Acute carbon tetrachloride feeding induces damage of large but not small cholangiocytes from BDL rat liver

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LeSage, Gene D., Shannon S. Glaser, Luca Marucci, Antonio Benedetti, Jo Lynne Phinizy, Rebecca Rodgers, Alessandra Caligiuri, Emmanuela Papa, Ziga Tretjak, Anne-Marie Jezequel, Leigh A. Holcomb, and Gianfranco Alpini. Acute carbon tetrachloride feeding induces damage of large but not small cholangiocytes from BDL rat liver. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1289–G1301, 1999.—Bile duct damage and/or loss is limited to a range of duct sizes in cholangiopathies. We tested the hypothesis that CCl₄ damages only large ducts. CCl₄ or mineral oil was given to bile duct-ligated (BDL) rats, and 1, 2, and 7 days later small and large cholangiocytes were purified and evaluated for apoptosis, proliferation, and secretion. In situ, we measured apoptosis by morphometric and TUNEL analysis and the number of small and large ducts by morphometry. Two days after CCl₄ administration, we found an increased number of small ducts and reduced number of large ducts. In vitro apoptosis was observed only in large cholangiocytes, and this was accompanied by loss of proliferation and secretion in large cholangiocytes and loss of choleretic effect of secretin. Small cholangiocytes de novo express the secretin receptor gene and secretin-induced cAMP response. Consistent with damage of large ducts, we detected cytochrome P-4502E1 (which CCl₄ converts to its radicals) only in large cholangiocytes. CCl₄ induces selective apoptosis of large ducts associated with loss of large cholangiocyte proliferation and secretion.

INTRAHEPATIC BILE DUCT epithelial cells or cholangiocytes form the intrahepatic biliary tree, a ramified network of interconnecting conduits of different sizes and functions within the liver (1, 3, 7–8, 10). Cholangiocytes play an important role in the secretion of ductal bile under basal and hormone-regulated conditions (1–3, 5–10, 17, 18, 20, 23). For example, secretin stimulates ductal bile secretion (1, 3, 5–10, 18, 23) by interaction with specific receptors expressed only by cholangiocytes (9) through an increase in intracellular levels of cAMP and activation of Cl⁻/HCO₃⁻ exchanger and Cl⁻ channel opening (5, 7–8, 10, 17, 18), which induces bicarbonate-rich choleresis in vivo (5, 18).

We have previously shown in both normal and bile duct-ligated (BDL) rats that cholangiocytes are morphologically heterogeneous with small cholangiocytes lining small bile ducts and large cholangiocytes lining large bile ducts (1, 3, 8, 10). We have also shown, using distinct subpopulations of cholangiocytes and intrahepatic bile duct units (IBDU) of different sizes, that secretin-regulated ductal secretion is present in large (>15 µm in diam) but not small (<15 µm in diam) IBDU (1, 3, 8, 10).

Cholangiocytes are the target cells in a number of experimental models of ductal hyperplasia (1, 3, 5, 7, 9, 18, 23). Cholangiocyte proliferation after BDL is closely associated with an increase in DNA synthesis, numbers of intrabiliary bile ducts, and ductal secretory activity (1, 3–5, 7, 9, 18). In the BDL rat model, cholangiocyte proliferation and increased ductal bile secretion occur in large cholangiocytes or large IBDU but are absent in small cholangiocytes or small IBDU (1, 10).

It is well known that administration of a single dose of CCl₄ to rats causes acute necrosis of hepatocytes in zone 3 of the hepatic lobule (15, 26), resulting in complete regeneration of hepatocytes after 7 days (26). However, recent studies (33) have shown that in addition to necrosis a substantial number of hepatocytes may undergo apoptosis in response to acute CCl₄ treatment. CCl₄ hepatotoxicity depends on the dehalogenation of CCl₄ catalyzed by cytochrome P-4502E1 in the liver cell endoplasmic reticulum (15, 29). Treatment with CCl₄ in vivo activates oxygen by the P-4502E1 catalyzed reaction via an NADPH-dependent mechanism (15, 29). Subsequent dissociation of superoxide radicals from the P-4502E1 substrate complex generates free radicals that react with micromosal membranes to induce lipid peroxidation that leads to cell membrane damage (15, 29). The cytochrome P-4502E1 is an enzyme endowed to epithelial cells originating from the embryonic mid gut because it has been localized in the rat in pancreas (25) and both duodenal and jejunal villous cells (34).

No information exists with regard to the effect of CCl₄ on proliferative and secretory processes of different segments of the intrahepatic biliary epithelium. Inasmuch as the loss and/or damage of large interlobular...
ducts by degenerative or inflammatory processes is thought to be a major factor in the pathogenesis of human liver diseases (e.g., primary biliary cirrhosis and primary sclerosing cholangitis) (7), we sought to develop an animal model of selective bile duct damage that is limited to large hormone-responsive bile ducts. Rats with ductal hyperplasia induced by BDL for 1 wk were used in these studies inasmuch as, in contrast to normal rats, secretin-induced bicarbonate-rich cholestasis can also be measured in vivo (5, 18). Furthermore, with an increase in the number of bile ducts after BDL, a reduction in cholangiocyte proliferative capacity due to injury can be measured more easily. We found that a single dose of CCl₄ in 1-wk BDL rats induces a transient cholangiocyte loss only in large ducts by cholangiocyte apoptosis, and this was associated with a reduction of proliferative and secretory processes in large cholangiocytes. In contrast, small cholangiocytes were resistant to apoptosis, proliferate in response to CCl₄, and transiently acquire phenotypes of large cholangiocytes.

MATERIALS AND METHODS

Animal Model

Male Fisher 344 rats (125–150 g) were purchased from Charles River (Wilmington, MA), maintained in a temperature-controlled environment (20–22°C) with a 12:12-h light-dark cycle, and fed ad libitum standard rat chow. Rats had free access to drinking water. Our studies were carried out in seven groups of animals, including 1-wk BDL control rats, 1-wk BDL rats subsequently treated by gavage with a single dose of CCl₄-mineral oil (1:1, 0.5 ml/100 g body wt) (26), and 1-wk BDL rats subsequently treated with a single dose of CCl₄ or mineral oil (0.5 ml/100 g body wt); the treated animals were killed on day 1, 2, and 7 after CCl₄ or mineral oil administration. Before each experimental procedure, the animals were anesthetized with pentobarbital sodium (50 mg/kg ip). All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee. When necessary, rats were euthanized with an overdose of pentobarbital sodium (200 mg/kg body wt ip), according to the regulations of the panel on euthanasia of the American Veterinary Medical Association. In all animals used, body weight, wet liver weight, and wet liver weight-to-body weight ratio were determined.

Materials

Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Porcine secretin was purchased from Peninsula (Belmont, CA). The substrate for γ-glutamyltranspeptidase (γ-GT), N-(γ-l-glutamyl)-4-methoxy-2-naphthylamide, was purchased from Polysciences (Warrington, PA). The nuclear dye 4’,6-diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes (Eugene, OR). The mouse anti-cytokeratin 19 (CK-19) antibody was purchased from Santa Cruz (CA), and the rabbit anti-vimentin (2) and antivimentin/Cajal bodies (21) were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Porcine secretin was purchased from Polypeptide Laboratories (St. Louis, MO) unless otherwise indicated. Porcine secretin was purchased from Polysciences (Warrington, PA). The nuclear dye 4’,6-diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes (Eugene, OR). The mouse anti-cytokeratin 19 (CK-19) antibody was purchased from Santa Cruz (CA), and the rabbit anti-vimentin (2) and antivimentin/Cajal bodies (21) were purchased from Sigma (St. Louis, MO) unless otherwise indicated.
normal bovine serum in PBS. The sections were subsequently incubated for 1 h at 37°C with terminal deoxynucleotidyl transferase and nucleotide mixture with fluorescin-dUTP in reaction buffer. After several washes in PBS, the specimens were resaturated (30 min at room temperature) in 3% BSA and 20% normal bovine serum in PBS and subsequently for 30 min at 37°C with an anti-fluorescein-conjugated antibody (Fab fragment from sheep) conjugated with horseradish peroxidase followed by 3,3'-diaminobenzidine tetrahydrochloride color reaction. In each liver section, the number of apoptotic bodies present in cholangiocytes and the diameter (distance between basement membrane) of the bile duct (in which apoptosis was observed) were evaluated. In each liver section, at least 50 nonoverlapping fields were analyzed. The incidence of apoptosis was expressed as number of apoptotic bodies for 100 cholangiocytes.

DAPI staining. In freshly isolated small and large cholangiocytes from 1-wk BDL rats and 1-wk BDL rats treated with CCl₄ or mineral oil, apoptosis was morphologically assessed by nuclear staining with DAPI as described by Que et al. (28). After incubation with DAPI (1 μM for 10 min at 22°C), small and large cholangiocytes were placed on coverslips and transferred to the stage of a Nikon Diaphot microscope equipped with fluorescence and differential interference optics where DAPI fluorescence was visualized with the use of excitation and emission filters of 380 and 460 nm, respectively. Apoptosis was determined by observation of nuclear fragmentation as described by Que et al. (28). In agreement with other studies (28), we used normal rat cholangiocytes treated with beaovericin (10 μM) for 4 h at 37°C as a positive control.

Assessment of Cholangiocyte Proliferation

In situ morphometric analysis of bile duct mass. In coded frozen liver sections (n = 6 for each group) from 1-wk BDL rats and 1-wk BDL rats treated with CCl₄ or mineral oil, morphometric analysis of small and large ducts was performed by point count analysis (37) by determining the number of small (<15 μm in diam) and large (>15 μm in diam) bile ducts stained for CK-19, a specific marker for cholangiocytes in rat liver (6, 7). Immunohistochemistry for CK-19 was performed as previously described (23). After staining for CK-19, liver sections were counterstained with hematoxylin and examined in a coded fashion with a Zeiss microscope. Point counting analysis (37) has also been applied by us to measure the number of intrahepatic bile ducts in normal and proliferating liver (23). The duct diameter was estimated from the distance between basement membranes. The data were expressed for each duct as the mean of two diameters taken perpendicular to each other. As previously shown in normal rats (13), there is a close association between bile duct diameter and the number of cholangiocytes.

In vitro molecular analysis. The genetic expression of H₃ histone, a specific marker of DNA synthesis (2), was assessed in lysate obtained from tissues and/or organs (−100 mg) and small and large cholangiocytes (3.0 × 10⁶ cells) using the lysate ribonuclease protection assay kit (Direct Protect, Ambion) (18, 23) according to the instructions supplied by the vendor. We used, for each cell sample, 45 μl of lysate containing 4.5 × 10⁵ purified cholangiocyte subpopulations. We used the following ³²P-labeled antisense riboprobes: a 204-bp riboprobe encoding the H₃ histone gene was transcribed from linearized pGEM42-H₃ histone cDNA (a gift from Dr. S. Gupta, Albert Einstein Hospital, Bronx, NY) and a 316-bp GADPH mRNA probe, the housekeeping gene (2, 8–10, 18). Rat spleen and yeast transfer RNAs were the positive and negative controls for H₃ histone, respectively; and rat kidney and yeast transfer RNAs were the positive and negative controls for GAPDH, respectively.

Assessment of Ductal Secretory Activity

Secretion receptor gene expression. In small and large cholangiocytes purified from 1-wk BDL rats and 1-wk BDL rats treated with CCl₄ or mineral oil, secretin receptor (SR) gene expression was assessed by the lysate ribonuclease protection assay (Direct Prot, Ambion) (18, 23). A 318-bp riboprobe encoding the message for SR was transcribed from pGEM42-SR (a gift from N. F. LaRusso, Mayo Clinic, Rochester, MN) and a 316-bp GAPDH mRNA probe, the housekeeping gene (2, 8–10, 18).

Assessment of intracellular cAMP levels. Basal and secretin-induced intracellular cAMP synthesis of small and large cholangiocytes from 1-wk BDL rats and 1-wk BDL rats treated with CCl₄ or mineral oil were determined as previously described (1–3, 8, 10, 18, 20, 23). After isolation, pure preparations of small and large cholangiocytes were incubated for 1 h at 37°C to restore surface proteins damaged by treatment with proteolytic enzymes (20). The two subpopulations (1 × 10⁶ cells) were stimulated with secretin (10⁻⁷ M) in the presence of 0.2% BSA or 0.2% BSA (control) for 5 min at 22°C (1–3, 8, 10, 18, 20, 23). After ethanol extraction, cAMP levels were measured by RIA using a commercially available kit (Amersham).

Studies of in vivo biliary physiology. After anesthesia with pentobarbital sodium, rats were surgically prepared for bile collection as previously described by us (5). When steady-state bile flow was achieved, secretin (10⁻⁷ M) was infused for 30 min followed by a final infusion of Krebs-Henseleit bicarbonate solution (KRH) for 60 min. Bile was collected at 10-min intervals, placed in preweighed tubes, and immediately stored at −70°C before determining bicarbonate concentration. Bicarbonate concentration (measured as total CO₂) in bile from BDL and CCl₄-treated BDL rats was determined by a Natelson microgramosimeter apparatus (Scientific Industries, Bohemia, NY).

Immunohistochemical Analysis of Cytochrome P-4502E1

Liver tissue obtained from BDL rats was snap frozen and sectioned at 7 μm using a cryostat. Frozen liver sections were placed on polylysine-coated slides and allowed to air dry. Liver sections were fixed in methanol for 10 min at −20°C before staining for cytochrome P-4502E1. After a brief rinse of slides in PBS, endogenous peroxidase was blocked by 15-min incubation in methanol-hydrogen peroxide. Endogenous biotin was blocked using an Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's procedure. Slides were incubated with a polyclonal antibody, which recognizes the rat P-450 CYP2E1 (1:100, Chemicon International, Temecula, CA), plus goat serum (1:25 dilution) for 2 h at room temperature followed by a 2-h incubation in a biotinylated goat anti-rabbit IgG antibody (1:500 dilution, Vector Laboratories). The immunoreactivity for the rat P-450 CYP2E1 was detected with the indirect peroxidase procedure by using the ABC system (Santa Cruz Biotechnology, Santa Cruz, CA) according to the instructions provided by the manufacturer. All slides were well rinsed with PBS between each stage of the immunohistochemical procedure. Slides were briefly stained with hematoxylin and dehydrated in a graded ethanol series before clearing with xylene and coverslipping using Permount. Slides were examined with a microscope (Olympus Optical BX 40).
Western blot analysis of cytochrome P-4502E1

Western blot analysis of cytochrome P-4502E1 was performed in microsomes purified from rat liver (positive control, Chemicon), normal rat hepatocytes (positive control, 1 × 10⁷), or small and large cholangiocytes (1 × 10⁷ each) obtained from 1-wk BDL rats and 1-wk BDL rats treated with a single dose of CCl₄ or mineral oil. Microsomes were prepared from hepatocytes or small and large cholangiocytes as previously described (21). Briefly, cell preparations (1 × 10⁶ cells) were resuspended in ice-cold 250 mM sucrose, 100 mM Tris-HCl, 1 mM EDTA, pH 7.2, containing 0.5% Triton X-100, and 8% (vol/vol) protease inhibitors (Complete, Boehringer Mannheim, Mannheim, Germany) and sonicated on ice (30 s bursts) to lyse the cells. The cell lysate was successively centrifuged at 800 g for 10 min, at 13,500 g for 20 min, and at 105,000 g for 60 min at 4°C. The microsomal pellet was suspended in 100 mM sodium phosphate, 10 mM MgCl₂, and 20% glycerol, pH 7.4, and immediately processed for Western blot analysis. The total protein concentration of hepatocytes and small and large cholangiocytes was determined by the protein assay from Bio-Rad Laboratories (Hercules, CA). Equal amounts of protein from microsomes purified from normal rat hepatocytes or small and large cholangiocytes were resolved by SDS-10% PAGE and transferred onto a nitrocellulose filter (Bio-Rad). The filter was blocked using a 5% solution of nonfat dry milk in Tris-buffered saline (TBST, composed of 50 mM Tris, 150 mM NaCl, and 0.05% Tween 20). The filter was washed again five times with TBST, and proteins were visualized using chemiluminescence (Amersham Life Science). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low-light imaging system (Alpha Innotech).

Statistical Analysis

All data are expressed as means ± SE. The differences between groups were analyzed by Student's t-test when two groups were analyzed or ANOVA if more than two groups were analyzed.

RESULTS

Characterization of CCl₄-Induced Hepatotoxicity in BDL Rats

The effects of acute CCl₄ or mineral oil treatment on body weight, wet liver weight, and wet liver-to-body weight ratio in BDL rats are shown in Table 1. The effects of CCl₄ or mineral oil treatment on transaminase serum levels are shown in Table 2. Consistent with the concept that acute CCl₄ treatment induces damage of liver cells, following CCl₄ treatment there was a marked increase in the serum levels of AST and ALT compared with 1-wk BDL rats and 1-wk BDL rats treated with mineral oil (Table 2). The increased serum enzyme levels (observed on days 1 and 2 after CCl₄ treatment) returned to BDL control values on day 7 after CCl₄ treatment (Table 2). As further evidence for hepatocellular damage, we found that the levels of albumin mRNA markedly decreased on both days 1 (−5.6-fold, P < 0.05 vs. control values) and 2 (−3.0-fold, P < 0.05 vs. control values) after CCl₄ treatment before returning to values similar to that of 1-wk BDL rats on day 7 (Fig. 1). No significant changes in albumin gene expression were found in total RNA samples from 1-wk BDL rats treated with mineral oil compared with 1-wk BDL rats (Fig. 1). Densitometric analysis of albumin mRNA was normalized to GAPDH gene expression (Fig. 1, right).

Purification of Small and Large Cholangiocytes

By counterflow elutriation we isolated two distinct and pure (by γ-GT histochemistry, results not shown) subpopulations of small (mean diam 8 µm) and large (mean diam 14 µm) cholangiocytes from 1-wk BDL rats and 1-wk BDL rats treated with a dose of CCl₄ or mineral oil.
mineral oil. Viability of the two cholangiocyte subpopulations was always greater than 99%. We did not detect hepatocytes (by glucose 6-phosphatase histochemistry) (36) or mesenchymal cells (by immunohistochemistry for vimentin) (2) in our preparations of small and large cholangiocytes (results not shown). The message for α-fetoprotein was not present in purified cholangiocytes from CCl4- or oil-treated BDL rats (results not shown).

In Situ Quantitative Evaluation of Apoptosis in Small and Large Intrahepatic Bile Ducts

Liver morphology. The livers of 1-wk BDL rats and 1-wk BDL rats treated with a single dose of mineral oil showed marked proliferation of bile ducts with a pattern similar to that of previous studies (5). In agreement with previous studies (15, 26), 1 day after CCl4 treatment we observed necrosis in the parenchyma, scarce inflammatory infiltrate, and fatty acid changes of the surviving hepatocytes. Proliferating bile ductules (<15 µm in diam) were often seen in close proximity to or surrounded by necrotic hepatocytes and showed vacuolization of the cytoplasm without evidence of advanced necrosis. Two days after CCl4 treatment necrosis was usually restricted to zone 3, whereas liver morphology 7 days after CCl4 treatment was similar to 1-wk BDL rats or 1-wk BDL rats treated with mineral oil.

Quantitative evaluation of apoptosis. Morphological analysis of the intrahepatic biliary epithelium in 1-wk BDL rats (control) and 1-wk BDL rats on day 1 after CCl4 treatment showed a few apoptotic bodies, which appeared as small acidophilic globules often containing nuclear material localized between two adjacent cholangiocytes, engulfed within their cytoplasm, or released into the biliary lumen without an associated inflammatory response. The number of apoptotic bodies/100 cholangiocytes observed in large (diam >15 µm) bile ducts significantly increased in BDL rats 2 days after CCl4 treatment (Fig. 2) with respect to BDL rats 2 days after oil treatment (0.46 ± 0.03 vs. 0.20 ± 0.05 apoptotic bodies/100 cholangiocytes in CCl4-treated and oil-treated rats, respectively, P < 0.001). Seven days after CCl4 treatment the number of apoptotic bodies was similar to that of BDL rats 7 days after oil treatment (0.19 ± 0.09 vs. 0.16 ± 0.04, P > 0.05) in CCl4-treated and oil-treated rats, respectively. The increase in the number of apoptotic bodies observed in large bile ducts 2 days after CCl4 treatment compared with BDL oil-treated rats (P < 0.001) was also confirmed by TUNEL analysis. The number of apoptotic bodies, when present, for each bile duct ranged between 1 and 4. Apoptosis was never observed in small (diam <15 µm) bile ducts from 1-wk BDL rats and 1-wk BDL rats treated with CCl4 or mineral oil. Acute CCl4 treatment did not result in morphological evidence of necrosis in cholangiocytes. We did not detect a significant increase of hepatocyte apoptosis with respect to controls after acute CCl4 treatment.
Quantitative measurement of apoptosis by DAPI staining in small and large cholangiocytes. Nuclear fragmentation, determined by nuclear staining by DAPI (28), was employed as criteria for assessing apoptosis in pure preparations of small and large cholangiocytes from 1-wk BDL rats and 1-wk BDL rats treated with CCl₄ or mineral oil. In small and large cholangiocytes from BDL (control) rat livers, we did not detect apopto-

Fig. 2. Light microscopic analysis of rat liver section (5 µm thick) obtained from 1-wk BDL rat 2 days after administration of single dose of CCl₄. Large bile duct located in portal space shows evidence of apoptosis (arrows) in epithelium lining bile ducts. Sections were stained with hematoxylin and eosin. Original magnification, ×250.

Fig. 3. A: fluorescent assessment of nuclear fragmentation by 4',6-diamidino-2-phenylindole (DAPI) staining in pure preparations of small and large cholangiocytes from 1-wk BDL rats (control) and 1-wk BDL rats treated with single dose of CCl₄. No nuclear fragmentation was observed in small and large cholangiocytes from 1-wk BDL rats (a and d, respectively). On day 2 after acute CCl₄ treatment, apoptosis was present in large (e) but not small (b) cholangiocytes that showed uncondensed homogeneous chromatin. No apoptosis was detected in small (c) or large (f) cholangiocytes on day 7 after acute CCl₄ treatment. Original magnification, ×250. B: quantitative expression of percentage of cholangiocyte apoptosis in CCl₄- or mineral oil-treated BDL rats. *P < 0.05 vs. percentage of cholangiocyte apoptosis of small cholangiocytes.
sis (Fig. 3A, a and d); cells showed homogeneous distribution of chromatin with intact nuclei. In contrast, large cholangiocytes from CCl4-treated BDL rats (Fig. 3A, e) displayed morphological characteristics of apoptosis evidenced by chromatin condensation and nuclear fragmentation (28) on day 2 after CCl4 treatment. No apoptosis was detected in small cholangiocytes on day 2 after acute CCl4 treatment (Fig. 3A, b). Quantitative assessment of percentage of cholangiocytes with nuclear fragmentation showed that CCl4 induced apoptosis in large (but not small) cholangiocytes on day 1 and 2 after acute CCl4 treatment before returning to BDL control levels on day 7 (Fig. 3B). No apoptosis was seen in small or large cholangiocytes from 1-wk BDL rats treated with mineral oil (Fig. 3B). Normal cholangiocytes treated with 10 µM beauvericin (the positive control, results not shown) displayed the same morphological features of apoptosis that we observed in cholangiocytes from CCl4-treated BDL rats.

Measurement of Cholangiocyte Proliferative Capacity

In situ morphometric analysis. By in situ morphometry, we measured the number of small and large intrahepatic bile ducts estimated from the percentage of tissue section occupied by bile ducts smaller and greater than 15 µm in diameter, respectively (Fig. 4). These studies showed that the percent volume of large bile ducts in liver markedly decreased on day 2 after CCl4-treatment compared with 1-wk BDL rats but returned to values similar to that of 1-wk BDL control rats on day 7 after CCl4 treatment (Fig. 4). In contrast to the CCl4-induced loss of large bile ducts, we found an increase in percent volume in the liver of small (<15 µm in diam) bile ducts on day 2 after CCl4 treatment compared with 1-wk BDL liver. The percent volume of small bile ducts in liver returned to values comparable to that of 1-wk BDL control rat liver on day 7 after CCl4 treatment (Fig. 4). No significant changes in the number of large ducts were observed in BDL rats on days 1 and 2 after acute oil treatment (Fig. 4). We found a significant (P < 0.05) increase in the number of large (but not small) ducts on day 7 after oil administration compared with 1-wk BDL control rats (Fig. 4), which is consistent with our previous observations that BDL stimulates proliferative capacity in large but not small cholangiocytes (4).

In vitro assessment of cholangiocyte proliferative capacity. The transcript for the H3 histone gene was absent in small cholangiocytes from 1-wk BDL rats (Fig. 5). In contrast, H3 histone gene expression (normal...
ized to the genetic expression of GAPDH) markedly increased in small cholangiocytes on both days 1 (~70% increase) and 2 (~90% increase) before declining to undetectable levels on day 7 after CCl₄ treatment (Fig. 5). In contrast, in large cholangiocytes H₃ histone gene expression decreased 10% on day 1 and 50% on day 2 before returning to BDL control values 7 days after CCl₄ treatment (Fig. 5). The expression of the housekeeping gene GAPDH was similar among small and large cholangiocytes from BDL, CCl₄-, and oil-treated 1-wk BDL rats (Fig. 5). No changes in H₃ histone gene expression were seen in large cholangiocytes on days 1, 2, and 7 after control oil treatment compared with large cholangiocytes from 1-wk BDL control rats (Fig. 5). The message for H₃ histone was absent in small cholangiocytes from oil-treated rats. Comparability of RNA used was assessed by hybridization for GAPDH (housekeeping gene). Autoradiograms were quantified by densitometry. Densitometric values are means of 2 experiments.

Measurement of Ductal Secretory Activity

SR gene expression. In large cholangiocytes SR gene expression decreased 50% on day 1 and was absent on day 2 after CCl₄ treatment (Fig. 6). In large cholangiocytes, the genetic expression of SR mRNA returned to 1-wk BDL control values on day 7 after CCl₄ treatment (Fig. 6). SR mRNA was absent in small cholangiocytes from BDL rats but there was de novo expression of SR on day 1, which increased further on day 2 before returning to undetectable values on day 7 after acute CCl₄ treatment (Fig. 6). No changes in SR gene expression were seen in large cholangiocytes on days 1, 2, and 7 after control oil treatment compared with large cholangiocytes from 1-wk BDL control rats (Fig. 6). The message for SR was absent in small cholangiocytes on days 1, 2, and 7 following acute oil treatment (Fig. 6).

Intracellular cAMP levels. Ductal secretion was estimated in purified cholangiocytes from measurement of secretin-stimulated cAMP synthesis. In large cholangiocytes from 1-wk BDL rats, secretin markedly increased intracellular cAMP levels (Fig. 7). On day 2 after CCl₄ treatment, the genetic expression of GAPDH markedly increased in small cholangiocytes on both days 1 and 2 before declining to control values on day 7 after CCl₄ treatment. In contrast, H₃ histone gene expression (which was absent in small cholangiocytes from BDL rats) was markedly expressed by small cholangiocytes on days 1 and 2 before returning to control undetectable values on day 7 after CCl₄ treatment. Administration of mineral oil to 1-wk BDL rats did not alter H₃ histone gene expression in purified large cholangiocytes. Transcript for H₃ histone gene was absent in small cholangiocytes from oil-treated rats. Comparability of RNA used was assessed by hybridization for GAPDH (housekeeping gene). Autoradiograms were quantified by densitometry. Densitometric values are means of 2 experiments.
treatment, secretin-induced cAMP levels markedly decreased in large cholangiocytes before returning to values similar to 1-wk BDL rats 7 days after acute CCl4 treatment (Fig. 7). No increase in secretin-induced cAMP synthesis was observed in small cholangiocytes from 1-wk BDL rats (Fig. 7). But on days 1 and 2 after acute CCl4 treatment secretin-stimulated cAMP synthesis appears in small cholangiocytes (Fig. 7). The de novo secretin-induced cAMP synthesis in small cholangiocytes disappeared on day 7 after CCl4 treatment (Fig. 7). The control oil treatment did not affect basal or secretin-stimulated cAMP levels in large cholangiocytes or induce secretin-stimulated cAMP synthesis in small cholangiocytes (Fig. 7).

![Fig. 6. Quantitative expression of secretin receptor (SR) gene in small and large cholangiocytes purified from 1-wk BDL rats and 1-wk BDL rats treated by gavage with single dose of CCl4 or mineral oil. SR gene expression in large cholangiocytes decreased on days 1 and 2 before returning to control values on day 7 after CCl4 treatment. Message for SR was absent in small cholangiocytes from BDL rats but was markedly expressed by small cholangiocytes on days 1 and 2 before returning to control undetectable values on day 7 following CCl4 treatment. Administration of mineral oil to 1-wk BDL rats did not alter H3 histone gene expression in purified large cholangiocytes. Transcript for SR gene was absent in small cholangiocytes from oil-treated rats. Comparability of RNA used was assessed by hybridization for GAPDH (housekeeping gene). Autoradiograms were quantified by densitometry. Densitometric values are means of 2 experiments.](image1)

![Fig. 7. Basal and secretin-stimulated cAMP levels were determined in pure subpopulations of small (left) and large (right) cholangiocytes from 1-wk BDL rats and 1-wk BDL rats treated with CCl4 or mineral oil. Cholangiocytes were stimulated at 22°C for 5 min with secretin (10^{-7} M), and cAMP levels were determined by RIA. * Secretin-induced cAMP levels of small and large cholangiocytes differing from basal cAMP levels are indicated by P < 0.05.](image2)
BDL values on bile flow and bicarbonate secretion returned to control Concomitant with restoration of functions of large cholangiocytes from 1-wk BDL control rats (Fig. 9). P-4502E1 in large cholangiocytes compared with large marked decrease in the expression of cytochrome 1 and biliary bicarbonate secretion was reduced on both marked (Table 3). Similarly, secretin-induced cAMP synthesis in large purified cholangiocytes (Figs. 6 and 7 and Table 3).

Table 3. Effect of acute CCl4 or mineral oil (control) treatment on bile flow and biliary bicarbonate secretion of BDL rats

<table>
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<tr>
<th>Treatment</th>
<th>Bile Flow</th>
<th>Bicarbonate Secretion</th>
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<tr>
<td></td>
<td>Basal, µl·min⁻¹·kg⁻¹</td>
<td>Secretin, µl·min⁻¹·kg⁻¹</td>
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<tr>
<td>BDL 1 wk</td>
<td>102.03±9.42</td>
<td>160.59±16.28*</td>
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<tr>
<td>BDL 1 wk + CCl4 1 day</td>
<td>107.20±6.21</td>
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<td>BDL 1 wk + mineral oil 1 day</td>
<td>93.56±10.27</td>
<td>151.33±6.26*</td>
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<td>BDL 1 wk + CCl4 2 days</td>
<td>128.05±9.31</td>
<td>129.75±12.10</td>
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<td>BDL 1 wk + mineral oil 2 days</td>
<td>113.75±9.15</td>
<td>152.54±7.73*</td>
</tr>
<tr>
<td>BDL 1 wk + CCl4 7 days</td>
<td>92.03±4.14</td>
<td>201.97±6.45*</td>
</tr>
<tr>
<td>BDL 1 wk + mineral oil 7 days</td>
<td>97.57±9.50</td>
<td>160.18±14.65*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats. *P < 0.05 vs. corresponding value of basal bile flow. †P < 0.05 vs. corresponding value of basal biliary bicarbonate secretion. Statistical analysis was performed by both Student’s unpaired t-test and ANOVA.

Studies of biliary physiology. Secretin significantly (P < 0.05) increased both bile flow and bicarbonate secretion in 1-wk BDL rats (control) and 1-wk BDL rats on days 1, 2, and 7 after acute mineral oil treatment (Table 3). In CCl4-treated rats, the choleretic effect of secretin on days 1 and 2 after CCl4 treatment was markedly reduced (Table 3). Similarly, secretin-induced biliary bicarbonate secretion was reduced on both days 1 and 2 after CCl4 treatment. Both secretin-induced bile flow and bicarbonate secretion returned to control BDL values on day 7 after CCl4 treatment (Table 3), indicating restoration of ductal secretory activity. After CCl4 administration, the transient loss of ductal secretion in vivo (i.e., secretin-induced bile flow and bicarbonate secretion) occurred at the same time period as the loss of SR gene expression and secretin-stimulated cAMP synthesis in large purified cholangiocytes (Figs. 6 and 7 and Table 3).

Immunohistochemical and Immunoblotting Analysis of P-4502E1

Immunohistochemistry of frozen liver sections from BDL rats showed positive staining for cytochrome P-4502E1 in bile ducts (Fig. 8). A positive reaction was also observed in hepatocytes. Immunoblotting analysis shows that a signal (migrating at 52 kDa) for cytochrome P-4502E1 was expressed in large but not small cholangiocytes purified from BDL rats (Fig. 9). We found on both days 1 and 2 after CCl4 treatment a marked decrease in the expression of cytochrome P-4502E1 in large cholangiocytes compared with large cholangiocytes from 1-wk BDL control rats (Fig. 9). Concomitant with restoration of functions of large damaged bile ducts, the expression of cytochrome P-4502E1 returned to values similar to that of 1-wk BDL control rats (Fig. 9). Mineral oil treatment did not alter the expression of cytochrome P-4502E1 in large cholangiocytes compared with large cholangiocytes from 1-wk BDL control rats (Fig. 9). Cytochrome P-4502E1 protein expression remained absent in small cholangiocytes purified from CCl4- or oil-treated BDL rats (Fig. 9).

DISCUSSION

The major findings of these studies relate to the development and morphological, biochemical, molecular, and functional characterization of an animal model of selective bile duct damage that is restricted to large hormone-responsive ducts. In 1-wk BDL rats subsequently treated by gavage with a single dose of CCl4, we found that 2 days after CCl4 administration, the number of large (>15 µm in diam) bile ducts decreased. Consistent with the finding of bile duct loss limited to large ducts, 2 days after acute CCl4 treatment, apoptosis was detected in large ducts and cholangiocytes, respectively. DNA synthesis transiently decreased in large cholangiocytes. Inasmuch as large isolated cholangiocytes are localized in large bile ducts in BDL rats (1), the loss of proliferative capacity in large isolated cholangiocytes corresponds with the reduction of numbers of large ducts in 2-day CCl4-treated rats. Finally, consistent with CCl4-induced bile duct damage limited to large hormone-responsive ducts, there was ablation of secretin-induced bicarbonate-rich choleresis in CCl4-treated BDL rats and a marked decrease in SR gene expression and secretin-induced cAMP levels in large purified cholangiocytes. The inhibitory effects of CCl4...
on large cholangiocyte growth and secretion were transient, with restoration to BDL control values by day 7. We have previously shown (1, 3, 8, 10) that small cholangiocytes and small bile ducts are morphologically and functionally different from the remainder of the biliary tree due to their lack of secretin receptor and absence of proliferative response to BDL. The present study shows that small cholangiocytes in small ducts are also different in their pathophysiological response to CCl4. First, we did not observe apoptosis in small purified cholangiocytes or small ducts in situ. Second, small cholangiocytes had increased DNA synthesis leading to an increased number of small bile ducts. Finally, small cholangiocytes developed de novo SR gene expression and secretin-stimulated cAMP synthesis. These studies further extend our previous observations (1, 3, 4, 8, 10) that not only are cholangiocytes in different-sized ducts physiologically heterogeneous with regard to proliferative and secretory processes, they are also fundamentally different in their responses to pathological injury. Moreover, the studies represent the first evidence for a physiological role of small cholangiocytes that do not respond to secretin (1, 3, 8, 10) and are mitotically inactive after BDL (1) but de novo develop secretory activity and begin to proliferate after acute CCl4 treatment. To explain the different sensitivities of small and large cholangiocytes to CCl4 toxicity, we demonstrated that cytochrome P-4502E1, the enzyme that initiates CCl4 hepatotoxicity (15, 19), is present in large but not small cholangiocytes. The loss of duct function and cholangiocyte loss by apoptosis limited to large bile ducts may be initiated by cytochrome P-4502E1 metabolism of CCl4 in large cholangiocytes. Small bile ducts are resistant to damage and to induction of apoptosis due to absence of CCl4 metabolism by cytochrome P-4502E1 in small cholangiocytes. The different sensitivity of small and large cholangiocytes to CCl4 based on cytochrome P-4502E1 presence is completely analogous to the CCl4-induced centrilobular hepatocellular necrosis due to higher cytochrome P-4502E1 in centrilobular region (12). In support of our findings, other investigators have shown cytochrome P-4502E1 in large but not small human bile ducts (22), which would indicate that toxins like CCl4 have the potential to directly injure the human biliary tree as well. Other previous studies (38) have shown selective toxicity of a number of chemicals (e.g., organic arsenicals and chlorpropamide) toward large intrahepatic ducts. These studies, however, failed to quantitate bile duct function in response to injury and did not elucidate the mechanisms of duct injury as we accomplished in the present studies.

Employing a number of end points of cholangiocyte proliferation and secretion, we demonstrated that acute CCl4 treatment induces transient damage to large hormone-responsive ducts in BDL rats, which is associated with a decrease in proliferative capacity and secretory processes of large cholangiocytes. The greatest injury to large ducts occurs on day 2 after acute CCl4 treatment, concomitant with the peak of hepatocellular injury (increased transaminases and decreased albumin gene expression). At the peak of hepatotoxicity on day 2, cytochrome P-4502E1 expression in large cholangiocytes diminishes. This loss of P-4502E1 at the time of maximum toxicity may provide a protective effect from further CCl4-induced damage. Like the transient effects of CCl4 on hepatocyte function (15, 26), large cholangiocytes and large bile ducts respond with proliferation and complete restoration of the numbers of intrahepatic bile ducts, duct secretory function, and cytochrome P-4502E1 expression on day 7 following acute CCl4 treatment. Over the course of CCl4-induced hepatotoxicity, we evaluated the morphological evidence of apoptosis in both in situ liver sections and purified cholangiocytes. We found apoptosis to be low in 1-wk BDL control rats, which indicates that BDL alone does not induce apoptosis in cholangiocytes. On days 1 and 2 after acute CCl4 treatment, we detected by in situ morphometry apoptosis in large but not small bile ducts. Similarly, on days 1 and 2 after CCl4 treatment, we found (by DAPI staining) apoptosis in large but not small isolated cholangiocytes with nuclear fragmentation that was identical to our control beauvericin-induced cholangiocyte apoptosis (28). Although our observations of apoptosis in cholangiocytes contrast with a number of studies showing hepatocyte necrosis after acute CCl4
treatment, it does agree with other models of bile duct loss such as primary biliary cirrhosis or vanishing bile duct syndrome where cholangiocytes are lost by apoptosis (27). Other studies have shown that in addition to necrosis hepatocytes undergo apoptosis in response to acute CCl4 treatment (33).

In addition to showing damage of large bile ducts, the studies demonstrated that small cholangiocytes not only are resistant to CCl4 injury without morphological features of apoptosis but also de novo proliferate beginning 1 day after acute CCl4 treatment. Proliferation of large cholangiocytes after acute CCl4 treatment is delayed, and we postulate that this delay in proliferation is due to selective apoptosis in large cholangiocytes. In support of this concept, apoptosis has been shown to either delay or prevent proliferative response of other cell types (16). The explanation for the resistance of small cholangiocytes to apoptosis may be due to the absence of cytochrome P-4502E1 in small cholangiocytes or perhaps to their more undifferentiated nature (32, 35), similar to the chemical toxin resistance of other precursor cells (30).

Although SR gene expression and secretin-induced cAMP levels are increased in small cholangiocytes on day 2 after CCl4 treatment, secretin did not increase bile flow in vivo. On day 2 after CCl4 treatment small proliferating bile ducts are either inadequate in number or not fully differentiated at this early time point and thus not able to generate bile in response to secretin in vivo. Moreover, the presence and the increase of both SR gene expression and secretin-induced cAMP synthesis are not necessarily associated with enhanced secretin-stimulated ductal bile flow (5, 18, 23). For example, in normal rat liver, SR is present on cholangiocytes (9) and secretin increases cAMP levels in purified cholangiocytes (8, 10, 18, 23). However, secretin still does not increase bile flow.

Proliferation of small bile ducts (which occurs on days 1 and 2 after acute CCl4 treatment) contrasts with the BDL model, where only large cholangiocytes proliferate (4), but is similar to our findings in partial hepatectomized rats where we found that small cholangiocytes transiently proliferate to replace the lost ducts (24). Both CCl4 hepatotoxicity and partial hepatectomy represent models of transient bile duct loss. It is reasonable to speculate that small cholangiocytes proliferate in these models to restore the biliary tree. An extension of this hypothesis there is the potential of migration of small proliferating cholangiocytes into larger ducts, which has been proposed to occur in normal rat liver at a very slow rate (11). Further studies are needed, using labeled cholangiocytes to determine if migration of small cholangiocytes into larger ducts occurs in animal models of bile duct loss, including CCl4-induced toxicity. Although it has been previously shown (35) that undifferentiated cells in terminal bile ducts may rarely be activated by transdifferentiation to hepatocytes, in BDL rats acutely treated with CCl4 we did observe glucose-6-phosphatase expression in our cholangiocyte subpopulations. Our observation that small cholangiocytes transiently proliferate only when large cholangiocyte function is impaired also suggests the possibility that large cholangiocytes may signal small cholangiocytes to begin to proliferate. Further studies, however, are necessary to examine the potential of signaling (cross talk) between cholangiocyte subpopulations of different sizes.

We believe that our novel studies have an important pathophysiological relevance. This is the first study that morphologically and functionally characterizes a model of selective apoptosis restricted to the large hormonally responsive segments of the intrahepatic biliary tree. Although an in vitro model of cholangiocyte apoptosis has been previously described (28), our studies are the first to develop and pathophysiologically characterize an in vivo model of cholangiocyte apoptosis. Our studies showing cholangiocyte apoptosis in a bile duct injury animal model are consistent with previous findings that cholangiocytes are lost by apoptosis in human liver disease (e.g., primary biliary cirrhosis and primary sclerosing cholangitis). Understanding the mechanisms by which specific cholangiocyte subpopulations (originating from different portions of the biliary tree) differentially respond to pathological perturbations (e.g., BDL) or hepatotoxic agents (e.g., CCl4) may increase our knowledge of the selective involvement of certain sized ducts in human cholestatic liver diseases (e.g., primary biliary cirrhosis and primary sclerosing cholangitis).

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REFERENCES


CCL4-INDUCED APOPTOSIS OF CHOLANGIOCYTES


