Regression of cholangiocyte proliferation after cessation of ANIT feeding is coupled with increased apoptosis

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1Departments of Internal Medicine and 2Medical Physiology, 3Division of Research and Education, Scott & White Hospital and The Texas A&M University System Health Science Center, College of Medicine and 4Central Texas Veterans Health Care System, Temple, Texas 76504; 5Third Department of Internal Medicine, Tohoku University School of Medicine, Aobaku, Sendai, Japan 980-8574; and 6Division of Gastroenterology, University of Rome, La Sapienza, Rome, Italy 00100

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LeSage, Gene, Shannon Glaser, Yoshiyuki Ueno, Domenico Alvaro, Leonardo Baiocchi, Noriatsu Kanno, Jo Lynne Phinizy, Heather Francis, and Gianfranco Alpini. Regression of cholangiocyte proliferation after cessation of ANIT feeding is coupled with increased apoptosis. Am J Physiol Gastrointest Liver Physiol 281: G182–G190, 2001.—Cholangiocyte proliferation and loss through apoptosis occur in cholestatic liver diseases. Our aim was to determine the mechanisms of apoptosis in an animal model of ductal hyperplasia. Rats were fed α-naphthylisothiocyanate (ANIT) for 2 wk and subsequently fed normal chow for 1, 2, and 4 wk. Proliferation was assessed in sections by morphometry and in small and large cholangiocytes by proliferating cellular nuclear antigen immunoblots and measurement of cAMP levels. Apoptosis and reactive oxygen species (ROS) levels were also assessed. ANIT feeding increased small and large cholangiocyte proliferation and apoptosis. Cessation of ANIT feeding was associated with decreased proliferation and a further increase in apoptosis in small and large cholangiocytes. Cholangiocytes from ANIT-fed rats or exposed to ANIT in vitro showed increased apoptosis and ROS generation. ANIT-induced duct injury results in enhanced proliferation and apoptosis in small and large cholangiocytes. The mechanism of ANIT-induced apoptosis may be due to ROS generation induced directly by ANIT. Our model has implications for understanding the pathophysiology of cholangiopathies (characterized by the coexistence of cholangiocyte apoptosis and proliferation).

IN THE NORMAL STATE, CHOLANGIOCYTES are mitotically quiescent (3, 32); however, they proliferate in response to injury/toxins, including bile duct ligation (BDL) (4–6), CCl4 (33, 35), and α-naphthylisothiocyanate (ANIT) feeding (5), resulting in ductal hyperplasia or damage (3–6, 33, 35). A number of studies (25, 26, 38) have shown that ANIT treatment alters hepatic glutathione levels. In human liver diseases characterized by chronic cholestasis, cholangiocytes markedly proliferate (6, 14–16, 44). Proliferation may be triggered by local inflammation (39), increased ductal pressure (47), increased bile acids (2), or alteration of portal nerve function or hormone signaling (31). Cholangiocyte loss, due to apoptosis leading to ductopenia, is also observed in these diseases (11, 12, 27, 31, 33, 35, 39). Apoptosis may stimulate cholangiocyte proliferation to compensate for loss of ductal mass and function (33, 35). For instance, acute CCl4 administration induces cholangiocyte loss through apoptosis, which subsequently results in compensatory cholangiocyte proliferation (33, 35). The CCl4 model of cholangiocyte apoptosis is dissimilar to human liver diseases in which apoptosis and proliferation coexist (14, 15). The cellular mechanisms resulting in cholangiocyte apoptosis in animal models and human liver diseases remain obscure.

The intrahepatic biliary tree is heterogeneous regarding morphology and secretory, proliferative, and apoptotic activities (1, 3, 7, 8, 28, 33, 35). Specifically, small ducts (<15 μm in diameter) are lined by small cholangiocytes (8 μm in size), whereas larger ducts (>15 μm in diameter) are lined by larger cholangiocytes (13 μm in size) (1, 7). Large ducts are the major anatomic sites of secretin-stimulated ductal secretion (1, 7, 28), whereas small ducts do not respond to secretin (1, 7, 28). Following BDL, cholangiocyte proliferation is restricted to large cholangiocytes (3, 33). In contrast, after 70% hepatectomy (34) or CCl4-induced duct damage (33, 35), small and large ducts proliferate, contributing to the regrowth of the biliary tree. The function of small cholangiocytes remains undefined due to the unavailability of experimental models to study the pathophysiology of small cholangiocytes.

One of the mechanisms thought to trigger apoptosis is the accumulation of reactive oxygen species (ROS) in
cells (37). The increase in intracellular ROS levels correlates with cell death caused by a variety of proapoptotic stimuli, including drugs (30). Antioxidants protect cells from apoptosis, and \( \text{H}_2\text{O}_2 \) induces apoptosis in a variety of cells (13). Although a role for ROS in transducing apoptotic signals from the extracellular environment has been suggested, recent studies suggest that ROS may act downstream of the caspase system (48).

The aim of this study was to develop a model of coexisting cholangiocyte proliferation and apoptosis and to determine if ROS plays a role in cholangiocyte apoptosis. We first fed normal rats 0.1% ANIT or control diet for 2 wk and subsequently fed them normal chow for 1, 2, and 4 wk. At the selected feeding times, we measured proliferation and apoptosis in isolated small and large cholangiocytes. To evaluate the mechanisms by which small and large cholangiocytes undergo ANIT-induced apoptosis, we measured both in vivo and in vitro ROS in small and large cholangiocytes.

**MATERIALS AND METHODS**

**Animal model.** Male Sprague-Dawley rats (150–175 g) were purchased from Charles River (Wilmington, MA). The animals were kept in a temperature-controlled environment (22°C) with a 12:12-h light/dark cycle. The studies were conducted in 1) rats fed 0.1% ANIT or control diet (AIN 76) for 2 wk and 2) rats first fed 0.1% ANIT for 2 wk and subsequently fed normal chow for 1, 2, and 4 wk. Before each procedure, animals were anesthetized with sodium pentobarbital (50 mg/kg ip). In all of the animals used, we measured wet liver weight, body weight, and wet liver weight-to-body weight ratio.

**Materials.** Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Rat chow was prepared by Dyets (Bethlehem, PA). Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA). RIA kits for the determination of intracellular cAMP levels were purchased from Amersham (Arlington Heights, IL). The mouse anti-cytokeratin 19 (CK-19) antibody was purchased from Amersham (Arlington Heights, IL). The monoclonal mouse antibody against the secretin receptor is upregulated in all of the models of cholangiocyte hyperplasia (2, 3, 9, 18, 32, 33, 35) and downregulated during depressed cholangiocyte proliferation (33, 35). In situ immunohistochemistry for PCNA is an approach that has previously been used for evaluating cellular proliferation in a number of cell types (17, 21, 41). Western blot analysis was performed as follows. Rat spleen (100 µg) and BSA (100 µg) were the positive and negative controls, respectively. Small and large cholangiocytes (3.0 × 10^6) were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 µM aprotinin, 1 µM phenylmethylsulfonyl fluoride, and 1 M leupeptin) and sonicated six times (30-s bursts). Proteins (100 µg/lane) were resolved by SDS-7.5% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose filter. After blocking, the filter was incubated overnight at 4°C with a rabbit anti-PCNA antibody (1:200) followed by incubation with an anti-rabbit biotinylated anti-mouse immunoglobulin (ECL Kit; Amersham Life Science, Little Chalfont, England) diluted 1:100,000 with 50 mM Tris, 150 mM NaCl, and 0.05% Tween 20. Following several washes, the filter was visualized using chemiluminescence (ECL Plus kit; Amersham). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low light imaging system (Alpha Innotech, San Leandro, CA).

As an independent marker of proliferation, we measured basal and secretin-stimulated cAMP levels in purified cholangiocytes. The secretin receptor is upregulated in all of the models of cholangiocyte hyperplasia (2, 3, 9, 18, 32, 33, 35) and downregulated during depressed cholangiocyte proliferation (33, 35). Intracellular cholangiocyte cAMP levels were determined by RIA (7, 8, 32, 33, 35) by using a commercially available kit (Biotrak-RPA509, Biotrak-cAMP [125I] assay, Amersham). Small and large cholangiocytes were incubated for 1 h at 37°C and stimulated with 0.2% BSA (basal) or 100 nM secretin for 5 min at 22°C (7, 8, 32, 33, 35). Following extraction with ethanol, intracellular cAMP levels were measured.

**Effect of ANIT feeding on proliferation and apoptosis.** Cholangiocyte proliferative capacity was evaluated in liver sections by quantitative localization of PCNA in small and large intrahepatic bile ducts. PCNA is a nonhistone nuclear protein that plays an important role in DNA replication and cellular proliferation by interacting with DNA polymerase-δ (29). In situ immunohistochemistry for PCNA was performed as follows. After blocking of endogenous peroxidase activity by methanol-peroxidase solution, nonspecific binding was blocked by incubation with normal goat serum for 30 min at room temperature. Paraffin-embedded sections (5 µm thick) were incubated with anti-PCNA antibody (clone PC-10; Oncogene Research Products, Cambridge, MA) at 4°C overnight. After several washes with cold PBS, biotin-labeled secondary antibody at a dilution of 1:100 (DAKO) was added for 1 h at room temperature. To detect this reaction, a peroxidase-labeled avidin/biotin complex with diaminobenzidine (Dojin Chemical, Kumamoto, Japan) was used as a substrate. Over 100 small and large cholangiocytes were counted in three different fields for each group of animals. Sections were counterstained with hematoxylin and examined with a microscope (Olympus BX 40; Olympus Optical, Tokyo, Japan).

**Morphometry of liver sections.** The percentage of liver sections with positive immunoreactivity for PCNA was determined by point count analysis (49) by determining the number of small and large ducts stained for CK-19 (6, 32). This technique has previously been used by us (32, 33, 35) to measure intrahepatic ductal mass in both normal and rat livers with ductal hyperplasia. Sections were examined with an Olympus BX 40 microscope.

Proliferative capacity was also assessed by measurement of protein expression (by Western blot analysis) for PCNA in small and large purified cholangiocytes. Western blot analysis for PCNA is an approach that has previously been used for evaluating cellular proliferation in a number of cell types (17, 21, 41). Western blot analysis was performed as follows. Rat spleen (100 µg) and BSA (100 µg) were the positive and negative controls, respectively. Small and large cholangiocytes (3.0 × 10^6) were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 µM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 M leupeptin) and sonicated six times (30-s bursts). Proteins (100 µg/lane) were resolved by SDS-7.5% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose filter. After blocking, the filter was incubated overnight at 4°C with a rabbit anti-PCNA antibody (1:200) followed by incubation with an anti-rabbit biotinylated anti-mouse immunoglobulin (ECL Kit; Amersham Life Science, Little Chalfont, England) diluted 1:100,000 with 50 mM Tris, 150 mM NaCl, and 0.05% Tween 20. Following several washes, the filter was visualized using chemiluminescence (ECL Plus kit; Amersham). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low light imaging system (Alpha Innotech, San Leandro, CA).

**Purification of small and large cholangiocytes and ducts.** Pure small and large cholangiocytes (by γ-glutamyltranspeptidase histochemistry (46)) were purified by immunoaffinity bead separation (7, 8, 35) by using a mouse monoclonal oval cell-2 antibody (IgM; kindly provided by Dr. R. Faris, Brown University, Providence, RI) against an unidentified membrane antigen expressed by all rat intrahepatic cholangiocytes (23). Cell viability was determined by RIA (7, 8, 32, 33, 35) and downregulated during depressed cholangiocyte proliferation (33, 35). Intracellular cholangiocyte cAMP levels were determined by RIA (7, 8, 32, 33, 35) by using a commercially available kit (Biotrak-RPA509, Biotrak-cAMP [125I] assay, Amersham). Small and large cholangiocytes were incubated for 1 h at 37°C and stimulated with 0.2% BSA (basal) or 100 nM secretin for 5 min at 22°C (7, 8, 32, 33, 35). Following extraction with ethanol, intracellular cAMP levels were measured.
were measured by a commercially available kit (Amersham) according to the vendor’s instructions.

Apoptosis. Small and large cholangiocytes purified from control or ANIT-fed rats and rats fed ANIT followed by normal chow for 1, 2, and 4 wk were analyzed for apoptosis by annexin-V staining [a specific marker of early-stage apoptosis (20)] as previously described by us (31). Briefly, purified small and large cholangiocytes \((1 \times 10^6)\) were incubated for 10 min with a biotinylated antibody reacting with annexin-V 1:50 dilution in HEPES buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 5 mM CaCl\(_2\)) and spun down with a cytopin device (100 g for 5 min) onto glass slides. Cell smears were soaked in methanol/ethanol 1:1 for 90 s and incubated for 1 h at room temperature with streptavidin-horseradish peroxidase conjugate (1:1,000 dilution in HEPES buffer). Following washes with HEPES buffer, cell smears were incubated with diaminobenzidine (1:9 solution in H\(_2\)O\(_2\)) solution for 15 min, counterstained for 10 s with hematoxylin solution, and examined by light microscopy with an Olympus BX 40 microscope equipped with a camera. At least 300 cells/slide were counted in a coded fashion.

Does ANIT in vitro induce cholangiocyte apoptosis? We next evaluated in purified pooled cholangiocytes [which are composed of small and large cholangiocytes (3, 7, 8)] from normal rats whether ANIT induces in vitro cholangiocyte apoptosis and if the oxygen scavenger ethyl ester glutathione prevents the increase in cholangiocyte apoptosis due to in vitro ANIT treatment. Purified pooled cholangiocytes from normal rats were treated for 2 h at 37°C with ANIT (1–10 \(\mu\)M), H\(_2\)O\(_2\) (100 \(\mu\)M), or ANIT plus ethyl ester glutathione (2

Table 1. Liver weight, body weight, and liver weight-to-body weight ratio in rats fed first with 0.1% ANIT or control diet for 2 wk and subsequently with normal chow for 1, 2 and 4 wk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver Weight, g</th>
<th>Body Weight, g</th>
<th>Ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7.50 ± 0.11</td>
<td>162.20 ± 1.91</td>
<td>4.61 ± 0.60</td>
</tr>
<tr>
<td>ANIT 2 wk</td>
<td>7.27 ± 0.34</td>
<td>122.50 ± 3.70</td>
<td>5.88 ± 0.14*</td>
</tr>
<tr>
<td>ANIT 2 wk + Normal Diet 1 wk</td>
<td>12.25 ± 0.67</td>
<td>206.93 ± 8.89</td>
<td>5.93 ± 0.23*</td>
</tr>
<tr>
<td>ANIT 2 wk + Normal Diet 2 wk</td>
<td>14.03 ± 0.64</td>
<td>261.09 ± 10.70</td>
<td>5.38 ± 0.09*</td>
</tr>
<tr>
<td>ANIT 2 wk + Normal Diet 4 wk</td>
<td>19.29 ± 0.76</td>
<td>390.61 ± 6.87</td>
<td>4.90 ± 0.14*</td>
</tr>
</tbody>
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Values are means ± SE of 17–36 rats. ANIT, \(\alpha\)-naphthylisothiocyanate. *\(P < 0.05\) vs. corresponding values in control rats.

Fig. 1. Top: immunohistochemistry for proliferating cellular nuclear antigen (PCNA) in liver sections from rats fed \(\alpha\)-naphthylisothiocyanate (ANIT) (B) or control diet (A) for 2 wk. No PCNA-positive cells were observed in control rats. The number of PCNA-positive cholangiocytes (arrows) increased in liver sections from ANIT-fed rats. At cessation of ANIT feeding, there was a decrease in the number of PCNA-positive cholangiocytes that, however, remained higher than controls. Original magnification, \(\times 1,000\). Bottom: data from small (C) and large (D) ducts are shown. Data are means ± SE of 3 experiments.
Subsequently, cells were incubated with 1 µM 4,6-diamidino-2-phenylindole (DAPI) for 10 min at 37°C 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. Nuclei were visualized from the fluorescent DNA binding dye DAPI (40). Cholangiocytes were considered apoptotic if nuclear invagination, condensation, and fragmentation were observed (40); 300 cells were analyzed for each experimental group. Apoptosis was expressed as total of the cells counted. We used as a positive control normal cholangiocytes treated with beauvericin (10 µM) for 4 h at 37°C (33, 35, 40).

**Measurement of ROS.** We next evaluated intracellular ROS concentration in 1) small and large cholangiocytes purified from rats fed control or ANIT diet and rats fed ANIT followed by normal food for 4 wk and 2) small and large normal cholangiocytes treated in vitro at 37°C with ANIT (10 µM for 2 h), H₂O₂ (100 µM for 2 h), or ethyl ester glutathione (2 µM for 15 min) + ANIT (10 µM for 2 h). The H₂O₂ concentration in cholangiocytes was measured by employing the ROS indicator dye 2',7'-dichlorofluorescein (DCF; Molecular Probes, Eugene, OR). DCF is nonionized and membrane permeant and is thus able to diffuse into cells (24). Within the cell, the acetate groups are removed by intracellular esterase, resulting in a polar molecule that is trapped within cells. DCF fluoresces when it is oxidized by H₂O₂ or lipid peroxides (24). The level of DCF fluorescence in cells is related linearly to the amount of peroxides present within the cell. DCF stock (10⁻³ M) was prepared in DMSO and diluted to 10⁻⁵ M to add to cells. After 30 min of incubation, excess extracellular DCF was removed by washing and cells were mounted on the stage of a Nikon fluorescence microscope. The fluorescence was quantitated by a cooled charge-coupled device camera (Photometerics), and data were analyzed by employing Isee software (Envision, Durham, NC). The regions of interest, defined by cell cytoplasm, were identified, and total fluorescence for each cell was quantitated.

**Statistical analysis.** All data are expressed as means ± SE. The differences between two groups were analyzed by Student's t-test.

**RESULTS**

**Liver weight and liver weight-to-body weight ratio.** The wet liver weight-to-body weight ratio was higher in ANIT-fed rats compared with controls (Table 1).
Following cessation of ANIT feeding, the wet liver weight-to-body weight ratio decreased but remained higher than in control-fed rats (Table 1). The number of PCNA-positive small and large cholangiocytes significantly \((P < 0.05)\) increased following ANIT feeding (Fig. 1). At cessation of ANIT feeding, there was a gradual decrease in the number of PCNA-positive cholangiocytes, which, however, remained higher than control values (Fig. 1).

Two weeks after ANIT feeding, we found an increased number of small and large ducts compared with control rats (Fig. 2). Cessation of ANIT feeding induced a gradual decrease in the number of small and large ducts (Fig. 2). Although decreased, the number of small and large ducts remained higher than that of control rats even 4 wk after cessation of ANIT feeding (Fig. 2).

DNA replication was active in large (but not small) cholangiocytes from control-fed rats (Fig. 3). ANIT feeding induced a significant \((P < 0.05)\) increase in PCNA protein expression in large cholangiocytes and de novo DNA replication in small cholangiocytes. Following cessation of ANIT feeding, DNA replication of small and large cholangiocytes declined but remained higher than that of control cholangiocytes even after 4 wk of normal feeding (Fig. 3).

Consistent with increased cholangiocyte proliferation, ANIT feeding induced an increase in secretin-stimulated cAMP levels in large cholangiocytes (Fig. 4). ANIT feeding induced the de novo expression of secretin-stimulated cAMP levels in small cholangiocytes (Fig. 4), which are constitutively unresponsive to secretin (1, 3, 7, 8, 35). Secretin-stimulated cAMP levels of small and large cholangiocytes remained higher than controls even 4 wk after cessation of ANIT feeding (Fig. 4).

**Apoptosis.** We evaluated whether alterations in cholangiocyte proliferation are associated with temporal and reciprocal changes in cholangiocyte apoptosis. We found that ANIT-induced increases in pro-

Fig. 3. Measurement of PCNA protein expression in small and large cholangiocytes from rats first fed ANIT or control diet for 2 wk and subsequently fed normal food for 1, 2, and 4 wk. Autoradiograms were quantified by densitometry. Data are means \(\pm\) SE of 4 experiments. *\(P < 0.05\) vs. corresponding values in control-fed rats.

Fig. 4. Measurement of cAMP levels in small (A) and large (B) cholangiocytes from rats first fed ANIT or control diet and subsequently fed normal food for 1, 2, and 4 wk. Data are means \(\pm\) SE of 3–12 experiments. *\(P < 0.05\) vs. corresponding basal values.
liferative capacity of small and large cholangiocytes were associated with apoptosis of these cells (Fig. 5). Regression of cholangiocyte proliferation (at cessation of ANIT feeding) was associated with a further increase of apoptosis of small and large cholangiocytes (Fig. 5). However, at 4 wk after cessation of ANIT feeding, apoptotic activity, although lowered, remained higher than in control cholangiocytes (Fig. 5). The direct effects of ANIT on cholangiocyte apoptosis were first determined in vitro in pooled cholangiocytes, which include small and large cholangiocytes (3, 7, 8). Dose-dependent studies showed that 1, 2, and 10 μM ANIT induced apoptosis in 0.8 ± 0.2, 2.1 ± 0.6, and 6.5 ± 1.1% of cholangiocytes, respectively. Since the 10-μM concentration induced apoptosis to a similar degree to that observed in vivo, we employed this concentration for the studies. ANIT increased cholangiocyte apoptosis; however, pretreatment of cholangiocytes with ethyl ester glutathione reduced ANIT-induced apoptosis (Fig. 6).

Measurement of ROS. Small and large cholangiocytes were isolated from control, ANIT-fed rats and ANIT-fed rats subsequently fed normal food for 4 wk. DCF fluorescence, which is proportional to intracellular ROS concentration, was higher in ANIT-fed rats, as shown by the shift of the fluorescence histogram to the right (higher fluorescence) in both small and large cholangiocytes (Fig. 7). In rats fed ANIT and then control diet, ROS generation decreased but was higher than in control rats (Fig. 7).

Intracellular ROS was increased in both small and large cholangiocytes treated in vitro with ANIT or H₂O₂ (Fig. 8). In ANIT- and H₂O₂-treated cells there was a shift of the fluorescence histogram to the right in both small and large cholangiocytes (Fig. 8), indicating increased ROS generation. The oxygen scavenger ethyl ester glutathione prevented ROS production due to ANIT (Fig. 8).

**DISCUSSION**

These studies show that ANIT administration to rats induces coexisting cholangiocyte apoptosis and proliferation. During ANIT feeding, both small and large cholangiocytes (lining small and large ducts, respectively) proliferate and undergo apoptosis. During ANIT feeding, proliferation is the predominant event, since we observed an increase in duct mass. After cessation of ANIT feeding, apoptosis became the predominant event, with a progressive decline in proliferation but increase in cholangiocyte apoptosis, resulting in a reduction of duct mass. In vitro, we found that ANIT directly increased apoptosis and ROS levels in cholangiocytes and inhibition of ROS prevented ANIT-induced apoptosis. In vivo, ROS generation in cholangiocytes from ANIT-fed rats was greater than control both during and after ANIT feeding. These studies show that ANIT-induced cholangiopathy is similar to human cholestatic liver diseases, with the presence of apoptosis coexisting with proliferation (14, 15). Also, the overall duct mass resulted from a balance between proliferation and apoptosis. Finally, these studies establish a role for ROS in a drug-induced model of duct injury for both the induction of apoptosis and the persistence of apoptosis after drug withdrawal.

Small or large ducts may be exclusively involved in human cholestatic liver disease (3, 6, 33, 35). In primary biliary cirrhosis, larger intrahepatic bile ducts show apoptosis, whereas in drug-induced ductopenia, smaller ducts have apoptotic cholangiocytes (14–16).
Similarly, in animal models, proliferation or apoptosis is restricted to certain sizes of ducts (3, 31, 33, 35). BDL induces proliferation only in large bile ducts (3, 33). CCl₄ induces de novo proliferation only in small cholangiocytes (33, 35), whereas partial hepatectomy causes proliferation of both small and large cholangiocytes (34). CCl₄ induces cholangiocyte apoptosis like ANIT; however, apoptosis is restricted to large ducts (33, 35). Thus the ANIT model is unique among animal models of cholangiopathies, with both coexisting apop-

![Fig. 7. ANIT feeding increases intracellular ROS in both small (A) and large (B) cholangiocytes. Small and large cholangiocytes were isolated from control, ANIT-fed rats, and ANIT-fed rats subsequently fed with normal food for 4 wk. ROS generation was detected by the fluorescent indicator 2',7'-dichlorofluorescein (DCF) as described in MATERIALS AND METHODS. The histograms show number of cells vs. cellular fluorescence. Consistent with increased ROS generation in cholangiocytes from ANIT-fed rats, there was a shift of the fluorescence histogram to the right in both large and small cholangiocytes. In rats fed ANIT, then control diet, ROS generation was similar to control rats.]

![Fig. 8. ANIT increased cholangiocyte intracellular ROS. Small (B) and large (A) isolated rat cholangiocytes were pretreated with ethyl ester glutathione for 15 min, then treated with ANIT (10 µM) or H₂O₂ (100 µM) for 2 h. ROS generation in cholangiocytes was detected by DCF (1 µg/ml) added 30 min before visualization. The fluorescence intensity of DCF is proportional to levels of ROS. In ANIT- and H₂O₂-treated cells there was a shift of the fluorescence histogram to the right in both large and small cholangiocytes, indicating increased ROS generation. The oxygen scavenger ethyl ester glutathione prevented ROS production due to ANIT.]

G188 ANIT INDUCTION OF CHOLANGIOCYTE GROWTH AND APOPTOSIS
osis and proliferation present in small and large ducts.

The effect of ANIT on cholangiocyte apoptosis was persistent even 4 wk after cessation of treatment. This finding contrasts with other models of ductal hyperplasia (BDL, partial heptectomy, and acute CCl4 treatment), in which cholangiocyte proliferation is transient (32–35) or completely normalizes with removal of injury (10). Although cholangiocyte apoptosis may completely disappear after 4 wk, this represents the first described model of persistent cholangiocyte apoptosis. Also, the model has relevance to drug-induced ductopenic syndromes, in which, even after removal of the drug, cholestasis, once acquired, remains persistent for periods of up to 1 yr (16).

The apoptotic process consists of an activation and an execution phase (45). Previous studies (22) have suggested that ROS may be a key element in the activation of apoptosis in many cell types, including cholangiocytes (42). ROS levels in cells correlate with the presence of cell death (22). Antioxidants protect cells from apoptosis, and experimentally increased ROS levels induce apoptosis (22). Increased Bcl-2 expression is protective against apoptosis, and Bcl-2 may at least partially function as an intracellular antioxidant (19). Other mechanisms for ROS activation of apoptosis may involve activation of transcriptional factors or kinases (42). We demonstrated that ANIT or H2O2 in vitro induces ROS formation in small and large cholangiocytes. The ANIT-induced increase in ROS and apoptosis in purified cholangiocytes is prevented by pretreatment with the antioxidant ethyl ester glutathione. The data show that ANIT can directly induce apoptosis, and we propose that the initial increase in apoptosis during ANIT feeding is due at least in part to the direct effects of ANIT on cholangiocyte ROS production. Since ANIT has been shown to alter hepatic glutathione levels (25, 26, 38), low cholangiocyte glutathione may predispose cholangiocytes to ROS generation and ROS-mediated apoptosis in this model. In vivo, ROS is increased in both small and large cholangiocytes while rats are on the ANIT diet. Subsequent to removal of ANIT from the diet, cholangiocyte ROS levels decrease but remain at values higher than controls. The persistence of high ROS production after ANIT removal may be due to persistence of portal inflammation that occurs in the ANIT model, with cytokine release from periductular inflammatory cells inducing ROS production. The observation of a persistent ROS increase after ANIT removal is potentially important, since it suggests that antioxidant drugs could be therapeutic in the recovery from a prolonged phase of drug-induced duct injury (6, 44) or in chronic liver diseases with persistent ductopenia (6, 44).

Cholangiocyte proliferation was evaluated by several complementary independent methods. Similar to our previous studies (2, 3, 18, 32, 35) in rats with BDL, partial heptectomy, and fed bile acids, we have found increased cholangiocyte PCNA expression, increased number of PCNA-positive cholangiocytes and CK-19-positive ducts, and increased secretin-stimulated cAMP levels in proliferating cholangiocytes. Our data are consistent with previous studies demonstrating that ANIT feeding induces cholangiocyte proliferation (5, 10) and other studies showing that (following ANIT withdrawal) cholangiocytes undergo apoptosis (10). However, the previous studies (5, 10) did not show that apoptosis coexists with proliferation, determine the size of intrahepatic bile ducts affected by ANIT, or demonstrate the involvement of ROS in cholangiocyte apoptosis as we did in this study.

In summary, these studies demonstrate the relationship between cholangiocyte proliferation and apoptosis as they determine bile duct structure in a drug-induced bile duct injury model. The studies may be important to better understand the course of cholestatic liver diseases characterized by the coexistence of cholangiocyte damage and proliferation, the latter representing a mechanism of bile duct repair, which determines the outcome of the disease (14, 15). These studies for the first time implicate increased ROS generation in the initiation and persistence of bile duct injury in an animal model, which could also happen in human cholangiopathies. The potential for cytoprotective action through antioxidant therapy in cholangiopathies should be explored. Finally, the study underscores the importance of evaluating the zonal nature of different forms of bile duct injury (6).

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