Human and Experimental Evidence Supporting a Role for Osteopontin in Alcoholic Hepatitis

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We identified, in the transcriptome analysis of patients with alcoholic hepatitis (AH), osteopontin (OPN) as one of the most up-regulated genes. Here, we used a translational approach to investigate its pathogenic role. OPN hepatic gene expression was quantified in patients with AH and other liver diseases. OPN protein expression and processing were assessed by immunohistochemistry, western blotting and enzyme-linked immunosorbent assay. OPN gene polymorphisms were evaluated in patients with alcoholic liver disease. The role of OPN was evaluated in OPN−/− mice with alcohol-induced liver injury. OPN biological actions were studied in human hepatic stellate cells (HSCs) and in precision-cut liver slices. Hepatic expression and serum levels of OPN were markedly increased in AH, compared to normal livers and other types of chronic liver diseases, and correlated with short-term survival. Serum levels of OPN also correlated with hepatic expression and disease severity. OPN was mainly expressed in areas with inflammation and fibrosis. Two proteases that process OPN (thrombin and matrix metalloproteinase 7) and cleaved OPN were increased in livers with AH. Patients with AH had a tendency of a lower frequency of the CC genotype of the +1239C single-nucleotide polymorphism of the OPN gene, compared to patients with alcohol abuse without liver disease. Importantly, OPN−/− mice were protected against alcohol-induced liver injury and showed decreased expression of inflammatory cytokines. Finally, OPN was induced by lipopolysaccharide and stimulated inflammatory actions in HSCs. Conclusion: Human and experimental data suggest a role for OPN in the pathogenesis of AH. Further studies should evaluate OPN as a potential therapeutic target. (HEPATOLOGY 2013;58:1742-1756)

Alcoholic liver disease (ALD) is a major cause of advanced liver disease worldwide. Despite its relevance as an etiological factor of liver cirrhosis and liver-related mortality, there are not major advances in the treatment of this disease. In particular, there are no targeted therapies that attenuate liver injury and fibrosis in patients with alcohol abuse. The development of such therapies is hampered by the

Abbreviations: Ab, antibody; ABIC, age, serum bilirubin, international normalized ratio, and serum creatinine score; AH, alcoholic hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; HCV, hepatitis C virus; H&E, hematoxylin and eosin; HSC, hepatic stellate cell; HVPG, high venous pressure gradient; IHC, immunohistochemistry; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; MELD, Model for End-Stage Liver Disease; MMP, matrix metalloproteinase; MPO, myeloperoxidase; mRNA, messenger RNA; NAFLD, nonalcoholic steatohepatitis; NASH, nonalcoholic steatohepatitis; OPN, osteopontin; PDGF, platelet-derived growth factor; PH, portal hypertension; qPCR, quantitative real-time polymerase chain reaction; rh, recombinant human; SE, standard error; SNP, single-nucleotide polymorphism; SPP1, secreted phosphoprotein 1; TG, triglyceride; TGF-β, transforming growth factor beta; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha; UTR, untranslated region; WT, wild type.

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poor knowledge of disease mechanisms in humans. One of the scenarios in which new therapies is urgently needed is alcoholic hepatitis (AH), a clinical condition characterized by inflammation, massive hepatic steatosis, pericellular fibrosis, and severe hepatocellular damage. The development of episodes of AH heavily affects the progression and severity of ALD. In its severe cases, AH carries a high short-term mortality (20%-30% of mortality at 3 months). Patients with severe AH are prompted to develop liver failure, portal hypertension (PH), and severe bacterial infections. Unfortunately, current therapies (i.e., corticosteroids) fail in many patients, and attempts to develop targeted therapies, such as the use of tumor necrosis factor alpha (TNF-α)-blocking agents, have not been successful. Because rodents are resistant to develop advanced stages of ALD, human studies are required to identify new targets for therapy. In our laboratory, we previously found that CXC chemokines are markedly overexpressed in patients with AH and their hepatic expression correlates with disease severity. Moreover, we recently performed a functional analysis of a high-throughput transcriptome study, which revealed that genes encoding extracellular matrix (ECM) proteins are differentially expressed in livers with AH. Among these proteins, osteopontin (OPN) was the most up-regulated. Because OPN is a highly active molecule that can act as a true cytokine, the current study was aimed at specifically investigating the role of OPN in AH as a potential target for therapy.

OPN, or secreted phosphoprotein 1 (SPP1), is an ECM protein that can also act as a neutrophil-attracting chemokine. It is synthesized by multiple tissues and secreted into body fluids. In target cells, OPN binds to a high number of integrins and CD44 to promote profibrogenic and inflammatory actions. Importantly, OPN undergoes a high number of post-translational modifications, including serine phosphorylation, O-glycosylation, and proteolytic processing. Importantly, the proteolytic actions of proteases, such as thrombin or matrix metalloproteinase (MMP)-7, yield a more active form of OPN.

There is growing evidence that OPN plays a major role in the wound-healing response to acute and chronic injury in many organs, including the lung and the kidney. In addition, recent studies indicate that OPN participates in the pathogenesis of hepatic steatosis, inflammation, and the resulting fibrosis in different types of liver diseases. In particular, OPN seems to play a major role in the pathogenesis of non-alcoholic steatohepatitis (NASH), a condition that resembles the histological findings of ALD, including steatosis and neutrophil infiltration. The mechanisms by which OPN regulates steatohepatitis include Hedgehog-signaling pathway stimulation. At the cellular level, OPN is highly secreted by hepatic stellate cells (HSCs) to induce cell cytokine release and collagen synthesis.

To investigate the role of OPN in AH, we performed a translational study using different approaches, including gene expression, protein content, and DNA polymorphisms analysis, in patients with AH as well as experimental studies in mice lacking OPN, in precision-cut liver slices and in cultured liver cells.

Materials and Methods

Selection of Patients With Alcoholic Hepatitis and Other Liver Diseases and Control Livers. Patients admitted to the Liver Unit of the Hospital Clinic of Barcelona (Barcelona, Spain) with clinical, analytical, and histological features of AH from 2007 to 2010 were prospectively included in the study (n = 48). Inclusion criteria were described previously. Patients with hepatocellular carcinoma or any other potential cause of liver disease were excluded from the study. Liver biopsy was obtained using a transjugular approach, and portal pressure gradient was measured. All patients received nutritional as well as psychological support for achieving alcohol abstinence.

Three control groups were also included. In all cases, a liver biopsy was available. The first group comprised patients with hepatitis C virus (HCV)-induced liver disease (genotype 1; n = 28). No patient had previous antiviral therapy. The second group comprised patients with compensated cirrhosis resulting from HCV or past history of alcohol abuse (in all
cases, patients were abstinent for at least 6 months; n = 29). The third group comprised patients with morbid obesity who underwent a laparoscopic liver biopsy during bariatric surgery and had criteria of NASH (n = 47), according to Kleiner’s criteria.17 Finally, fragments of normal liver tissue were obtained from optimal cadaveric liver donors (n = 3) or resection of liver metastases (n = 4), as described in detail previously.6

All liver specimens were analyzed by an expert pathologist, and a part of the biopsy was submerged into an RNA stabilization solution (RNAlater). The protocol was approved by the ethics committee of the Hospital Clinic, and all patients gave informed consent.

Selection of Patients for Genetic Studies. Patients were included from the University Hospital of Salamanca (Salamanca, Spain) and Amiens University Hospital (Amiens, France). Patients with compensated alcoholic liver cirrhosis (n = 102), alcoholic patients without liver disease (n = 196), and patients with biopsy-proven AH (n = 100) were included.18 Patients with cirrhosis had either histological diagnosis or clinical, analytical, and imaging signs of compensated cirrhosis.19 Alcoholic patients without ALD had no clinical evidences of liver disease, normal liver blood tests, and normal liver ultrasonography. Finally, patients with AH were diagnosed following the same criteria used in the current study. All patients were negative for viral hepatitis and other known causes of chronic liver disease. In addition, sex-matched healthy volunteers (n = 152) were included in the study as negative controls. All of them consumed less than 10 g of ethanol per day and none had a history of alcohol abuse or alcohol dependence and neither did their first- or second-degree relatives. The study was approved by both ethical committees, and all patients gave informed consent.

Real-Time Polymerase Chain Reaction Analysis. RNA isolation was performed on liver biopsies from patients with AH (n = 44), HCV (n = 23), NASH (n = 31), and compensated cirrhosis (n = 12) as well as from fragments of normal livers (n = 7). Quantitative real-time polymerase chain reaction (qPCR) was performed as described in the Supporting Materials.

OPN Serum-Level Analysis. Serum samples were obtained from peripheral and suprahepatic blood and stored at −80°C. OPN serum levels were measured in patients with AH (n = 26), HCV (n = 22), NASH (n = 23), and compensated alcoholic cirrhosis (n = 17) as well as from healthy volunteers (n = 5) using the Quantikine Human Osteopontin Immunoassay Kit (R&D Systems, Minneapolis, MN).

Immunohistochemistry. Paraffin-embedded sections were incubated with the primary antibody (Ab), anti-human OPN (Abcam, Cambridge, UK), following the immunohistochemistry (IHC) protocol described in the Supporting Materials.

Western Blotting Studies. Processed OPN was analyzed in serum proteins from patients with AH (n = 16), compensated alcoholic cirrhosis (n = 16), and healthy volunteers (n = 7) using a specific Ab to detect MMP-cleaved OPN by western blotting (catalog no.: ab8448; Abcam). Western blotting studies were performed in human and cellular samples, as described in the Supporting Materials.

OPN Single-Nucleotide Polymorphism Analysis. Genomic DNA extraction was performed in nucleated peripheral blood using standard methods and stored at −20°C. Samples were mixed on PCR plates for simultaneous analysis in a blind fashion. Four single-nucleotide polymorphisms (SNPs) of the SPP1 gene (PubMed references: rs28357094, rs9138, rs11730582, and rs2728127) were analyzed in the selected population. Genotyping was carried out using specific TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA), and PCR reactions were read in a sequence detection system in an ABI PRISM 7000 instrument (Applied Biosystems-Life Technologies Corporation, Carlsbad, CA).

Studies in Experimental ALD in Mice. Male OPN-deficient (OPN−/−) mice were generated by Dr. Gao’s laboratory (National Institutes of Health, Bethesda, MD), and ethanol was administered following the chronic-binge model, as described previously.20 Briefly, OPN−/− and wild-type (WT) mice were divided into two groups (n = 10). Ethanol groups were fed with a liquid diet containing 5% ethanol for 10 days, whereas the control group received a pair-fed diet. After this period, ethanol groups were gavaged with single doses of ethanol (5 g/kg body weight, 20% ethanol), whereas control group mice were gavaged with isocaloric dextrin maltose. Mice were then sacrificed 9 hours after gavage, and sample collection was performed. All animal experiments were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee.

Precision-Cut Liver Slices From Mice and Cell Cultures. High-precision-cut slices (250 μm) were obtained from freshly isolated mice livers using a vibratome instrument (VT1000S; Leica Microsystems, Wetzlar, Germany). The optimized cutting procedure and the incubation conditions were followed as described previously.6 Human primary HSCs were isolated from healthy fragments of liver donors following
the Nycozenz gradient protocol. Additionally, three different cell lines were used in a subset of experiments, including LX-2 cells (a kind gift from Scott L. Friedman, Mount Sinai Hospital, New York, NY), a human HSC line, RAW264 cells, a mouse macrophage line, and EL4, a mouse T-type lymphoma line. Both liver slices and cells were led to stabilize and further treated with lipopolysaccharide (LPS), recombinant human (rh) or mouse TNF-α, rh or mouse transforming growth factor beta (TGF-β), rhOPN, rh interleukin (IL)−1β, and rh platelet-derived growth factor (PDGF), all purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis. Results of quantitative variables are expressed as mean ± standard error (SE), unless otherwise specified. The differences between groups were analyzed using nonparametric tests (Mann-Whitney’s U test) for continuous variables and the chi-square test for categorical variables. Correlations between variables were evaluated using Spearman’s rho. Cox’s regression analysis was employed to identify variables associated with mortality. Survival curves by Kaplan-Meier’s method were created and compared using the log-rank test. Regarding SNP analysis, allele and genotype frequencies of patients were compared by means of the chi-square test or Fisher’s exact test, when necessary, and a logistic regression analysis was employed to adjust for potential confounders. Bonferroni’s correction for multiple testing was applied to SNP analysis, by which only those comparisons that generated a P value <0.05/n (n = total number of comparisons) were considered significant. Statistical analysis was performed using SPSS version 14.0 for Windows (SPSS, Inc., Chicago, IL).

Results

General Characteristics of Patients With AH. Forty-eight patients were included in the study with clinical, analytical, and histological characteristics of AH. Overall, 90-day mortality was 20%. Seventy-nine percent (n = 38) of patients had severe AH at admission, as defined as an age, serum bilirubin, international normalized ratio, and serum creatinine (ABIC) score >6.71.16 Patients were predominantly male (63%) and the mean age was 49 years. The majority of patients had severe sinusoidal PH (mean high venous pressure gradient [HVPG]: 19.5 ± 0.8 mmHg). The main epidemiological, clinical, and analytical characteristics of patients are shown in Table 1.

Mean length of biopsy specimens was 6.0 ± 0.6 mm, the mean number of fragments was 5.0 ± 0.4, and the mean number of portal spaces was 6 ± 1. The majority of patients had moderate or marked steatosis (69%) of diffuse distribution, marked hepatocyte ballooning (61%), marked necroinflammation (37%), and cirrhosis (62%).

**Table 1. Clinical, Analytical, and Hepatic Hemodynamic Characteristics of Patients With AH**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean ± SE or %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>49 ± 1.12</td>
</tr>
<tr>
<td>Male, %</td>
<td>63</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.69 ± 1.04</td>
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<tr>
<td>Alcohol intake, g/day</td>
<td>107.29 ± 5.17</td>
</tr>
<tr>
<td>90-day mortality, %</td>
<td>20</td>
</tr>
<tr>
<td>Analytical and hemodynamic parameters</td>
<td></td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>109 ± 5</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.83 ± 0.06</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>157 ± 12</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>GGT, U/L</td>
<td>588 ± 81</td>
</tr>
<tr>
<td>Bilirubin, mg/dL</td>
<td>12.7 ± 1.27</td>
</tr>
<tr>
<td>Albumin, mg/dL</td>
<td>26.2 ± 0.58</td>
</tr>
<tr>
<td>Platelet count, ×10⁹/L</td>
<td>13.2 ± 1.36</td>
</tr>
<tr>
<td>Leucocyte count, ×10⁹/L</td>
<td>9.9 ± 0.78</td>
</tr>
<tr>
<td>INR</td>
<td>1.87 ± 0.11</td>
</tr>
<tr>
<td>HEPG, mmHg</td>
<td>19.49 ± 0.84</td>
</tr>
<tr>
<td>Cirrhosis, %</td>
<td>62</td>
</tr>
<tr>
<td>Scoring systems</td>
<td></td>
</tr>
<tr>
<td>Maddrey's DF</td>
<td>60.48 ± 6.27</td>
</tr>
<tr>
<td>MELD score</td>
<td>22 ± 0.9</td>
</tr>
<tr>
<td>ABIC score</td>
<td>7.63 ± 0.19</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; GGT, gamma-glutamyl transpeptidase; INR, international normalized ratio; DF, discriminant function.

* n = 48.

OPN Is Overexpressed in Patients With AH. We previously detected, by microarray analysis, that OPN was extremely up-regulated in AH.6 Confirmatory gene expression analysis revealed that OPN was almost 150 times overexpressed in patients with AH, compared to normal livers (P < 0.001; Fig. 1A). Among other types of liver diseases, patients with NASH—but not with HCV—had a moderate increase in hepatic OPN expression (P < 0.01). Importantly, OPN was up-regulated in liver with compensated cirrhosis (P < 0.05), but to a lesser extent than in patients with AH (P < 0.001).

Next, we found that OPN serum levels were much higher in patients with AH, compared to patients with other liver diseases and healthy controls (P < 0.001). Patients with both HCV and compensated cirrhosis also had increased OPN levels, compared to healthy controls (P < 0.01 and P < 0.001, respectively; Fig. 1B). The gender of patients did not influence either hepatic expression or serum levels of OPN. Importantly, OPN hepatic messenger RNA (mRNA) and serum levels correlated in patients with AH (P < 0.05;
Furthermore, suprahepatic serum levels paralleled also with OPN measured in peripheral veins ($P < 0.01$; Fig. 1D). Taken together, these results indicate that OPN is markedly overexpressed in AH and could be useful as a disease biomarker. Moreover, the damaged liver seems to be a source of circulating OPN in patients with AH.
Finally, OPN protein expression and deposition in liver tissue was assessed by IHC. The results revealed a prominent OPN staining mainly focused in fibrotic areas of livers of patients with AH, whereas it was barely detected in normal livers and in patients with NASH and HCV (Fig. 1E). These findings strongly support the hypothesis that OPN is overexpressed in patients with AH, both at the RNA and protein levels.

OPN Hepatic Expression and Serum Levels Correlate With Disease Severity in Patients With AH. We next explored whether OPN gene and serum levels correlate with disease severity, which was assessed using the ABIC score. Patients were stratified into nonsevere AH (ABIC < 6.71; n = 10) and severe AH (ABIC > 6.71; n = 38). OPN hepatic gene expression and serum levels were significantly higher in patients with severe AH than in those with nonsevere AH (P < 0.05 in both; Fig. 2A).

Furthermore, short-term mortality was assessed at 90 days. OPN mRNA and serum levels were higher in patients who did not survive (P < 0.05 in both; Fig. 2B). Additionally, Kaplan-Meier’s curve approach was used for determining whether both hepatic OPN expression and OPN serum levels are good parameters for prediction of patient short-term mortality. Receiver operating curve analysis was used to identify a cut-off value of 112-fold expression to define patients with low and high hepatic OPN gene expression and a cut-off value of 240 ng/mL to define patients that had low or high circulating OPN levels (P < 0.05 in both; Supporting Fig. 1). Kaplan-Meier’s curves showed that both parameters predict mortality in patients with AH (P < 0.05), because the group of patients that had increased OPN hepatic expression and serum levels also had increased number of deaths during the 90-day period (Fig. 2C,D). These results strongly indicate that OPN hepatic expression predicts disease severity in patients with AH.

We finally investigated whether OPN expression correlates with expression of other neutrophil-attractive chemokines involved in the pathogenesis of AH, such as the CXC family. We found that OPN gene expression correlated with hepatic gene expression of different CXC chemokines (P < 0.05 and P < 0.001), especially IL-8 (Supporting Fig. 2).

OPN Posttranscriptional Modifications in AH. Because thrombin (PAR-2) and MMP-7 are known to process OPN and amplify its pathogenic activity, we then explored whether the resulting cleaved OPN is increased in AH. We first found, by qPCR analysis, that both thrombin and MMP-7 were overexpressed in patients with AH, compared to normal livers (P < 0.001 and P < 0.05, respectively; Fig. 3A). Furthermore, because the amount of proteins obtained in a transjugular biopsy is highly limited and western blotting analysis could not be performed, the analysis performed on serum proteins revealed that patients with AH had increased levels of MMP-cleaved fragments of OPN—but not full-length fragments—in serum, compared to healthy controls (P < 0.001). Furthermore, patients with compensated alcoholic cirrhosis had more cleaved OPN than healthy controls in serum (P < 0.05), but to a lesser extent than patients with AH (Fig. 3B,C). Interestingly, these results suggest that the 10-fold increase of OPN serum levels found in patients with AH, compared to healthy controls, as measured by enzyme-linked immunosorbent assay (Fig. 1B), is mainly a result of the increased content of the MMP-cleaved form of OPN.

OPN Single-Gene Polymorphisms in AH. To gain more insight in the potential functional role of OPN, we next investigated whether OPN gene variations predispose patients with alcohol abuse to develop AH. An SNP analysis of the OPN gene was performed in a cohort of alcoholic patients with different degrees of ALD, including patients with alcohol abuse without liver disease (n = 196), patients with compensated alcoholic liver cirrhosis (n = 102), and patients with AH (n = 100). The main characteristics of the patients with AH are shown in Supporting Table 1. Furthermore, a group of healthy controls (n = 152) were added in the study. Four SNPs were analyzed, including three within the promoter region (−1748 A/G, −443 T/C, and −66 T/G) and one within the 3′ untranslated region (UTR; +1239 A/C). All these SNPs were associated with pathological features in different diseases.

The results of the SNP analysis are shown in Table 2. Patients with compensated cirrhosis did not show changes in the genotype frequencies of the four SNP analyzed, compared with alcoholics without liver disease and healthy controls. Patients with AH did not show different genotype frequencies of the −443 T/C and −66 T/G SNPs, but had a lower frequency of the CC genotype of the 3′ UTR +1239 A/C SNP compared with alcoholics without liver disease and healthy controls (P < 0.05). However, this result was nonsignificant when multiple correction testing was applied for the 16 comparisons performed in Table 2 (α = 0.003). Importantly, although there were no differences in the frequencies of the −1748 A/G SNP among alcoholics with or without liver disease, we found that the presence of the AA genotype in patients with AH was associated with increased mortality at 1, 3, and 6 months. Specifically, patients...
Fig. 2. (A) Association between OPN hepatic gene expression and OPN levels in peripheral serum with disease severity (measured by the ABIC score) in patients with AH and control groups (*\(P < 0.05\)). (B) Association between OPN hepatic gene expression and OPN levels in peripheral serum with 90-day mortality in patients with AH and control groups (*\(P < 0.05\)). (C) Kaplan-Meier’s curve showing 90-day mortality according to OPN hepatic gene expression in patients with AH (*\(P < 0.05\)). (D) Kaplan-Meier’s curve showing 90-day mortality according to OPN levels in peripheral serum in patients with AH (*\(P < 0.05\)).
with AH and the AA genotype of this SNP had a 75% mortality at 6 months, whereas patients with AH and AG or GG genotype had a 50% mortality ($P < 0.05$). Importantly, the presence of AA genotype remained an independent predictor factor of 6-month mortality after adjustment by age, sex, and Model for End-Stage Liver Disease (MELD) score ($P = 0.046$; odds ratio: 2.65 [1.02-6.9]). This result may suggest that the presence of genetic variances in the OPN gene could modulate the prognosis of patients with ALD.

**Attenuated Susceptibility to Alcohol-Induced Liver Injury in Mice Lacking OPN.** We next explored whether OPN plays a pathogenic role in an experimental model of ALD. WT and OPN$^{-/-}$ maltose-fed groups did not show differences in both hepatic and serum parameters. In contrast, WT ethanol-fed mice
had higher hepatic triglyceride (TG) levels, compared to WT-fed mice \( (P<0.05) \), but TG levels were reduced in OPN\(^{-/-}\) ethanol-fed mice \( (P<0.05) \). Hepatic cholesterol levels did not change between groups (Fig. 4A). OPN hepatic gene expression was increased after ethanol feeding in WT mice \( (P<0.05; \text{Fig. } 4B) \). Furthermore, liver inflammation was quantified by analyzing hepatic gene expression of proinflammatory cytokines, such as TNF-\(\alpha\), monocyte chemotactic protein 1 (MCP-1), and IL-6. All three genes were highly increased in WT ethanol-fed mice, compared to the maltose-fed group \( (P<0.05) \), whereas their expression was almost suppressed in the OPN\(^{-/-}\) ethanol-fed group \( (P<0.05; \text{Fig. } 4B) \).

Regarding serum parameters, alanine aminotransferase (ALT) serum levels were highly increased in WT ethanol-fed mice, compared to the maltose-fed group \( (P<0.01) \), but were reduced in OPN\(^{-/-}\) ethanol-fed mice \( (P<0.01) \). However, no differences were observed in serum aspartate aminotransferase (AST), and serum TGs were increased in OPN\(^{-/-}\) ethanol-fed mice \( (P<0.05; \text{Fig. } 4C) \). In addition, no differences were noted in cytokine serum profile between WT and the OPN\(^{-/-}\) ethanol-fed groups (Fig. 4D).

Hematoxylin and eosin (H&E) staining of paraffin liver sections showed that ethanol administration increased degree of microsteatosis and hepatocellular injury in WT mice. However, OPN\(^{-/-}\) ethanol-fed mice had reduced steatosis and hepatic parenchyma was more conserved than in WT ethanol-fed mice (Fig. 5A). IHC studies performed for assessment of hepatic inflammation, injury, and immune cell infiltration revealed that ethanol administration increased the number of p65-positive cells in WT and OPN\(^{-/-}\) mice, compared to the maltose-fed groups, but was slightly lower in OPN\(^{-/-}\) mice (Fig. 5B). Furthermore, WT ethanol-fed mice also had an increased number of infiltrating neutrophils, as shown by myeloperoxidase (MPO) staining, that was partially attenuated in OPN\(^{-/-}\) ethanol-fed mice (Fig. 5C). However, macrophage infiltration, tested by F4/80 staining, remained unaltered after ethanol administration in both WT and in OPN\(^{-/-}\) mice (Fig. 5D).

Taken together, these results suggest that OPN mediates, at least in part, inflammatory response to ethanol, as shown by decreased hepatic expression of proinflammatory cytokines, decreased nuclear factor kappa B activation of inflammatory cells, and decreased neutrophil infiltration in OPN\(^{-/-}\) ethanol-fed mice. As a result, mice deficient in the OPN gene are partially, but not completely, protected against alcohol-induced liver injury.

**Mechanisms Underlying Increased OPN Synthesis and Pathogenic Effects.** We next analyzed the production of OPN in different in vitro approaches, including primary human HSCs, mouse precision-cut liver slices, and RAW264 and EL4 immune cell lines. Among different mediators of liver injury, we focused on LPS, a pathogenic factor in ALD\(^{24}\) known to regulate OPN production and secretion.\(^{25,26}\)

Western blotting studies showed that MMP-cleaved fragments of OPN were detected both intra- and extracellularly in activated primary HSCs (Fig. 6A, western blotting panel). OPN was highly produced under basal (untreated) conditions, and intracellular synthesis remained unaltered after incubation with LPS or other key inflammatory mediators of the pathogenesis of AH, including TNF-\(\alpha\), PDGF, IL-1\(\beta\), and TGF-\(\beta\). However, there was a marked increase in the secretion of MMP-

**Table 2. Genotyping Frequencies of OPN Gene Polymorphisms**

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Genotype</th>
<th>Cirrhosis Versus AWLD</th>
<th>P Value</th>
<th>Cirrhosis Versus Controls</th>
<th>P Value</th>
<th>AH Versus AWLD</th>
<th>P Value</th>
<th>AH Versus Controls</th>
<th>P Value</th>
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<td>AA</td>
<td>47 (46.1) 95 (48.7)</td>
<td>0.548</td>
<td>47 (46.1) 72 (47.7)</td>
<td>0.327</td>
<td>46 (50.0) 95 (48.7)</td>
<td>0.633</td>
<td>46 (50.0) 72 (47.7)</td>
<td>0.390</td>
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<tr>
<td>−1748 A/G</td>
<td>AG</td>
<td>48 (47.1) 81 (41.5)</td>
<td></td>
<td>48 (47.1) 61 (40.4)</td>
<td></td>
<td>40 (43.5) 81 (41.5)</td>
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<td>GG</td>
<td>7 (6.9) 19 (9.7)</td>
<td></td>
<td>7 (6.9) 18 (11.9)</td>
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<td>6 (6.5) 19 (9.7)</td>
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<td>6 (6.5) 18 (11.9)</td>
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<tr>
<td>rs11730582</td>
<td>TT</td>
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<td>0.307</td>
<td>26 (25.5) 32 (21.1)</td>
<td>0.709</td>
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Data are presented as absolute frequencies (%). Genotyped patients for each SNP do not equal the total number of subjects listed in each category, because some SNPs could not be genotyped for technical reasons in some patients.

Abbreviation: AWLD, alcoholic patients without liver disease.
cleaved fragments of OPN in supernatants of HSCs treated with TNF-α, LPS, IL-1β, and TGF-β (P < 0.05; Fig. 6A). These results suggest that activated HSCs could be a source of processed OPN after liver injury.

Furthermore, mouse precision-cut liver slices incubated with LPS had increased gene expression of OPN at 48 hours (P < 0.05; Fig. 6B), but not after incubation with TNF-α and TGF-β (data not shown).
Similarly, LPS increased OPN gene expression in RAW264 cells—a macrophage cell line—in a dose-dependent manner ($P<0.05$; Fig. 6C), but not in EL4 cells—a T-cell line—(data not shown). These results suggest that, in addition to activated HSCs, the hepatic parenchyma and the resident and infiltrating
Fig. 6. (A) OPN protein expression and secretion to cell supernatant in HSC incubated with mediators of liver injury up to 48 hours (*P < 0.05 versus basal). (B) OPN gene expression in mouse liver slices incubated with LPS up to 48 hours (*P < 0.05 versus LPS 0 μg/mL). (C) OPN gene expression in RAW264 cells incubated with LPS up to 24 hours (*P < 0.05 versus LPS 0 ng/mL). (D) MCP-1 and ICAM-1 gene expression in HSC incubated with recombinant OPN up to 24 hours (*P < 0.05 versus OPN 0 μg/mL; **P < 0.01 versus rhOPN 0 μg/mL). (E) Activation of intracellular signaling pathways in HSC incubated with recombinant OPN up to different short times (*P < 0.05 versus time 0 minutes).
macrophages could be also a source of OPN in a damaged liver.

We next explored the biological effects of recombinant OPN in primary HSCs and LX-2 cells. In HSCs, OPN induced gene expression of cell adhesion molecules, such as intercellular cell adhesion molecule 1, and proinflammatory cytokines, such as MCP-1, in a dose-dependent manner \( (P < 0.05; \text{Fig. 6D}) \). In addition, OPN induced protein kinase B (Akt) and extracellular signal-regulated kinase (ERK) \( 1 \), but not ERK-2, phosphorylation in LX-2 cells at short incubation times \( (P < 0.05; \text{Fig. 6E}) \), whereas neither c-Jun N-terminal kinase–signaling nor p38-signaling pathways were induced (data not shown). Taken together, these results suggest that OPN may participate in HSC activation, inducing proinflammatory effects.

Discussion

The current study provides evidence that OPN is overexpressed in AH and may play a pathogenic role in this severe clinical condition. This assumption is based on two main results: First, OPN hepatic gene expression paralleled with disease severity in patients with well-characterized AH and, second, mice lacking OPN are resistant to develop ethanol-induced liver damage. Our results are in accord with previous experimental studies showing that OPN plays a role in liver injury and fibrogenesis in different experimental models of acute and chronic liver damage.\(^{13-15}\) Moreover, our results confirm a recent study showing that OPN is overexpressed in nonalcoholic steatohepatitis (NAFLD) and could play a pathogenic role.\(^ {14}\) Because NAFLD shares many histological features with AH, including fat accumulation, polymorphonuclear infiltration, and hepatocellular damage, it is conceivable that OPN is a major pathogenic factor in steatohepatitis, regardless of the causative agent. However, it is important to stress that AH represents the most severe form of ALD. Whether OPN is also overexpressed in mild forms of ALD is unknown and deserves further investigation.

The most relevant result of this study is that OPN serum levels are particularly elevated in AH. It is well known that OPN can serve as a serum marker of advanced cirrhosis because its serum levels correlated with the degree of fibrosis in different human diseases, such as chronic hepatitis C and NAFLD.\(^ {14,15}\) Our study confirmed this hypothesis, because OPN serum levels were found to be increased in compensated cirrhosis, compared to controls. Interestingly, OPN levels were much more elevated in patients with AH, suggesting that the accelerated fibrosis and inflammation in these patients results in an increased release of OPN. This hypothesis is supported by the finding that serum and suprahepatic OPN levels correlate with hepatic gene expression. Further studies should be performed to explore whether serum OPN, together with other proteins, can be used as serum markers of AH. This is a clinically relevant issue because many centers do not perform transjugular biopsies and the development of serum markers of AH would be of great clinical interest.

Because increased OPN hepatic expression could be a consequence, rather than a mediator, in AH, two different functional studies were employed. First, we challenged mice lacking OPN to ethanol-induced liver injury. OPN-deficient mice developed less-severe liver injury and proinflammatory gene expression than WT mice. These results indicate that OPN is not merely a structural ECM protein produced by myofibroblasts that passively distorts the liver architecture, but that it actually participates in the inflammatory response to injury. Our \textit{in vitro} studies, showing that OPN induces proinflammatory effects in primary HSCs, support this assumption. However, we found that the reduced hepatic expression of proinflammatory cytokines and the hepatic content of TGs found in OPN-deficient mice after ethanol feeding did not correlate with their
increased serum levels. There are some possible explanations to address this question. First, alcohol not only induces liver inflammation, but also a systemic inflammatory response and inflammatory changes in other organs, such as the heart, kidney, and nervous system. In addition, it also increases fatty acid mobilization from extrahepatic sources, including visceral adipose tissue. Therefore, circulating levels of cytokines do not necessarily correlate with intrahepatic expression. We previously demonstrated that intrahepatic expression of TNF, a key circulating cytokine in alcohol-induced organ damage, was not increased in livers from patients with AH. Moreover, inflammatory cytokines frequently act in an auto- and paracrine manner and locally regulate parenchymal function and wound-healing response to injury. In this line, previous studies from our lab late parenchymal function and wound-healing response with AH. Our second functional approach expression of cytokines predicts disease outcome in to injury. In this line, previous studies from our lab demonstrated that intrahepatic, rather than systemic, expression of cytokines predicts disease outcome in patients with AH. Our second functional approach was to explore whether gene variations of OPN modulate response to alcohol abuse and predisposes patients to develop severe forms of AH. We found that patients with AH had a lower frequency of the CC genotype of the 3′ UTR +1239 A/C SNP, compared to alcoholics without liver disease. However, this result did not reach statistical significance after correction for multiple testing because of the relatively small sample size. Larger studies in the setting of international networks are needed to elucidate whether OPN gene variations influence individual susceptibility to develop an episode of AH among heavy drinkers. Interestingly, the presence of the AA genotype of the −1748 A/G SNP in patients with AH was associated with increased mortality at 1, 3, and 6 months. The presence of AA genotype remained a predictive factor of 6-month mortality after adjustment for age, sex, and MELD score. Although this result suggests that the genetic variations of OPN could influence the clinical severity of AH, it should be considered exploratory, and larger epidemiological studies have to be performed to confirm this finding. Also, mutagenic analysis should determine the influence of these SNPs in expression of OPN by liver cells, its affinity to its receptors, and its susceptibility to undergo posttranscriptional regulation.

Finally, we explored, in cultured HSCs and immune cells and in precision-cut liver slices, the potential mechanisms implicated in the increased OPN expression in livers with AH. Two results were particularly relevant. First, TNF-α, a known mediator of ALD, did not induce any expression of OPN. In contrast, LPS dose dependently stimulated OPN expression. These results suggest that OPN could mediate, at least in part, the inflammatory and fibrogenic effects of increased LPS in the liver. Future experimental studies should test this hypothesis. In addition, we found that HSCs and infiltrating inflammatory cells could be an important source of OPN in a damaged liver. This assumption is reinforced by a previous study in our lab that compared gene expression profiles of freshly isolated HSCs from cirrhotic and normal livers by microarray analysis. This study showed that OPN was 16-fold up-regulated in HSCs activated in cirrhotic, compared to normal, livers.

In summary, our study demonstrated that OPN is markedly overexpressed in patients with AH, especially in those with more-severe disease, and that OPN may play a role in experimental ethanol-induced liver injury in mice. Collectively, these results suggest that OPN may be an appealing target for therapy in these patients. However, there are several issues that should be addressed in future studies. First, the precise cellular source of OPN in AH is unknown. Second, it is unclear whether OPN also pays a role in hepatic fibrogenesis in patients with AH. The results of two recent studies suggest that hepatic expression of OPN correlates with degree of hepatic fibrosis in patients with alcoholic and nonalcoholic steatohepatitis. Also, OPN is expressed in areas of myofibroblast accumulation and exerts fibrofilmic properties in cultured HSCs. Although we did not find a direct correlation between degree of fibrosis and expression of OPN, these recent studies strongly suggest that OPN could play a fibrogenic role in patients with steatohepatitis, regardless of its cause. And, most important, future preclinical studies should explore whether targeting OPN is safe and effective as a therapeutic option in patients with AH.

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References