) prevented the increase in cell surface Fas (Fig. 8). Consistent with our previous study (15), chelerythrine also blocked GCDC-induced apoptosis (Fig. 8). These data mechanistically link increases in PKC activity by toxic bile salts to Fas plasma membrane trafficking.

Does GCDC render cells more sensitive to apoptosis by the J O-2 Fas agonistic antisera? We reasoned that by increasing cell surface expression of Fas, GCDC-treated cells may become more susceptible to apoptosis induced by Fas agonists. Indeed, apoptosis by the J O-2 Fas agonistic antisera was significantly increased in GCDC-treated cells (Fig. 9). The ability of GCDC to sensititize hepatocytes to apoptosis by Fas stimulation was inhibited by both brefeldin A and nocodazole (Fig. 9), an observation suggesting that Fas trafficking from the cell interior to the surface was necessary for GCDC sensitization of hepatocytes to Fas-stimulated apoptosis. These data demonstrate that the increased cell surface expression of Fas observed in GCDC-treated cells is functional in regard to transducing death signals.

DISCUSSION

The principal findings of this study relate to the cellular mechanisms of Fas-dependent, Fas ligand-independent apoptosis by toxic bile salts. The results demonstrate that 1) Fas is predominantly intracellular

![Graph A](image1)

![Graph B](image2)
in hepatocytes; 2) GCDC cytotoxicity is associated with an increase in cell surface Fas; 3) inhibition of protein trafficking with brefeldin A, nocodazole, or chelerythrine attenuates the increase in cell surface Fas and apoptosis during treatment with GCDC; and 4) and GCDC-treated hepatocytes are sensitized to cell death by Fas agonistic antisera. Toxic bile salts appear to effect a Fas-dependent apoptotic cascade by a process requiring an increase in the cell surface density of this death receptor.

Because of the propensity for the death domains of death receptors to spontaneously associate (3), the density of cell death receptors on the plasma membrane must be tightly regulated by the cell. One post-transcriptional mechanism for regulating the plasma membrane density of surface receptors is to sequester receptors within intracellular pools. The receptors can then be shuttled to the plasma membrane by appropriate stimuli in a regulated fashion. Consistent with these concepts, the death receptors Fas and tumor necrosis factor receptor-1 can be largely intracellular, sequestered within the Golgi complex (5, 14). Recently, it has been demonstrated that ganciclovir- and transcription-independent, p53-mediated apoptosis are mediated by a redistribution of cytoplasmic Fas to the cell surface (5). In our current study, we found an increase in cell surface Fas during treatment by toxic concentrations of GCDC. The increase in cell surface Fas was blocked by the protein secretion inhibitor brefeldin A and the microtubule poison nocodazole. These data suggest that bile salts are able to effect shuttling of Fas death receptors from intracellular stores utilizing a classic protein secretion pathway.

Activation of PKC appears to contribute to the shuttling of intracellular Fas to the plasma membrane. Indeed, we have shown that the PKC inhibitor chelerythrine blocks the increase in cell surface Fas and bile salt-induced apoptosis. These observations mechanistically link our previously published results showing attenuation of bile salt cytotoxicity by PKC inhibitors to our current observations demonstrating the role of Fas in bile salt-mediated apoptosis (15). However, we cannot exclude additional mechanisms of bile salt stimulation of Fas trafficking to the plasma membrane. Bile salts may also perturb Golgi signaling. Morphological studies employing radiolabeled bile salts, a fluorine-containing bile salt analog, and fluorescently labeled glycodeoxycholate have all demonstrated bile salt localization with the Golgi apparatus (8, 18, 27). Association of toxic bile salts with the Golgi network may potentially initiate intracellular stress signals, resulting in the vesicular trafficking of Fas to the plasma membrane.
We have previously demonstrated Fas oligomerization independent of Fas ligand by GCDC treatment of hepatocytes (10). The results of this study suggest that an increase in cell surface Fas is required for this oligomerization event. However, it is unclear whether an increase in cell surface density of Fas receptors alone is sufficient for their spontaneous oligomerization or if other additional processes are involved in this model of toxin-induced apoptosis. Interestingly, a protein inhibitor of tumor necrosis factor receptor-1 oligomerization has been identified and referred to as silencer of death domain (SODD) (13). Because of the similarities in signaling by death domain-containing receptors, it is likely that a similar protein may exist for Fas. Bile salts could also potentially facilitate Fas oligomerization by binding to such inhibitory proteins and disabling their binding to Fas. Toxic bile salts may also facilitate binding of Fas-associated death domain protein (FADD) or the recently described FADD-like interleukin-1β-converting enzyme (FLICE)-associated huge protein (FLASH) to plasma membrane-associated Fas (12). Direct measurements of bile salts on binding of the death-inducing signaling complex proteins to the Fas receptor will be needed to address these questions. Finally, we cannot exclude the existence of an undiscovered Fas-binding ligand in this process.

Our observations may have relevance to the clinical observation that colchicine, an agent that depolymerizes microtubules, is salutary in human cholestatic liver diseases (29). Perhaps colchicine is beneficial by preventing Fas from translocation to the plasma membrane by inhibiting microtubule transport in these liver diseases. We were unable to directly test colchicine in our in vitro model because it unexpectedly induced cytotoxicity in the MnTcpx24 cell line. However, our data with nocodazole, also a microtubule-depolymerizing agent, support this concept.

In summary, the data in the current study suggest that toxic bile salts cause cell death, in part, by effecting translocation of Fas from the cytoplasm to the cell surface. The shuffling of Fas and the resulting apoptosis can be inhibited by the Golgi-disrupting agent brefeldin A and the microtubule poison nocodazole. By inference, a Golgi-associated and microtubule-dependent pathway appears to be involved in the trafficking of Fas to the cell surface during bile salt cytotoxicity. The increase in density of cell surface Fas receptors promotes their oligomerization, initiating a caspase-dependent death-signaling pathway (10). These results provide a new model for toxin-induced liver injury, namely a Fas-dependent but Fas ligand-independent mechanism for liver injury. The implications of these results for potential therapy of cholestatic liver diseases are currently under investigation.

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