Spontaneous Development of Liver Tumors in the Absence of the Bile Acid Receptor Farnesoid X Receptor

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Abstract
Farnesoid X receptor (FXR, NR1H4) is a member of the nuclear hormone receptor superfamily, which plays an essential role in regulating bile acid, lipid, and glucose homeostasis. Both male and female FXR−/− mice spontaneously developed liver tumors; however, no other tumors were developed after 15 months of age. In contrast, no liver tumors were observed in wild-type mice of the same age. Histologic analyses confirm that tumors were hepatocellular adenoma and carcinoma. Although there was no obvious tumor at ages 9 to 12 months, FXR−/− livers displayed prominent liver injury and inflammation. Strong labeling of apoptotic hepatocytes and liver differentiation–induced compensatory regeneration were observed. Deregluation of genes involved in bile acid homeostasis in FXR−/− mice was consistent with abnormal levels of bile acids presented in serum and liver. Genes involved in inflammation and cell cycle were up-regulated in aging FXR−/− mice but not in wild-type controls. Increasing the bile acid levels by feeding mice with a 0.2% cholic acid diet strongly promoted N-nitrosodiethylamine–initiated liver tumorigenesis, whereas lowering bile acid pool in FXR−/− mice by a 2% cholestyramine feeding significantly reduced the malignant lesions. Our results suggest an intriguing link between metabolic regulation and hepatocarcinogenesis. [Cancer Res 2007;67(3):863–7]

Introduction
Farnesoid X receptor (FXR, NR1H4) is a member of the nuclear hormone receptor superfamily. Members from this superfamily regulate diverse physiologic processes, including development, differentiation, metabolism, and homeostasis (1). FXR is highly expressed in liver, intestine, kidney, and adrenal gland. The identification of bile acids as bona fide FXR endogenous ligands unravels an essential function of FXR in controlling bile acid metabolism (2–4). A large body of evidence indicates that the major function of FXR is to control bile acid homeostasis and to prevent bile acid–induced liver toxicity. Bile acids are end products from cholesterol catabolism and are critical for normal absorption of cholesterol, lipids, and fat-soluble vitamins in the intestine. However, because of the intrinsic toxicity of bile acids, their levels need to be strictly regulated. By regulating the expression of genes involved in bile acid synthesis, conjugation, and transportation, FXR turns out to be the master regulator of bile acid homeostasis (5–7). Moreover, FXR was recently shown to mediate the effects of bile acid signaling on normal liver regeneration (8). Activation of FXR by bile acids in intestine protects the tissue from bacterial-induced mucosal injury (9). In addition to acting as a bile acid sensor, FXR also helps to regulate other metabolic pathways such as lipid and glucose metabolism (10–13). Mice lacking FXR moderately show higher levels of bile acids and lipids in serum and liver (14).

At a young age, FXR−/− mice display no general liver toxicity, although they show higher sensitivity to exogenously applied bile acid–induced liver damage (14). However, the long-term effects of the disruption of bile acid and other metabolic pathways in FXR−/− mice have not been studied. Here, we report that both male and female FXR−/− mice spontaneously developed liver tumors when they were 15 months of age. Before tumors emerged, liver damage and subsequent compensatory proliferation were observed in aging FXR−/− mice but not in the wild-type controls. Genes involved in inflammation and cell cycle were up-regulated in the livers of aging FXR−/− mice. We also provide evidence indicating that sustained high levels of bile acids may contribute to liver tumorigenesis in FXR−/− mice.

Materials and Methods
Animal maintenance and treatments. The wild-type and FXR−/− mice that have been extensively crossed to C57BL/6 background (14) were held in a pathogen-free animal facility under standard 12:12-h light/dark cycle. Mice were fed standard rodent Chow and water ad libitum. Wild-type mice 4 weeks of age were injected i.p. with a single dose of liver carcinogen, N-nitrosodiethylamine (DEN, 90 mg/kg) or PBS, followed by feeding a standard diet or a 0.2% cholic acid (CA) diet for 30 weeks. Female FXR−/− mice at 11 months old were fed with a 2% cholestyramine diet for 3 months. All procedures followed the NIH guidelines for the care and use of laboratory animals.

Liver histology. When experiments terminated, livers were removed, and small pieces containing either normal or tumor regions were fixed in 4% formaldehyde-PBS solution, embedded in paraffin, sectioned at 5 μm, and stained with H&E. For immunohistochemical staining, the sections were first incubated with an anti-a-fetoprotein antibody and then followed by an anti-immunoglobulin G secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using standard immunohistochemistry procedures. For 5-bromo-2-deoxy-uridine (BrdU) staining, mice were injected i.p. with the BrdU solution (10 mg/mg body weight) 2 h before being euthanized. Liver sections were prepared and stained using a BrdU staining kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. The same sections were used for terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) staining by a TUNEL kit (Roche).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). F. Yang and X. Huang contributed equally to this work. Requests for reprints: Wendong Huang, Department of Gene Regulation and Drug Discovery, Beckman Research Institute, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010; Phone: 626-256-4673, ext. 65203; Fax: 626-256-8704; E-mail: whuang@coh.org. ©2007 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-06-1078

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RNA preparation and reverse transcription-PCR. Total RNAs from the livers of wild-type and FXR−/− mice were isolated using TRI reagents (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 3 g of RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen, San Diego, CA). mRNA was quantified by real-time quantitative PCR using Moloney murine leukemia virus reverse transcriptase (Invitrogen, San Diego, CA). The following specific forward (F) and reverse primers were used: mINFForest City, CA). The following specific forward (F) and reverse primers were used: mINF (R) were used: mINF

TGGACCACTCGGATGAGCTCATTG; mTNFα F, TCCTGCAGAGCCAGATTATCTC; mTNFα R, GGTTGTCTTTGAGATCCATGCCGT; CYP7a F, CAAGAACTTCGGGGTGATC; mTNFα R, GGTTGTCTTTGAGATCCATGCCGT; CYP7a R, CACTTCTTCAGAGGCTGCTTTC; and small heterodimer promoter 12α-hydroxylase (CYP8b) F, TAGCCCTGTAGGTGTGTGTGC; CYP8b R, AGTCGAGATCTTCCCTGAACCTG; and small heterodimer promoter (SHP) F, GTCTTTCTGGACCTTTGAGCTG; SHP R, GTAGAGGC-CATGAGGAGATTG. The quantity of mRNA was normalized by 18S rRNA. The primers of 18S RNA were from QuantumRNA 18S Internal Standard kit (Ambion, Austin, TX).

Table 1. Relative liver weight, tumor incidence, serum, and liver total bile acids, and ALT in wild-type and aging FXR−/− mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Liver/body weight</th>
<th>Tumor incidence (%)</th>
<th>Serum bile acids (μmol/L)</th>
<th>Liver bile acids (μmol/L)</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT male</td>
<td>8</td>
<td>4.5 ± 0.7</td>
<td>0</td>
<td>21 ± 7</td>
<td>0.41 ± 0.08</td>
<td>63 ± 17</td>
</tr>
<tr>
<td>WT female</td>
<td>9</td>
<td>4.7 ± 0.6</td>
<td>0</td>
<td>23 ± 3</td>
<td>0.39 ± 0.10</td>
<td>45 ± 11</td>
</tr>
<tr>
<td>FXR−/− male</td>
<td>15</td>
<td>6.9 ± 2.5*</td>
<td>100</td>
<td>101 ± 24</td>
<td>0.67 ± 0.16</td>
<td>831 ± 213</td>
</tr>
<tr>
<td>FXR−/− female</td>
<td>12</td>
<td>6.5 ± 3.4*</td>
<td>100</td>
<td>89 ± 36†</td>
<td>0.72 ± 0.14†</td>
<td>916 ± 191</td>
</tr>
</tbody>
</table>

*P < 0.05 when compared to the same-sex wild-type mice.
†P < 0.01 when compared to the same-sex wild-type mice.
‡P < 0.05 when compared to the same-sex wild-type mice.

Bile acid and alanine aminotransferase analysis. Serum was isolated and total bile acids were measured using bile acid LSK assay kit according to the manufacturer’s instructions (Diagnostic Chemicals Limited, CT). Total liver bile acids were measured according to the previous description (8). Serum alanine aminotransferase (ALT) was measured at the City of Hope Helford Research Hospital.

Statistical analyses. Statistical analyses results were carried out using two-tailed Student's t-test.

Results and Discussion

Although FXR−/− mice have severe defects to tolerate exogenously applied bile acid–induced liver damage, no obvious liver toxicity was observed in young FXR−/− mice fed a standard diet (11). To investigate the potentially deleterious long-term effects of FXR knock-out on liver, we monitored the liver morphology of FXR−/− mice up to 15 months of age and observed the development of liver tumors in these knock-out mice between 13 and 15 months of age. At 15 months of age, all of the mice of both sexes developed liver tumors with varying severity (Fig. 1B). In contrast, no tumors were found in wild-type mice of the same age (Fig. 1A), suggesting a causal relationship between the absence of FXR and liver tumorigenesis. Liver sections stained with H&E indicate typical hepatocellular adenoma and carcinoma (Fig. 1C), which was further confirmed by the positive staining of α-fetoprotein (Fig. 1D). The ratios of liver weight to body weight in aging FXR−/− mice were significantly higher than those of wild-type controls, indicating a phenotype of hepatomegaly in aging FXR−/− liver (Table 1). This liver enlargement is not completely due to the tumor formation because we observed the same hepatomegaly even before tumors were observed.

ALT is a commonly used marker of liver damage. The levels of ALT in aging FXR−/− mice were much higher than those in wild-type mice of the same age (Table 1). A major defect of FXR−/− mice is the disruption of FXR-mediated control of bile acid homeostasis. We measured the total bile acid levels in serum and livers from aging FXR−/− mice and wild-type mice and discovered both serum and liver bile acids were significantly higher in FXR−/− mice compared with the wild-type controls (Table 1). Because bile acids
have been implicated in the promotion of colon cancer, we also examined whether tumors were present in the intestine or other tissues in FXR-/− mice; however, tumors were not found in any tissues other than liver. These results indicate a specific effect of FXR deficiency in liver carcinogenesis.

To investigate the mechanism contributing to the formation of liver tumors in FXR-/− mice, we first monitored the liver morphology of FXR-/− mice at different ages. Starting at 9 months of age, some mice displayed preneoplasms, and at 12 months of age, small foci became obvious. Histologic analysis at these ages indicated clearly liver damage, including many vacuoles due to lipid deposits (Fig. 2A-a), vacuolation due to cell damage (Fig. 2A-b), inflammation (Fig. 2A-c), and focal necrosis (Fig. 2A-d). None of these pathologic changes were found in wild-type mice or in young FXR-/− mice. In addition, TUNEL staining of the same liver sections indicated that some hepatocytes go to apoptosis in liver as FXR-/− age, whereas this was not observed in wild-type mice of the same age (Fig. 2B). Significant amounts of BrdU-positive cells were detected around the damaged regions in sections of liver from aging FXR-/− mice, which suggests that a compensatory regenerative proliferation may be initiated (Fig. 2C).

To further confirm whether aging FXR-/− mice have defects in bile acid metabolism, we checked the expression levels of several important genes involved in bile acid metabolism and which were previously identified as target genes of FXR. CYP7a and CYP8b are two major enzymes in the bile acid synthesis pathways, and their gene expressions were previously shown to be negatively regulated by FXR. In the absence of FXR, these two genes are moderately increased in young FXR-/− mice (14). As expected, expression of CYP7a and CYP8b increased 4- and 2-fold, respectively, in aging FXR-/− mice (Fig. 3A). NTCP is a basolateral bile acid transporter that pumps bile acids into liver, and its expression is inhibited by FXR activation. In the absence of FXR in aging mice, NTCP expression increased (Fig. 3A), similar to a previous report in young FXR-/− mice (14). SHP is a primary target of FXR and is a key factor that mediates the down-regulation of CYP7a gene. The level of SHP expression was much lower in aging FXR-/− mice (Fig. 3A). These gene expression analyses were consistent with the higher levels of total bile acids in serum and liver from aging FXR-/− mice.

We observed an increase in the number of apoptotic cells and focal necrosis, which result in the compensatory cell proliferation in livers from aging FXR-/− mice. We thus check the expression of two cyclin genes required for cell cycle progression. The results indicate that both cyclin D1 and cyclin E1 expressions were strongly increased in the livers from aging FXR-/− mice compared with the wild-type controls (Fig. 3).

Inflammation is known to stimulate cell death and increase cell turnover, thus promoting liver tumorigenesis (15–17). Therefore, we checked the gene expression of several cytokines using the liver RNAs prepared from peritumor regions of livers of 15-month-old FXR-/− mice. As expected, mRNA levels of proinflammation factors, interferon γ, tumor necrosis factor-α, and interleukin-6, were significantly higher than those in wild-type controls (Fig. 3B). In contrast, the expression of cyclooxygenase-2 does not change (data not shown). The same pattern of gene expressions was observed in tumor regions from the same livers or livers from 9-month-old FXR-/− mice (data not shown). The results suggest that the increased expression of proinflammatory genes as FXR-/− mice age may eventually contribute to liver tumor formation in these mice.

Disruption of bile acid homeostasis is the major defect in FXR-/− mice. Bile acids have been shown to promote liver tumor in a hepatitis B virus transgenic mouse model and are considered to induce inflammation and liver tumorigenesis in mdr-2 knockout mice (18, 19). To test if bile acids contribute to the liver tumor formation in FXR-/− mice, we fed wild-type mice with a 0.2% CA diet for 30 weeks with or without preinjection of a liver carcinogen, DEN. The results indicate that, although 0.2% CA diet by itself did not induce liver tumor at 30 weeks, it strongly promoted liver tumors in those mice that were pretreated with a single dose of DEN (Supplementary Table S1). CA diet also increased the expression of tumor necrosis factor-α and interleukin-6 genes (Supplementary Fig. S1), suggesting that increased levels of bile acids are sufficient to induce the expression of proinflammatory cytokines. We also fed the FXR-/− mice with a diet containing 2% cholestyramine, a bile acid–sequestering resin, for 3 months starting at 11 months of age when the mice had no tumor. Compared with the standard diet, cholestyramine feeding

Figure 2. Liver H&E, TUNEL, and BrdU staining of FXR−/− mice. A, a–d, H&E staining of liver tissues from FXR−/− mice at 9 to 10 months of age (40×). Arrows, inflammatory cells; arrowheads, necrotic regions. B, TUNEL staining of liver sections from the same mice showing apoptotic cells in FXR−/− livers, but not in wild-type livers (20×). C, BrdU staining of the same liver sections as (B) showing positive BrdU-stained cells in FXR−/− livers but not in wild-type controls (40×).
significantly reduced the number and size of liver malignant lesions in aging FXR−/− mice (Supplementary Table S2). All these experiments suggest that chronically higher levels of bile acids in FXR−/− mice may contribute to the liver tumor formation as these animals age. However, other metabolic defects in addition to higher levels of bile acids may also play important roles for the overall liver tumorigenesis in FXR−/− mice.

Liver cancer is one of the most common forms of cancers worldwide, and both genetic and environmental factors contribute to hepatocarcinogenesis (20). Our findings indicate that metabolic defects such as chronically higher levels of bile acids can promote liver tumor formation, thus suggesting an intriguing link between metabolic regulation and hepatocarcinogenesis.

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References

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