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Progesterone stimulates the proliferation of female and male cholangiocytes via autocrine/paracrine mechanisms

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sion, progesterone may be an important autocrine/paracrine regulator of cholangiocyte proliferation.

bile ducts; gastrointestinal hormones; intrahepatic biliary epithelium

CHOLANGIOCYTES ARE THE TARGET cells in cholangiopathies including primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) (4, 8). During the progression of cholangiopathies, proliferation/loss of cholangiocytes is critical for the maintenance of biliary secretory function and intrahepatic ductal mass (2, 4, 8). In recent years, considerable progress has been made in demonstrating that cholangiocytes differentially respond to cholestasis and liver injury induced by hepatotoxins with alterations in proliferation and secretion (4, 8, 26, 27, 43, 44, 48). In fact, proliferating cholangiocytes serve as a neuroendocrine organ during liver disease pathogenesis and, as such, secrete and respond to a number of hormones and neuropeptides contributing to the autocrine and paracrine pathways, which modulate liver inflammation and fibrosis (8). In rat models of experimental cholestasis including bile duct ligation (BDL), there is increased cholangiocyte growth and ductal secretory activity (1, 2, 8).

Progesterone is a steroid hormone synthesized by the ovaries and adrenal glands, by the corpus luteum during pregnancy, and in the central and peripheral nervous system (16, 50). The first step in the biosynthesis of steroids is the conversion of cholesterol to pregnenolone, which is the precursor of all steroid hormones (63). The rate-limiting step of steroidogenesis is the rate of cholesterol transport from intracellular stores to the inner mitochondrial membrane, which is the location of cytochrome P450 side-chain cleavage (p450scc) (63). The steroidogenic acute regulatory protein (StAR) mediates this rate-limiting step of steroidogenesis by regulating the translocation of cholesterol from the outer to the inner mitochondrial membrane (46). Once in the inner mitochondrial membrane, P450scc catalyzes the transformation of cholesterol to preg-

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nenolone (46, 74). Pregnenolone moves from the mitochondria to the microsomal compartment where it is converted to the Δ 4,3-keto steroid progesterone by 3β -hydroxysteroid dehydrogenase (3β -HSD) (72).

The biological responses triggered by progesterone are mediated by both genomic and nongenomic mechanisms. The genomic action of progesterone is mediated by two progesterone receptor (PR) isoforms, PR-A and PR-B, that are members of the nuclear receptor family of ligand-dependent transcription factors (45). The progesterone gene contains multiple promoters from which PR-A and PR-B are transcribed (45). PR-B differs from PR-A only in that PR-B contains an additional stretch of 164 amino acids at the NH₂ terminus of the protein (45). The binding of progesterone to the PR promotes its translocation from the cytoplasm to the nucleus and initiates receptor dimerization, DNA binding, and subsequent transcription of genes containing progesterone response elements (41). In most cases, PR-A and PR-B have distinct transcription activities despite having similar steroid and DNA-binding activities (19). PR-B is a strong activator of gene transcription, whereas PR-A can act as a ligand-dependent trans-repressor of PR-B (19). The large majority of PR target genes have been identified in breast cancer cells (60). Most of these target genes were regulated by PR-B, which included upregulation of the expression of proteins regulating cell adhesion, cell growth, membrane bound receptors, transcription and protein processing (60). In addition, progesterone can activate the gene transcription of vascular endothelial growth factor (VEGF) in endometrial adenocarcinoma cells (52). Another mechanism has also recently been uncovered by which PR can mediate nongenomic responses through their ability to directly recruit and activate c-Src (11). In addition to genomic actions, progesterone has been recently shown to activate nongenomic membrane-initiated signaling mechanisms (54, 57). These effects are mediated by the progesterone receptor membrane component protein (PGRMC1 and PGRMC2) (12, 38). Activation of progesterone membrane receptors has been shown to stimulate increased intracellular Ca²⁺ in oocytes (75). However, the downstream signaling mechanisms have not been fully explored. The human homolog of PGRMC1, hpr6, plays a role in decreasing the susceptibility of cancer cells to chemotherapeutic drugs (17, 18). PGRMC1 is a dioxin-inducible gene, which suggests a possible role in stress responses and has been implicated in mediating progesterone's neuroprotective effects (29, 39, 64).

A number of studies have shown that sex hormones regulate cholangiocyte functions. Estrogens enhance proliferation and secretion of male cholangiocytes by interaction with estrogen receptors (7). We have also shown (6) that 1) ovariectomy to BDL female rats induced a decrease in the number of bile ducts and 2) administration of 17- β estradiol to BDL, ovariectomized rats prevented the decrease in intrahepatic ductal mass. In addition, we have shown that estrogens in male cholangiocytes activate the Ras/Raf/Src/Shc/ERK1/2 pathway, thereby synergizing the effects of different growth factors including nerve growth factor (NGF) and insulin-like growth factor I (9, 26). In addition, we have shown that prolactin stimulates cholangiocyte proliferation of female rats by activation of the Ca²⁺/PKC signaling pathway (70). Studies have demonstrated the uptake of (7 α -³H) progesterone by mouse liver tissue (71). In adult female prairie dogs, progesterone impairs gallbladder

emptying in response to cholecystokinin (73). Furthermore, progesterone has been shown to induce cell proliferation and an abnormal mitotic process in male rat liver (10). However, very little information is known concerning the effects of progesterone on the biliary system and more specifically, the effects of progesterone on cholangiocyte proliferation of female and male rats have not been evaluated.

The present study was performed to address 1) the presence of progesterone receptors in both female and male cholangiocytes and 2) the role of progesterone in the regulation of cholangiocyte proliferation of both female and male normal and BDL rats. We found that cholangiocytes express both the nuclear (PR-A and PR-B) receptors and the PGRMC1 and PGRMC2 membrane receptors and the newly identified mPR α (13). In both female and male rats, we demonstrated that chronic progesterone treatment increases bile duct mass of normal rats, whereas administration of antiprogestrone to rats with extrahepatic cholestasis induced by BDL prevents cholestasis-induced proliferation. In addition, since we demonstrated that cholangiocytes possess the enzymatic pathway for steroidogenesis, we propose that the proliferative effects induced by progesterone on cholangiocytes are regulated by an autocrine pathway (in addition to paracrine mechanisms).

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The monoclonal mouse antibody reacting with proliferating cellular nuclear antigen (PCNA) was purchased from DAKO (Kyoto, Japan). The substrate for γ -glutamyl-transpeptidase (γ -GT), *N*-(γ -L-glutamyl)-4-methoxy-2-naphthylamide was purchased from Polysciences (Warrington, PA). Progesterone EIA kits for the measurement of progesterone levels in serum and cholangiocyte supernatant from the selected groups of animals were purchased from Cayman Chemical, Ann Arbor, MI. The rabbit polyclonal antibody for the nuclear progesterone receptor PR (C-20) (PR-A and PR-B), the rabbit polyclonal antibody for StAR (FL-285), the goat polyclonal antibody for 3 β -HSD (C-18), and the goat polyclonal antibody for cytokeratin-19 (CK-19; G-14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody for the membrane progesterone receptor component (PGRMC1) was purchased from Atlas Antibodies (Stockholm, Sweden). The mouse monoclonal antibody for PGRMC2 was purchased from Affinity Bioreagents (Golden, CO). The rabbit polyclonal antibody for p450ccc was purchased from Chemicon-Millipore (Temecula, CA). The mouse monoclonal antibody for CK-19 was purchased from Novocastra Laboratories (Newcastle, UK).

Animals. Female and male 344 Fischer rats (150–175 g) were purchased from Charles River (Wilmington, MA) and maintained in a temperature-controlled environment (20–22°C) with a 12:12-h light-dark cycle. Animals were fed ad libitum standard rat chow and had free access to drinking water. To evaluate the *in vivo* effect of progesterone on cholangiocyte growth, normal female and male rats were treated with a daily intraperitoneal (IP) injection of 1) NaCl or 2) progesterone (50 mg/kg body wt per day, a dose similar to that used in other studies in rats) (10) for 1 wk. We evaluated the effect of *in vivo* administration of antiprogestrone (a sheep polyclonal antibody to antiprogestrone, Abcam, Cambridge, MA) on cholangiocyte proliferation of female and male BDL rats. Immediately after BDL (2), rats received daily IP injections of either nonimmune serum or a polyclonal neutralizing progesterone antibody (6.5 nmol/dose), the same dose used in other studies in pregnant hamsters (30), for 7 days. Before each experimental procedure, animals were injected with pentobarbital sodium (50 mg/kg body wt ip) following the regulations of the panel on euthanasia of the American Veterinarian Medical

Association and local authorities. Study protocols were performed in compliance with institutional guidelines and were approved by the IACUC of Scott and White Hospital.

Purification of cholangiocytes. Cholangiocytes were isolated by immunoaffinity separation (24, 27, 33), by using a mouse monoclonal antibody (IgM, kindly provided by Dr. R. Faris, Brown University, Providence, RI) that recognizes an unidentified antigen expressed by all intrahepatic rat cholangiocytes (33). The purity (98–99%) of cholangiocytes was evaluated by γ -GT histochemistry (62). Cell viability (by Trypan blue exclusion) ranged from 95 to 98%. Normal male rat intrahepatic cholangiocyte cultures (NRC) were developed, characterized, and maintained in culture as described by us (3).

Evaluation of progesterone receptor expression. Nuclear (PR-A/B) and membrane (PGRMC1 and PGRMC2) progesterone receptor expression was evaluated by 1) immunofluorescence in frozen liver sections from female and male normal and BDL rats and male NRC; and 2) RT-PCR in total RNA (0.75 μ g) from uterus (positive control) or purified cholangiocytes from female and male normal and BDL rats and male NRC. Since antibodies are not available, we evaluated the expression of mPR α in total RNA (0.75 μ g) isolated cholangiocytes and male NRC by RT-PCR. PR-A/B expression was also evaluated by immunoblotting of protein (10 μ g) from whole cell lysate from uterus (positive control) or isolated cholangiocytes from female and male normal and BDL rats and male NRC.

Immunofluorescence and immunohistochemistry. For immunofluorescence, liver sections (20 μ m thick; $n = 3$ per each group of animals) from normal and BDL female and male rats were fixed in 4% paraformaldehyde (in 1 \times PBS) for 10 min, followed by tissue permeabilization in PBST (1 \times PBS with 0.2% Triton X-100). Sections were then blocked in 4% BSA (in PBST). Primary antibodies for mouse anti-CK-19 (1:50), goat anti-CK-19 (1:10 used for PGRMC2 costaining), rabbit anti-PR (1:10), rabbit anti-PGRMC1 (1:50), and mouse anti-PGRMC2 (1:10) were diluted in 1% BSA (in PBST). Sections were incubated overnight at 4°C and washed three times for 10 min each with 1 \times PBST at room temperature. Sections were incubated with appropriate secondary antibodies [Cy2 anti-mouse (1:50), Cy2 anti-goat (1:50), Cy3 anti-mouse (1:50), and Cy3 anti-rabbit (1:50)] (Jackson Immunochemicals, West Grove, PA) for 2 h at room temperature protected from light. Following incubation, the slides were washed in PBST at room temperature and coverslipped with Antifade gold containing 4,6-diamidino-2-phenylindole (DAPI) as a counterstain (Molecular Probes, Eugene, OR). Images were visualized with an Olympus IX-71 (Tokyo, Japan) confocal microscope.

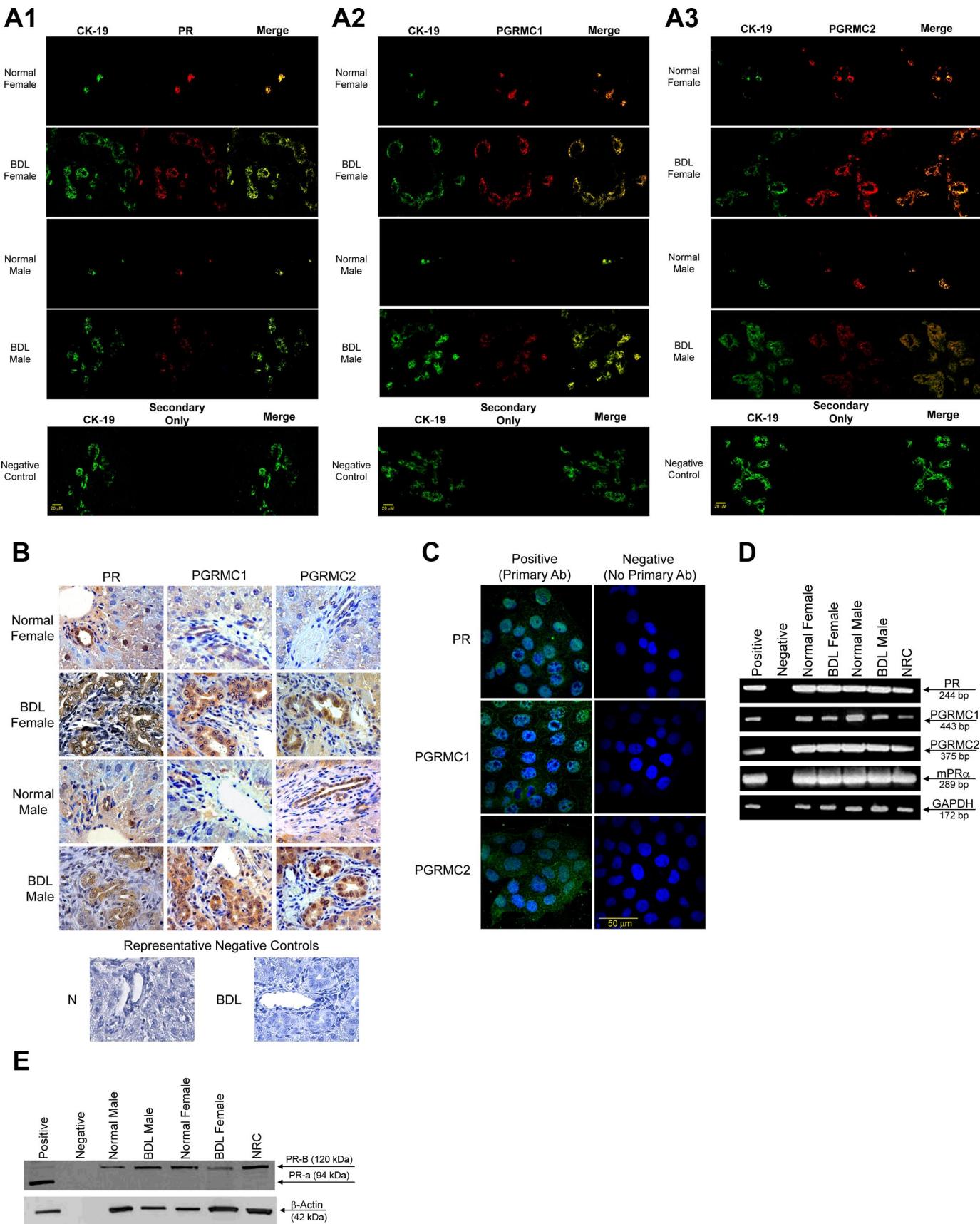
Liver sections from normal and BDL female and male rat (fixed in Bouin solution) were mounted on glass slides coated with acetone aminopropyltriehtoxylan (2%) solution. After deparaffination, endogenous peroxidase activity was blocked by incubation (30 min) in methanolic hydrogen peroxide (2.5%). Sections were hydrated in graded ethanol and rinsed in 1 \times PBS. The endogenous biotin was blocked by the Biotin Blocking System (Dako Cytomation, Glostrup, Denmark). Following washes in 1 \times PBS, sections were incubated overnight at 4°C with antibodies for PR, PGRMC1, and PGRMC2 (1:100 dilution). Samples were rinsed with 1 \times PBS, incubated for 10 min at room temperature with a secondary biotinylated antibody

(Dako Cytomation LSAB Plus System-HRP), then with Dako ABC (Dako Cytomation LSAB Plus System-HRP), developed with 3,3'-diaminobenzidine, and counterstained with hematoxylin. For all immunoreactions negative controls (preimmune serum substituted for primary antibody) were included. Observations and light microscopy photographs of liver sections were taken by Leica Microsystems DM 4500 B Light Microscopy (Wetzlar, Germany) with a Jenoptik Prog Res C10 Plus Videocam (Jena, Germany).

For immunofluorescence in male NRC, cells were seeded on coverslips in a six-well plate (500,000 cells/well) and allowed to adhere overnight. The coverslips were transferred into a new six-well plate containing cold PBS and washed for 5 min at room temperature. Next, the coverslips were washed three times for 10 min each at room temperature in PBST (1 \times PBS with 0.2% Triton) and blocked 1 h at room temperature in 4% BSA in 1 \times PBS. The blocking solution was removed and the coverslips were incubated with the primary antibodies for PR, PGRMC1, and PGRMC2 diluted in 1% BSA/PBS 24 h 4°C. The next day, the coverslips were washed three times for 10 min at room temperature in PBST. Next, they were incubated with a Cy2-conjugated anti-rabbit or mouse secondary antibody 1:50 (Jackson Immunochemicals, West Grove, PA) in 1% BSA/PBS at room temperature for 2 h and washed three times for 10 min each with PBST. Following incubation, the coverslips were mounted into microscope slides with Antifade gold containing DAPI as a counterstain (Molecular Probes). Images were taken on an Olympus IX71 fluorescence microscope with a DP70 digital camera.

RT-PCR. We evaluated by RT-PCR the expression of the message for the nuclear receptor [PRA/B (common) and PRB], membrane progesterone receptor components (PGRMC1 and PGRMC2) and the newly identified mPR α (13) in total RNA (0.75 μ g) from uterus (positive control), male and female cholangiocytes from normal and BDL rats, and male NRC. We first performed RT-PCR for the nuclear (PRA/B and PRB) membrane progesterone receptor components (PGRMC1 and PGRMC2) and the newly identified mPR α to determine that cholangiocytes express the expected molecular weight band for these four receptor isoforms and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the housekeeping gene (1). Total RNA was extracted utilizing the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the instructions provided by the vendor. RNA was extracted from normal and BDL cholangiocytes (1×10^5) and reverse transcribed with the Reaction Ready First Strand cDNA synthesis kit (SuperArray). These reactions were used as templates for the PCR assays. Primers PR-A/B, PGRMC1, PGRMC2 and mPR α were synthesized by Integrated DNA Technologies (Coralville, IA). The primers for common PR-A/B were previously reported by others (28, 53). The primers used for the common PR-A and PR-B amplification region that corresponds to the hormone binding domain of the receptor were 5'-CTC CTG GAT GAG CCT GAT GGT G-3' (sense) and 5'-CAC CAT GCC CGC CAG GAT CTT G-3' (antisense), which generate a 283-bp amplification product. The primers for the membrane components of the progesterone receptor PGRMC1 and PGRMC2 were designed according to the following sequences: PGRMC1 (NCBI GenBank Accession no. NM 021766) (49) and PGRMC2 (NCBI GenBank Accession no. NM 001008374) (68). The primers for PGRMC1 were 5'-CTC TCA ACC TGC TGC TCC TT-3'

Fig. 1. *A:* immunofluorescence for progesterone receptors (PR, PGRMC1, and PGRMC2) in liver sections from normal and bile duct-ligated (BDL) female and male rats demonstrates that bile ducts express these receptors (red staining). Colocalization with CK-19 (green staining, a cholangiocyte-specific marker) of the bile ducts expressing the progesterone receptor is also visible. Bar = 20 μ m. *B:* immunohistochemistry for progesterone receptors (PR, PGRMC1, and PGRMC2) in liver sections from normal and BDL female and male rats confirms receptor expression by bile ducts. Low-level expression of the progesterone receptor was expected in hepatocytes (36). *C:* by immunofluorescence, progesterone receptors (PR, PGRMC1, and PGRMC2) were expressed by normal rat intrahepatic cholangiocyte cultures (NRC). Specific immunoreactivity is shown in green and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). No staining was visible when primary antibodies were omitted. Bar = 50 μ m. *D:* RT-PCR in pure cholangiocytes from normal and BDL female and male rats and NRC shows that cholangiocytes express the PR-A/B and PGRMC1, PGRMC2, and mPR α . The expression of GAPDH, the housekeeping gene, was similarly expressed by freshly isolated cholangiocytes and NRC. *E:* by immunoblotting, cholangiocytes from normal and BDL female and male rats and NRC express the PR-B (but not PR-A). The expression of β -actin, the housekeeping gene, was similar in freshly isolated cholangiocytes and NRC. PR, progesterone receptor; PGR, progesterone receptor membrane component protein.



(sense) and 5'-CGC TCC TTC AAG CAG TTT TC-3' (antisense), which generate a 443-bp amplification product. The primers for PGRMC2 were 5'-AGT CTG GCT GTG ATG CTC CT-3' (sense) and 5'-AAC AGG ATC CCT TGC TCC TT-3' (antisense), which generate a 408-bp amplification product. The primer set for mPR α was previously described by others (13). The primers for mPR α 5'-CTG GAA GCC GTA CAT CTA TGC-3' (sense) and 5'-GAA GCT GTA ATG GCC AGA ACT C-3' (antisense), which generate a 289-bp amplification product. The primer for GAPDH was purchased from SuperArray. Standard RT-PCR conditions were used with 0.75 μ g of total mRNA (35 step cycles: 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C). The PCR samples for progesterone receptors were run on agarose gels, and the bands excised and removed from the gel with the Qiaquick Gel Extraction Kit (Qiagen). The purified fragments were subsequently sequenced by Davis Sequencing (Davis, CA).

Immunoblots. We evaluated the expression of the nuclear (PR-A/B) progesterone receptor by immunoblots in protein (10 μ g) from whole cell lysates from rat uterus (positive control) and cholangiocytes from normal and BDL female and male rats and male NRC. Blots were normalized by β -actin (5). The intensity of the bands was determined by scanning video densitometry using the phospho-imager, Storm 860, Amersham Biosciences (Piscataway, NJ) using the ImageQuant TLV 2003.02 (Little Chalfont, Buckinghamshire, UK).

Effect of in vivo administration of progesterone to normal rats or antiprogestrone antibody to BDL rats on portal inflammation, lobular damage, necrosis, progesterone serum levels, and cholangiocyte apoptosis and proliferation.

We evaluated the effect of chronic in vivo administration of 1) saline or progesterone (50 mg/kg body wt per day) (10) to normal female or male rats in the absence or presence of RU-486 (15 mg/kg body wt), a nuclear progesterone receptor antagonist (40), and 2) nonimmune serum or antiprogestrone antibody to male or female BDL rats (immediately after BDL) on portal inflammation, lobular damage, necrosis, cholangiocyte apoptosis and proliferation, and serum progesterone levels. Paraffin-embedded liver sections (5 μ m, 3 sections analyzed per group) were stained with hematoxylin and eosin (H&E), and lobular damage, necrosis, and the degree of portal inflammation were evaluated in a coded fashion. A score of 0 was used when inflammatory cells were found in only one to two portal tracts, a score of 1 when less than 25% of the total portal tract presented inflammatory cells, a score of 2 if less than 50% of the portal tracts was infiltrated by inflammation, and a score of 3 when we found inflammation in more than 50% of the total portal tract.

Measurement of cholangiocyte apoptosis was evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) analysis (42) using a commercially available kit (Wako Chemicals, Tokyo, Japan). Following staining, sections (5 μ m, 3 slides analyzed per group) were evaluated in coded fashion with a microscope (Olympus Optical, U-PMTVC, Tokyo, Japan); 200 cells per slide were counted in a coded fashion in 10 nonoverlapping fields.

Cholangiocyte proliferation was evaluated by quantitative evaluation of the number of PCNA- and CK-19-positive cholangiocytes in paraffin-embedded liver sections (5 μ m, 3 slides analyzed for each group). Following staining, sections were counterstained with hematoxylin and examined with a microscope (Olympus Optical, U-PMTVC, Tokyo, Japan). Over 100 cholangiocytes were counted in a random, blinded fashion in three different fields for each group of animals. Data were expressed as number of PCNA- or CK-19-positive cholangiocytes per each 100 cholangiocytes. Serum progesterone levels of normal, BDL, normal + progesterone-, and BDL + anti-progesterone-treated male and female rats were determined by ELISA by commercially available kits (Cayman Chemical, Ann Arbor, MI).

Evaluation of expression of the message and protein for p450scc, 3 β -HSD, and StAR and secretion of progesterone by cholangiocytes. To evaluate the pathway of progesterone steroidogenesis in cholangiocytes (which we propose regulates intrahepatic biliary growth by an autocrine pathway), we evaluated 1) the expression of p450scc

(which initiates the biosynthesis of all steroid hormones) (59, 61) and 3 β -HSD (which converts pregnenolone to progesterone), two key enzymes in the steroidogenesis pathway of progesterone (59, 61), and StAR (which mediates the rapid increase in pregnenolone synthesis stimulated by trophic hormones) (46) by immunofluorescence, RT-PCR, and real-time PCR; and 2) the amount of progesterone secreted by primary cultures (6 h) of normal and BDL female and male cholangiocytes and male NRC.

Immunofluorescence for StAR, p450scc, and 3 β -HSD in liver sections and male NRC was performed as described for progesterone receptor with the difference that other primary antibodies were used: rabbit anti-StAR (1:20), rabbit anti-p450scc (1:200), and goat anti-3 β -HSD (1:20) diluted in 1% BSA (in PBST). Immunohistochemistry for StAR, p450scc, and 3 β -HSD in liver sections was performed as described for progesterone receptors with the difference that other primary antibodies were used: StAR, p450scc, and 3 β -HSD (all at 1:100).

The expression of the messages for p450scc, 3 β -HSD, and StAR was evaluated by RT-PCR in total RNA (0.75 μ g) from cholangiocytes isolated from normal and BDL female and male rats and male NRC. Specific oligonucleotide primers for the rat StAR were designed based on the rat StAR sequence (35) (sense 5'-AGCTCGGCTCA-GAGTAGCAG-3' and antisense 5'-ACAGCCATCAGGAGCAC-TCT-3', 417 bp). The primers for the rat p450scc were purchased from SuperArray. Specific oligonucleotide primers for the rat 3 β -HSD were designed based on the rat 3 β -HSD sequence (32) (sense 5'-CTCCATAGCCAACCCAGTGT-3' and antisense 5'-CCCTCTTC-CCATCATTGAGA-3', 480 bp). The comparability of the RNA used was assessed by RT-PCR for the housekeeping gene, GAPDH (Superarray). Standard RT-PCR conditions were used with 1 μ g of total mRNA (35 step cycles: 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C). The PCR samples for StAR and 3 β -HSD were run on agarose gels, and the bands were excised and removed from the gel with the Qiaquick Gel Extraction Kit (Qiagen). The purified fragments were sequenced by Davis Sequencing.

The amount of progesterone secreted by cholangiocytes was evaluated as previously described by us (25, 26, 48). Briefly, purified cholangiocytes from normal and BDL female and male rats and male NRC were incubated at 37°C for 0 and 6 h. Thereafter, cells were centrifuged at 1,500 rpm for 10 min, and the supernatant was transferred to a tube and stored at -70°C before analysis for progesterone levels by ELISA using commercially available kits (Cayman Chemical). Cholangiocyte progesterone secretion was calculated as the difference between the amount of progesterone detected at 6 h and the amount detected at time zero.

To evaluate whether progesterone secreted by cholangiocytes stimulates cholangiocyte proliferation, the supernatant of isolated cholangiocytes (after 6 h of incubation at 37°C) from normal and BDL female and male rats was transferred into plates containing NRC (3). In addition, NRC was also pretreated with antiprogestrone antibody for 30 min prior to the addition of cholangiocyte supernatant. After 24 h of incubation, cholangiocyte proliferation was evaluated by utilizing a commercially available ViaLight Plus High Sensitivity Cell Proliferation Kit (Lonza Rockland, Rockland, ME) (31). This is the same approach that we have used for evaluating the stimulatory effects of NGF on the proliferation of cholangiocytes (26).

Inhibition of progesterone steroidogenesis by aminoglutethimide. To further provide evidence that progesterone regulates cholangiocyte proliferation by an autocrine pathway, we propose studies to demonstrate that inhibition of cholangiocyte progesterone steroidogenesis by aminoglutethimide (AMG) (51) induces a decrease in cholangiocyte replication evaluated by measurement of PCNA protein expression (27) by immunoblots (27). Briefly, NRC were plated in a six-well plate (800,000 cells per well) and cells were incubated overnight at 37°C for 24 h with 0.2% BSA (basal) or AMG (100 nM, a dose used in other studies) (65), which inhibits cytochrome p450scc (51).

Subsequently, cholangiocyte proliferation was assessed by PCNA immunoblots (27).

Statistical analysis. All data are expressed as means \pm SE. Differences between groups were analyzed by the Student's unpaired *t*-test when two groups were analyzed and ANOVA when more than two groups were analyzed, followed by an appropriate post hoc test. A value of $P < 0.05$ was considered significant.

RESULTS

Cholangiocytes express progesterone receptors. By immunofluorescence in liver sections from normal and BDL female and male rats, intrahepatic bile ducts express progesterone receptors; PR, PGRMC1, and PGRMC2 (Fig. 1A). Colocalization with CK-19 (a cholangiocyte-specific marker) (42) of the bile ducts expressing the progesterone receptor is visible in Fig. 1A. Immunoreactivity for PR, PGRMC1, and PGRMC2 was also confirmed by immunohistochemistry in liver sections (Fig. 1B). Low-level expression of the progesterone receptor was expected in hepatocytes (36). Similarly, progesterone receptors (PR, PGRMC1, and PGRMC2) were expressed by male NRC (Fig. 1C).

By RT-PCR, we demonstrated that freshly isolated cholangiocytes and NRC express the mRNAs for PR-A/B (with a primer that recognizes both sequences), PGRMC1, PGRMC2, and mPR α (Fig. 1D). The expression of GAPDH mRNA was similar in freshly isolated cholangiocytes and NRC (Fig. 1D). The sequence of the PCR fragment for rat PR-B (both A/B) was 100% homologous to the rat PR sequence (32) (NM022847). The sequence of the PCR fragment for rat PGRMC1 was 100% homologous to the rat PGRMC1 sequence (35) (NM021766). The sequence of the PCR fragment for rat PGRMC2 was 100% homologous to the rat PGRMC2 sequence (35) (NM001008374). By immunoblots, the nuclear PR-B (but not PR-A) was detected in normal and BDL male and female freshly isolated cholangiocytes and male NRC (Fig. 1E).

Effect of *in vivo* administration of progesterone to normal rats or antiprogestrone antibody to BDL rats on portal inflammation, lobular damage, necrosis, and cholangiocyte apoptosis and proliferation. Light microscopy of H&E-stained paraffin-embedded liver sections demonstrated that there were no significant differences in the degree of portal inflammation, necrosis, apoptosis, and lobular damage in all groups of animals (Table 1). Administration of progesterone to normal female or male rats increased cholangiocyte proliferation evidenced by the increased number of PCNA- (Table 1) and CK-19-positive (Table 1 and Fig. 2) cholangiocytes compared with the corresponding normal female or male rats treated with saline. The administration of the nuclear progesterone receptor antagonist RU-486 (40) to normal female or male rats partly prevented the progesterone-induced increase in cholangiocyte growth (Table 1 and Fig. 2).

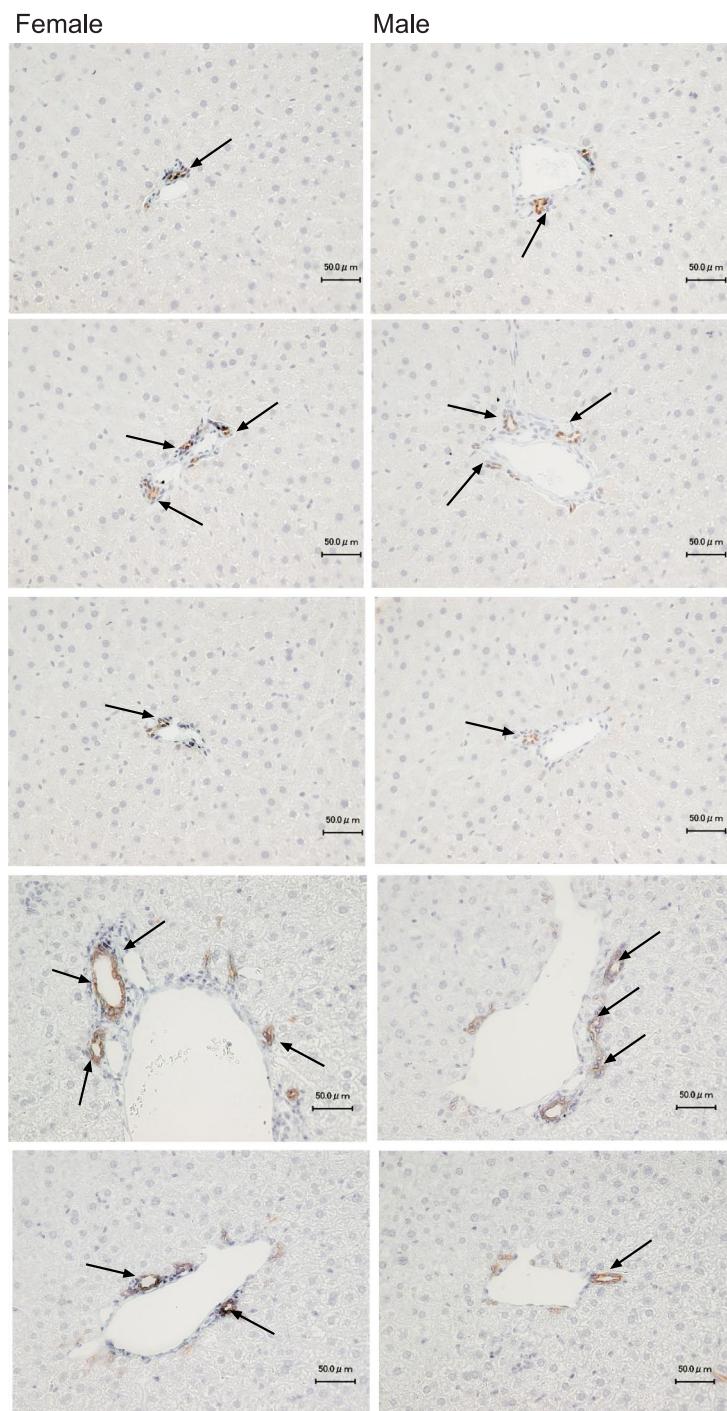
Administration of antiprogestrone antibody to female or male BDL rats (immediately after BDL) decreased the number of PCNA- (Table 1) and CK-19-positive-positive cholangiocytes (Table 1 and Fig. 2) compared with liver sections from the corresponding normal female or male BDL rats treated with nonimmune serum. The decrease in cholangiocyte growth (by the administration of antiprogestrone antibody to BDL rats) was associated with enhanced cholangiocyte apoptosis (demonstrated by TUNEL staining) compared with BDL rats treated with nonimmune serum (Table 1).

Table 1. Measurement of portal inflammation, necrosis, lobular damage, apoptosis and cholangiocyte proliferation

| Parameters | Female | | Male | |
|---|--------------------|--------------------|----------------------------|--------------------------|
| | Normal + Saline | Normal + Prog | BDL + Nonimmune Serum | BDL + Anti-Prog Antibody |
| Lobular damage ($n = 5$) | Not detected | 0.1 \pm 0.1 | 1.4 \pm 0.1 | Not detected |
| Necrosis ($n = 5$) | Not detected | 0.2 \pm 0.1 | 1.8 \pm 0.1 | Not detected |
| Portal inflammation ($n = 5$) | Not detected | 1.4 \pm 0.1 | 1.5 \pm 0.1 | Not detected |
| Cholangiocyte apoptosis ($n = 5$) | 0.1 \pm 0.1 | 1.6 \pm 0.1 | 3.8 \pm 0.5 \ddagger | 0.1 \pm 0.1 |
| PCNA-positive cholangiocytes ($n = 5$) | 0.7 \pm 0.2 | 5.2 \pm 1.0 $*$ | 22.6 \pm 1.2 | 0.5 \pm 0.1 |
| CK-19-positive cholangiocytes ($n = 5$) | 25.6 \pm 1.8 | 38.0 \pm 2.4 $*$ | 131.0 \pm 3.5 | 30.2 \pm 2.0 |
| Normal + RU-486 + Prog | | | | |
| Normal + Prog | 30.8 \pm 1.4 $*$ | | | |
| RU-486 + Prog | | | 45.2 \pm 5.2 $*$ | 28.3 \pm 1.2 $*$ |
| Normal + RU-486 + Prog | | | | |
| Normal + Prog | 131.0 \pm 3.5 | | 111.2 \pm 3.2 \ddagger | 128.2 \pm 4.0 |
| RU-486 + Prog | | | 30.2 \pm 2.0 | 28.3 \pm 1.2 $*$ |
| Normal + BDL + Nonimmune Serum | | | | |
| Normal + Prog | 1.2 \pm 0.1 | | 1.2 \pm 0.1 | 1.2 \pm 0.1 |
| RU-486 + Prog | 1.2 \pm 0.1 | | 1.2 \pm 0.1 | 1.2 \pm 0.1 |
| BDL + Anti-Prog Antibody | 1.4 \pm 0.1 | | 1.4 \pm 0.2 | 1.4 \pm 0.2 |

Values are means \pm SE of cumulative values obtained from the evaluation of 10 portal tracts for each group. BDL, bile duct ligation; CK-19, cytokeratin-19; PCNA, proliferating cellular nuclear antigen; Prog, progesterone. * $P < 0.05$ vs. corresponding values of normal rats treated with saline for 1 wk. $\ddagger P < 0.05$ vs. corresponding values of BDL rats treated with nonimmune serum for 1 wk.

Fig. 2. Measurement of the number of cytokeratin-19 (CK-19)-positive cholangiocytes in liver sections from the selected groups of animals. *A*: female rats. *B*: male rats. Administration of progesterone to normal female or male rats increased the number of CK-19-positive cholangiocytes compared with the corresponding normal female or male rats treated with saline. The administration of the nuclear progesterone receptor antagonist RU-486 to normal female or male rats prevented progesterone-induced increase in cholangiocyte growth. In addition, administration of antiprogestrone antibody to female or male BDL rats (immediately after BDL) decreased the number of CK-19-positive cholangiocytes compared with liver sections from the corresponding female or male BDL rats treated with nonimmune serum. Original magnification, $\times 10$. Data are means \pm SE of 5 values obtained from the 3 slides evaluated per each group of animal.



Cholangiocytes express StAR, p450scc, and 3 β -HSD and secrete progesterone: possible regulation of cholangiocyte proliferation by an autocrine mechanism. By immunofluorescence in liver sections, cholangiocytes from normal and BDL female and male rats express the proteins for StAR, p450scc, and 3 β -HSD (Fig. 3A). Immunoreactivity for StAR, p450scc, and 3 β -HSD was also confirmed by immunohistochemistry in liver sections (Fig. 3B). Immunohistochemistry indicates that hepatocytes may also express the proteins required for steroidogenesis, which will need to be evaluated in future studies. NRC also expressed StAR, p450scc, and 3 β -HSD by immuno-

nofluorescence (Fig. 3C). By RT-PCR, purified cholangiocytes from normal and BDL female and male rats and NRC expressed the mRNA messages for StAR, p450scc, and 3 β -HSD (Fig. 3D). The expression of GAPDH mRNA was similar in freshly isolated cholangiocytes and NRC (Fig. 3D). The sequence of the PCR fragment for rat 3 β -HSD was 100% homologous to the rat 3 β -HSD sequence (32) (NM017265). The sequence of the PCR fragment for rat StAR was 100% homologous to the rat StAR sequence (35) (NM031558).

During short-term (6 h) culture, normal and BDL female and male cholangiocytes secrete progesterone (Fig. 4A). The levels

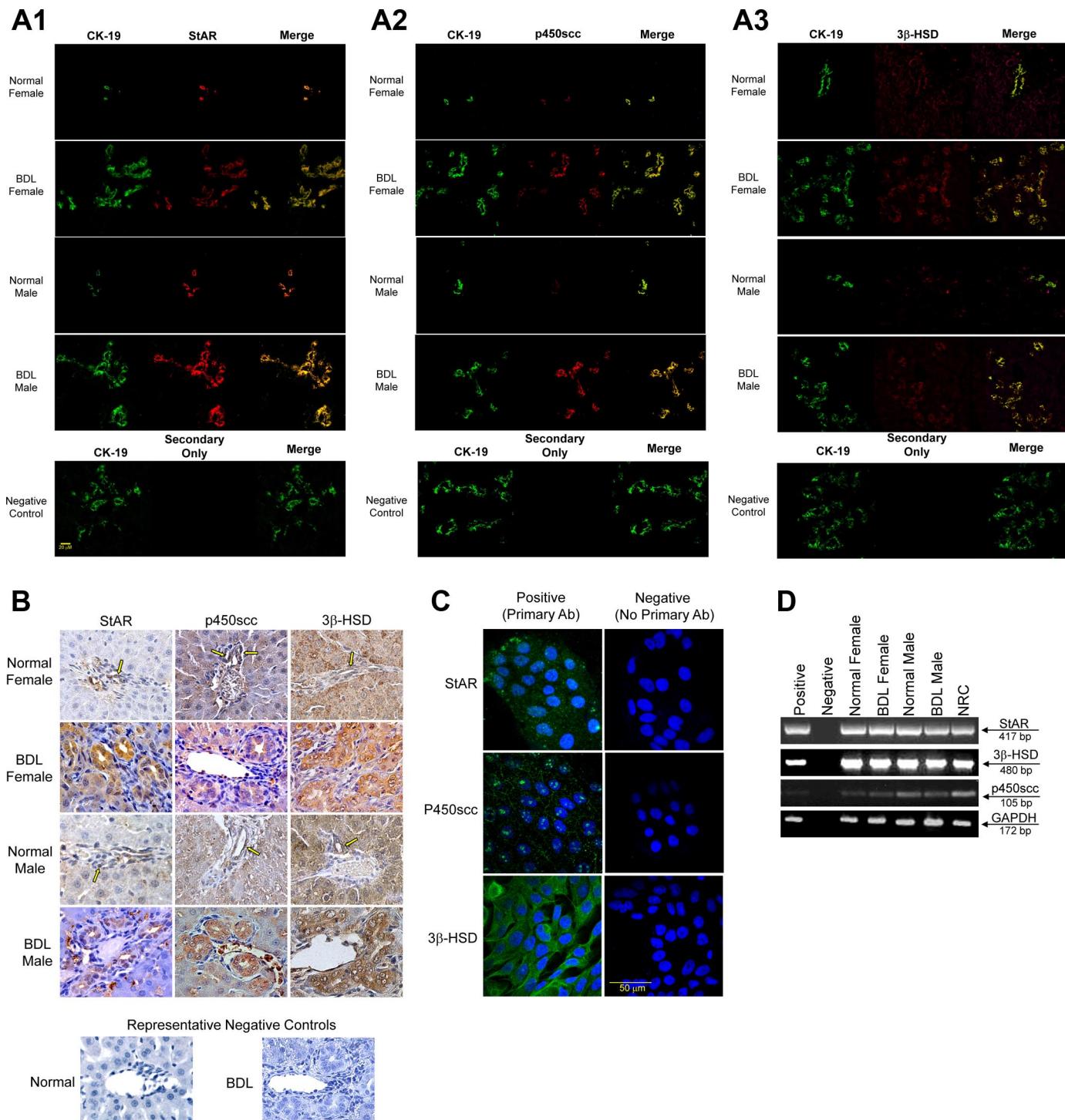


Fig. 3. A: immunofluorescence for key proteins in the progesterone steroidogenesis pathway [steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage (p450scc), and 3 β -hydroxysteroid dehydrogenase (3 β -HSD)] in liver sections from normal and BDL female and male rats demonstrates that bile ducts express these steroidogenesis pathway proteins (red staining). Colocalization with CK-19 (green staining, a cholangiocyte-specific marker) of the bile ducts expressing StAR, p450scc, and 3 β -HSD is also visible. Bar = 20 μ m. B: immunohistochemistry in liver sections from normal and BDL female and male rats for StAR, p450scc, and 3 β -HSD confirms bile duct expression, with expression of some components in hepatocytes. C: by immunofluorescence, NRC expressed StAR, p450scc, and 3 β -HSD, key proteins in the progesterone steroidogenesis pathway. Specific immunoreactivity is shown in green and nuclei were stained with DAPI (blue). No staining was visible when primary antibodies were omitted. Bar = 50 μ m. D: RT-PCR in pure cholangiocytes from normal and BDL female and male rats and NRC shows that cholangiocytes express mRNA messages for StAR, p450scc, and 3 β -HSD.

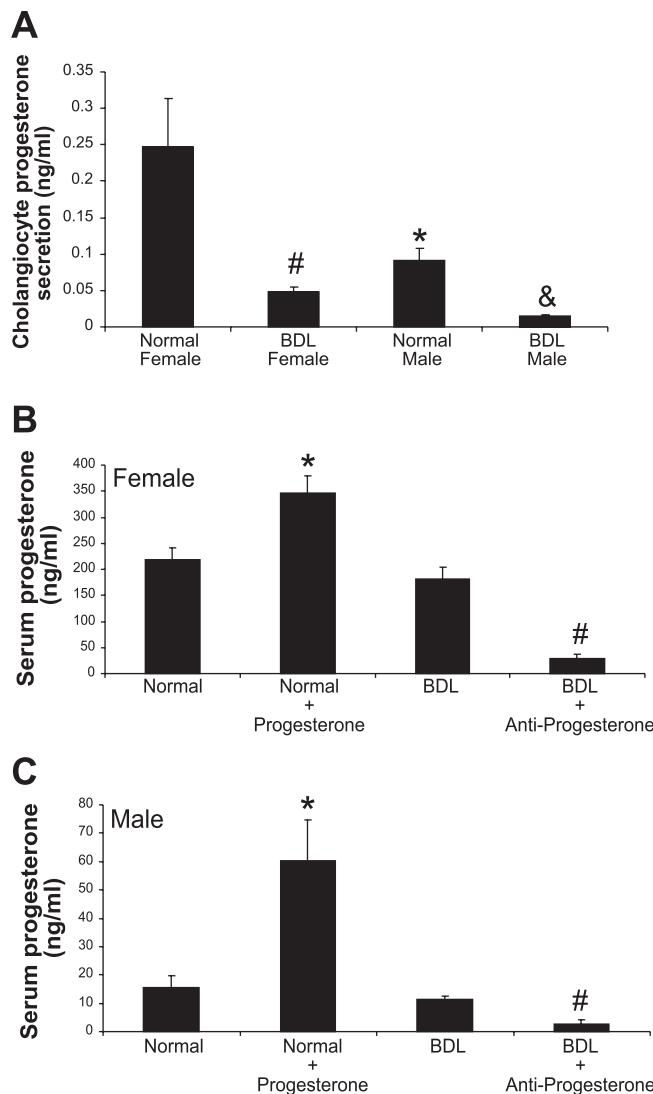


Fig. 4. Progesterone levels in cholangiocyte supernatants and serum from normal and BDL female and male rats. A: progesterone levels in normal female cholangiocyte supernatants were significantly higher than those of normal male rats. Following BDL, there is a significant decrease in progesterone in the supernatants of both female and male rat cholangiocytes compared to normal cholangiocytes. Data are means \pm SE of 5 experiments. * $P < 0.05$ normal female vs. normal male. # $P < 0.05$ normal female vs. BDL female. & $P < 0.05$ normal male vs. BDL male. B and C: serum progesterone levels were significantly higher in normal female rats compared with normal male rats. BDL had no significant effects on serum progesterone levels. Chronic administration of progesterone to normal male and female rats significantly elevated serum progesterone levels. Administration of antiprogestrone significantly reduced serum progesterone levels in both BDL female and male rats. Data are means \pm SE of 5 experiments. * $P < 0.05$ normal vs. normal + progesterone. # $P < 0.05$ BDL vs. BDL + antiprogestrone.

of progesterone were significantly higher in the supernatant of normal female cholangiocytes compared with the supernatant of normal male cholangiocytes (Fig. 4A). However, we observed a significant decrease in progesterone levels in supernatants from female and male BDL cholangiocytes compared with their corresponding normal cholangiocytes (Fig. 4A). Serum progesterone levels were significantly higher in normal female rats compared with normal male rats (Fig. 4, B and C). BDL had no significant effects on serum progesterone levels.

Chronic administration of progesterone to normal male and female rats significantly elevated serum progesterone levels (Fig. 4, B and C). Administration of antiprogestrone significantly reduced serum progesterone levels in both BDL female and male rats (Fig. 4, B and C).

Consistent with the concept that cholangiocytes can regulate their own growth by an autocrine mechanism, the supernatant from primary cultures of normal and BDL female and male cholangiocytes stimulated the proliferation of NRC compared with basal, unstimulated NRC (Fig. 5, A and B). As expected, supernatants from BDL female and male rats stimulated proliferation to a lesser extent as that of the normal female and male supernatants. In vitro pretreatment of NRC with antiprogestrone antibody, prior to stimulation with cholangiocyte supernatant, partly inhibited progesterone-stimulated cholangiocyte proliferation (Fig. 5, A and B).

Inhibition of progesterone steroidogenesis by aminoglutethimide prevents NRC proliferation. Progesterone induced a dose-dependent increase in NRC. An increase in the proliferative response was observed with varying doses of progesterone (Fig. 6). Furthermore, inhibition of progesterone steroidogenesis by in vitro treatment of NRC with AMG prevented basal cholangiocyte proliferative activity evaluated by immunoblots for PCNA (Fig. 7).

DISCUSSION

The present study provides the following new findings: 1) female and male rat cholangiocytes express both nuclear and membrane receptors that bind progesterone (PR, PGRMC1, PGRMC2, and mPR α); 2) chronic administration of progesterone to normal female and male rats stimulates biliary proliferation, which can be partly prevented by the simultaneous administration of the nuclear progesterone receptor antagonist RU-486 (40); 3) biliary proliferation stimulated by BDL (1, 2, 27) in female and male rats can be partially prevented by the administration of a neutralizing antiprogestrone antibody (30); and 4) cholangiocytes from both female and male rats possess the enzymatic pathway for the steroidogenesis of progesterone and secrete progesterone, which indicates that in addition to a paracrine pathway cholangiocytes regulate their own growth in an autocrine mechanism.

It is well known that cholangiocyte proliferation is coordinately regulated by a number of hormones, neuropeptides, and other factors such as bile acids during normal and cholestatic states (4, 7, 8, 23, 24, 27). With regard to sex hormones, we have shown that estrogens play a key role in the regulation of hyperplastic and neoplastic cholangiocyte growth (7, 8). Furthermore, the hormone prolactin stimulates the proliferation of normal female cholangiocytes by an autocrine mechanism (70). In support of the stimulatory effect of progesterone on cell growth (10, 14, 34), the chronic administration of progesterone stimulates a significant increase in biliary mass of normal female and male rats. As expected, chronic administration of progesterone induced a significant increase in serum progesterone levels.

To evaluate the potential role of progesterone during cholestasis, female and male rats with BDL were treated with a neutralizing antiprogestrone antibody for 1 wk (30). Treatment with antiprogestrone antibody significantly lowered serum progesterone levels of male and female rats with BDL.

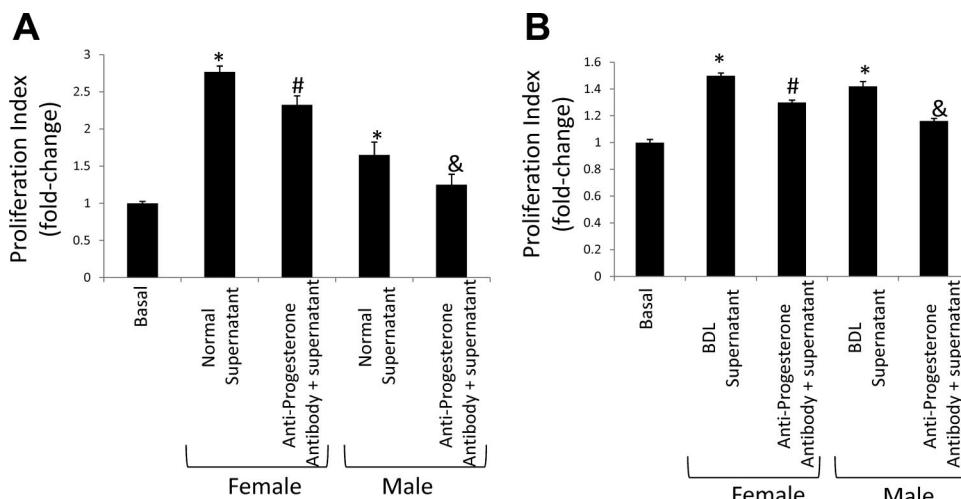


Fig. 5. Effect of normal and BDL cholangiocyte supernatant (in the absence or presence of antiprogestrone antibody) on NRC proliferation. *A*: consistent with the concept that cholangiocytes regulate their own growth by an autocrine mechanism, the supernatant from primary cultures of normal female and male cholangiocytes stimulated the proliferation of NRC compared with basal unstimulated NRC. In vitro pretreatment of NRC with antiprogestrone antibody, prior to stimulation with cholangiocyte supernatant, partly inhibited progesterone-stimulated cholangiocyte proliferation. Data are means \pm SE of 5 experiments. *B*: in a similar fashion, the supernatant from primary cultures of BDL female and male cholangiocytes also stimulated the proliferation of NRC compared with basal unstimulated NRC (although to a smaller degree than normal supernatant). The proliferation was also partly inhibited by antiprogestrone. Data are means \pm SE of 5 experiments. * P < 0.05 vs. corresponding basal value. # P < 0.05 vs. NRC treated with supernatant from normal (or BDL) female cholangiocytes. & P < 0.05 vs. NRC treated with supernatant from normal male (or BDL) cholangiocytes.

compared with both normal and BDL controls, which indicates effective immunoneutralization of progesterone (21). Administration of antiprogestrone significantly diminished intrahepatic biliary mass compared with control-treated animals. These findings are parallel with our previous studies showing that administration of neutralizing antibodies for prolactin, NGF, and VEGFs decreases the circulating levels of these proteins, thus reducing intrahepatic ductal mass (25, 26, 70). Consistent with previous studies (25, 26, 42), this decrease of intrahepatic biliary mass was associated with an increase in cholangiocyte apoptosis.

Consistent with progesterone-induced alterations in cholangiocyte proliferation, we found that both normal and BDL female and male cholangiocytes express PGRMC1, PGRMC2, and mPR α receptors. The membrane receptors have been associated with rapid nongenomic signaling in several cell types, which involve the activation of intracellular calcium signaling mechanisms (54, 56, 57). In addition to the recently discovered progesterone membrane receptors, cholangiocytes also express the nuclear PR-B receptor. Nuclear progesterone receptors are members of the nuclear receptor family of ligand-

dependent transcription factors (45). In some contexts, cytoplasmically located PR interact directly with c-Src and activate downstream signaling mechanisms (66). Activation of PR has been shown to increase the proliferation of a number of normal and neoplastic cell types (10, 14, 34). In our study, we demonstrate that progesterone-induced biliary proliferation in normal female and male rats can be prevented by administration of the nuclear PR antagonist, RU-486 (mifepristone). RU-486 acts as a competitive receptor antagonist at the nuclear progesterone receptor and does not affect the membrane progesterone receptor (22). Because of the blockage of progesterone-induced biliary proliferation with in vivo administration of RU-486, we speculate that the proliferative signaling mechanism occurs predominantly via PR-B. However, the present study cannot evaluate or rule out the exact contribution of the

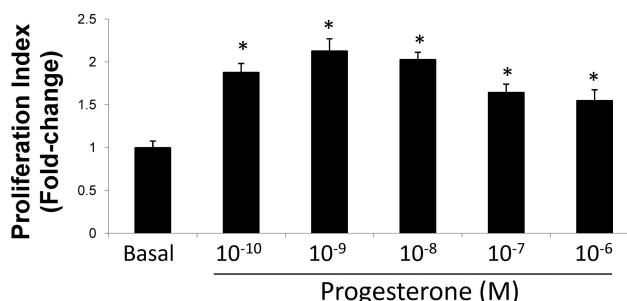


Fig. 6. Dose-dependent effects of progesterone (10^{-10} to 10^{-6} M for 24 h) on NRC proliferation. Progesterone stimulates a dose-dependent increase in NRC proliferation. Data are means \pm SE of 5 experiments. * P < 0.05 vs. basal.

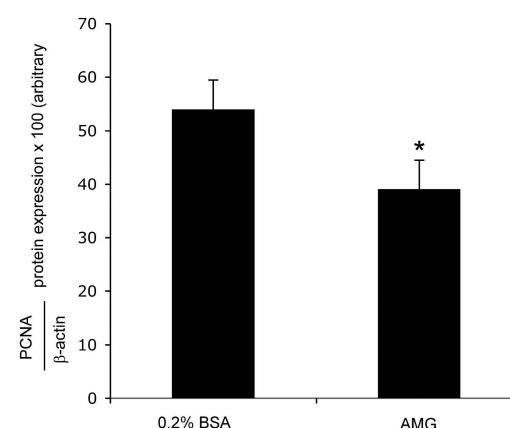


Fig. 7. Effect of aminoglutethimide (AMG) inhibition of cholangiocyte steroidogenesis on NRC proliferation. Inhibition of progesterone steroidogenesis by in vitro treatment of NRC with AMG prevented cholangiocyte proliferation evaluated by immunoblots for PCNA. * P < 0.05 vs. basal. Data are means \pm SE of 4 experiments. * P < 0.05 vs. basal.

PGRMC1, PGRMC2, and mPR α in the stimulation of cholangiocyte proliferation. The exact signaling mechanism of progesterone-induced proliferation was beyond the scope of the present study and will be delineated in future studies.

In addition to progesterone receptor expression, we demonstrate the novel findings that cholangiocytes express the enzymatic pathway (StAR, p450scc, and 3 β -HSD) for the synthesis of progesterone. In confirmation of cholangiocytic expression of the progesterone steroidogenesis pathway, we also show that cholangiocytes from normal and BDL female and male rats and NRC secrete progesterone. These findings are similar to our previous studies demonstrating that cholangiocytes synthesize and secrete a number of growth promoting factors such as NGF, VEGF, prolactin, and serotonin (25, 26, 48, 70). Therefore, our present findings indicate that progesterone can play a key role in the regulation of cholangiocytic proliferation by not only by paracrine but also autocrine signaling mechanisms. We predict that the local autocrine secretion of progesterone by cholangiocytes augments the overall paracrine effects of circulating progesterone. To shed light on the potential autocrine signaling mechanism, we demonstrate that supernatants from normal and BDL male and female cholangiocytes stimulate NRC proliferation. The proliferative response stimulated by normal and BDL cholangiocytic supernatants was partially blocked by an antiprogestrone antibody. The partial, but not full, blockage of proliferation is most likely due to other factors secreted by cholangiocytes into the supernatant that we have previously demonstrated to be growth promoting for cholangiocytes, such as NGF, prolactin, and VEGF (25, 26, 70). BDL supernatants stimulated proliferation to a lesser extent than supernatants from normal rats, which confirms our finding that progesterone levels are lower in BDL cholangiocytic supernatants. However, the BDL supernatants significantly induced cholangiocytic proliferation, which was partially blocked by antiprogestrone, indicating that although the level of progesterone in the supernatant was low progesterone remains a critical factor in cholangiocytic proliferation *in vitro*. We postulate that the decrease in cholangiocytic synthesis by cholangiocytes was due to a counterregulatory mechanism acting to downregulate or modulate cholangiocytic proliferation in the chronic model of BDL where proliferation is leveling off at day 7 and thereafter. In further confirmation of an autocrine signaling mechanism, we demonstrate that inhibition of the synthesis of progesterone with AMG, a p450scc inhibitor, prevents NRC proliferation. A number of studies have shown that progesterone has previously been shown to regulate proliferation and protect cells from apoptosis in autocrine/paracrine mechanisms (15, 47, 55). Since cholangiocytic secretion of progesterone is downregulated by BDL, serum levels of progesterone (which are considerably elevated in serum compared with cholangiocytic supernatants) indicate that the paracrine signaling mechanism may play a role during cholestasis.

In contrast to a previous report that serum progesterone levels are elevated during BDL in rabbits (20), we observed that serum progesterone levels remained unchanged in female and male rats with BDL compared with normal control animals. We postulate that our observed findings are merely due to species differences and decreased cholangiocytic progesterone secretion. We also observed that there was a significant downregulation of progesterone secretion in cholangiocytes isolated from BDL rats. The finding is in contrast to our

previous publications that demonstrated that other growth promoting factors are upregulated during cholestasis (8, 25, 26). We believe that the downregulation of progesterone secretion during cholestasis may be a counterregulatory mechanism to regulate biliary proliferation, since cholangiocytes have less need to secrete the prostimulatory factor, progesterone, to sustain cholangiocytic proliferation. We hypothesize that progesterone synthesis is reduced during cholestasis by shunting of pregnenolone to the synthesis of other steroid hormones, such as estrogen. In fact, estrogens prevent the increase of cholangiocytic apoptosis and loss of cholangiocytic proliferation induced by the biliary-digestive diversion in the BDL rat (69). Steroids such as progesterone and estrogen have been shown to have neuroprotective and antiapoptotic effects in a number of cell types (37, 58, 67). Regulation of steroid production by cholangiocytes may be a key mechanism in controlling cholangiocytic growth and responses to injury and deserves further evaluation. Antiprogestrone therapy might benefit patients in conditions with cholangiocytic proliferation such as conditions during extrahepatic cholestasis.

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