Castration inhibits biliary proliferation induced by bile duct obstruction: novel role for the autocrine trophic effect of testosterone

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Yang F, Priester S, Onori P, Venter J, Renzi A, Franchitto A, Munshi MK, Wise C, Dostal DE, Marzioni M, Saccomanno S, Ueno Y, Gaudio E, Glaser S. Castration inhibits biliary proliferation induced by bile duct obstruction: novel role for the autocrine trophic effect of testosterone. Am J Physiol Gastrointest Liver Physiol 301: G981–G991, 2011. First published September 8, 2011; doi:10.1152/ajpgi.00061.2011.—Increased cholangiocyte growth is critical for the maintenance of biliary mass during liver injury by bile duct ligation (BDL). Circulating levels of testosterone decline following castration and during cholestasis. Cholangiocytes secrete sex hormones sustaining cholangiocyte growth by autocrine mechanisms. We tested the hypothesis that testosterone is an autocrine trophic factor stimulating biliary growth. The expression of androgen receptor (AR) (AR antagonist) was determined in liver sections, male cholangiocytes, and cholangiocyte cultures [normal rat intrahepatic cholangiocyte cultures (NRICC)]. Normal or BDL (immediately after surgery) rats were treated with testosterone or antitestosterone antibody or underwent surgical castration (followed by administration of testosterone) for 1 wk. We evaluated testosterone serum levels; intrahepatic bile duct mass (IBDM) in liver sections of female and male rats following the administration of testosterone; and secretin-stimulated cAMP levels and bile secretion. We evaluated the expression of 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3), the enzyme regulating testosterone synthesis in cholangiocytes. We evaluated the effect of testosterone on the proliferation of NRICC in the absence/presence of flutamide (AR antagonist) and antitestosterone antibody and the expression of 17β-HSD3. Proliferation of NRICC was evaluated following stable knock down of 17β-HSD3. We found that cholangiocytes and NRICC expressed AR. Testosterone serum levels decreased in castrated rats (prevented by the administration of testosterone) and rats receiving antitestosterone antibody. Castration decreased IBDM and secretin-stimulated cAMP levels and ductal secretion of BDL rats. Testosterone increased 17β-HSD3 expression and proliferation in NRICC that was blocked by flutamide and antitestosterone antibody. Knock down of 17β-HSD3 blocks the proliferation of NRICC. Drug targeting of 17β-HSD3 may be important for managing cholangiopathies.

biliary epithelium; biliary secretion; 17β-hydroxysteroid dehydrogenase 3; secretin; sex hormones

IN ADDITION TO PLAYING A KEY role in the ductal secretion of water and bicarbonate (mainly regulated by secretin) (3, 30), cholangiocytes are the target cells in a number of chronic cholestatic liver diseases (termed cholangiopathies), including primary sclerosing cholangitis and primary biliary cirrhosis (PBC) (5, 8). During the progression of cholangiopathies, the balance between the proliferation/loss of cholangiocytes is critical for the maintenance of biliary secretory function and intrahepatic bile ductal mass (5, 8, 34, 36). Cholangiocytes respond to cholestasis and liver injury with changes in proliferation and ductal bile secretion (2, 3, 18, 22). In response to bile duct ligation (BDL), there is enhanced bile duct hyperplasia and secretin-stimulated choleresis (a functional marker of cholangiocyte growth) (2, 3, 18, 22), whereas, following the administration of hepatotoxins (e.g., carbon tetrachloride), there is loss of cholangiocyte mass and secretory function (36). Indeed, proliferating cholangiocytes serve as neuroendocrine cells secreting and responding to a number of hormones and neuropeptides contributing to the autocrine and paracrine pathways that modulate the homeostasis of the biliary epithelium (8, 16, 18, 19, 22, 23, 39).

Cholangiocyte growth/apoptosis is regulated by a number of factors, including gastrointestinal hormones, the second messenger system, cAMP, and sex hormones, including estrogens, prolactin, follicle-stimulating hormone, and progesterone (7, 8, 17, 23, 39, 49). Regarding sex hormones, estrogens have been shown to sustain cholangiocyte proliferation and reduce cholangiocyte apoptosis (6, 7). Prolactin is expressed and secreted by cholangiocytes and stimulates the growth of cholangiocytes by an autocrine mechanism (49). Progesterone enhances the proliferative activity of female and male cholangiocytes via autocrine mechanisms, since cholangiocytes possess the enzymatic pathway for steroidogenesis (23). Follicle-stimulating hormone increases cholangiocyte proliferation by an autocrine mechanism via cAMP-dependent phosphorylation of ERK1/2 and Elk-1 (39).

Testosterone is an anabolic steroid that is primarily secreted in the testes of males and the ovaries of females, although small amounts are also secreted by the adrenal glands. Testosterone is thought to predominantly mediate its biological effects through binding to the androgen receptor (AR) (43). Similar to other members of the nuclear receptor superfamily, ARs function as a ligand-inducible transcription factor. ARs are expressed in the liver by hepatocellular carcinoma, hepato-
tocyt in areas of ductular metaplasia, and in a number of metastatic adenocarcinomas, including bile duct cholangiocarcinoma (26, 44, 47). However, no information exists regarding the expression and role of testosterone and its receptors in the regulation of cholangiocyte growth and ductal secretory activity in cholestasis. The rate-limiting step in the synthesis of testosterone from androstenedione (a product of dehydroepiandrosterone and progesterone, both of which are products of pregnenolone and cholesterol) depends on the enzyme 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3) (20). On the basis of these findings, we performed experiments aimed to demonstrate that: 1) reduction of testosterone serum levels decreases cholangiocyte proliferation; 2) testosterone administration stimulates biliary growth and prevents the decrease in biliary hyperplasia induced by castration; 3) cholangiocytes express 17β-HSD3 and secrete testosterone regulating biliary hyperplasia induced by castration; 4) cholangiocytes express 17β-HSD3 and secrete testosterone regulating biliary hyperplasia; 5) cholangiocytes express 17β-HSD3 and secrete testosterone regulating biliary hyperplasia; and 6) silencing of 17β-HSD3 (the enzyme regulating testosterone synthesis) (20) decreases biliary proliferation in vitro. The data suggest an autocrine compensatory role of testosterone in sustaining cholangiocyte proliferation in cholestasis, a pathological condition characterized by testicular atrophy and lowered serum testosterone levels (14, 33, 54, 57).

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated. The monoclonal mouse antibody (clone PC-10) reacting with proliferating cellular nuclear antigen (PCNA) and the goat polyclonal antibody (antibody raised against a peptide mapping within an internal region of AR of human origin) reacting with both subtypes of AR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The AR antibody (G-13) is recommended for the detection of AR of mouse, rat, and human origin. The substrate for γ-glutamyltranspeptidase (γ-GT), N-(γ-γ,γ-triamyl)-4-methoxy-2-naphthylamide, was purchased from Polysciences (War-lington, PA). The enzyme-linked immunosorbent assay (ELISA) kit used for measuring testosterone levels in serum and supernatant of primary cultures (after 6 h of incubation at 37°C) of isolated cholangiocytes from the selected groups of rats and primary cultures of rat intrahepatic cholangiocyte cultures [normal rat intrahepatic cholangiocyte cultures (NRICC)] from normal male rats (4) was purchased from New England Biolabs (BE, MA). A modified reverse transcriptase (RT)-PCR (1) and fluorescence-activated cell sorter (FACS) analysis (45) in cholangiocytes and hepatocytes from normal and BDL male rats. In NRICC, the expression of AR was also evaluated by RT-PCR and immunofluorescence (39). For all immunoreactions, negative controls (with normal serum from the same species substituted for the primary antibody) were included.

With regard to RT-PCR (performed using the GeneAmp RNA PCR kit from Perkin-Elmer, Branchburg, NJ), specific oligonucleotide primers were based on the sequence of the rat AR (11) (sense, 5′-CCCTTGTTGTGTCAGCTAGAA-3′ and antisense, 5′-TAGACA-GGATCCTGCGCTGCT-3′), with an expected fragment length of 247 bp; we used RNA (1 μg) from rat testes and yeast-transfer RNA as positive and negative controls, respectively. The comparability of the RNA used was assessed by RT-PCR for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense 5′-GT-GACGTTCAACGGAATCTCCATT-3′ and antisense 5′-GTTAG-TGGGGCCCTGGAATTGTTA-3′), with an expected fragment length of 294 bp; primers were based on the rat GAPDH sequence (15); rat kidney and yeast transfer RNA were the positive and negative controls for the GAPDH gene, respectively. Standard RT-PCR conditions were used, consisting of 35 step cycles: 30 s at 94°C, 30 s at 60°C for AR and 52°C for GAPDH for 45 s at 72°C. FACS analysis for AR was performed in isolated cholangiocytes and hepatocytes from normal and BDL rats using a C6 flow cytometer and analyzed by CFlow Software (Accuri Cytometers, Ann Arbor, MI). At least 20,000 events in the light scatter (side scatter/forward scatter) were acquired. The expression of AR was identified and gated on fluorescence channel 1-A (FL1-A) count plots. The relative quantity of AR (mean selected protein fluorescence) was expressed as mean FL1-A (samples) per mean FL1-A (secondary antibodies only). The standard errors were calculated as (CV FL1-A) × (mean FL1-A)/SQR(count 1), where CV is coefficient of variation and SQR is square root. We evaluated the expression of 17β-HSD3 (37) by: 1) immunofluorescence (16) in frozen liver sections (4–5 μm thick) and immunohistochemistry (39) in paraffin-embedded liver sections (4–5 μm thick) from normal and BDL male rats; and 2) RT-PCR (1) and fluorescence-activated cell sorter (FACS) analysis (45) in cholangiocytes and hepatocytes from normal and BDL male rats. In BDL, the expression of AR was also evaluated by RT-PCR and immunofluorescence (39). For all immunoreactions, negative controls (with normal serum from the same species substituted for the primary antibody) were included.

Purification of cholangiocytes and hepatocytes. Virtually pure cholangiocytes (~98% by γ-GT histochemistry) (46) were isolated by immunoaffinity separation (27) 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 (27). Cell viability (by trypan blue exclusion) was ~98%. Hepatocytes were isolated from normal and BDL male rats as described (27). Male NRICC, which display morphological, phenotypic, and functional phenotypes similar to that of freshly isolated cholangiocytes, were developed, characterized, and maintained in culture as described by us (4).

Expression of AR and 17β-HSD3. The presence of AR was evaluated by: 1) immunofluorescence (16) in frozen liver sections (4–5 μm thick) and immunohistochemistry (39) in paraffin-embedded liver sections (4–5 μm thick) from normal and BDL male rats; and 2) RT-PCR (1) and fluorescence-activated cell sorter (FACS) analysis (45) in cholangiocytes and hepatocytes from normal and BDL male rats. In NRICC, the expression of AR was also evaluated by RT-PCR and immunofluorescence (39). For all immunoreactions, negative controls (with normal serum from the same species substituted for the primary antibody) were included.

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FACS analysis (45) (see above) in freshly isolated male cholangiocytes; and 3) real-time PCR and immunofluorescence (16) in male NRICC. For all immunoreactions, negative controls (with normal serum from the same species substituted for the primary antibody) were included. Following immunofluorescence for AR and 17β-HSD3, images were visualized using an Olympus IX-71 confocal microscope. Light microscopy photographs of liver sections stained for AR and 17β-HSD3 were taken by Leica Microsystems DM 4500 B Light Microscopy (Wetzlar, Germany) with a Jenoptik Prog Res C10 Plus Videocam (Jena, Germany). The positivity of bile ducts for AR and 17β-HSD3 was evaluated as described by us (25). When 0–5% of bile ducts were positive for AR and 17β-HSD3, we assigned a negative score; a ± score was assigned when 6–10% of bile ducts were positive; a + score was assigned when 11–30% of bile ducts were positive; a ++ score was assigned with 31–60% of bile ducts positive; and a +++ score was assigned when >61% of bile ducts were positive. Two pathologists performed the evaluations in a blinded manner. For real-time PCR, total RNA was extracted from cholangiocytes by the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using the Reaction Ready First-Strand cDNA synthesis kit (SuperArray, Frederick, MD). These reactions were used as templates for the PCR assays using a SYBR Green PCR master mix and specific primers designed against the rat 17β-HSD3 NM_054007 (51) and GAPDH, the housekeeping gene (SuperArray), in the real-time thermal cycler (ABI Prism 7900HT sequence detection system). A ΔΔCt (delta delta of the threshold cycle) analysis was performed using normal cholangiocytes as the control sample. Data are expressed as fold change of relative mRNA levels ± SE (n = 6).

Fig. 1. A: image showing that intrahepatic bile ducts from normal and bile duct ligation (BDL) male rats express androgen receptor (AR) (red staining). Colocalization with CK-19 (green staining) of bile ducts expressing the AR (red staining) is also visible. Bar = 50 μm. B: by immunohistochemistry, normal cholangiocytes and hepatocytes express low levels of AR, expression that increased following BDL (A and B; see Table 1). Original magnification, ×40. C: by RT-PCR, the message for AR was expressed by freshly isolated cholangiocytes from normal (NR) and BDL rats and normal rat intrahepatic cholangiocyte cultures (NRICC); the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was expressed similarly by these cells. MW, mol wt. D: by immunofluorescence, NRICC also expressed the protein for AR. Bar = 20 μm. E and F: fluorescence-activated cell sorter (FACS) analysis shows the presence of the protein for AR in freshly isolated cholangiocytes and hepatocytes from normal and BDL male rats; the expression of AR seemed up-regulated (* P < 0.05) in BDL cholangiocytes compared with normal cholangiocytes. Data are means ± SE of 3 determinations.
Evaluation of testosterone serum levels, cholangiocyte apoptosis, and intrahepatic bile duct mass in liver sections. Testosterone serum levels in female and male rats were measured by commercially available ELISA kits (Cayman Chemical). Cholangiocyte apoptosis was evaluated in paraffin-embedded liver sections (4–5 μm thick) by a quantitative terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) kit (Apoptag; Chemicon International) (39). The percentage of TUNEL-positive cholangiocytes was counted in six nonoverlapping fields (magnification ×40) for each slide; the data are expressed as the percentage of TUNEL-positive cholangiocytes. The number of intrahepatic bile ducts in frozen liver sections (4–5 μm thick) was determined by the evaluation of intrahepatic bile duct mass (IBDM) by point-counting analysis (35, 56). IBDM was measured as the percentage area occupied by γ-GT-bile duct/total area × 100 (35, 56). Morphometric data were obtained in six different slides for each group; for each slide, we performed the counts in six nonoverlapping fields (n = 36). Following the selected staining, sections were counterstained with hematoxylin and eosin and analyzed for each group using a BX-51 light microscopy (Olympus, Tokyo, Japan).

Measurement of basal and secretin-stimulated cAMP levels and bile secretion. At the functional level, cholangiocyte proliferation was evaluated by measurement of basal and secretin-stimulated cAMP levels in purified cholangiocytes by RIA (17, 22, 32) and bile and bicarbonate secretion in bile fistula rats (3), two functional indexes of cholangiocyte proliferation (3, 17, 22). For the measurement of cAMP levels (17, 22), purified cholangiocytes (1 × 10⁵) were incubated for 1 h at 37°C and incubated for 5 min at room temperature with 0.2% BSA (basal) or 100 nM secretin with 0.2% BSA (basal) or 100 nM secretin (17, 22, 32). Following anesthesia with pentobarbital sodium, rats were surgically prepared for bile collection (3). When steady-state bile flow was reached [60–70 min from the infusion of Krebs-Ringer-Henseleit (KRH) solution], the animals were infused by a jugular vein with secretin (100 nM) for 30 min followed by a final infusion of KRH for 30 min. Bile was collected every 10 min in preweighed tubes that were used for determining bicarbonate concentration. Bicarbonate concentration (measured as total CO₂) in the selected bile sample was determined by an ABL 520 Blood Gas System (Radiometer Medical, Copenhagen, Denmark).

Effect of testosterone on the expression of 17β-HSD3 and proliferation of NRICC: Effect of pharmacological inhibition and molecular silencing of 17β-HSD3 on NRICC proliferation. To obtain a dose-response curve, NRICC were treated with vehicle (1% methanol, where testosterone is dissolved, basal value) or testosterone (10⁻⁵ to 10⁻¹¹ M for 7 days in 1% methanol) before evaluating cell proliferation by MTS assays (16). We also evaluated the effects of testoster-

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<th>Cholangiocytes</th>
<th>Normal</th>
<th>BDL</th>
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<td>Hepatocytes</td>
<td>±</td>
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AR, androgen receptor; BDL, bile duct ligation. Grading scale: - = 0–5%; ± = 6–10%; + = 11–30%; ++ = 31–60%; +++ = >61%.

Table 1. Semiquantitative analysis of AR expression in liver sections from normal and BDL male rats

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
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<tr>
<td>Cholangiocytes</td>
<td>+</td>
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<td>Hepatocytes</td>
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Fig. 2. Measurement of serum testosterone levels in the selected groups of animals. Testosterone serum levels were lower in female and male BDL rats compared with their corresponding normal rats. The administration of testosterone increased testosterone serum levels in normal and BDL female and male rats. Castration significantly decreased testosterone serum levels in normal and BDL rats. Administration of neutralizing anti-testosterone antibody decreased testosterone serum levels in both normal and BDL rats compared with rats treated with nonimmune serum. The administration of testosterone to BDL castrated rats partly prevented castration-induced reduction of testosterone serum levels. Data are means ± SE of 12 cumulative determinations. *P < 0.05 compared with the corresponding values of normal rats. #P < 0.05 compared with the corresponding values of normal rats. &P < 0.05 compared with the corresponding values of BDL rats.
Table 2. Measurement of IBDM in the experimental groups of animals

<table>
<thead>
<tr>
<th>Treatments</th>
<th>IBDM, %</th>
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<tr>
<td><strong>Male</strong></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>BDL</td>
<td>6.32 ± 0.67</td>
</tr>
<tr>
<td>BDL + testosterone</td>
<td>10.4 ± 0.80</td>
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<tr>
<td>BDL + castration + testosterone</td>
<td>5.48 ± 0.63</td>
</tr>
<tr>
<td>BDL + testosterone antibody</td>
<td>1.8 ± 0.14</td>
</tr>
<tr>
<td>BDL - castration + testosterone</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.77 ± 0.14</td>
</tr>
<tr>
<td>BDL</td>
<td>4.01 ± 0.21</td>
</tr>
<tr>
<td>BDL + testosterone antibody</td>
<td>6.68 ± 0.89</td>
</tr>
</tbody>
</table>

Values are means ± SE. IBDM, intrahepatic bile duct mass. P < 0.05 vs. IBDM of normal rats (a) and vs. IBDM of BDL rats (b).

**RESULTS**

**Expression of AR.** By both immunofluorescence and immunohistochemistry in liver sections, we demonstrated that intrahepatic bile ducts from normal and BDL male rats express AR (Fig. 1, A and B). Colocalization of CK-19 (a cholangiocyte-specific marker) (18) with intrahepatic bile ducts expressing AR is visible in Fig. 1A. By immunohistochemistry, normal cholangiocytes and hepatocytes express low levels of AR, expression that increased following BDL (Fig. 1, A and B, and Table 1). By RT-PCR, the message for AR (247 bp) was expressed by freshly isolated cholangiocytes and hepatocytes from normal and BDL male rats and NRICC (Fig. 1C); the housekeeping gene, GAPDH mRNA (294 bp), was similarly expressed by these cells (Fig. 1C). By immunofluorescence, NRICC also expressed the protein for AR (Fig. 1D). We also demonstrated by FACS analysis the presence of AR in freshly isolated cholangiocytes and hepatocytes from normal and BDL male rats (Fig. 1, E and F); the expression of AR was upregulated in BDL compared with normal cholangiocytes (Fig. 1E).

**Evaluation of testosterone serum levels, cholangiocyte apoptosis, and IBDM in liver sections.** Parallel to previous studies (53, 54), testosterone serum levels were lower in female and male BDL rats compared with their corresponding normal rats (Fig. 2A). The serum levels of testosterone were lower in female rats compared with the corresponding values of male rats (Fig. 2B). The administration of testosterone increased testosterone serum levels in normal and BDL male rats (Fig. 2, A and B). Castration significantly decreased testosterone serum levels in normal and BDL male rats (Fig. 2B) (31). Similarly, administration of neutralizing antitestosterone antibody decreased testosterone serum levels in both normal and BDL male rats compared with rats treated with nonimmune serum (Fig. 2B). The administration of testosterone to BDL castrated rats partly prevented castration-induced reduction of testosterone serum levels (Fig. 2B). In female and male BDL rats, there

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**Fig. 3.** Measurement of basal and secretin-stimulated cAMP levels in purified cholangiocytes. In normal and BDL rats (without castration), secretin increases cAMP levels of purified cholangiocytes. In purified cholangiocytes from normal and BDL castrated rats, there was ablation of the stimulatory effects of secretin on cAMP levels. *P < 0.05 vs. the corresponding basal cAMP levels. #P < 0.05 vs. basal cAMP levels of normal cholangiocytes. Data are means ± SE of 6 experiments.
was increased IBDM compared with their corresponding normal rats (Table 2) (3). Also, testosterone increased IBDM in normal (data not shown) and BDL female and male rats (Table 2) compared with rats treated with vehicle. Consistent with the concept that testosterone is a trophic factor for cholangiocyte growth, castration (which reduces serum testosterone levels) (31) decreased IBDM in both normal (data not shown) and BDL rats compared with rats without castration (Table 2). We next demonstrated that administration of an antitestosterone antibody (which reduces the circulating levels of testosterone) decreased IBDM compared with control BDL rats (Table 2), and administration of testosterone partly prevented castration-induced loss of IBDM (Table 2). In BDL castrated rats and BDL rats treated with antitestosterone antibody, there was an increase in apoptosis compared with BDL rats (data not shown).

Castration inhibits secretin-stimulated cAMP levels and bile secretion. Secretin increased cAMP levels of cholangiocytes from normal but not normal castrated rats (Fig. 3). Basal cAMP levels of cholangiocytes from BDL rats were higher than cAMP levels of normal cholangiocytes (Fig. 3). As expected (3, 35), secretin did not induce changes in bile and bicarbonate secretion in normal rats with or without castration (Table 3). In agreement with previous studies (2, 3), in BDL rats (without castration), secretin increased cAMP levels in purified cholangiocytes (Fig. 3) and bicarbonate-rich cholestasis in bile fistula rats (Table 2). After castration to BDL rats, the stimulatory effects of secretin on cAMP levels in purified cholangiocytes (Fig. 3) and bile and bicarbonate secretion in bile fistula rats (Table 2) were ablated. Chronic administration (1 wk) of testosterone to BDL castrated rats restored the functional secretory activity of cholangiocytes, since secretin was able to stimulate bile and bicarbonate secretion (Table 3) in these rats.

Expression of 17β-HSD3 in liver sections and cholangiocytes: Evaluation of testosterone secretion in NRICC and determination of the effect of pharmacological inhibition and molecular silencing of 17β-HSD3 on NRICC growth. By immunofluorescence and immunohistochemistry in liver sections, 17β-HSD3 was expressed by intrahepatic bile ducts from normal and BDL male rats (Fig. 4, A and B). The immunoreactivity was higher in bile ducts from male BDL rats compared with their corresponding normal rats. mRNA (by real-time PCR) and protein (by FACS) for 17β-HSD3 was expressed by normal male cholangiocytes and increased following BDL (Fig. 4, C and D). In purified cholangiocytes from male BDL rats with castration or receiving antitestosterone antibody, the expression of 17β-HSD3 mRNA was similar to or higher than that of BDL cholangiocytes (Fig. 4C), which is likely due to a compensatory mechanism by cholangiocytes in response to decreased testosterone serum levels after castration or the administration of antitestosterone antibody to BDL rats. We have also demonstrated that normal cholangiocytes and NRICC secrete testosterone in the supernatant, and the levels of testosterone increased in the supernatant of BDL cholangiocytes compared with normal cholangiocyte supernatant (Fig. 4E). In purified cholangiocytes from BDL rats with castration or receiving antitestosterone antibody, the secretion of testosterone was similar to that of BDL cholangiocytes (Fig. 4E). By
immunofluorescence, NRICC express the protein for 17β-HSD3 (Fig. 4F).

Similar to what is shown in vivo (Table 2), testosterone (10^{-11} to 10^{-5} M) in vitro increased the proliferation (by MTS) of NRICC compared with the corresponding basal value (Fig. 5A). Also, testosterone (100 nM) increased the mRNA expression of 17β-HSD3 (the key enzyme regulating testosterone synthesis) (20) in NRICC compared with the
corresponding basal value (Fig. 5A). Consistent with the concept that testosterone (synthesized and secreted by freshly isolated cholangiocytes and hepatocytes from normal and BDL rats, and male NRICC express testosterone receptors. We have also shown that: 1) testosterone serum levels were lower in female and male BDL compared with normal rats and 2) castration and administration of neutralizing antitestosterone antibody significantly decreased testosterone serum levels in normal and BDL male rats. The administration of testosterone increased testosterone serum levels in both normal and BDL male rats and partly prevented castration-induced reduction of testosterone serum levels. We have also demonstrated that: 1) testosterone increased IBDM of both normal and BDL female and male rats; 2) castration and administration of antitestosterone antibody decreased IBDM in BDL male rats; and 3) administration of testosterone partly prevented castration-induced loss of IBDM in BDL male rats. At the functional level, in BDL castrated rats, there was ablation of secretin-stimulated cAMP levels and bicarbonate-rich choleresis, two functional indexes of biliary growth (2, 3, 22, 36). Similar to in vivo, in in vitro studies, we have demonstrated that testosterone increased the proliferation of male NRICC. We have also

DISCUSSION

The present study demonstrates the paracrine and autocrine role of testosterone in the stimulation of cholangiocyte growth in normal and cholestatic states. We first demonstrated that intrahepatic bile ducts and hepatocytes (in liver sections) and freshly isolated cholangiocytes and hepatocytes from normal and BDL rats, and male NRICC express testosterone receptors. We have also shown that: 1) testosterone serum levels were lower in female and male BDL compared with normal rats and 2) castration and administration of neutralizing antitestosterone antibody significantly decreased testosterone serum levels in normal and BDL male rats. The administration of testosterone increased testosterone serum levels in both normal and BDL male rats and partly prevented castration-induced reduction of testosterone serum levels. We have also demonstrated that: 1) testosterone increased IBDM of both normal and BDL female and male rats; 2) castration and administration of antitestosterone antibody decreased IBDM in BDL male rats; and 3) administration of testosterone partly prevented castration-induced loss of IBDM in BDL male rats. At the functional level, in BDL castrated rats, there was ablation of secretin-stimulated cAMP levels and bicarbonate-rich choleresis, two functional indexes of biliary growth (2, 3, 22, 36). Similar to in vivo, in in vitro studies, we have demonstrated that testosterone increased the proliferation of male NRICC. We have also
shown that: 1) intrahepatic cholangiocytes and NRICC express 17β-HSD3 (the key enzyme regulating testosterone synthesis) (20) and secrete testosterone; 2) the biliary expression of 17β-HSD3 and secretion of testosterone increases after BDL and in NRICC after in vitro treatment with testosterone; and 3) stable transfection of 17β-HSD3 in NRICC decreases the proliferative activity of these cells. The finding that testosterone stimulates biliary proliferation by an autocrine mechanism may be an important compensatory mechanism for sustaining biliary growth and ductal secretory activity in ductopenic pathologies of the biliary epithelium.

Cholangiopathies, which specifically target cholangiocytes, are characterized by dysregulation of the balance between biliary growth/damage (5). Thus, studies aimed to understand the intracellular mechanisms of this phenomenon are necessary. In this context, the cholestatic BDL rodent model has been used widely to pinpoint the mechanisms of biliary growth/apoptosis (2, 3, 6, 18, 19, 22, 34, 49). A number of studies have demonstrated that 1) gastrointestinal and sex hormones, bioactive amines, and neuropeptides regulate the growth of bile ducts and 2) in the course of chronic cholestasis, cholangiocytes acquire neuroendocrine phenotypes regulating biliary functions by both autocrine and paracrine pathways (2, 3, 5–8, 18, 19, 22, 34, 49). Our study provides the first evidence that ARs are expressed by cholangiocytes and testosterone stimulates biliary growth and secretion during cholestasis. We propose that testosterone is important in sustaining biliary proliferation and ductal secretory activity in pathological conditions associated with functional damage of the biliary epithelium.

We first demonstrated in male rats the expression of functional testosterone receptors in bile ducts, cholangiocytes, hepatocytes, and NRICC. Supporting our findings, a number of studies have shown the presence of functional ARs in liver cells, including hepatocytes and bile ducts from PBC patients (26, 29, 38). Also, the AR mRNA has been detected in human liver biopsy samples, fetal liver, and HepG2 cells (48). In the next sets of experiments, we demonstrated that enhanced testosterone serum levels correlate with increased IBDM in male rats and decreased testosterone serum levels are associated with reduction of the number of intrahepatic bile ducts in male rats. We first validated our models, demonstrating that testosterone serum levels decreased in cholestatic BDL male rats (compared with normal rats) and in castrated rats and increased in rats chronically treated with testosterone. The decrease in testosterone serum levels is likely due to hypothyroidism of seminal vesicles as observed in rats during puberty and BDL-induced cholestasis (33, 54, 57). Testicular atrophy has been observed in cirrhotic patients (52), whereas lower serum testosterone levels have been shown in patients with PBC (14). Contrasting results exist regarding serum testosterone levels in other liver diseases. For example, while a study has demonstrated lowered serum testosterone levels in cirrhotic rats (53), other studies have shown elevated serum testosterone may promote the development of hepatocellular carcinoma in cirrhosis (50). The decrease in serum testosterone levels observed in our animals is supported by a number of studies in rodents (31, 55) in which the decrease is prevented by the chronic administration of testosterone (55). Similar to our finding, other studies have demonstrated that chronic administration of testosterone increased testosterone serum levels (9). A number of studies have shown that sex hormones, including estrogens, prolactin, follicle-stimulating hormone, and progesterone, sustain biliary proliferation by both paracrine and autocrine mechanisms (6, 7, 23, 39, 49). However, no information exists regarding the role of testosterone in modulating the balance between cholangiocyte growth/loss. The fact that testosterone increases biliary hyperplasia and prevents the loss of biliary growth and function (following castration) supports the concept that androgens can be important for ameliorating the cholestatic conditions associated with testicular hypotrophy and ductopenic conditions associated with decreased testosterone levels as occurs in PBC (14). These findings and the fact that the administration of neutralizing antigonad steroid antibodies reduces testosterone serum levels and biliary hyperplasia introduce the concept that the administration of testosterone receptor antagonists or antigonad steroid antibodies may be new therapeutic approaches for decreasing the aberrant growth of cholangiocytes. At the functional level, we demonstrated that the decrease in testosterone serum levels was associated with ablation of secretin-stimulated cAMP synthesis in cholangiocytes and bicarbonate-rich choleresis in bile fistula male rats, two functional parameters that were restored by the chronic administration of testosterone. Indeed, enhanced secretin receptor expression and secretin-stimulated ductal secretory activity is associated with enhanced biliary hyperplasia (2, 3, 22), whereas reduced secretory capacity in response to secretin is an index of functional cholangiocyte damage (34, 36).

We next performed experiments in male rats aimed at demonstrating that: 1) purified cholangiocytes and NRICC express 17β-HSD3 (the key enzyme regulating testosterone synthesis) (20) and secrete testosterone and 2) testosterone stimulates in vitro the growth of NRICC by directly interacting with ARs on cholangiocytes by an autocrine mechanism stimulating the expression of 17β-HSD3. The higher expression of 17β-HSD3 and the enhanced secretion of testosterone by purified cholangiocytes from BDL rats and BDL rats treated in vivo with testosterone (that proliferate at higher rates) support the concept that testosterone (secreted by cholangiocytes) is a trophic autocrine hormone for sustaining biliary hyperplasia. The fact that testosterone stimulates biliary growth by an autocrine fashion is also supported by the fact that testosterone increases the mRNA expression of 17β-HSD3 in vitro in NRICC and flutamide (a specific antagonist of AR) and antitestosterone antibody inhibit the growth of NRICC in vitro. Conclusive evidence that testosterone is an important autocrine trophic factor sustaining biliary growth is also supported by the finding that, in conditions of lowered serum testosterone levels (after castration and administration of neutralizing antitestosterone antibody to BDL rats), there is enhanced testosterone secretion likely by a compensatory mechanism. The possible role of testosterone as a key autocrine trophic was also supported by knock down of 17β-HSD3, which caused a marked decreased in NRICC proliferation. In support of our findings, a number of studies demonstrate the presence of 17β-HSD3 in the liver (41) and show that this enzyme isoform modulates the stimulatory effects on the mitosis of a number of cells, including cholangiocytes. This idea is supported by recent studies showing that specific inhibitors of 17β-HSD3 have been shown to inhibit hormone-dependent prostate cancer and benign prostate hyperplasia (12). The novel concept that cholangiocytes are secretory cells synthesizing a number of factors (including
testosterone) regulating the homeostasis of the biliary epithelium is supported by a number of studies. For example, cholangiocytes synthesize a number of neuroendocrine factors, such as progestosterone, prolactin, vascular endothelial growth factor, and nerve growth factor, that stimulate biliary growth (19, 21, 23, 49). Also, serotonin inhibits biliary hyperplasia in BDL rats, since cholangiocytes secrete serotonin, the blockade of which enhances cholangiocyte proliferation in the course of cholostasis (42).

In summary, we have demonstrated the presence of functional ARs on cholangiocytes and that testosterone stimulates biliary growth by an autocrine mechanism by increasing the expression of 17β-HSD3 in cholangiocytes. We have also demonstrated that testosterone prevents the decrease in biliary hyperplasia typical of BDL after castration and the administration of neutralizing antitestosterone antibody. We propose that drug targeting of 17β-HSD3 may be important to regulate the balance between biliary proliferation/loss and ductal secretory activity in cholangiopathies.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


