Upregulation of osteopontin expression is involved in the development of nonalcoholic steatohepatitis in a dietary murine model

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Sahai, Atul, Padmini Malladi, Hector Melin-Aldana, Richard M. Green, and Peter F. Whittington. Upregulation of osteopontin expression is involved in the development of nonalcoholic steatohepatitis in a dietary murine model. Am J Physiol Gastrointest Liver Physiol 287: G264–G273, 2004. First published March 25, 2004; 10.1152/ajpgi.00002.2004.—The pathogenesis of nonalcoholic steatohepatitis (NASH) is poorly defined. Feeding mice a diet deficient in methionine and choline (MCD diet) induces experimental NASH. Osteopontin (OPN) is a Th1 cytokine that plays an important role in several fibroinflammatory diseases. We examined the role of OPN in the development of experimental NASH. A/J mice were fed MCD or control diet for up to 12 wk, and serum alanine aminotransferase (ALT), liver histology, oxidative stress, and the expressions of OPN, TNF-α, and collagen I were assessed at various time points. MCD diet-fed mice developed hepatic steatosis starting after 1 wk and inflammation by 2 wk; serum ALT increased from day 3. Hepatic collagen I mRNA expression increased during 1–4 wk, and fibrosis appeared at 8 wk. OPN protein expression was markedly increased on day 1 of MCD diet and persisted up to 8 wk, whereas OPN mRNA expression was increased at week 4. TNF-α expression was increased from day 3 to 2 wk, and evidence of oxidative stress did not appear until 8 wk. Increased expression of OPN was predominantly localized in hepatocytes. Hepatocytes in culture also produced OPN, which was stimulated by transforming growth factor-β and TNF-α. Moreover, MCD diet-induced increases in serum ALT levels, hepatic inflammation, and fibrosis were markedly reduced in OPN−/− mice when compared with OPN+/+ mice. In conclusion, our results demonstrate an upregulation of OPN expression early in the development of steatohepatitis and suggest an important role for OPN in signaling the onset of liver injury and fibrosis in experimental NASH.

fatty liver; fibrosis; oxidative stress; inflammation

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) and nonalcoholic steatohepatitis (NASH) form a spectrum of disease from simple accumulation of excess fat in the liver to cirrhosis and end-stage liver disease (1, 3). Whereas the disease can occur in many clinical settings, it is frequently associated with obesity, Type II diabetes, and the metabolic syndrome (34, 37, 40). An estimated 30 million adults and 1.6 million children in the United States have NAFLD, and 30% of them develop NASH (1). Little is known about the pathogenesis of NASH and the mechanisms of progression from simple fatty liver through steatohepatitis to cirrhosis.

Studies in patients and in experimental models of NASH suggest that oxidative stress and peroxidative injury may play a role in the progression of disease (19, 38, 40). These studies have led to a “two-hit” theory regarding the pathogenesis of NASH. The first hit involves accumulation of fat in the liver. This provides increased substrate for mitochondrial β-oxidation, which increases oxidative stress, the second hit. However, oxidative stress has not been examined in hepatic steatosis before the development of NASH, and there is currently little insight into the cellular signaling that leads to activation of inflammation and fibrosis in NASH. Patients with NASH exhibit elevated serum and hepatic levels of TNF-α, and an anti-TNF-α antibody has been shown to reduce inflammation in animal models of NAFLD (6, 18, 21, 48). However, the role of TNF-α in the pathogenesis of NASH remains undefined.

Osteopontin (OPN) is a Th1 cytokine that plays an important role in the pathogenesis of various inflammatory and fibrotic diseases including renal tubulointerstitial injury and atherosclerosis (4, 5, 7, 8, 15, 28). It is synthesized and secreted by a variety of immune cells as well as epithelial, endothelial, and smooth muscle cells (4, 7, 28). It is present as a native 78-kDa protein in various cell systems, whereas the 66-kDa secreted form of OPN is the predominant active form of OPN involved in many pathophysiological processes (7, 28). OPN stimulates T cell proliferation and induces T cells and macrophages to express other Th1 cytokines (2, 33). It is also a key cytokine in granulomatous inflammation (32). Although the function of OPN is not completely defined, it is involved in macrophage recruitment during inflammation, acts as a survival or mitogenic factor for epithelial and vascular cells, and is associated with renal extracellular matrix synthesis and fibrosis (23, 24, 28, 31, 35, 41–43).

The role of OPN in liver diseases is undefined. Carbon tetrachloride administration in the rat has been shown to increase OPN expression in liver where it was localized mainly to Kupffer cells, macrophages, andstellate cells (16). Recombinant OPN also stimulated hepatic macrophage migration in vitro. These data suggest that OPN could play an important role in recruiting inflammation to the liver. Recent studies have also shown markedly increased OPN gene expression in human hepatocellular carcinoma, which suggests that hepatocytes can synthesize OPN (13, 49). These limited findings led us to consider the hypothesis that OPN may play a role in the pathogenesis of NASH. Of interest, increased expression of OPN in the vasculature and kidney is observed in obesity and diabetes and major risk factors in the development of NASH (10, 42–45). In the present study, we examined the role of OPN in the development of steatohepatitis and liver fibrosis in...
a murine nutritional model of NASH and the relationship of OPN expression to other potential mediators of disease progression, such as TNF-α and oxidative stress.

MATERIALS AND METHODS

Animals and experimental protocol. Female A/J mice were purchased from Jackson Laboratory (Bar Harbor, ME), and diets were initiated at age 7–8 wk. OPN knockout (OPN−/−) and their wild-type littersmates (OPN+/+) were generously provided by Dr. David Denhardt (Rutgers University). All animal protocols were approved by the Northwestern University Animal Care and Use Committee (ACUC) and the Lakeside Veterans Affairs Medical Center and conformed to standard procedures set out by ACUC.

In pilot studies, we examined the effect of a diet deficient in methionine and choline (MCD diet) in eight strains of mice (C57BL/6, C57L/J, A/J, FVB, DBA, SJL, AKR/J, and ob/ob) and identified A/J mice as the most sensitive strain, as evident by the highest serum alanine aminotransferase (ALT) levels and histology. Thus all subsequent experiments were performed with A/J mice. These mice were fed MCD or CHOW diet or MCD diet supplemented with methionine and choline (ICN Biochemicals, San Diego, CA) for up to 12 wk. These initial experiments demonstrated no differences in serum ALT levels or histology between supplemented MCD and CHOW diets. Therefore, subsequent studies used CHOW as the control diet. Groups of three to five mice from control and MCD diets were killed at days 1–3 and weeks 1, 2, 4, 8, and 12. Body weights were recorded at the start and the end of each experimental period. Blood was collected by cardiac puncture, and ALT levels were determined in fresh serum using a spectrophotometric kit procedure (cat. no. 505P; Sigma Diagnostics, St. Louis, MO). Livers were rapidly excised, rinsed in ice-cold saline, and weighed. Aliquots of liver were snap-frozen in liquid nitrogen and kept at −80°C until analyzed. A portion of each liver was fixed in 10% formalin for histology.

In a separate experiment, we examined the effect of MCD diet on liver injury and fibrosis in 129/SvJ Black Swiss OPN−/− and OPN+/+ mice. Because this strain was not included among the eight strains tested in our initial pilot studies and is the background strain of the only OPN knockouts available to us, we first examined the effect of MCD diet in 129/SvJ Black Swiss mice (Jackson Laboratory) over a time course of 2–8 wk on serum ALT, liver inflammation, and fibrosis. This strain developed somewhat higher serum ALT levels than the OPN−/− mice (data not shown). By 4 wk, inflammation and fibrosis were equivalent to that seen at 8 wk in A/J mice. Therefore, OPN−/− and OPN+/+ mice were fed the MCD or control diets for 4 wk, and serum ALT levels and histology were assessed.

Histology and immunohistochemistry. Formalin-fixed liver tissue was processed and 5-μm-thick paraffin sections were stained with hematoxylin and eosin and Masson’s trichrome for histological analysis. A hepatopathologist (H. Melin-Aldana) that was blinded to the experimental conditions examined all sections for steatosis, inflammation, necrosis, and fibrosis.

OPN immunostaining was performed using a Vectastain Elite ABC kit and 3,3’-diaminobenzidine (DAB; Vector laboratories, Burlingame, CA). Briefly, sections were deparaffinized, washed, and preincubated in blocking solution followed by incubation with a monoclonal anti-OPN antibody (MPIIIB10; Development Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Sections were then incubated with biotinylated secondary antibody, washed, covered with DAB, and counterstained with hematoxylin.

Measurement of liver triglyceride, thiobarbituric acid-reactive substances, and collagen levels. Liver samples were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 1 μM PMSE. Liver triglyceride contents were measured by a spectrophotometric kit procedure (cat. no. 2750–500; Thermo DMA, Arlington, TX) and expressed as milligrams of triglyceride per gram wet liver weight. Thiobarbituric acid-reactive substance (TBAR) concentration (as nanomoles of malondialdehyde produced per gram liver weight) was assessed by a spectrophotometric kit procedure (cat. no. 0801192; Zepptomix, Buffalo, NY). Reduced glutathione levels were measured in liver homogenates prepared in 5% metaphosphoric acid per kit instructions (cat. no. 354102; Calbiochem, San Diego, CA) and expressed as micromoles of glutathione per gram liver weight.

Western blot analysis. Protein levels of OPN and CYP2E1 were assessed by Western blot analysis as previously described (41). Liver samples were homogenized in a lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 25 mM EDTA, 5 mM EGTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, and 1 mM DTT) containing protease inhibitor cocktail (Calbiochem). Homogenates were centrifuged at 12,000 g for 5 min at 4°C. Samples were mixed with 5× reducing electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, 1% β-mercaptoethanol, and 0.02% bromophenol blue) and heated for 5 min at 95°C. Samples containing 10–25 μg protein were resolved by 10% SDS polyacrylamide gel electrophoresis and then transferred overnight onto nitrocellulose membranes by electrophoresis. CYP2E1 protein was detected using a monoclonal rabbit anti-human CYP2E1 antibody (1:2,000 dilution, Chemicon, Temecula, CA). OPN protein was detected using a monoclonal anti-OPN antibody MPIIIB10 (1:10 dilution). β-Actin antibody (Sigma Diagnostics, St. Louis, MO) was used to confirm equal protein loading among samples. The bound primary antibodies were detected with a horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution, Amersham, Arlington Heights, IL) and visualized with an enhanced chemiluminescence method. Quantitation of protein levels was performed by densitometric analysis using an Eagle Eye II video system (Strategene, La Jolla, CA).

ELISA. TNF-α protein levels were measured in tissue homogenates by ELISA using a commercial kit (Biosource International, Camarillo, CA) and are expressed as picograms per milliliter.

Real-time PCR analysis. The mRNA expression of TNF-α and collagen I were assessed by real-time PCR. Total RNA was isolated from liver samples using TRIzol reagent (GIBCO-BRL, Grand Island, NY). One microgram of total RNA was reverse transcribed using 50 U of SuperScript II RNase− reverse transcriptase and 50 ng of random hexamers (Invitrogen, Carlsbad, CA). Real-time PCR was performed using 4 μl of the total cDNA in a 50-μl reaction volume containing QuantiTect SYBRgreen PCR Master Mix ( Qiagen, Valencia, CA) with the specific primers for mouse TNF-α, collagen I, and the housekeeping gene GAPDH (Integrated DNA Technologies, Coralville, IA). The primer sequences used are TNF-α (forward 5’-CAC GCT CTT CGT TCT ACT GAA-3’; reverse 5’-GGC TAC AGG CTT GTC ACT CGA-3’), collagen I (forward 5’-ATG TTC AGC TTT GTG GAC CTC-3’; reverse 5’-TCC CTC GAC TAC ATC TTC-3’), and GAPDH (forward 5’-GTC GTG GAT CTC TGC C-3’; reverse 5’-TCC CTC GGG CAC CTT CCT-3’). Amplification was performed in duplicate for each sample in an ABI Prism 5700 Sequence Detector (PE Applied Biosystems) with denaturation for 15 min at 95°C followed by 40 PCR cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The amount of mRNA was calculated using GAPDH as the endogenous control.

Northern blot analysis. Osteopontin mRNA expression was analyzed by Northern blot analysis as previously described (41). Total RNA (5–10 μg) was electrophoresed in 1% formaldehyde agarose gels and transferred to nitrocellulose membrane for overnight. Membranes were then hybridized with [32P]-labeled cDNA probe of rat OPN (2B7) by random priming following kit instructions (GIBCO-BRL). The membrane was washed and probed with [32P]-labeled cDNA probe of mouse TNF-α (R&D Systems, Minneapolis, MN), GAPDH (forward 5’-GTC GTG GAT CTC TGC C-3’; reverse 5’-TCC CTC GGG CAC CTT CCT-3’). Quantification of mRNA expression was performed by densitometric analysis. The data are expressed as fold increase in OPN/18S mRNA expression in MCD diet over controls.
Cell culture experimental protocol. The mouse hepatocyte cell line AML-12 was obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells exhibit differentiated, nontransformed hepatocyte phenotype and have been previously used in studies of hepatocyte injury (17). Cultures were grown in 1:1 mixture of DME/Ham’s F-12 medium supplemented with 10% fetal bovine serum, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and antibiotics. Cultures were maintained in 75-cm² flasks in their growth medium in 5% CO₂-95% air at 37°C. To assess the effect of transforming growth factor (TGF)-β and TNF-α on OPN expression, cells were subcultured in 100-mm dishes in growth medium until the cultures reached 70–80% confluence. At this time, cells were made quiescent by incubation for 24 h in serum-and hormone-free DME/Ham’s F-12 medium. Quiescent cultures were exposed to TGF-β (1 and 10 ng/ml), TNF-α (1 and 10 ng/ml), or control (vehicle PBS) at equivalent concentrations of the reagents for 18 h at 37°C. At the end of incubation, conditioned medium was removed and concentrated using Ultrafree-4 centrifugal filter units (Millipore, Billerica, MA). The concentrated medium was mixed with 5× electrophoresis sample buffer, heated for 5 min at 95°C, and analyzed for OPN protein expression by Western blot analysis.

Table 1. Effect of MCD diet on relative liver weight, liver triglyceride content, serum ALT levels, TBARs, reduced glutathione, and Cyp2E1 protein expression in A/J mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative Liver Wt, liver/body wt ×100</th>
<th>Liver TG, mg/g wet liver</th>
<th>Serum ALT, IU/l</th>
<th>TBARs, nmol/g wet liver</th>
<th>Reduced Glutathione, μmol/g wet liver</th>
<th>Cyp2E1 (fold increase)</th>
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<tr>
<td>Control</td>
<td>3.63±0.10</td>
<td>10±2.5</td>
<td>27±5.2</td>
<td>0.67±0.08</td>
<td>8.50±0.61</td>
<td>1.00±0.10</td>
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<tr>
<td>MCD, day 1</td>
<td>4.10±0.20</td>
<td>11±1.9</td>
<td>38±8.1</td>
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<tr>
<td>MCD, day 2</td>
<td>4.44±0.24*</td>
<td>16±3.6</td>
<td>33±5.5</td>
<td></td>
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</tr>
<tr>
<td>MCD, day 3</td>
<td>4.45±0.28*</td>
<td>22±4.2*</td>
<td>78±7.4*</td>
<td></td>
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<tr>
<td>MCD, 1 wk</td>
<td>5.73±0.30*</td>
<td>40±4.5*</td>
<td>108±2.4*</td>
<td>0.91±0.12</td>
<td>8.51±0.45</td>
<td>1.22±0.10</td>
</tr>
<tr>
<td>MCD, 2 wk</td>
<td>5.99±0.24*</td>
<td>42±6.1*</td>
<td>128±15*</td>
<td>0.81±0.07</td>
<td>8.10±0.55</td>
<td>1.11±0.23</td>
</tr>
<tr>
<td>MCD, 4 wk</td>
<td>7.15±0.36*</td>
<td>51±5.0*</td>
<td>211±12*</td>
<td>0.78±0.09</td>
<td>8.72±0.52</td>
<td>1.31±0.15</td>
</tr>
<tr>
<td>MCD, 8 wk</td>
<td>5.10±0.12*</td>
<td>72±6.3*</td>
<td>258±16*</td>
<td>1.00±0.09*</td>
<td>6.21±0.38*</td>
<td>3.01±0.22*</td>
</tr>
<tr>
<td>MCD, 12 wk</td>
<td>6.84±0.25*</td>
<td>78±8.0*</td>
<td>250±14*</td>
<td>1.20±0.08*</td>
<td>5.02±0.59*</td>
<td>2.55±0.18*</td>
</tr>
</tbody>
</table>

Data is means ± SE of 3–5 mice in each group. *P < 0.01 compared with respective control value. MCD, diet deficient in methionine and choline; ALT, alanine aminotransferase; TBAR, thiobarbituric acid-reactive substance; TG, triglyceride.

RESULTS

Effect of MCD diet on relative liver weight, liver triglyceride content and serum ALT levels. Mice fed the MCD diet showed a significant increase in liver weight relative to body weight starting at day 2, which remained elevated up to 12 wk (Table...
The MCD diet also induced a twofold increase in liver triglyceride levels by day 3, fourfold increase by 1–2 wk, and eightfold increase by 12 wk (Table 1). Serum ALT levels were increased from day 3, reaching a peak of 258 ± 16 IU/l at 8 wk (Table 1).

Effect of MCD diet on the development of steatosis, steatohepatitis, and liver fibrosis. Histological examination demonstrated macrovesicular steatosis involving lobular zones 2 and 3 after 1 wk of the MCD diet (Fig. 1C). Panlobular steatosis developed by 2 wk (Fig. 1D), and the degree of steatosis further increased during 4–8 wk of the MCD diet (Fig. 1, E and F). The histological changes paralleled the increases in liver triglyceride content (Table 1). Minimal focal lobular inflammation appeared at 1 wk (Fig. 1C) and increased at 2 wk (Fig. 1D). Inflammation progressively advanced in intensity and distribution and included portal areas by 4 wk of the diet (Fig. 1E). Both lobular and portal inflammation further worsened at 8 wk, when there was intense panlobular and portal acute and chronic inflammatory infiltrate observed (Fig. 1F). Trichrome staining showed increased collagen deposition by 8 wk in the portal tract (Fig. 2C), around the central vein (Fig. 2D), and in the lobule in a pericellular distribution (Fig. 2, C and D) but not at earlier time points.

Effect of MCD diet on collagen I expression. Real-time PCR demonstrated an increase in collagen I mRNA expression between 1 and 4 wk of the MCD diet, which was maximally increased to fourfold at 4 wk and declined thereafter (Fig. 3). Therefore, the induction of collagen I gene expression preceded the accumulation of collagen protein deposition observed at 8 wk of MCD diet (Fig. 2).

Effect of MCD diet on oxidative stress. Table 1 shows that mice fed the MCD diet exhibited a 50–80% increase in hepatic TBARs concentrations at 8 and 12 wk of examination. MCD diet caused a 30% reduction of glutathione levels at 8 and 12
CYP2E1 has been shown to be involved in lipid peroxidation, and its levels are increased in a nutritional model of NASH (47). In this study, MCD diet resulted in a threefold increase in CYP2E1 protein expression at 8 and 12 wk but had no effect at earlier time points (Table 1).

**Effect of MCD diet on TNF-α expression.** TNF-α contributes to oxidative stress and is thought to be involved in the pathogenesis of NASH (6, 9, 18, 21, 48). Therefore, we examined the effect of MCD diet on TNF-α expression during the development of NASH in A/J mice. As shown in Fig. 4A, TNF-α mRNA expression increased over threefold at day 3 of MCD diet, with smaller increases (2-fold) observed at 4 and 8 wk. TNF-α protein levels were increased 1.5- to 2-fold from day 3 to 2 wk of dietary treatment (Fig. 4B).

Northern blot analysis showed significant constitutive OPN mRNA expression in the control mice (Fig. 6). Dietary treatment increased OPN mRNA expression 11-fold at 4 wk and 13-fold at 8 wk but had little effect from day 1 to 2 wk of the diet (Fig. 6). These results suggest that the MCD diet stimulates OPN expression by both posttranscriptional and transcriptional mechanisms.
The results of OPN immunohistochemistry are shown in Fig. 7. Significant increases in OPN protein staining were observed in MCD diet-fed mice beginning at day 3, which further increased by 8 wk. OPN protein expression was predominantly localized to hepatocytes (Fig. 7, B and C, arrow).

**66 kDa osteopontin secretion by cultured hepatocytes.** TGF-β is implicated in liver fibrosis (11, 14), and TNF-α is thought to play a role in the development of NASH (6, 18, 21, 48). In addition, both TGF-β and TNF-α increase OPN expression in other cell types (7, 28). Therefore, we examined the effect of TGF-β and TNF-α on OPN protein expression in the AML-12 hepatocyte cell line. Figure 8 shows the results of Western blot analysis of culture medium obtained from the cultured hepatocyte exposed for 18 h to TGF-β or TNF-α, each at concentrations of 1 and 10 ng/ml. Small amounts of OPN protein levels were detected in the medium from cultured hepatocytes under control conditions. Incubation with either TGF-β or TNF-α resulted in three- to fourfold increases in OPN protein expression compared with controls (Fig. 8). TGF-β and TNF-α also stimulated OPN protein expression in primary cultures of rat hepatocytes as well as in H35 rat hepatoma cells (data not shown).

**Effect of MCD diet on liver injury and fibrosis in OPN knockout mice.** We further examined whether the complete absence of OPN expression would alter the development of liver injury and fibrosis using OPN knockout (OPN−/−) mice. Liver samples from the OPN−/− mice showed complete absence of OPN gene expression, and feeding OPN+/+ mice the MCD diet resulted in increased OPN protein expression similar in magnitude to A/J mice (data not shown). MCD diet-treated OPN+/+ mice had substantial increases in serum ALT levels (204 ± 34 IU/l), which were reduced to 86 ± 15 IU/l in OPN−/− mice (Fig. 9A). Consistent with the reduction in serum ALT levels, hepatic lobular and portal inflammation were significantly less in OPN−/− mice compared with OPN+/+ mice (Fig. 9B). However, MCD diet induced similar degree of macrovesicular steatosis in OPN+/+ and OPN−/− mice (Fig. 9B), and the diet-induced increases in liver triglyceride content were not different between OPN+/+ and OPN−/− mice (Fig. 9C).

MCD diet caused a sixfold increase in collagen I mRNA expression (Fig. 10A) and marked perivenular, pericellular, and portal fibrosis in OPN+/+ mice as assessed by trichrome staining (Fig. 10B). Both collagen I mRNA expression and histological fibrosis were markedly reduced in OPN−/− mice on the MCD diet (Fig. 10, A and B).

**DISCUSSION**

Our results demonstrate marked increases in OPN expression early in the evolution of steatohepatitis in a mouse nutritional model of NASH. Mice fed an MCD diet developed steatosis within 1 wk, which progressed by 4 wk to steatohepatitis and subsequent hepatic fibrosis. Increased OPN expression preceded increases in TNF-α and oxidative stress, and OPN-deficient mice demonstrated significantly less liver injury and fibrosis in response to the MCD diet. Our findings suggest an important role for OPN in the progression of experimental NASH.

The experimental model of NASH used in the present study involves restriction of intake of the essential nutrients choline and methionine. Mice fed the MCD diet as well as mice lacking methionine adenosyltransferase have been shown to develop liver disease similar to human NASH (19, 20, 25, 26, 39, 47). We examined the course of liver disease in this nutritional model beginning at day 1 and extending to 12 wk to determine the course of events leading to steatohepatitis and fibrosis. We observed liver pathology much earlier than has been previously demonstrated in this nutritional model (19, 47). By 1 wk, there was histological macrovesicular steatosis, which progressed to panlobular macrovesicular steatosis by 2 wk. Minimal inflammation appeared at 1 wk and progressed to significant hepatitis by 4

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**Fig. 7.** Immunohistochemistry of OPN expression. Paraffin-embedded sections of controls (A) and MCD diet-fed mice at day 3 (B) and 8 wk (C) were analyzed for OPN protein expression by immunohistochemistry using anti-OPN antibody MPIIIB10. The arrows point to the localization of OPN within hepatocytes. (Original magnifications: ×100 in A and B; ×400 in C; n = 3)

**Fig. 8.** Effect of transforming growth factor (TGF)-β and TNF-α on OPN protein expression in cultured hepatocytes. Quiescent hepatocytes were exposed in a serum-free medium either to TGF-β or TNF-α at concentrations of 1 and 10 ng/ml for 18 h. At the end of respective incubations, conditioned medium was analyzed for OPN protein expression by Western blot analysis. β-Actin antibody was used to confirm equal protein loading among the samples (n = 5). Veh., vehicle.
Thus there was evidence of progression from steatosis to NASH from 1 to 4 wk in this model.

It has been presumed that inflammation incites fibrosis in NASH (1, 3). We found induction of collagen 1 transcription, indicating stellate cell activation, before significant histological inflammation could be demonstrated. Type 1 collagen mRNA showed over twofold induction at 1 wk and fourfold increase at 2 wk of MCD diet, coincident with the appearance and progression of inflammation. Increased collagen deposition as indicated by trichrome staining was observed at 8 wk. Thus the findings in our model were entirely consistent with the histopathological findings in human NASH. However, we observed increased collagen 1 mRNA expression before the appearance of significant histological inflammation. These findings suggest that inflammation may not be the only inciting agent for fibrosis.

Our data suggest that OPN is an important cytokine for intrahepatic signaling in NAFLD/NASH. In cell cultures, we
demonstrated that it is synthesized and secreted by hepatocytes. Secretion of the 66-kDa protein increased severalfold in response to TNF-α and TGF-β, cytokines that are known to play a role in liver diseases. Studies from other organs and tissues indicate that the 66-kDa protein is the predominant active and secreted form of OPN (4, 7, 28). These observations indicate that hepatocytes have the capacity to synthesize an active form of OPN in response to various stimuli.

We demonstrated marked increases in 66-kDa OPN protein expression very early in this nutritional model of NASH. Small constitutive levels of 66-kDa OPN were observed in the control liver. The MCD diet induced a 16-fold increase in 66-kDa OPN protein levels by day 1 and a 40-fold increase by day 3. Expression of the 78-kDa native form of OPN, for which there is greater constitutive expression, was also increased, although to a lesser degree. In the two-hit hypothesis, the accumulation of fat in hepatocytes is thought to be an important early event in the progression to NASH. Our findings suggest that alterations in hepatocyte metabolism and/or accumulation of microvesicular fat may contribute to the early increase in OPN, as is the case in diabetes-associated atherosclerosis (45). We did not observe any significant changes in OPN mRNA levels during day 1 to 2 wk of the diet, suggesting a posttranscriptional mechanism in the early increase in OPN. However, both OPN protein and mRNA levels were significantly upregulated by 4 wk of MCD diet. Therefore, different signaling pathways may be involved in the diet-induced increases in OPN expression by posttranscriptional and transcriptional mechanisms.

The initial increase in OPN expression was accompanied by a modest increase in TNF-α mRNA and protein expression from day 3 to 1 wk, during which time we could discern no histological evidence of inflammation in the liver. Because in our studies TNF-α stimulated OPN expression in cultured hepatocytes, there is a possible positive feedback loop for OPN synthesis that could accelerate an inflammatory response. Throughout the course of disease, OPN was localized mainly to hepatocytes and not in inflammatory cells, as assessed by immunohistochemistry. This feedback from TNF-α could play a role in maintaining the production of OPN by hepatocytes and possibly inducing transcriptional upregulation, which was observed at 4 wk. Because collagen I expression was increased as early as 1 wk of the diet, the upregulation in OPN expression may play a key role in both the development of inflammation and fibrosis. Indeed, our results with OPN knockout mice demonstrate an important role for OPN in the development of liver injury and fibrosis, because MCD diet-induced increases in serum ALT levels, hepatic inflammation, and fibrosis were markedly reduced in OPN+/− mice in comparison with OPN+/+ mice. However, histological steatosis and liver triglyceride levels were unchanged between OPN+/+ and OPN+/− mice, suggesting that mechanisms other than the induction of OPN are involved in the development of steatosis in the MCD-diet model of NASH. The mechanisms by which hepatocyte-derived OPN potentially induces liver injury and fibrosis is not defined by our studies but may include interactions with other cytokines and growth factors involved in hepatic fibrosis.

Oxidative injury has been proposed to play a role in the progression of hepatic steatosis to NASH (19, 38, 40). In our dietary model, some lipid peroxidation was observed, but well after the onset of OPN upregulation. Hepatic TBARs concentrations increased by 50 and 80% at 8 and 12 wk of MCD diet, respectively, but not at earlier time points. We did not observe any significant changes in CYP2E1 expression or glutathione levels during 1–4 wk of MCD diet, but a threefold increase in CYP2E1 expression and 30% reduction in glutathione levels were observed beginning at 8 wk, similar to levels reported in C57BL/6 and ob/ob mice fed an MCD diet (19, 20). The magnitude of changes in TBARs in this nutritional model is similar to those reported in models of carbon tetrachloride- and ethanol-induced liver injury (30, 46). The changes in oxidative stress in this nutritional model, which temporally correlated with the evolution from fatty liver to steatohepatitis, are consistent with the idea that oxidative injury plays a role in the progression of hepatic steatosis to NASH. However, this is the first study to examine oxidative injury at earlier time points in a murine MCD-diet model. Our observation that oxidative stress occurs after many other changes that could affect the progression of steatosis to NASH provides reason to question whether oxidative stress is the key second hit leading to the progression of NASH.

In summary, we have identified OPN as a cytokine that is synthesized by hepatocytes, and its expression is increased early in the course of experimental steatohepatitis. Upregulation of OPN expression preceded increases in TNF-α and collagen I expression, and oxidative stress was observed only later in the development of steatohepatitis. The absence of OPN expression in OPN knockout mice blunted the liver injury and fibrosis induced by the MCD diet. Together, these findings suggest that OPN plays an important role in signaling the progression of liver injury and fibrosis in this experimental model of NASH.

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