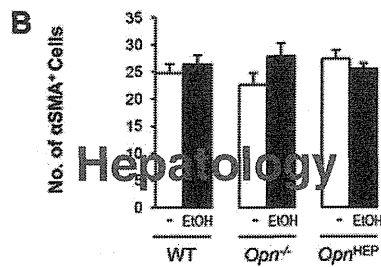
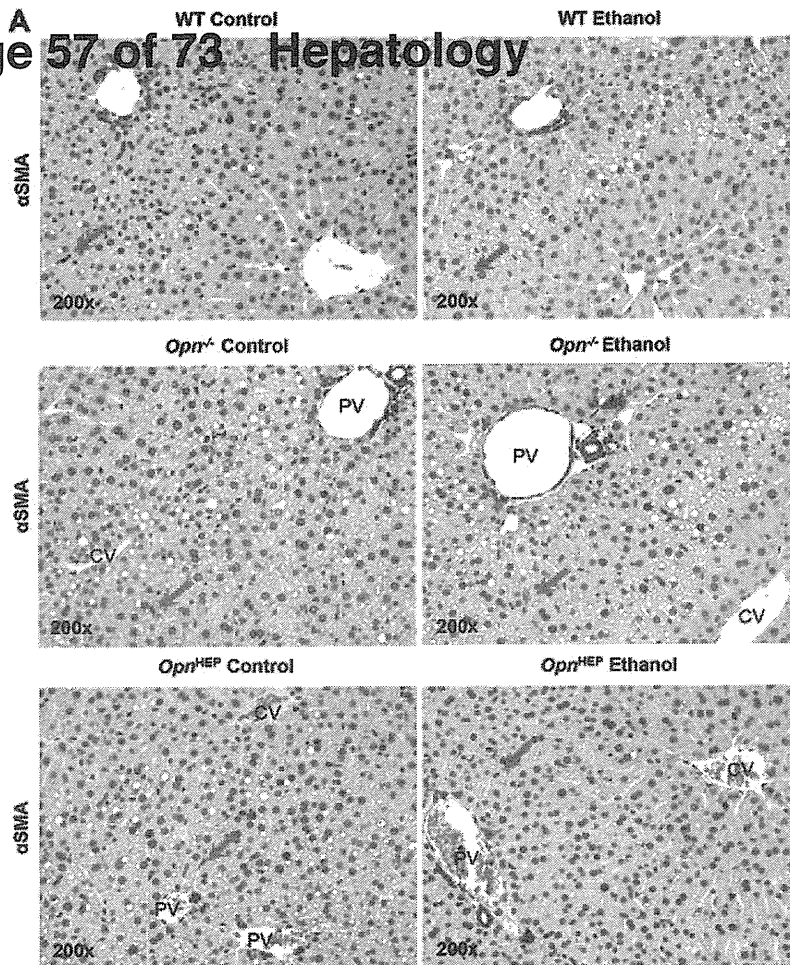
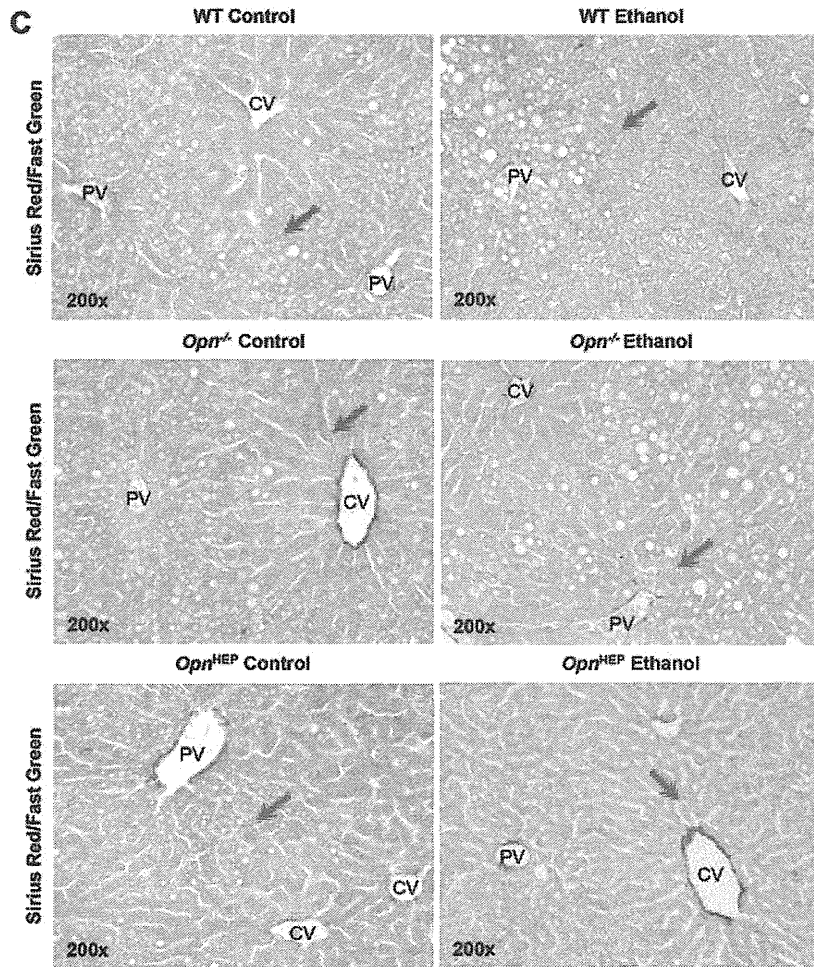


Supplementary Figure 2 Continuation

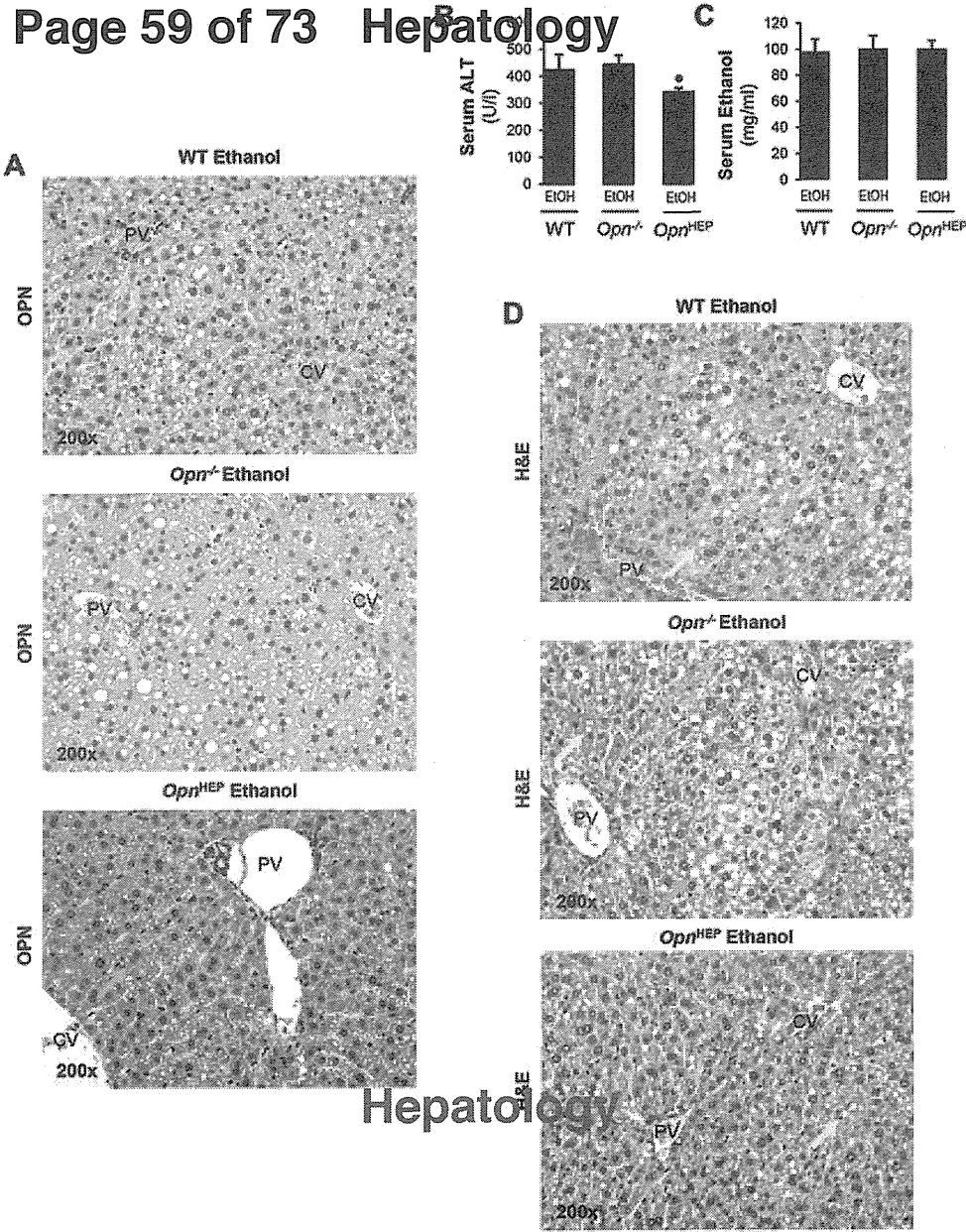


Supplementary Figure 3

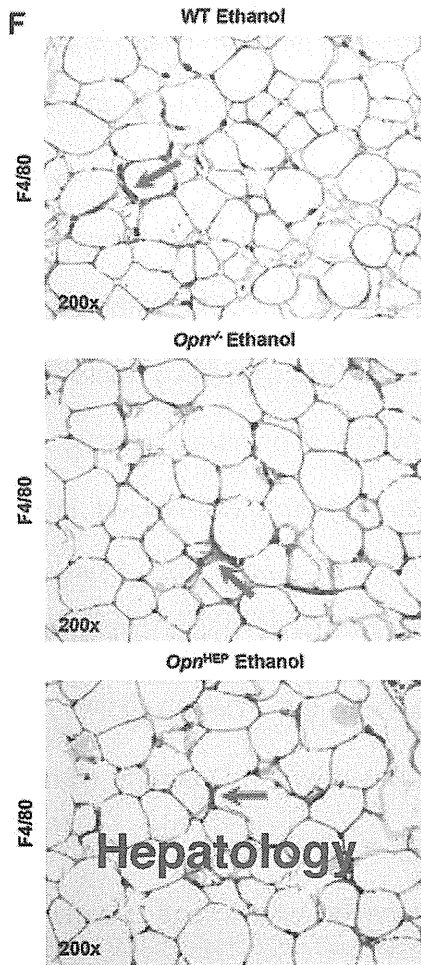
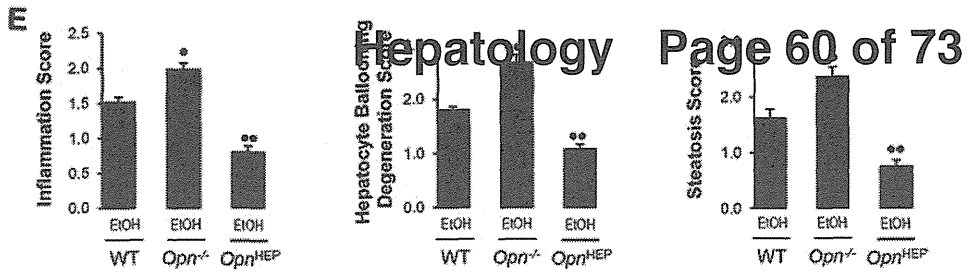


Hepatology

Supplementary Figure 3 Continuation



Supplementary Figure 4



Supplementary Figure 4 Continuation

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

General methodology. Plasma, biliary and fecal OPN was measured by ELISA (IBL, Minneapolis, MN). ALT activity, alcohol, TG and glucose were analyzed using kits (Pointe Scientific, Canton, MI). LDL and VLDL were determined with a kit (BioVision, Mountain View, CA). Insulin was measured by ELISA (Merckodia Inc, Winston Salem, NC). Total RNA extraction and qRT-PCR analysis was performed as previously (1, 2) using the following primers: *Opn* forward 5'-gagaccgtcactgctagtagacaca-3', *Opn* reverse 5'-gactccttagactcaccgctctt-3', *Tnfa* forward 5'-ccagtgtgggaagctgtcct-3', *Tnfa* reverse 5'-aagcaaaagaggaggcaaca-3', *Calnexin* forward 5'-gcctgaagattgggatgaaa-3', *Calnexin* reverse 5'-caatcctctggcttctctgc-3', *16S* rRNA forward 5'-agagttgatcctggctcag-3' and *16S* rRNA reverse 5'-tgctgcctcccgtaggagt-3'. The primers to specifically detect the most abundant Gram-negative bacteria *16S* rDNA in feces were forward 5'-ctcctacgggaggcagcag-3' and reverse 5'-gwattaccgcgkcgctg-3' and were selected based on published literature (3). The following Abs were used in Western blot analysis: CYP2E1 provided by Dr. Lasker (Puracyp Inc., Carlsbad, CA), TNF α (Fitzgerald, Acton, MA), Catalase (EMD Millipore, Billerica, MA), ADH, pIRS1, IRS1, pAkt1/2/3, Akt1/2/3, Calnexin and Actin (Santa Cruz Biotechnology, Santa Cruz, CA). The 2A1 OPN Ab used in this and in our previous studies (4) was generated and donated by Dr. Denhardt (Rutgers University, NJ) (5). Ponceau red staining was performed using a kit (Sigma). The ECL reaction was developed using a Fujitsu Las4000 scanner and quantified with Image J software. ADH activity was measured according to the method of Haseba *et al* (6). Extracellular nitrites and nitrates were determined by the Griess method using a kit (Sigma). Blood and liver LPS were measured using a kit (Lonza, Walkersville, MD). Primary human Kupffer cells were isolated as previously (2). Details on general methodology such as CYP2E1 and catalase activities, Sirius red/fast green staining

as well as intra- and extracellular hydroperoxides can be found in our previous publications (4, 7-10).

Lieber-DeCarli model of early alcohol-induced liver injury. To provoke early alcohol-induced liver injury, we employed the control and the ethanol Lieber-DeCarli diets (Bio-Serv Inc., Frenchtown, NJ), which are equicaloric and have the same composition with respect to fat (42% of calories) and protein (16% of calories). Due to the presence of a high percentage of fat in these diets, steatosis, which is amplified by ethanol feeding, is expected to occur both in the control (less apparent) and in the ethanol group after a long feeding period. The content of carbohydrates is 42% of total calories (dextrin-maltose) in the control diet and 12% of total calories in the ethanol diet, where up to 30% of carbohydrate calories are replaced by ethanol (11). 10 wks old male WT, *Opn*^{-/-} and *Opn*^{HEP} Tg were used in the Lieber-DeCarli model. Male mice were chosen to avoid the effect of estrogens regulating OPN expression (12). Mice were acclimatized to the liquid diet regime by feeding them the control diet for 3 d. The percentage of ethanol-derived calories was progressively increased from 10% (1 wk) to 20% (1 wk), 25% (2 wks) and 30% (3 wks). Mice were pair-fed and body weight was monitored throughout the experiment. Liver and body weight were recorded at the point of sacrifice to calculate the liver-to-body weight ratio. Blood was collected by submandibular bleeding under anesthesia and livers were removed for further analysis. All animals received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health.

Assessment of the therapeutic potential of m-OPN. To evaluate the therapeutic potential of m-OPN, 10 wks old male mice were fed the ethanol Lieber-DeCarli diets for 3 wks. This time was chosen to compare with the results from our previous publication where m-OPN was administered throughout the entire feeding period (13). The percentage of ethanol-derived

calories was progressively increased from 10% (1 wk) to 20% (1 wk) and to 30% (1 wk). Next, half of the mice received m-OPN at a concentration similar to the physiological concentration in human milk (200 µg/ml) (14, 15) dissolved in water and incorporated into the ethanol diet for 2 wks while ethanol feeding continued. Equal concentration of LPS-free BSA was used to correct for exogenous protein intake in the control group. Samples were collected as above.

The chronic-plus-single-binge ethanol feeding model of alcohol-induced liver injury. The model was performed according to (16). 10 wks old male mice were acclimatized to the control Lieber-DeCarli diet for 5 days following which they were fed ethanol for 10 days. The percentage of ethanol-derived calories was 30%. An acute administration of ethanol (5 g/kg b. wt.) was given by oral gavage the last day and mice were sacrificed 8 hours later. Samples were collected as above.

Pathology. The entire left liver lobe and the omental adipose tissue were resected and fixed in 10% neutral-buffered formalin and processed into paraffin sections for H&E staining or IHC. Blind analysis by a liver pathologist according to the Brunt classification (17) was used to determine the activity scores. Ten 100x fields per liver section from the same mouse were examined for necroinflammatory activity, which was scored as follows: 0 for none, 1 for <2 foci per 100x field, 2 for 2-4 foci per 100x field, 3 for 5-10 foci per 100x field and 4 for >10 foci per 100x field. The density of the necroinflammatory activity was also calculated per mm² per 100x fields. Ballooning degeneration was identified when hepatocytes were enlarged in most cases to more than twice the size of its neighboring cells. In addition, the cytoplasmic membrane became rounded instead of the usual hexagonal shape of normal hepatocytes. Most of the cytoplasm appeared empty except for a few irregular wisps of pink material representing damaged cytoplasmic content. Livers were also embedded in OCT compound, sectioned at 5 µM and stained with oil red O using standard procedures followed by morphometric assessment. Image-

Pro plus 7.0 Software (Media Cybernetics, Bethesda, MD) was used to measure the adipocyte diameter.

Immunohistochemistry. The 2A1 OPN Ab was donated by Dr. Denhardt (Rutgers University, NJ) (5). The Ab was also tested in livers from *Opn*^{-/-} mice in C57BL/6J or in 129sv genetic background to ensure specificity (4). The Abs for 4-hydroxynonenal, F4/80 and TNF α were from Abcam (Cambridge, MA), EMD Millipore and Fitzgerald, respectively. The Ab for α SMA was from Sigma. Immunochemical reactions were developed using the Histostain Plus detection system (Invitrogen, Carlsbad, CA). For the computer-assisted morphometric assessment, the integrated optical density (IOD) was calculated in 10 random fields per section containing similar size portal tract or central vein at 100x and using Image-Pro plus 7.0 Software (Media Cybernetics, Bethesda, MD). The results were averaged and expressed as fold-change over the controls. The TNF α , F4/80 and α SMA indexes were calculated by counting the number of positively stained cells per field in 20 fields per slide at 200x magnification. In the adipose tissue, crown-like structures were determined by the presence of aggregates of single or fused macrophages (F4/80⁺ cells).

Source of OPN. rOPN was obtained from R&D Systems (Minneapolis, MN). M-OPN was purified from human milk or from bovine milk by anion-exchange chromatography as described elsewhere (18) (US Patent 7,259,243). The m-OPN used in our experiments is more than 97% pure OPN as analyzed by Edman sequencing, RP-HPLC and MS analysis. Bovine m-OPN has been thoroughly characterized with respect to post-translational modifications and the fragmentation pattern (19). The preparation used in the current study is glycosylated and phosphorylated. It contains 28 phosphorylation sites, which are phosphorylated by 90-100%. Likewise, the five O-glycosylation sites are fully occupied (19). The form of bovine m-OPN used is significantly processed by the endogenous milk protease plasmin. A significant part of the

OPN is present as the N-terminal fragment representing residues 1-145/147, which is in accordance with the way OPN is found in milk.

Flow cytometry. RAW 264.7 cells (mouse leukemic monocyte macrophages, 3×10^5 cells) were incubated with 200 ng/ml FITC-LPS and 0-0.2 μ M rOPN in 1 ml of DMEM-F12 for 30 min at room temperature. In some experiments TLR4 and MD2 neutralizing Abs (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Cells were washed twice with DMEM-F12 and analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and the side scatter parameter to gate the signal.

Statistical analysis. Data were analyzed by a two-factor analysis of variance and results were expressed as mean \pm standard error of the mean ($n=10$ /group for the *in vivo* experiments and at least $n=6$ for the *in vitro* experiments).

SUPPLEMENTARY RESULTS

The alcohol metabolizing enzymes are similar in the livers from ethanol-treated mice.

Since alcohol levels were comparable in all ethanol-treated mice, we evaluated whether similar ethanol metabolism occurred as well. This was confirmed evaluating both protein expression and activity of CYP2E1 (**Supplementary Fig. 1A-1B**), ADH (**Supplementary Fig. 1A and 1C**) and catalase (**Supplementary Fig. 1A and 1D**), the liver alcohol metabolizing enzymes. All ethanol-treated mice displayed comparable CYP2E1, ADH and catalase expression and enzymatic activity henceforth attesting for similar ethanol metabolism.

Alcohol-induced lipid peroxidation remains basal in Opn^{HEP} Tg mice but increases in WT

and $Opn^{-/-}$ mice. Because of the significant role of lipid peroxidation-end products in the development of ALD (20), we performed IHC to evaluate for 4-hydroxynonenal, an α,β -unsaturated hydroxylalkenal produced by lipid peroxidation in many cells. IHC and morphometric analysis showed greater 4-hydroxynonenal staining in ethanol-treated WT and $Opn^{-/-}$ compared to Opn^{HEP} Tg mice (not shown); hence, natural induction plus overexpression of OPN in hepatocytes decreases lipid peroxidation preventing alcohol hepatotoxicity.

Alcohol-induced insulin resistance is similar in all ethanol-fed mice.

Because of the close association between alcohol consumption and insulin resistance in the development of liver injury, we performed H&E staining in the adipose tissue from the alcohol-fed mice (**Supplementary Fig. 2A**). The number of crown-like structures (**Supplementary Fig. 2B**) and the average adipocyte diameter (**Supplementary Fig. 2C**) were similar in all mice fed alcohol and serum glucose and insulin remained unchanged (**Supplementary Fig. 2D-2E**). Moreover, hepatic insulin signaling determined by measuring IRS1 and Akt phosphorylation was similar in

all ethanol-fed mice (**Supplementary Fig. 2F**). Thus, the differences in liver injury in ethanol-treated WT, *Opn*^{-/-} and *Opn*^{HEP} Tg mice are not due to changes in insulin resistance.

αSMA and Sirius red/Fast green staining are similar in the ethanol-treated mice. To determine whether differences in hepatic stellate cell activation occurred under ethanol feeding, we performed IHC and morphometry analysis for αSMA, a marker of hepatic stellate cell activation (**Supplementary Fig. 3A-3B**), and Sirius red/Fast green staining for collagenous proteins (**Supplementary Fig. 3C**). Both markers remained quite similar in all ethanol-fed mice.

A mouse model of chronic plus binge ethanol feeding (the NIAAA model) also shows that natural induction plus forced overexpression of OPN in hepatocytes protects from alcohol-induced liver injury and steatosis whereas natural induction of OPN does not suffice to confer full protection. In addition to the Lieber-DeCarli model of chronic alcohol feeding, we also used a second model of alcoholic liver injury due to chronic ethanol feeding plus a single ethanol binge. This protocol has been recently described to synergistically induce liver injury, inflammation and fatty liver in addition to mimic acute-on-chronic alcoholic liver injury in patients (16). Analysis by IHC showed enhanced OPN⁺ staining in ethanol-fed *Opn*^{HEP} Tg compared to WT mice (**Supplementary Fig. 4A**). Serum ALT activity was lower in ethanol-treated *Opn*^{HEP} Tg mice but it was elevated in WT and in *Opn*^{-/-} mice (**Supplementary Fig. 4B**). All groups of ethanol-treated mice showed comparable serum ethanol concentration (**Supplementary Fig. 4C**). H&E staining (**Supplementary Fig. 4D**) and the activity scores (**Supplementary Fig. 4E**) revealed significant protection from alcohol-induced liver injury in *Opn*^{HEP} Tg compared to WT followed by *Opn*^{-/-} mice. Last, H&E (not shown) and F4/80 IHC from adipose tissue also demonstrated similar number of crown-like structures and adipocyte diameter in all the ethanol-fed mice (**Supplementary Fig. 4F**).

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SUPPLEMENTARY LEGEND TO FIGURES

Supplementary Figure 1. Analysis of CYP2E1, ADH and catalase in livers from control and ethanol-treated WT, $Opn^{-/-}$ and Opn^{HEP} Tg mice. Western blot analysis (A) along with enzymatic activities of hepatic CYP2E1 (B), ADH (C) and catalase (D). The quantification of the intensity of the signal for each protein corrected by calnexin is indicated under the blots. $n=10$ /group; $**p<0.01$ for ethanol vs control.

Supplementary Figure 2. Changes in insulin resistance does not account for the differences in ethanol-induced liver injury in WT, $Opn^{-/-}$ and Opn^{HEP} Tg mice. WT, $Opn^{-/-}$ and Opn^{HEP} Tg mice were fed 7 wks either the control or the alcohol Lieber-DeCarli diet. H&E staining from adipose tissue (A). The number of crown-like structures in the adipose tissue is similar in all the ethanol-treated mice (B). Computer-assisted morphometric analysis showing similar adipocyte diameter in all the ethanol-treated mice (C). Serum glucose (D) and insulin (E) levels. Western blot analysis of pIRS1 and pAkt1/2/3. The quantification of the intensity of the signal for each protein corrected by the non-phosphorylated isoform and by calnexin is indicated under the blots (F). $n=10$ /group; $*p<0.05$ for ethanol vs control and $*p<0.05$ for Opn^{HEP} Tg vs WT.

Supplementary Figure 3. α SMA expression and Sirius red/Fast green staining remains similar in all ethanol-fed mice. WT, $Opn^{-/-}$ and Opn^{HEP} Tg mice were fed 7 wks either the control or the alcohol Lieber-DeCarli diet. IHC depicts a similar number of α SMA⁺ cells (red arrows) in all ethanol-treated mice (A). Computer-assisted morphometric analysis of the α SMA IHC calculated as the number of α SMA⁺ cells in 20 fields at 200x magnification (B). Sirius red/Fast green staining for collagenous proteins (red arrows) shows no major difference among groups (C). $n=10$ /group.

Supplementary Figure 4. A mouse chronic-plus-single-binge ethanol feeding model also shows that natural induction plus forced overexpression of OPN in hepatocytes protects from alcohol-induced liver injury and steatosis whereas natural induction of OPN does not suffice to confer full protection. 10 wks old male mice were fed ethanol according to the chronic-plus-single-binge ethanol feeding model (16). IHC shows increased OPN staining (green arrows) by ethanol feeding in WT and more in Opn^{HEP} Tg mice (A). Serum ALT activity (B) and ethanol (C). H&E staining shows less inflammation, hepatocyte ballooning degeneration, micro- (yellow arrows) and macrovesicular (green arrows) steatosis in ethanol-treated Opn^{HEP} Tg followed by WT and by $Opn^{-/-}$ mice. PV: portal vein. CV: central vein (D). The scores for inflammation, hepatocyte ballooning degeneration and steatosis are lower in ethanol-treated Opn^{HEP} Tg followed by WT and $Opn^{-/-}$ mice (E). F4/80 IHC in adipose tissue showing similar crown-like structures (red arrows) and average adipocyte diameter in all ethanol-fed mice (F). $n=10$ /group; * $p<0.05$ and ** $p<0.01$ for any genotype vs WT mice.

Supplementary Table 1. *F4/80⁺ TNF α ⁺ cells in mouse liver*

	Control	EtOH
WT	0.126 \pm 0.025	0.252 \pm 0.024*
<i>Opn</i> ^{-/-}	0.220 \pm 0.032 [•]	0.358 \pm 0.052* [•]
<i>Opn</i> ^{HEP} Tg	0.123 \pm 0.015	0.153 \pm 0.028 [•]

**p* < 0.05 for ethanol vs control

[•]*p* < 0.05 for *Opn*^{-/-} and *Opn*^{HEP} Tg vs WT mice

Special Report

Etiology and prognosis of fulminant hepatitis and late-onset hepatic failure in Japan: Summary of the annual nationwide survey between 2004 and 2009

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Aim: To summarize the annual nationwide survey on fulminant hepatitis (FH) and late-onset hepatic failure (LOHF) between 2004 and 2009 in Japan.

Methods: The annual survey was performed in a two-step questionnaire process to detail the clinical profile and prognosis of patients in special hospitals.

Results: Four hundred and sixty ($n = 227$ acute type; $n = 233$ subacute type) patients had FH and 28 patients had LOHF. The mean age of patients with FH and LOHF were 51.1 ± 17.0 and 58.0 ± 14.4 years, respectively. The causes of FH were hepatitis A virus in 3.0%, hepatitis B virus (HBV) in 40.2%, other viruses in 2.0%, autoimmune hepatitis in 8.3%, drug allergy-induced in 14.6% and indeterminate etiology in 29.6% of patients. HBV reactivation due to immunosuppressive therapy was observed in 6.8% of FH patients. The short-term survival rates of patients without liver transplantation (LT)

were 48.7% and 24.2% for the acute and subacute type, respectively, and 13.0% for LOHF. The prognosis was poor in patients with HBV reactivation. The implementation rate for LT in FH patients was equivalent to that in the previous survey. The short-term survival rates of total patients, including LT patients, were 54.2% and 40.8% for the acute and subacute type, respectively, and 28.6% for LOHF.

Conclusion: The demographic features and etiology of FH patients has gradually changed. HBV reactivation due to immunosuppressive therapy is problematic. Despite advances in therapeutic approaches, the prognosis of patients without LT has not improved.

Key words: acute liver failure, fulminant hepatitis, Japan, liver transplantation, viral hepatitis

INTRODUCTION

IN JAPAN, FULMINANT hepatitis (FH) is defined as having hepatitis, when grade II or worse hepatic

encephalopathy develops within 8 weeks of the onset of disease symptoms, with a prothrombin time of 40% or less.^{1,2} FH is further classified into two subtypes, acute and subacute types, in which encephalopathy occurs within 10 days and later than 11 days, respectively, of the onset of the disease symptoms. Patients showing a prothrombin time of 40% or less, with hepatic encephalopathy developing between 8 and 24 weeks of disease onset are classified as having late-onset hepatic failure (LOHF).³ Etiologies with hepatitis present in the histology, such as viral infection, autoimmune hepatitis and drug allergy-induced liver injury are defined as causes of

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FH and LOHF. In contrast, acute liver failure due to other causes with the absence of hepatitis in the histology, such as drug toxicity, circulatory disturbance and metabolic disease, are excluded as causes of FH and LOHF. Recently, a novel diagnostic criteria for acute liver failure in Japan was established by the Intractable Hepato-Biliary Disease Study Group.^{4,5} These criteria included other causes with liver damage without the absence of hepatitis in the histology in addition to the present criteria.

Among viral infection, hepatitis B virus (HBV) is a major cause of FH in Japan.^{6,7} HBV infection is classified into transient HBV infection type and acute exacerbation in an HBV inactive carrier. With advances in cytotoxic chemotherapy and immunosuppressive therapy, reactivation of hepatitis B is becoming a clinical problem.⁸ Moreover, recent introduction of rituximab plus steroid combination therapy for non-Hodgkin's lymphoma has been associated with HBV reactivation in transiently infected patients, namely, *de novo* hepatitis. However, the prevalence of HBV reactivation in patients with FH and LOHF is unknown.

Advances in therapeutic strategies for FH and LOHF have improved the prognosis. Since 1988, living-donor liver transplantation (LT) has been adopted in patients who are beyond the supportive care of a critical unit.⁶ Recently, artificial liver support with high-flow or on-line hemodiafiltration (HDF) has been used. Since 2006, a nucleoside analog, entecavir, has been used as a substitute for lamivudine, as an antiviral agent for HBV. However, it is unknown whether these new treatments improve the prognosis of FH.

The Intractable Hepato-Biliary Diseases Study Group has annually performed a nationwide survey of patients with FH and LOHF since 1983.⁶ Since 2000, approximately 600 hospitals have been enrolled in the survey. This report summarizes the results of the survey between 2004 and 2009 to address the trends in the etiology and prognosis of patients with FH and LOHF and compares them with the previous survey.⁷

METHODS

THE NATIONWIDE SURVEY was performed annually. The number of hospitals for survey has changed in each year. Maximum (608) was in 2007 and minimum (544) was in 2006, with active members of the Japan Society of Hepatology and the Japanese Society of Gastroenterology between 2005 and 2010. The survey was performed in a two-step questionnaire process to detail the clinical profile and prognosis of patients who were

diagnosed as FH or LOHF in the previous year. The recovery rate of the first and second questionnaire was 39–59% and 60–100%, respectively. Patients who met the diagnostic criteria for FH or LOHF were entered into the survey. Patients under 1 year of age, those with alcoholic hepatitis, those with chronic liver diseases and those with acute liver failure with no histological features of hepatitis were excluded from the analysis.

According to criteria described in previous reports,^{7,9} the etiology of FH and LOHF was classified into five categories: (i) viral infection; (ii) autoimmune hepatitis; (iii) drug allergy-induced liver injury; (iv) indeterminate etiology despite sufficient examinations; and (v) unclassified due to insufficient examinations. Patients with viral infection consisted of those with hepatitis A virus (HAV), HBV, hepatitis C virus (HCV), hepatitis E virus (HEV) and other viruses. The patients with HBV infection were classified into three subgroups according to serum markers of HBV, hepatitis B core antibody (HBcAb) and immunoglobulin (Ig)M-HBcAb: (i) transient HBV infection; (ii) acute exacerbation in HBV carriers; and (iii) indeterminate infection patterns. In the present study, we classified acute exacerbation in HBV carriers into three subgroups according to the new criteria:^{4,5} (i) inactive carriers, without drug exposure; (ii) reactivation in inactive carriers by immunosuppressant and/or anticancer drugs; and (iii) reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (i.e. *de novo* hepatitis). Because not every patient was examined for serological markers of transient HBV infection before the onset of FH and LOHF (with HBcAb and/or hepatitis B surface antigen [HBsAg] in serum), we defined HBV reactivation as that occurring in transiently infected patients when they developed HBV-related hepatitis due to immunosuppressive therapy or cytotoxic chemotherapy with reappearance of HBsAg in the serum and did not conform to the criteria of transient HBV infection.

The statistical significance of differences between groups was assessed using Student's *t*-test, Fisher's exact test or Kruskal-Wallis one-way ANOVA. Data are shown as mean \pm standard deviation. The study was conducted with the approval of the Ethical Committee of Kagoshima University Graduate School of Medical and Dental Sciences.

RESULTS

Demographic features and survival rates

FROM 2004–2009, 582 PATIENTS were enrolled in the survey. Ninety-four patients were excluded from