Steatohepatitis develops rapidly in transgenic mice overexpressing Abcb11 and fed a methionine-choline-deficient diet

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Submitted 8 October 2004; accepted in final form 11 January 2005

MATERIALS AND METHODS

Animal protocol. Male FVB/NJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male TTR-Abcb11 transgenic mice, in an FVB/NJ background, were bred from founder lines at Northwestern University or the Lakeside Veterans Affairs Medical Center.

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Am J Physiol Gastrointest Liver Physiol 288: G1321–G1327, 2005. First published January 13, 2005; doi:10.1152/ajpgi.00455.2004.—Nonalcoholic fatty liver disease (NAFLD) is the most common cause of abnormal liver chemistries in the United States and other developed countries and is a significant cause of cryptogenic cirrhosis (3). NAFLD represents a wide spectrum of liver injury, including simple steatosis, steatohepatitis, fibrosis, and cirrhosis. Hepatic steatosis occurs in up to 25% of individuals and can progress to cirrhosis in a significant minority of these patients (26). Hepatic steatosis is commonly associated with obesity, type 2 diabetes, and hypertriglyceridemia (25). The pathophysiological mechanisms leading to the progression from simple steatosis to nonalcoholic steatohepatitis (NASH), however, remain poorly elucidated.

A current hypothesis to help explain the progression from steatosis to NASH is the “two-hit hypothesis.” The first hit is chronic accumulation of excessive hepatic fat secondary to a metabolic disturbance, such as insulin resistance (14). The second hit has been suggested to be toxic lipid peroxidation and oxidative stress due to reactive oxygen species accumulation (7). Inflammatory cytokine-mediated liver injury has also been implicated as a second hit (7, 33, 49). NASH, however, is most likely a disease of multifactorial etiology, in which disease pathogenesis involves “multiple hits” or pathogenic factors.

Mice fed a diet deficient in methionine and choline (MCD) have been shown to develop steatosis, necroinflammation, and eventually progressive fibrosis. This MCD dietary model of NASH has been extensively used in previous studies since this nutritional model creates progressive, fibrosing steatohepatitis in rodents (19, 21, 23, 32, 33). Both methionine and choline are essential precursors of hepatic phosphatidylcholine (PC) synthesis, and the hepatic steatohepatitis induced by this diet may be due, in part, to impairments in PC synthesis. Figge et al. (13) recently developed the transthyretin-Abcb11 (TTR-Abcb11) transgenic mouse model, which overexpresses the hepatic canalicular ATP-dependent bile salt transporter Abcb11. Hepatobiliary secretion of bile salt is the primary driving force for bile formation, and enhanced canalicular expression of Abcb11 [often termed bile salt export pump (BSEP)] results in increased bile flow and increased biliary lipid secretion. In particular, TTR-Abcb11 mice exhibit an increase in biliary secretion of bile salts, cholesterol, and PC. In addition, when fed a lithogenic diet (high in fat, cholesterol, and cholic acid), these mice demonstrate significantly less hepatic steatosis than wild-type FVB/NJ controls (13).

Hepatobiliary secretion of PC is high, and it has been estimated that the entire hepatic PC content is secreted into bile each day (42, 43). Thus mice with enhanced biliary secretion of PC, such as TTR-Abcb11 mice, may be more susceptible to rapidly develop hepatic injury induced by the MCD diet. Steatosis, however, is likely an essential precursor for the development of steatohepatitis. Mice that are more resistant to the development of hepatic steatosis may be more resistant to oxidative stress, hepatocyte injury, and steatohepatitis. Therefore, the primary aim of this study was to employ TTR-Abcb11 transgenic mice to further determine the mechanisms responsible for the development of steatohepatitis.

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An investigator (R. M. Green) blinded to experimental conditions paraffin, and 5-formalin for histology.

Samples were mixed with 5 mM Tris-HCl buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, and 1 mM 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane. Liver triglyceride and cholesterol content was measured using a spectrophotometric assay from Sigma Diagnostics (St. Louis, MO) as per instructions from the manufacturer and was expressed as milligrams of lipid per milligrams of liver protein. Liver thiobarbituric acid reactive substance (TBARS) content was measured by spectrophotometric assay using a kit from Zeptometrix (Buffalo, NY) and expressed as nanomoles of TBARS per milligrams of protein. Reduced glutathione levels were measured in liver homogenate prepared in 5% metaphosphoric acid using a spectrophotometric assay purchased from Calbiochem (San Diego, CA). Real-time PCR was performed on five to seven mice in the experimental group and a minimum of three separate mice in the control group. Reported data reflect a minimum of two distinct runs on each mouse liver. Amplification was performed in duplicate for each sample in an ABI Prism 5700 sequence detector (PE Applied Biosystems). All data were normalized for GAPDH expression. Relative gene expression levels from real-time PCR data were analyzed as recently reported using the comparative threshold cycle method, as described in the Applied Biosystems Sequence Detection Systems instruction guide (2, 20, 32, 33).

Statistical analysis. Data are presented as means \pm SE. Comparison between groups was performed using Student’s t-test or ANOVA analysis.

RESULTS

The effect of the MCD diet on serum ALT levels. After 7 days on the MCD diet, TTR-Abcb11 mice had significantly elevated serum ALT levels (82.6 \pm 11.2 IU), which were higher than those observed in FVB/NJ mice (38.9 \pm 7.3 IU), \( P = 0.005 \). After 14 days on the MCD diet, serum ALT levels were significantly elevated to a comparable degree in both the TTR-Abcb11 mice (144.2 \pm 13.9 IU) and FVB/NJ (161.2 \pm 13.9 IU) controls. Histologically, TTR-Abcb11 mice also showed more inflammation after 14 days on the MCD diet (1.3 \pm 0.5 IU), compared with FVB/NJ mice (0.8 \pm 0.5 IU), \( P = 0.05 \). Elevated ALT levels persisted through 30 days in both TTR-Abcb11 (111.9 \pm 13.9 IU) and FVB/NJ (102.3 \pm 9.0 IU) mice. When fed a control diet for up to 30 days, both
TTR-Abcb11 and FVB/NJ wild-type controls maintained normal serum ALT levels (39.6 ± 8.1 and 37.3 ± 7.2 IU, respectively) (see Fig. 1).

The effect of the MCD diet on hepatic triglyceride and cholesterol levels. TTR-Abcb11 mice fed an MCD diet had lower hepatic triglyceride levels than FVB/NJ mice after 7 (0.16 ± 0.03 vs. 0.33 ± 0.06 mg/mg protein), 14 (0.2 ± 0.03 vs. 0.37 ± 0.06 mg/mg protein), and 30 days (0.34 ± 0.08 vs. 0.58 ± 0.2 mg/mg protein), \( P = 0.001 \). Consistent with this finding, histologically FVB/NJ mice also demonstrated more steatosis than TTR-Abcb11 mice at 7 (2.3 ± 0.5 vs. 1.7 ± 0.5 mg/mg protein) and 14 (3.0 ± 0.6 vs. 1.5 ± 0.5 mg/mg protein) days, \( P < 0.05 \) (see Fig. 2). As illustrated in Fig. 3, both mouse strains demonstrate progressive hepatic triglyceride deposition over time, results that we confirmed histologically (\( P < 0.05 \)). Hepatic triglyceride content in mice fed a control chow diet for up to 30 days was minimal and similar in both TTR-Abcb11 (0.04 ± 0.006 mg/mg protein) and FVB/NJ (0.06 ± 0.02 mg/mg protein) mice. Serum triglyceride levels were unchanged.

FVB/NJ mice showed progressive increases in hepatic cholesterol content from baseline levels (0.03 ± 0.01 mg/mg protein) after both 7 days (0.05 ± 0.01 mg/mg protein) and 14 days (0.06 ± 0.01 mg/mg protein) on an MCD diet, \( P < 0.05 \). Hepatic cholesterol levels in TTR-Abcb11 mice, however, remained similar to baseline levels (0.04 ± 0.02 mg/mg protein) after either 7 days (0.05 ± 0.01 mg/mg protein) or 14 days (0.05 ± 0.01 mg/mg protein) on the MCD diet. Serum cholesterol levels were similar in chow-fed FVB/NJ (152.9 ± 33.1 mg/dl) and TTR-Abcb11 mice (175.3 ± 11.6 mg/dl). Serum cholesterol levels decreased after 7 days (127.1 ± 20.7 mg/dl)
Table 1. Hepatic gene expression of TTR-Abcb11 and FVB/NJ mice fed the MCD diet

<table>
<thead>
<tr>
<th>Relative Gene Expression</th>
<th>Mouse Strain</th>
<th>Control diet</th>
<th>MCD diet</th>
<th>Control diet</th>
<th>MCD diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>SREBP-1c</td>
<td>FVB/NJ</td>
<td>1.07±0.45</td>
<td>1.38±0.1*</td>
<td>1.17±0.42</td>
<td>1.18±0.32</td>
</tr>
<tr>
<td></td>
<td>TTR-Abcb11</td>
<td>1.92±0.32†</td>
<td>0.48±0.11*†</td>
<td>2.54±0.59†</td>
<td>0.82±0.08†</td>
</tr>
<tr>
<td>Fatty acid synthase</td>
<td>FVB/NJ</td>
<td>1.2±0.41</td>
<td>0.96±0.19</td>
<td>1.17±0.77</td>
<td>0.66±0.45</td>
</tr>
<tr>
<td></td>
<td>TTR-Abcb11</td>
<td>2.0±0.02</td>
<td>0.97±0.37</td>
<td>2.41±0.12†</td>
<td>0.93±0.37†</td>
</tr>
<tr>
<td>TNF-α</td>
<td>FVB/NJ</td>
<td>1.7±0.80</td>
<td>0.9±0.16</td>
<td>1.44±0.6</td>
<td>0.68±0.20†</td>
</tr>
<tr>
<td></td>
<td>TTR-Abcb11</td>
<td>0.3±0.03</td>
<td>7.0±3.89</td>
<td>0.32±0.04</td>
<td>2.06±0.46‡</td>
</tr>
<tr>
<td>IL-6</td>
<td>FVB/NJ</td>
<td>2.3±1.10</td>
<td>1.5±0.98</td>
<td>1.81±0.84</td>
<td>0.58±0.16‡</td>
</tr>
<tr>
<td></td>
<td>TTR-Abcb11</td>
<td>0.3±0.12</td>
<td>19.2±4.2</td>
<td>0.27±0.11</td>
<td>2.25±0.59‡</td>
</tr>
</tbody>
</table>

Data represent means ± SE. These data represent results from 5–7 separate mice in the experimental group and at least 3 mice in the control group, with all samples run at least in duplicate. Mice were fed a methionine-choline-deficient (MCD) diet for 7 or 14 days, and gene expression of selective lipid metabolic or cytokine genes was measured. Gene expression was assessed using real-time PCR and normalized for GAPDH gene expression. SREBP-1c, sterol regulatory element binding protein-1c; TTR, transthyretin. *P < 0.01 for TTR-Abcb11 vs. FVB/NJ MCD diet-fed mice; †P < 0.01 for TTR-Abcb11 chow vs. MCD diet-fed mice; ‡P < 0.05 for TTR-Abcb11 vs. FVB/NJ MCD diet-fed mice.

mg/dl) and 14 days (79.4 ± 12.7 mg/dl) on the MCD diet in FVB/NJ mice, P < 0.05. Similarly, serum cholesterol levels also decreased after 7 days (93.3 ± 13.3 mg/dl) and 14 days (95.7 ± 26.0 mg/dl) on the MCD diet in TTR-Abcb11 mice, P < 0.05.

Cytokine analysis using real-time PCR. Expression of TNF-α mRNA was assayed using real-time PCR. After 14 days on the MCD diet, TTR-Abcb11 mice demonstrated a threefold increase in TNF-α mRNA expression compared with FVB/NJ mice (P = 0.03). A 3.9-fold increase in IL-6 mRNA expression was also seen in TTR-Abcb11 compared with FVB/NJ mice after 14 days on the MCD diet. There was no significant difference in gene expression of both TNF-α and IL-6 mRNA at baseline in control diet-fed mice and in mice fed an MCD diet for only 7 days (see Table 1).

The effect of the MCD diet on oxidative stress. Hepatic TBARS, glutathione levels, and expression of CYP2E1 were analyzed as markers of oxidative stress. Minimally elevated TBARS occurred at 7 days on the MCD diet in both FVB/NJ (0.76 ± 0.24 nM/mg protein) and TTR-Abcb11 (1.34 ± 0.68 nM/mg protein) mice (P = not significant). By 14 days on the MCD diet, however, FVB/NJ mice had significantly greater TBARS compared with both control-fed mice and TTR-Abcb11 mice fed an MCD diet. TBAR levels were 4.05 ± 0.32 nM/mg protein in FVB/NJ mice vs. 1.79 ± 0.4 nM/mg protein in TTR-Abcb11 mice (see Fig. 4). After 30 days on the MCD diet, both FVB/NJ and TTR-Abcb11 mice had significantly elevated TBARS with no strain-specific differences. TBARS in control diet-fed mice were similar and minimal in both FVB/NJ and TTR-Abcb11 mice fed a chow diet.

Both mouse strains showed dramatic increases in CYP2E1 protein expression over time (P < 0.0005). After 14 days on the MCD diet, FVB/NJ mice showed a twofold increase in CYP2E1 protein expression, whereas this was increased fourfold in TTR-Abcb11 mice. This pattern persisted at 30 days on the MCD diet. On the chow diet, hepatic CYP2E1 protein expression showed no strain-specific differences (see Fig. 5).

Baseline hepatic glutathione levels in TTR-Abcb11 mice were higher (6.87 mM/mg protein) than in FVB/NJ mice (5.01 mM/mg protein), P < 0.05. Both mouse strains demonstrated minimal increases, rather than decreases, in hepatic glutathione over 30 days (see Fig. 6). The TTR-Abcb11 mice maintained higher levels of hepatic glutathione at all time points up to 30 days (P < 0.0001). Although increased glutathione production...
could account for these effects, we observed no corresponding increases of γ-GCS enzymatic activity or protein expression.

The effect of the MCD diet on SREBP-1c and FAS gene expression. SREBP-1c regulates many hepatic metabolic factors, including the rate of hepatic FAS (10, 16, 17, 34). After 7 days on the MCD diet, TTR-Abcb11 mice had a fourfold decrease in SREBP-1c mRNA expression (P < 0.001), and this effect persisted at 14 days (P = 0.002). Similar decreases of SREBP-1c mRNA expression were not observed in the FVB/NJ mice fed the MCD diet for up to 14 days. TTR-Abcb11 mice fed an MCD diet for 7 days had significantly lower SREBP-1c mRNA expression than FVB/NJ mice (P < 0.001). This difference between strains was not noted after 14 days of feeding of the MCD diet. Baseline expression of hepatic SREBP-1c mRNA was similar in FVB/NJ and TTR-Abcb11 mice fed a chow diet (see Table 1).

We also examined the expression of the SREBP-1c downstream target gene FAS. Consistent with SREBP-1c expression, after 7 days on the MCD diet, TTR-Abcb11 mice had a strong trend toward a decrease in FAS gene expression, with gene expression being reduced almost twofold (P = 0.07). By 14 days of MCD diet feeding, TTR-Abcb11 mice had a highly significant 2.6-fold decrease in FAS expression (P < 0.01). Baseline expression of hepatic FAS mRNA was similar in FVB/NJ and TTR-Abcb11 mice fed a chow diet (see Table 1).

DISCUSSION

The MCD diet induces the rapid development of hepatitis in TTR-Abcb11 transgenic mice, despite relative steatosis resistance. Both FVB/NJ strain controls and TTR-Abcb11 mice eventually manifest progressive hepatic triglyceride deposition, marked elevation of serum ALT, and histologically evident inflammation. A more pronounced increase in mRNA expression of both TNF-α and IL-6 was also observed in the TTR-Abcb11 mice at 1 and 2 wk compared with the FVB/NJ mice. In contrast, FVB/NJ mice exhibited elevated markers of oxidative stress sooner than the TTR-Abcb11 mice. A disassoc-

Fig. 5. Cytochrome P-450 2E1 (CYP2E1) expression in TTR-Abcb11 and FVB/NJ mice fed an MCD diet. Protein densitometry was performed on Western blots examining CYP2E1 expression and was normalized for tubulin expression. Both mouse strains showed dramatic increases in CYP2E1 protein expression over time, although it was greater in TTR-Abcb11 mice. P < 0.001 by ANOVA.

Fig. 6. Hepatic glutathione (GTH) levels in TTR-Abcb11 and FVB/NJ mice fed an MCD diet. Values are means ± SE. P < 0.001 by ANOVA.

ulation exists between the degree of oxidative stress and degree of early hepatitis. When followed over a longer period of time, however, both mouse strains show evidence of ongoing lipid peroxidation, as demonstrated by TBAR elevation and increased CYP2E1 protein expression. Alterations in FAS likely contribute to this phenomenon, as indicated by decreased mRNA expression of SREBP-1c and FAS in the TTR-Abcb11 mice on the MCD diet.

TTR-Abcb11 mice fed an MCD diet demonstrate a relative resistance to steatosis. This is the second steatogenic dietary model in which the TTR-Abcb11 mouse shows resistance to steatosis (13, 31). Steatosis induced by the MCD diet provides the “first hit” in NASH pathogenesis. Choline is an essential substrate for PC synthesis, the primary phospholipid in liver and bile. The MCD diet likely induces hepatic injury by restricting PC synthesis via the Kennedy pathway and preventing rescue by the phosphatidylethanolamine N-methyltransferase pathway. In fact, phosphatidylethanolamine N-methyltransferase knockout mice fed a choline-deficient diet develop lethal hepatic injury and failure (1). TTR-Abcb11 mice hypersecrete PC and thus have an enhanced need for PC synthesis. They may be particularly susceptible to early injury and hepatocyte damage when placed on an MCD diet. Our observations further emphasize the critical role of phospholipid metabolism in hepatocyte viability.

Abcb11 encodes for the liver BSEP, the major canalicular bile salt transporter (28, 41). TTR-Abcb11 mice not only overexpress BSEP, but have increased hydrophobicity of the bile salt pool and enhanced secretion of bile salts into bile that is coupled with increases in biliary phospholipids and cholesterol secretion (5, 13, 44). The increased PC excretion in this transgenic strain would be expected to increase dependence on PC synthesis to maintain hepatocyte stores. Unfortunately, we were technically unable to measure biliary PC secretion in TTR-Abcb11 mice on the MCD diet. Our observation that TTR-Abcb11 mice develop increased ALT levels more rapidly than control mice on the MCD diet serves to highlight the important role of PC metabolism in the pathogenesis of experimental steatohepatitis.
Hepatic steatosis is likely affected by SREBPs, transcription factors that regulate enzymes needed for fatty acid, triglyceride, and cholesterol synthesis (16). SREBP-1c controls the rate of hepatic FAS (10, 16, 17, 34). Overexpression of SREBP-1c enhances FAS and induces lipogenic enzymes like FAS that may control triglyceride accumulation and result in fatty liver disease (36, 37–39, 48). FAS expression is downregulated by higher levels of triglyceride accumulation. The hepatic steatosis and elevated hepatic triglyceride levels evident in the TTR-Abcb11 mice in our study correlate well with downregulation of the regulatory genes SREBP-1c and FAS mRNA. This suggests the physiological importance of a negative feedback system. This negative feedback likely works in conjunction with insulin to affect SREBP-1c expression (4).

Multiple studies have underscored the importance of inflammatory cytokine signaling and stimulation in the pathogenesis of NASH through mediation of hepatic inflammation, apoptosis, and necrosis (6, 9, 12, 15, 27, 29, 50). Patients with NASH demonstrate increased hepatic and serum TNF-α expression, a proinflammatory cytokine (8, 22). Experimental treatments against TNF-α or receptor deletions are known to attenuate the NASH disease process (18, 24, 50). Additionally, rodents on the MCD diet have higher TNF-α levels and are more sensitive to endotoxin-mediated injury (6). In our experimental model, TTR-Abcb11 mice fed the MCD diet demonstrated threefold increases in TNF-α RNA expression compared with wild-type controls. TNF-α may also trigger the production of other cytokines, such as IL-6 (15). The higher levels of cytokine expression resulting from 7 days compared with 14 days of MCD diet feeding may occur because, in this nutritional model, cytokines may be primarily elevated during the initial phases of inflammation. The upregulation of hepatic TNF-α and IL-6 mRNA seen in the TTR-Abcb11 mouse likely reflects the importance of this cytokine-mediated injury.

The substantial rise in TBARS seen after 14 days in the FVB/NJ mice and both strains after 30 days on the MCD diet emphasizes the role of lipid peroxidation in hepatic injury. It is not surprising that the TTR-Abcb11 mice initially manifest lower TBARS than their wild-type strain controls because they have less substrate (triglyceride) available for lipid peroxidation. In addition, there may be disassociation between the degree of steatosis and the severity of hepatitis (19). The TTR-Abcb11 mice, however, manifest significant hepatitis without concurrent elevations in TBARS after 7 days on the MCD diet. These data demonstrate a novel disassociation between the severity of hepatitis and the degree of oxidative stress.

NASH shares many pathophysiological characteristics with alcoholic steatohepatitis. In alcoholic steatohepatitis, evidence exists that upregulation of CYP2E1 results in lipid peroxidation and resultant accumulation of reactive oxygen species (7, 11, 30). Several studies have underscored the role of CYP2E1 in the pathogenesis of steatohepatitis (7, 23, 45, 46). Leclercq et al. (23) have further emphasized the importance of microsomal enzymes in the development of NASH by demonstrating their induction in a dietary model of NASH. The significant increase in CYP2E1 observed in TTR-Abcb11 mice fed an MCD diet is consistent with the role of CYP2E1 in the pathogenesis of steatohepatitis.

As reactive oxygen species are generated, antioxidant defense by glutathione may occur through scavenging of free radicals, and removing lipid peroxides (47). The imbalance of prooxidants, like microsomal CYP2E1, ROS release from mitochondria, and antioxidants like glutathione, results in hepatic damage (7). A reduction in glutathione may contribute to hepatic damage caused by oxidative stress, abrogated by secondary insults from cytokines such as TNF-α and IL-6. In our MCD dietary model of NASH, the expected decrease in glutathione activity or functional protein expression did not occur. The known antioxidant properties of glutathione could be responsible for the attenuated inflammation and injury seen in this model.

The TTR-Abcb11 transgenic mouse hypersecretes biliary lipids and is resistant to steatosis. It provides an animal model with which to investigate the pathogenic mechanisms of NASH. Our study is consistent with the observation in humans that the absolute amount of fat is not primarily responsible for the transition from bland hepatic steatosis to steatohepatitis. Rather, beyond a certain threshold of steatosis required for injury, the second hit becomes central to NASH development and progression. Therapy directed toward these latter pathological mechanisms could enhance the care of patients with NASH.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-59580 (to R. M. Green).

REFERENCES


AJP-Gastrointest Liver Physiol • VOL 288 • JUNE 2005 • www.ajpgi.org


