Liver asialoglycoprotein receptor levels correlate with severity of alcoholic liver damage in rats

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Our laboratory has previously shown that ethanol administration impairs multiple aspects of the process of receptor-mediated endocytosis (RME) in isolated hepatocytes, including decreased binding, internalization, degradation, and receptor recycling (2–7). A majority of these findings were demonstrated by using the hepatic asialoglycoprotein receptor (ASGP-R), one of the well-characterized receptors that undergo efficient endocytosis and recycling, as the model system. The ASGP-R recognizes glycoproteins with terminal galactose or N-acetylglalactosamine residues and removes these potentially harmful desialylated glycoproteins from the circulation (1). In addition to the removal of desialylated proteins, the ASGP-R has also been shown to be involved in the uptake of apoptotic cells (8), which was found to be significantly altered by ethanol administration (15), providing a potential physiological link between the ASGP-R and the progression of pathological changes associated with alcoholic liver injury.

The majority of studies that were performed concerning the function of the ASGP-R in relation to the development of alcohol-related liver pathology involved a technique in which ethanol was given orally as part of a total liquid diet (Lieber-DeCarli diet) (14). In this model of alcoholic liver disease (ALD), rats voluntarily drank either a liquid diet containing ethanol as 36% of calories or an isocaloric control diet. This mode of ethanol administration reproduced the early stages of ALD that are observed in humans in that significant liver lesions, such as steatosis (fatty liver) and apoptosis, were observed within 4 wk of administration (11). Corresponding functional changes in the ASGP-R over a time course of ethanol feeding were reported from our laboratory, demonstrating that RME by the ASGP-R is markedly impaired after as early as 1 wk, with decreases in ligand binding to the receptor observed without a corresponding change in receptor protein content. On prolonged ethanol feeding (5–7 wk), the decreased ligand binding to ASGP-R was caused by an actual decrease in receptor protein content that was accompanied by a loss of ASGP-R-specific mRNA (21). Because these studies were performed in rats that received the ethanol diet ad libitum and the animals developed only alcohol-induced steatosis, the present studies were performed to evaluate the impairments in ASGP-R content and expression levels in more severe forms of alcohol-related liver injury.

To assess the impairment on the ASGP-R in animals with a greater severity of alcohol-induced liver injury, we used the model of continuous intragastric infusion of alcohol. This model exhibits many of the pathological features of ALD that are observed in humans. In particular, it has been shown that, when unsaturated fats [i.e., fish oil (FO)] are included in the diet, the toxicity of ethanol is noted by the presence of fatty liver, inflammation, necrosis, and fibrosis (19, 20). In contrast, no pathological changes are observed in animals infused with an ethanol-containing diet given with fat in the form of medium-chain triglycerides (MCT). In the present study, liver tissue from animals that were intragastrically fed ethanol in the presence of FO or MCT were assessed for the level of the ASGP-R, as well as other proteins expressed by the liver. The purpose of these studies was to determine whether decreased ASGP-R content correlated with a degree of damage to the liver based on changes in liver pathology after different feeding regimens.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing between 275 and 300 g were fed a liquid diet by means of continuous intragastric infusion through permanently implanted gastric tubes for 1 mo, as previously described (16). The rats were given their total nutrient intake by intragastric

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infusion in which the percentage of calories derived from fat was 35% of total calories. Vitamins and minerals were given as described previously (9). The amount of ethanol administered to the animals started at 10 g kg⁻¹ day⁻¹ and was gradually increased over an 8- to 10-day period to 16 g kg⁻¹ day⁻¹ (full ethanol dose) as the animals developed tolerance. In addition, the ethanol concentration was modulated throughout to maintain high levels of blood ethanol (150–300 mg/dl) during the day. Control animals pair fed with ethanol-treated rats were given an isocaloric diet containing dextrose. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals. After 4 wk of full-dose ethanol administration, the animals were killed: a sample of liver was obtained for histopathological analysis; and the remainder of the liver was rapidly excised, washed with ice-cold potassium chloride, cut into small pieces, transferred to plastic vials to be frozen in liquid nitrogen, and stored at −80°C. Pieces of liver obtained in this manner were shipped to Dr. C. A. Casey’s laboratory to be used for Western and Northern blot analysis. The samples obtained for analysis were procured from animals fed MCT plus dextrose (MCTD) or MCT plus ethanol (MCTE) and FO plus dextrose or FO plus ethanol (FE). Liver tissue from some of these animals was also used in other studies.

**Histopathological analysis.** Hematoxylin-eosin staining, followed by light microscopy analysis, was performed on the liver samples that were removed at the time of euthanasia. The hepatic histopathology was assessed as follows: steatosis (the percentage of liver cells containing fat) was scored 1+ when <25% of the cells contained fat, 2+ when 26–50% of the cells contained fat, 3+ when 51–75% of the cells contained fat, and 4+ when >75% of the cells contained fat. Necrosis was quantified as the number of necrotic foci per square millimeter, and inflammation was scored as the number of inflammatory cells per square millimeter. At least three different sections were examined per liver sample, with the pathologist blinded to the identity of treatment group when assessing the histology.

**Measurements of alcohol and alanine aminotransferase in blood.** Blood was collected from the tail vein, and the ethanol concentration was measured by using an alcohol dehydrogenase kit from Sigma Chemical Co. (St. Louis, MO). The level of serum alanine aminotransferase (ALT; 2.6.1.2) activity was determined by using a commercial transaminase diagnostics kit (Sigma) with enzyme activity detected by an automated analyzer (Hitachi 747, Boehringer Mannheim, Indianapolis, IN).

**RNA isolation and Northern blot analysis.** Total RNA was isolated from frozen liver pieces (100–200 mg), according to the manufacturer’s instructions, by using TRReagent (Molecular Research Center, Cincinnati, OH). Liver RNA (20–30 μg) and 3 μg of a 0.24- to 9.5-kb RNA ladder (Life Technologies) were denatured in 50% formamide-16% formaldehyde in MOPS buffer at 65°C for 5 min, chilled on ice, and then fractionated in a 1% agarose-formaldehyde gel. After visualization of the 28S and 18S ribosomal subunits by ethidium bromide staining, the RNA was transferred to HyBond membrane (Amersham) by downward capillary action for 2.5 h by using the TurboBlotter assembly (Schleicher & Schuell). Once transferred, the membranes were hybridized (HybridMax Kit, Ambion) by using random primer (Ready-To-Go DNA Labeling Beads, Amersham Biosciences) probes for the major subunit [rat hepatic lectin-1 (RHL-1)] of the rat liver ASGP-R or GAPDH for normalization, as previously described (21).

**Western blot analysis.** Frozen liver pieces (50–100 mg) were homogenized in 0.25 M sucrose containing protease inhibitors (Sigma Protease Cocktail, catalog no. P-2714) and solubilized in Laemmli denaturing sample buffer (12). Aliquots of the suspension (10- to 25-μg protein) were resolved on a 10% SDS-polyacrylamide gel by using the Mini-Protean II Cell (Bio-Rad). After electrophoresis, the proteins were transferred at 10 V for 30 min onto 0.45-μm nitrocellulose by using the Trans-Blot System (Bio-Rad), followed by blocking of the nitrocellulose blots for 1 h with 5% milk in Tris-HCl, and 5% milk (pH 7.6). After electrotransfer, immunodetection of the various proteins of interest was performed as described here. Nitrocellulose blots were incubated overnight (4°C) or for 11 h at 23°C in blocking buffer containing 0.14 M NaCl, 50 mM Tris-HCl, and 5% milk (pH 7.6). The blots were then exposed (1 h at room temperature) in the same Tris-milk buffer that contained either nonimmune serum or the following antisera to the liver proteins of interest: polyclonal antiserum against ASGP-R (21), anti-Fas ligand antibody (mouse IgG; Transduction Laboratories), anti-caveolin (affinity purified rabbit antibody; Transduction Laboratories), and anti-clathrin heavy chain (mouse IgG1; Transduction Laboratories). Once incubation with the various antibodies was complete, the blots were washed in 0.5% Tween 20-20 mM Tris-HCl-150 mM NaCl buffer (pH 7.6), followed by incubation with 1 × 10⁵ counts-min⁻¹ ml⁻¹ of ¹²⁵I-labeled protein A or a 1:2,500 dilution of anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Promega, catalog no. W4011). After multiple washings, the blots were dried and exposed to KODAK X-OMAT film for 24 h at −70°C (for iodinated protein A) or detected by enhanced chemiluminescence (for HRP-conjugated antibody probe blots). Quantification of the autoradiograms or the enhanced chemiluminescence exposed blots was performed by using the Molecular Dynamics personal densitometer.

**RESULTS**

**General features of the model.** The purpose of the present study was to evaluate the relationship between ethanol-induced changes in the hepatic ASGP-R and the severity of liver pathology observed in animal-feeding models. For these studies, liver samples were obtained from animals intragastrically fed ethanol- or dextrose-containing diets, with either FO or MCT as the source of fat, for a period of 1 mo. Among the general features of this model, blood alcohol levels in the ethanol-fed groups (FE and MCTE) were measured to be in the range of 150 and 410 mg/dl, with an average blood ethanol concentration of 250 mg/dl. No significant difference was found in the level of alcohol in the blood between the two groups (MCTE: 228 ± 28 mg/dl; FE: 230 ± 26 mg/dl). In comparison, the concentration of alcohol measured in the blood of rats orally fed ethanol by using the Lieber-DeCarli liquid diet has been reported to be in the range of 100–150 mg/dl (13). No significant difference in weight gain was observed among the four groups of intragastrically fed animals (data not shown).

**Histopathology and measurement of serum ALT.** The assessment of pathological features observed in the liver samples obtained from the intragastrically fed animals revealed that the most severe levels of ALD (necrosis and inflammation) were only detected in the group fed ethanol in the absence of FO. In contrast, the MCTE group showed minimal fatty liver damage and no necrosis or inflammation. The control groups (dextrose- and no necrosis or inflammation among the four groups of intragastrically fed animals (data not shown).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Fatty Liver</th>
<th>Necrosis</th>
<th>Inflammation</th>
<th>ALT, U/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT-dextrose</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>MCT-ethanol</td>
<td>0.3 ± 0.4</td>
<td>0.03 ± 0.01</td>
<td>0.0</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Fish oil-dextrose</td>
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<td>0.0</td>
<td>0.0</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Fish oil-ethanol</td>
<td>4.0 ± 0.0</td>
<td>1.3 ± 0.3</td>
<td>24.3 ± 7.1</td>
<td>72 ± 11*</td>
</tr>
</tbody>
</table>

Values are means ± SE. ALT, alanine aminotransferase; MCT, medium-chain triglyceride. See **Histopathological analysis** under MATERIALS AND METHODS for definitions of fatty liver, necrosis, and inflammation scores. *P < 0.01 vs. dextrose-fed controls and MCT-ethanol.
ing liquid diet for at least 4 wk, the only liver damage that is reported is steatosis and apoptosis (11). The presence of severe liver lesions (necrosis and inflammation) and increased levels of serum ALT were found to be significantly higher only in the FE-fed animals compared with the other groups of rats after intragastric infusion of the diets (Table 1).

Measurement of ASGP-R protein content. We have previously reported that chronic (>5 wk) administration of ethanol to rats using the Lieber-DeCarli feeding model for ALD resulted in a decrease in ASGP-R binding to a representative ligand, which we determined was due to an actual decrease (25–40%) in ASGP-R content (21). In this study, we wanted to examine whether intragastric ethanol feeding, with its resultant pathological changes, caused correlative changes in ASGP-R protein levels. For these experiments, extractions of rat liver pieces obtained from the various intragastrically fed animals (MCT, MCTE, FO plus dextrose, FE) were resolved by 10% SDS-PAGE, probed with anti-ASGP-R antibody, and detected with either iodinated protein A or an HRP-conjugated enzyme system. Densitometric analysis of the immunoblots showed that ASGP-R content was decreased by an average of 30% in the MCTE group and by 55–60% in the FE group compared with their corresponding controls (Fig. 1). The decrease was significantly different between the two ethanol-fed groups and correlated with the increased presence of pathological changes in the FE group. These data confirm that receptor content is decreased after ethanol feeding in both the Lieber-DeCarli ad libitum model, as well as the intragastric model, and these changes were further potentiated when the animals were fed FE. The latter feeding, as pointed out previously, resulted in increased pathology in the liver, whereas the MCTE group showed only minimal fatty liver.

Western blot analysis of other liver proteins. In our next series of experiments, additional immunoblot analyses were performed to determine whether the intragastric feeding model for ALD affected other liver proteins. The proteins studied included Fas-ligand, caveolin, and clathrin. These proteins are involved in apoptotic and endocytic events in the liver that may or may not be associated with the activity of the ASGP-R. The results presented in Fig. 2 demonstrate that the most severe liver damage resulting from intragastric infusion of ethanol in the presence of FO correlated with alterations in ASGP-R content, whereas the other proteins remained unaffected. Significant changes in the level of Fas-ligand and clathrin were observed in the MCTE-fed animals compared with those fed MCTD, which may be reflective of damage that occurs early in ALD, such as increases in apoptosis and endocytic alterations.

Measurement of ASGP-R mRNA levels. To evaluate whether a relationship between ASGP-R expression and the observed decreases in ASGP-R protein content exists after intragastric feeding, Northern blot analysis was performed. For these studies, the expression of the ASGP-R receptor gene encoding the major component (RHL-1) of the rat ASGP-R was examined in the same groups of intragastrically fed animals. Fig. 3 shows a typical Northern blot for ASGP-R mRNA that was extracted from the livers of animals fed a liquid diet with dextrose or ethanol as the major source of carbohydrate and with MCT or FO as the source of fat. When the results of two to four pairs of animals were analyzed, the mRNA level for the RHL-1 subunit of the ASGP-R was significantly decreased by an average of 35% in the MCTE group and by 45% in the FE group compared with the dextrose-fed controls (Fig. 4).

**DISCUSSION**

Our laboratory (2–7, 21) has previously shown that chronic ethanol administration alters multiple aspects of the hepatic RME pathway. Specifically, it has been demonstrated that ethanol administration to rats by using a voluntary liquid animal model (the Lieber-DeCarli model) resulted in functional alterations in the hepatocyte-specific receptor, ASGP-R. In particular, ethanol treatment resulted in a decreased protein content of the receptor that was shown to be accompanied by
a loss of ASGP-R-specific mRNA and a decreased ability of the receptor to be synthesized from labeled amino acids (21). Elucidation of possible functional consequences resulting from the observed ethanol-induced impairments in ASGP-R function led to the discovery that the abundant liver receptor was involved in the clearance of cells dying as a result of a programmed (apoptotic) cell death. Our laboratory (15) has recently shown that the ASGP-R is involved in the in vitro clearance of apoptotic bodies and that the oral administration of ethanol by using the Lieber-DeCarli diet significantly impaired the ASGP-R-mediated uptake of apoptotic cells. As a result of these studies, we further hypothesize that the resultant accumulation of apoptotic cells generated in part by altered ASGP-R clearance may be involved in the enhancement of clinical alcohol-induced liver injury by initiating the release of proinflammatory mediators, the introduction of autoimmune responses, and inflammatory injury to the tissue.

In the present work using another animal model for ALD (the intragastric infusion of ethanol), we demonstrated that functional aspects of the ASGP-R (protein levels and mRNA gene expression) were decreased and that the changes were exacerbated when ethanol-induced pathological features were enhanced, as seen by changes in the animals that were intragastrically administered FE diets. In addition, these changes appeared to be specific for the ASGP-R. Therefore, it is attractive to speculate that, in the model of intragastric alcohol infusion compared with other models of ethanol administration, additional impairments to the ASGP-R may include further alterations in apoptotic cell clearance that may be

Fig. 2. Comparison of protein content levels in intragastrically fed rats. Liver samples from animals intragastrically fed liquid diets containing E or isocaloric D in the presence of either saturated fat (MCT) or FO were analyzed for the content of several liver proteins. Results are expressed as densitometric units normalized to protein concentration originally resolved by SDS-PAGE and are means ± SE for 4 pairs of animals in each treatment group. *Values that are significantly different from D-fed controls are indicated, P < 0.05. # Significance between the 2 E-fed groups, MCTE and FE, is indicated, P < 0.05.

Fig. 3. Northern blot analysis of ASGP-R mRNA in livers of rats fed E intragastrically for 4 wk. mRNA was obtained from livers of animals fed a D-containing control diet (MCTD or FD) or E diet (MCTE or FE) in the presence of saturated fat (MCT) or FO for 4 wk. The extracted mRNA was fractionated on agarose gels, transferred to nylon membranes, and hybridized with the random primer cDNA probe, as described in MATERIALS AND METHODS. The expression of the housekeeping gene GAPDH was also performed (data not shown) to indicate uniform loading of RNA samples into the gel. The results shown are 1 representative Northern blot from 3 independent experiments indicating visualization of the amount of ribosomal RNA loaded on the gel through ethidium bromide staining (A) and the detection of the expression of the major subunit (RHL-1) of ASGP-R (B).

Fig. 4. Determination of ASGP-R mRNA levels after intragastric E feeding. RNA from liver specimens obtained from the various experimental groups, animals intragastrically infused with diets containing MCTD or MCTE or diets containing FD or FE, were subjected to Northern blot analysis, as described in MATERIALS AND METHODS. Values are means ± SE. Results represent densitometric analysis of the blots and are expressed as the amount of RNA detected in the E-fed animals as a percentage of their D-fed controls. *Values that are significantly different from D-fed controls are indicated, P < 0.05.
involved in the accumulation of apoptotic cells in the liver. Indeed, it has been demonstrated in animals that were intragastrically administered ethanol that the number of apoptotic cells detected in the liver correlated to the development of ethanol-induced pathological liver injury (9, 22). Thus in animals that exhibit more significant pathological liver changes (i.e., FE-treated rats), the ASGP-R was found to be downregulated to a greater extent than when only steatosis is present, and the number of apoptotic cells present in the liver was found to be increased, which may be due, in part, to altered clearance of apoptotic cells by the ASGP-R. Furthermore, the accumulation of apoptotic cells may be involved in the development of pathological features, such as the formation of fibrotic lesions, as apoptotic cell-associated inflammatory injury to the tissue could potentially be linked to production of fibroin, a lymphokine whose expression has been shown to be increased in FE-fed rats (17). Additionally, ethanol-induced impairment to ASGP-R may be linked to the formation of alcohol-associated fibrosis, as fibronectin (one of the first extracellular matrix proteins to be elevated during fibrotic liver changes) has emerged as a natural ligand for ASGP-R (18). In the livers of animals treated with ethanol via models that result in lesser forms of liver injury without evidence of fibrosis (the Lieber-DeCarli model and the intragastric MCTE diet), the downregulation of functioning ASGP receptors may be involved in the expression or activity of specific nonparenchymal cell receptors (i.e., the mannose-specific receptor of endothelial cells, the galactosyl receptor on Kupffer cells, and the stellate cell scavenger receptor) that could be involved in a variety of adaptive functions, such as the removal of apoptotic cells when the ASGP-R is impaired, yet no histopathological liver injury is observed.

In conclusion, our data show that receptor content for the hepatic ASGP-R is decreased after chronic ethanol administration. These impairments occur in the absence of liver pathology (i.e., fatty liver only, no necrosis or inflammation) and are more dramatic when pathological liver damage occurs as in the intragastric feeding model. These changes in ASGP-R content were rather selective, as the contents of other proteins of interest were not altered during this feeding regimen. These results support the hypothesis that proper functioning of the ASGP-R is physiologically important in the liver. The mechanisms and consequences of impairments to the abundant receptor are of significant interest in elucidating the role of ASGP-R in alcohol liver injury, such as the concomitant alteration in the clearance of apoptotic cells and the receptor’s overall contribution in fibrogenic liver damage.

GRANTS

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REFERENCES


