Sex Hormone Influence on Hepatitis in Young Male A/JCr Mice Infected with *Helicobacter hepaticus*^{†‡}

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Hepatitis B virus (HBV), the leading cause of human hepatocellular carcinoma, is especially virulent in males infected at an early age. Likewise, the murine liver carcinogen *Helicobacter hepaticus* is most pathogenic in male mice infected before puberty. We used this model to investigate the influence of male sex hormone signaling on infectious hepatitis. Male A/JCr mice were infected with *H. hepaticus* or vehicle at 4 weeks and randomized into surgical and pharmacologic treatment groups. Interruption of androgen pathways was confirmed by hormone measurements, histopathology, and liver gene and Cyp4a protein expression. Castrated males and those receiving the competitive androgen receptor antagonist flutamide had significantly less severe hepatitis as determined by histologic activity index than intact controls at 4 months. Importantly, the powerful androgen receptor agonist dihydrotestosterone did not promote hepatitis. No effect on hepatitis was evident in males treated with the 5 α -reductase inhibitor dutasteride, the peroxisome proliferator-activated receptor- α agonist bezafibrate, or the nonsteroidal anti-inflammatory drug flufenamic acid. Consistent with previous observations of hepatitis-associated liver-gender disruption, transcriptional alterations involved both feminine (cytochrome P450 4a14) and masculine (cytochrome P450 4a12 and trefoil factor 3) genes, as well gender-neutral (H19 fetal liver mRNA, lipocalin 2, and ubiquitin D) genes. Hepatitis was associated with increased unsaturated C₁₈ long-chain fatty acids (oleic acid and linoleic acid) relative to saturated stearic acid. Our results indicate that certain forms of androgen interruption can inhibit *H. hepaticus*-induced hepatitis in young male mice, whereas androgen receptor agonism does not worsen disease. This raises the possibility of targeted hormonal therapy in young male patients with childhood-acquired HBV.

Chronic viral hepatitis is the most common cause of hepatocellular carcinoma (HCC) in humans. HCC, together with preneoplastic liver cirrhosis, accounts for nearly one million human deaths per year (27). Hepatitis B virus (HBV) infects 6% of all people, and hepatitis C virus (HCV) infects another 3% (23). Thus, nearly 10% of all humans are infected with at least one of these potentially tumorigenic viruses. In patients with chronic viral hepatitis, age is a significant prognostic factor. HBV typically causes more severe disease when acquired in childhood, whereas HCV is more virulent in mature adults (13, 17). Worldwide, HBV is the most common cause of liver cancer (14). Because severe sequelae, including cancer, are especially common in children, any advance in intervention for juvenile HBV infection should have a significant impact on overall disease burden.

Childhood-acquired HBV infections typically require three or more decades for full clinical expression. However, some HBV-infected children, mostly boys, develop cancer before the age of 21 (41, 43). Compared with girls, HBV-positive boys are significantly more likely to harbor circulating viral DNA, in-

dicative of active replication (29). HBV-associated liver tumors in children are more advanced at the time of diagnosis, and less responsive to treatment, than those in adults (8). Regardless of age, males are more than twice as likely as females to develop liver cancer (27). An increased understanding of how age and gender influence the natural history of chronic hepatitis in young males is needed to identify new strategies to protect this unusually vulnerable patient population.

Mice are used widely for chemical hepatocarcinogenesis studies. Unfortunately, investigation of viral hepatitis in rodents is hindered by resistance to infection with HBV and HCV. Genetically engineered mice that express viral transgenes have been developed, but because the inserted sequences are recognized as self, these animals ordinarily do not develop chronic hepatitis (31). The only well-characterized natural infectious liver carcinogen of mice is *Helicobacter hepaticus*. Susceptible mice infected at an early age develop progressive liver inflammation that closely resembles human chronic viral hepatitis, whereas delay of infection until adulthood results in attenuated disease (30). Importantly, tumorigenic hepatitis in *H. hepaticus*-infected mice exhibits the same male bias as in humans, making this an excellent model to investigate the influence of sex on disease outcomes (16, 31). Thus, *H. hepaticus* recapitulates the age and gender risk profiles of human HBV.

Numerous murine chemical and transgenic models have shown a reduction in liver tumors in males castrated at or before puberty (ca. 6 weeks of age) (39, 40). In contrast, delay

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of castration until after 20 weeks results in significantly diminished protection against HCC in male mice in a chemical model (38). We recently showed that 1-year-old male mice chronically infected with *H. hepaticus* were not protected from HCC following castration, nor did androgen receptor activation by dihydrotestosterone (DHT) promote tumors (32). Therefore, male liver cancer susceptibility in mice is maternally imprinted, and it becomes increasingly androgen insensitive with age. The window of opportunity for sex hormone-based intervention for liver cancer in male mice therefore appears to be limited to the juvenile and adolescent stages. Here we investigated the effect of peripubertal androgen intervention on *H. hepaticus* infection in male mice. We show that male sex hormones modulate the development of infectious hepatitis in young mice through both direct and indirect mechanisms. Surgical and pharmacologic androgen receptor inhibition reduced hepatitis severity, but androgen receptor agonism did not worsen disease. Interruption of DHT synthesis via 5 α -reductase inhibition had no effect on disease, and a similar lack of efficacy was noted for the peroxisome proliferator-activated receptor- α (PPAR α) agonist bezafibrate and the nonsteroidal anti-inflammatory drug (NSAID) flufenamic acid. Taken together, our results suggest that hormone therapy could prove effective against hepatitis in young male patients and that such treatments could be rationally designed to minimize impact on sexual development.

MATERIALS AND METHODS

Animals and bacteria. Male 3-week-old A/JCr mice ($n = 115$) were purchased from the National Cancer Institute (Frederick, MD). Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in static microisolator cages and were maintained under specific-pathogen-free conditions for 11 murine viruses, *Salmonella* spp., *Citrobacter rodentium*, ectoparasites, and endoparasites. Upon arrival, mice were confirmed to be free of known murine *Helicobacter* spp. by DNA PCR on pooled cage feces (15). At 4 weeks of age, mice were gavaged with *H. hepaticus* strain 3B1 (ATCC 51449) or vehicle as previously described (15). Briefly, bacteria were cultured on blood agar plates under microaerophilic conditions and resuspended in brucella broth at $\sim 10^8$ bacteria/ml as determined by spectrophotometry. At 4 weeks of age, mice received 0.2 ml of fresh inoculum or broth only via gavage every other day for a total of three doses. Animals were euthanized at 4 months of age via CO₂ inhalation. Protocols were compliant with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Massachusetts Institute of Technology Committee on Animal Care.

Surgical and pharmacologic treatments. Interventions targeting androgen and other pathways were instituted in the peripubertal period at 5 weeks of age. All surgeries were performed by a veterinarian (E.J.T.). Surgical castration was performed aseptically on mice anesthetized with 2% isoflurane gas delivered by nose cone. Continuous 90-day-release pellets containing the potent androgen receptor agonist DHT (5 mg; Innovative Research of America, Sarasota, FL) were implanted subcutaneously between the shoulders under isoflurane anesthesia. Topical disinfectants were allowed to dry, and the area was scrubbed with sterile phosphate-buffered saline to prevent exposure of the pellets to potentially damaging organic solvents. Pellets were inserted through a small skin incision, which was closed with one nonabsorbable suture that was removed 2 weeks later. Additional mice received one of the following drugs in food compounded by Bio-Serv, Inc. (Frenchtown, NJ): 0.0065% (wt/wt) dutasteride (Avodart; Smith-KlineBeecham), a 5 α -reductase inhibitor approved for human prostate disease; 0.6% (wt/wt) bezafibrate, a PPAR α agonist; or 0.6% (wt/wt) flufenamic acid, an NSAID that also inhibits 3-ketosteroid reductase activity in vitro (33). Food consumption and body condition were monitored weekly. A summary of interventions with physiologic effects is shown in Table 1. All groups contained 8 to 10 mice.

Treatment effects. (i) Serum hormones. Following euthanasia, blood samples were collected via cardiac puncture at necropsy. Serum was separated and frozen

TABLE 1. Surgical and pharmacologic treatments affecting androgen signaling and/or inflammatory pathways

Intervention	Action
Surgical castration.....	Loss of gonadal source of testosterone and dihydrotestosterone
Castration + DHT.....	No testosterone; high levels of androgen receptor agonist DHT
Dutasteride	5 α -reductase inhibitor; antagonizes conversion of testosterone to DHT
Flutamide	Competitive androgen receptor antagonist
DHT.....	Avid androgen receptor agonist
Bezafibrate	PPAR α agonist
Flufenamic acid	NSAID

at -20°C . Commercial radioimmunoassay (RIA) kits from Diagnostic Systems Laboratories (Webster, TX) were used according to the manufacturer instructions to quantitate testosterone (no. DSL-4100) and DHT (no. DSL-9600) in freshly thawed sera. RIA tubes were analyzed with a gamma counter, results entered into an Excel spreadsheet (Microsoft Corporation, Redmond, WA), and a logit-log curve generated from the calibration tubes. Sample values were determined by plotting intersects with the calibration curve. Multivariate parametric statistics across all groups were performed with one-way analysis of variance, and direct comparisons between equivalently treated infected and uninfected mice were assessed by an unpaired *t* test. All statistical analyses were performed using Prism 4.0 for Macintosh (GraphPad, San Diego, CA). *P* values of <0.05 were considered significant.

(ii) Androgen-responsive tissue morphology. At necropsy, selected tissues were fixed overnight in neutral-buffered 10% formalin and routinely processed for histology as described elsewhere (30). Kidneys and accessory sex organs (seminal vesicles, coagulating glands, and dorsal and ventral prostate glands) were processed for histology in order to confirm appropriate androgen-sensitive tissue responses to the surgical and pharmacologic treatments (26).

(iii) Hepatic mRNA and protein expression. RNA from liver was extracted using the RNeasy kit (Qiagen Inc., Valencia, CA). Quality and concentration were determined by spectrophotometry as described elsewhere (5). cDNA was generated from 3 μg template RNA using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). Assay-on-Demand reactions and Sybr green-based quantitative reverse transcription-PCRs (qRT-PCRs) (Applied Biosystems, Foster City, CA) with paired samples were performed as described previously (32). Results were normalized against the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene run in tandem. Genes were selected for analysis based on associations with *H. hepaticus*-induced hepatitis and/or gender dimorphism from previous studies in our laboratory (5, 32). Genes evaluated by Assay-On-Demand included the cytochrome P450 4a12 (*Cyp4a12*), *Cyp4a14*, hydroxysteroid dehydrogenase 3 β -V (*Hsd3b5*), H19 fetal liver mRNA (*H19*), trefoil factor 3 intestinal (*Tff3*), and ubiquitin D (*Ubd*) genes. Genes quantitated by Sybr green qRT-PCR were the B-cell leukemia/lymphoma 6 (*Bcl6*), *Cyp2a4*, *Cyp2d9*, epidermal growth factor receptor (*Egfr*), insulin-like growth factor-binding protein 2 (*Igfbp2*), and lipocalin-2 (*Lcn2*) genes. Primers used for Sybr green PCR have been published previously (32). Sexually dimorphic *Cyp4a* proteins were evaluated in castrated and intact mice by chemiluminescent Western blotting (ECL; Amersham, Buckinghamshire, United Kingdom) of whole-liver homogenates using polyclonal rabbit antibody raised against mixed rat *Cyp4a* antigens (Abcam, Cambridge, MA) or a cloned rat *Cyp4a1* polypeptide (19).

Outcome measures. (i) Hepatitis assessment. Formalin-fixed sections of all four liver lobes (median, left, right, and caudate) were stained with hematoxylin and eosin and graded on a scale of 0 to 4 for lobular, portal, and interface hepatitis by a comparative pathologist blinded to sample identity. A hepatitis histologic activity index (HAI) was generated from the sum of these scores, plus lobar distribution, as detailed elsewhere (32). Mice with an HAI of ≥ 4 were defined as having hepatitis. Nonparametric statistical analyses across groups were performed by Kruskal-Wallis analysis of variance, and pairwise comparisons between equivalently treated infected and uninfected mice were performed with the Mann-Whitney U test. Hepatitis incidences (proportion of mice with HAI of ≥ 4) were evaluated using Fisher's exact test.

(ii) Hepatitis-associated transcriptional changes. Transcriptional alterations in *H. hepaticus*-infected mice with and without hepatitis versus equivalently treated uninfected controls were measured and statistically evaluated for 12 genes as described above.

(iii) **Hepatic long-chain fatty acid (LCFA) profiles.** To evaluate whole-liver fatty acid profiles associated with androgen interruption and *H. hepaticus* infection, 0.3 g liver was thoroughly disrupted in a tissue homogenizer. The homogenate was resuspended in 1.3 ml phosphate-buffered saline containing Complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions, and 120 μ l 10% NP-40 (Calbiochem) detergent was added. After detergent solubilization, the sample was transferred to a siliconized microcentrifuge tube, agitated by vortexing, and cleared by 10 min of ultracentrifugation at 13,000 rpm. The supernatant with the lipid layer was transferred to a new siliconized microcentrifuge tube and dried under heat with a SpeedVac SVC 100 centrifugal evaporator (Savant Instruments, Holbrook, NY). Deconjugation was performed by addition of 1 ml 3% (vol/vol) type H2 *Helix pomatia* β -glucuronidase (Sigma) in 0.2 M (pH 5.0) sodium acetate, with agitation in an incubator/shaker at 39°C for 18 h. The deconjugated sample was transferred to a siliconized glass tube and extracted with 1 to 2 volumes diethyl ether. The supernatant was carefully pipetted and transferred to a second vial, and the extraction process was repeated three times. The combined supernatant was dried under a gentle stream of N_2 . Forty microliters of 40 μ l of acetonitrile was twice added and dried under stream of N_2 in order to remove a trace of water from the sample, which interferes with derivatization of the sample. The sample was then dissolved in hexane and derivatized online with *N,O*-bis(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (Pierce Biotechnology, Rockford, IL) by a sandwich technique as previously described (35) into an Agilent 5973N gas chromatography/mass selective detector (Agilent Technologies, Santa Clara, CA) equipped with a 30-m HP-5MS column. The injection port was operated in splitless mode with an injector temperature of 250°C and the transfer line at 280°C. The gas chromatograph was programmed at an initial temperature of 70°C for 1.5 min and then ramped to 280°C at a rate of 6°/min and from 280° to 310° at a rate of 25°C/min, with 4 min at the final temperature. Ion source parameters were as follow: ion source temperature, 250°C; quadrupole, 106°C; electron energy, 147 eV; and emission, 242 μ A. The mass selective detector was run on scan mode from *m/z* 50 to 650 at a rate of 2.48 scans/s in positive chemical ionization mode, using methane gas as a reagent gas. All the acquired data were processed with Agilent ChemStation software.

RESULTS

Treatment effects. (i) Serum hormones. To confirm that our surgical and/or pharmacologic interventions had the intended effect on androgen signaling, we first measured serum testosterone and DHT levels by RIA. As expected, circulating testosterone was reduced to undetectable or near-undetectable levels in surgically castrated mice (Fig. 1A). Both castrated and intact mice implanted with subcutaneous DHT pellets exhibited log-fold or higher circulating DHT levels compared with controls. In intact mice, DHT supplementation markedly suppressed endogenous testosterone. As expected, mice receiving dutasteride (a 5 α -reductase inhibitor that limits the conversion of testosterone to DHT) had decreased serum levels of DHT. Serum testosterone was increased in this group of mice, presumably reflecting decreased conversion to DHT. In contrast, hypertestosteronemia is not a common side effect in men taking dutasteride, suggesting species differences in excretion kinetics (9). The competitive androgen receptor antagonist flutamide significantly increased testosterone while producing widely divergent DHT levels in mice. A similar effect has been observed in men taking the drug (24). Neither the PPAR α agonist bezafibrate nor the NSAID flufenamic acid had any effect on serum testosterone or DHT (data not shown), even though the latter is known to inhibit 3 α - and 3 β -ketosteroid reductase enzyme activity in vitro (33).

(ii) Androgen-responsive tissue morphology. In addition to using serum hormone measurements, we validated the physiologic impact of the surgical and pharmacologic manipulations by histology of androgen-sensitive tissues, including kidneys and accessory sex organs. As expected, masculinization of

FIG. 1. Validation of androgen interruption in hormonally deprived and/or supplemented treatment groups. (A) Serum testosterone and DHT levels as determined by RIA. (B) Histology of androgen-sensitive tissues. Note the impact of castration and DHT supplementation on the masculine cuboidal phenotype of glomerular parietal cell epithelium (top row, arrows) and accessory sex gland secretions, including seminal vesicles (SV) and coagulating glands (CG) (bottom row). *, significantly increased ($P < 0.05$) versus intact controls; †, significantly decreased versus controls. Error bars indicate standard errors of the means.

these tissues, including hypertrophy of renal glomerular parietal epithelium and secretory stimulation of accessory sex organs, was accentuated in mice supplemented with DHT and lost in castrated animals (Fig. 1B) (26). Whereas accessory sex gland activity was predictably feminized by the androgen receptor antagonist flutamide and the DHT-lowering drug dutasteride, epithelial cells lining Bowman's capsule in the same animals maintained their masculine cuboidal phenotype (data not shown). This suggests an absolute dependence on androgen receptor signaling in accessory sex glands but not kidney. Taken together, the serum hormone measurements and histology of androgen-sensitive tissues confirmed that the surgical and pharmacologic interventions employed in this study had physiologically meaningful consequences in vivo.

(iii) Hepatic mRNA and protein expression. We and others have shown that >10% of liver genes in the adult mouse are expressed in a sexually dimorphic fashion (2, 10, 32). In order to survey the effect of our surgical and pharmacologic interventions on hepatic gene expression, we performed qRT-PCR for a 12-gene panel representing both sexually dimorphic and gender-neutral transcripts. As expected, surgical castration resulted in feminization characterized by increased message of female-predominant genes and decreased expression of masculine genes (see Fig. S1A in the supplemental material). The feminized gene expression profile was not reversed by DHT in castrated mice, confirming previous findings that liver masculinization is mediated indirectly through growth hormone pulsatility and not directly through androgen receptor signaling

(32, 37). To our surprise, the 5 α -reductase inhibitor dutasteride produced a feminine liver profile in spite of increased serum levels of aromatizable testosterone, the sex hormone that initiates the endocrine cascade resulting in pulsatile growth hormone secretion and hepatic masculinization (42). Bezafibrate increased the expression of both *Cyp4a* genes in our panel, in agreement with the known action of PPAR α agonists on this enzyme family (20). The NSAID flufenamic acid also upregulated both *Cyp4a* genes, albeit to a lesser extent. For reasons not immediately clear, flufenamic acid also significantly increased expression of the multifunctional acute-phase protein lipocalin-2 (34).

In order to corroborate the qRT-PCR results with sexually dimorphic liver enzyme expression at the protein level, we performed Cyp4a immunoblotting. Using a polyclonal antibody raised against multiple rat Cyp4a isoforms, liver microsome fractions from castrated mice produced an ill-defined band at ~55 kDa along with multiple indistinct lower-molecular-mass bands (see Fig. S1B in the supplemental material). In sexually intact males, the 55-kDa band was well delineated, and there were two distinct bands at ~50 and 48 kDa. Using an antibody raised against recombinant rat Cyp4a1 polypeptide, castrated mice exhibited a single band at ~55 kDa, whereas sexually intact males displayed a distinct couplet of slightly higher and lower molecular masses. These findings are consistent with sexually dimorphic Cyp4a banding patterns observed in other studies (6, 20). Taken together, our data demonstrate that the androgenic interventions were physiologically relevant at both the transcriptional and translational levels.

Outcome measures. (i) Hepatitis. Having validated the physiologic impact of our treatments on androgen signaling, we next evaluated the impact of these interventions on the development of *H. hepaticus*-induced hepatitis in young male mice. In agreement with previous observations (30, 32), our inoculation protocol resulted in hepatitis (defined as an HAI of ≥ 4) in approximately one-half of male mice (not shown). There was no significant difference in hepatitis incidence between groups regardless of androgenic intervention (range, 33 to 60%; $r^2 = 0.667$; $P = 0.12$). Therefore, none of the surgical or pharmacologic treatments resulted in the innate resistance to disease characteristic of *H. hepaticus*-infected females (30). Nevertheless, castration significantly reduced the mean HAI in mice with hepatitis, as did treatment with the competitive androgen receptor antagonist flutamide (Fig. 2). Whereas androgen receptor antagonism reduced hepatitis severity, agonism with the powerful androgen DHT did not increase disease severity. DHT supplementation did increase the mean HAI in castrated mice, but this was statistically insignificant, and disease scores remained lower than those of intact males. Neither the 5 α -reductase inhibitor dutasteride, the PPAR α agonist bezafibrate, nor the NSAID flufenamic acid had any influence on hepatitis severity. Our combined results demonstrated that androgen receptor inhibition impeded the development of hepatitis in young male mice but that 5 α -reductase antagonism, PPAR α agonism, and NSAID treatment did not. Importantly, androgen receptor agonism did not worsen disease. Taken together, our data showed that the influence of androgens on infectious hepatitis development in young male mice is complex and polyfactorial, involving both direct and indirect mechanisms.

FIG. 2. Effect of androgen and pharmacologic intervention on hepatitis severity in young male mice. (A) Semiquantitative morphological assessment of hepatitis severity as determined by HAI. Note the significantly decreased mean HAI in hepatitis-resistant treatment groups (castrated and flutamide) versus the remaining, hepatitis-sensitive groups. (B) Representative photomicrographs of mild, moderate, and severe hepatitis. Hematoxylin and eosin staining was used. Bar, 160 μ m. *, Significantly decreased ($P < 0.05$) versus intact controls. Error bars indicate standard errors of the means.

(ii) Hepatitis-associated transcriptional alterations. Next we explored gene expression profiles in mice that exhibited resistance to *H. hepaticus*-induced hepatitis (castrated and flutamide treatment groups) versus those that did not (intact, dutasteride, DHT, bezafibrate, and flufenamic acid treatment groups). In agreement with previous findings, the feminine liver gene *Cyp4a14* was significantly downregulated in sexually intact male mice with hepatitis as well as in most hepatitis-susceptible treatment groups (Fig. 3). Upregulation of the masculine genes *Cyp4a12* and *Tff3* was significantly associated with hepatitis in most groups. Of potential importance in lipid signaling, in all groups with hepatitis there was a significant increase in the *Cyp4a12/Cyp4a14* ratio (not shown). Three of the four gender-neutral genes selected for this panel, i.e., *H19*, *Lcn2*, and *Ubd*, were upregulated in mice with hepatitis. The masculine gene *Egfr* was increased in some but not all groups. Expression of the other genes in our panel (not shown) was unaltered in *H. hepaticus*-infected mice.

The two hepatitis-resistant groups, castrated and flutamide-treated mice, exhibited gene expression profiles different from

FIG. 3. Liver gene expression in *H. hepaticus*-infected mice with hepatitis (defined as having an HAI of ≥ 4) versus equivalently treated uninfected controls. Note the frequent downregulation of the feminine gene *Cyp4a14* and upregulation of the masculine genes *Cyp4a12* and *Tff3* as well as the gender-neutral genes *H19*, *Lcn2*, and *Ubd* in hepatitis-sensitive treatment groups compared with hepatitis-resistant (castrated and flutamide) groups. *, significantly increased ($P < 0.05$) versus intact controls; †, significantly decreased versus controls. Error bars indicate standard errors of the means.

those of the hepatitis-sensitive groups and from one another, suggesting unique mechanisms of disease protection (Fig. 3). Castrated mice with an HAI meeting our definition of hepatitis (≥ 4) exhibited increased levels of *Cyp4a12* relative to uninfected castrated mice, but this expression level did not approach that of sexually intact males. Moreover, because *Cyp4a14* expression was unchanged, there was no increase in the *Cyp4a12*/*Cyp4a14* ratio as seen in the hepatitis-sensitive treatment groups. None of the other genes in our panel were altered to a statistically significant degree in castrated mice with hepatitis. In flutamide-treated mice that met histologic criteria of hepatitis, *Cyp4a14* was significantly upregulated whereas all other genes were unchanged. It is interesting to note that the masculine gene *Tff3* was unchanged or downregulated in hepatitis-resistant treatment groups but markedly upregulated in hepatitis-sensitive mice. Viewed as a whole, the hepatitis-associated transcriptional profiles implicate multiple mechanistic pathways in disease propagation, including lipid

metabolism (*Cyp4a12* and *Cyp4a14*), tissue repair (*Tff3*), hepatocellular fetalization (*H19*), acute-phase responses (*Lcn2*), and increased protein turnover (*Ubd*). Moreover, the mixed pattern of masculine and feminine gene dysregulation agrees with our previous observation of liver-gender disruption in mice with chronic hepatitis and HCC and introduces a potentially novel biomarker of early liver dysfunction (32).

(iii) **LCFA profiles.** We previously showed by gas chromatography/mass spectrometry that the ratio of saturated to unsaturated microsomal C_{18} LCFAs was altered in aged male mice with chronic hepatitis (32). Here we extended this analysis to nine LCFAs, evaluated whole-liver tissue rather than isolated microsomes, and determined the effect of *H. hepaticus* infection on fatty acid composition in mice both with and without hepatitis. There were no significant differences in LCFA profiles between uninfected mice and *H. hepaticus*-infected mice without hepatitis (Fig. 4). In contrast, *H. hepaticus*-infected mice with hepatitis demonstrated a significant decline

FIG. 4. LCFA distribution in whole liver slices of uninfected and *H. hepaticus* (*Hh*)-infected males with and without hepatitis as determined by gas chromatography/mass spectrometry. Note the significantly elevated proportions of the unsaturated C_{18} LCFAs oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) in mice with hepatitis. *, significantly increased ($P < 0.05$) versus intact controls; †, significantly decreased versus controls. Error bars indicate standard errors of the means.

in palmitic acid as a fraction of the total LCFA pool and a higher proportion of unsaturated/saturated C₁₈ species. This increase in unsaturated C₁₈ products agrees with our previous documentation of a fivefold upregulation of stearoyl coenzyme A desaturase-2 in aged *H. hepaticus*-infected males with chronic hepatitis (32). These complementary results show that increased desaturation of stearic acid is a consistent finding in *H. hepaticus*-driven murine hepatitis. It is conceivable that this contributes to the steatosis seen in some mice with *H. hepaticus*-induced hepatitis and other chronic liver diseases.

DISCUSSION

HBV infection is the leading cause of human liver cancer worldwide (4). HBV acquired in infancy or early childhood is much more likely to produce liver failure and HCC than is infection of adults (21). Whereas most cases of childhood-acquired HBV infection occur in developing nations, modern immigration patterns make this a disease of global concern (36). Regardless of age at the time of infection, the clinical course of HBV is more severe in males than in females (1). Thus, because of their gender and developmental immaturity, young males are at greatest risk for significant HBV-associated morbidity and mortality. New therapeutic modalities are urgently needed for this patient population.

A/JCr mice infected with the enterohepatic bacterium *H. hepaticus* at a juvenile, but not at a mature, age develop chronic hepatitis and HCC in a male-predominant fashion, mimicking the age and gender risk profile of human HBV infection (30). We recently demonstrated that neither castration nor androgen receptor agonism with DHT in mature (1-year-old) male mice infected with *H. hepaticus* had a significant impact on hepatitis progression or HCC risk (32). This agrees with studies involving the chemical carcinogen diethylnitrosamine, in which delay of castration until 38 weeks was significantly less effective than castration at 20 weeks or earlier for preventing HCC in males (38). Therefore, susceptibility of male mice to both infectious and chemical hepatocarcinogens is maternally imprinted, becoming increasingly androgen insensitive with age.

In contrast to the androgen insensitivity of HCC progression in mature mice, numerous studies have shown that castration at or before puberty reduces liver cancer risk in male mice exposed to chemical carcinogens or expressing a protumorigenic transgene (39, 40). Here we have extended those observations to an infectious model of hepatocarcinogenesis. Our data indicate that surgical castration inhibits but does not prevent the development of chronic hepatitis in young male mice infected with *H. hepaticus*. This agrees with findings elsewhere that surgical or chemical castration of young male mice offers partial protection against chemical hepatocarcinogenesis without conferring a fully female phenotype (25). The competitive androgen receptor antagonist flutamide in our study also significantly reduced hepatitis sensitivity but was associated with a distinct gene expression profile compared with surgical castration, suggesting a different mechanism of activity. We were unable to determine whether the protective action of flutamide occurred directly within hepatocytes, indirectly through androgen receptor inhibition of nonliver parenchymal cells, or through an off-target effect (22). Importantly, whereas impair-

ment of sex hormone signaling decreased hepatitis severity, androgen receptor agonism with the powerful androgen DHT did not exacerbate disease. This suggests that androgens confer liver disease susceptibility predominantly through indirect mechanisms and raises the prospect of rationally designed hormone therapy to inhibit hepatitis progression with a minimal impact on male sexual development.

Cyp4a genes encode a group of cytochrome P450 enzymes involved in ω - and ω -1 LCFA hydroxylation and are involved in a variety of metabolic pathways affecting blood pressure and inflammation (7). Bezafibrate is a PPAR α agonist that modulates fatty acid metabolism and shows anti-inflammatory properties (18). We chose to evaluate this compound because of its known effect on *Cyp4a* expression as well as the documented accentuation of liver sexual dimorphism in PPAR α ^{-/-} mice (12). We administered a dose lower than that reported to induce liver tumors in mice, a species that is extraordinarily sensitive to PPAR α -induced hepatocarcinogenesis compared with humans (18). Nevertheless, we confirmed pharmacologic activity by demonstration of upregulated hepatic *Cyp4a* expression at both the message and protein levels. In the male mice in our study, bezafibrate preferentially upregulated the male-specific gene *Cyp4a12* relative to the female-predominant gene *Cyp4a14*. In spite of the reported anti-inflammatory properties in other systems, bezafibrate demonstrated no efficacy for reducing hepatitis severity in *H. hepaticus*-infected male mice.

In addition to bezafibrate, we administered to a group of mice the NSAID flufenamic acid. We selected this compound because in addition to its NSAID activity, it has been shown to act as a 3-ketosteroid reductase inhibitor *in vitro* (33). This could conceivably impede the clearance of DHT by preventing its conversion to a more hydrophilic and significantly less androgenic 3- α and 3- β -androstenediol. However, flufenamic acid treatment had no effect on circulating testosterone or DHT levels, nor did it demonstrate any efficacy against *H. hepaticus*-induced hepatitis.

The male rodent liver responds with widespread transcriptional feminization to a wide variety of disturbances (5, 11, 44). Upregulation of masculine liver genes, in contrast, typically does not occur. We recently reported a strong association between male HCC risk and liver-gender disruption, defined as the loss of a gender-identifying hepatic molecular signature in mice infected with *H. hepaticus* (32). This paradigm offers a solution to the paradox of male-predominant HCC risk in the absence of direct tumor promotion by androgenic hormones. In our model, proinflammatory cytokines associated with chronic hepatitis perturb the transcriptional regulation of sexually dimorphic liver genes, resulting in a loss of sex-specific function. In the present study as well as in previous reports, we observed upregulation of masculine genes and downregulation of feminine genes that was most apparent in sexually intact *H. hepaticus*-infected mice with the highest degree of hepatitis as measured by HAI. Thus, concurrent transcriptional hypermasculinization and feminization consistent with liver-gender disruption appears to be an early predictive biomarker for hepatitis progression in this model. To our knowledge there has been no systematic evaluation of sexually dimorphic liver genes in health or disease in humans, despite the known propensity of men versus women to develop HCC.

We have shown here and previously that genes associated with lipid metabolism are altered in the progression of hepatitis associated with *H. hepaticus* infection (32). In the present study, *H. hepaticus*-infected males with hepatitis, but not those without hepatitis, demonstrated lower levels of palmitic acid but higher levels of the mono- and polyunsaturated C₁₈ LCFAs oleic acid and linoleic acid. Liver inflammation in *H. hepaticus*-infected mice is associated with increased expression of the enzyme stearoyl coenzyme A desaturase-2 (Scd2) (32). Our results agree with a longitudinal study of men that demonstrated a positive correlation between SCD-1 expression and the proinflammatory marker C-reactive protein (28). Additional work is needed to determine the significance of C₁₈ desaturation in hepatitis and other chronic inflammatory diseases.

In conclusion, we have shown that peripubertal surgical castration and androgen receptor inhibition reduced the severity of hepatitis induced by the tumorigenic murine bacterium *H. hepaticus*. In contrast, neither 5 α -reductase inhibition, PPAR α agonism, nor NSAID treatment had any effect. Our results agree with the limited benefit of NSAID therapy for chronic viral hepatitis in humans (3) and suggest that hormone-targeted therapy may prove to be a more viable approach. To our knowledge, this is the first study to show that interruption of sex hormone signaling can impair the development of infectious hepatitis in mice. Whereas negative effects on male virilization represent an obvious hurdle to the application of this strategy in humans, our findings raise the possibility of targeted hormonal therapy in peripubertal male patients with acquired HBV infection. Additional studies are needed to identify the specific molecular pathways affected by androgens in a setting of chronic infectious hepatitis and to identify therapeutic modalities that could be translated to use in young male patients with childhood-acquired HBV.

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