LIVER ASIALOGLYCOPROTEIN RECEPTOR LEVELS CORRELATE WITH
SEVERITY OF ALCOHOLIC LIVER DAMAGE IN RATS

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**Running head:** Rat liver pathology correlates with receptor function

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ABSTRACT

It has been demonstrated that the oral administration of ethanol (Lieber-DeCarli liquid diet) to rats results in a decreased expression and content of the asialoglycoprotein receptor (ASGP-R) in the resultant fatty liver. In the current study, we wanted to determine whether the extent of impaired receptor content was correlated with the severity of liver pathology using the intragastric feeding model. When ASGP-R protein and mRNA levels were measured in animals infused with ethanol or dextrose in the presence of fish oil (FO) or medium-chain triglyceride (MCT) as the source of fat, more significant impairments to the ASGP-R were observed in the FO-ethanol group when compared with the MCT-ethanol group. Furthermore, only the FO-ethanol group showed pathological liver changes. These results demonstrate that a correlation exists between the progression of alcohol associated liver injury, as defined by the severity of liver pathology, and an ethanol-induced decline in ASGP-R content.

Key words: Receptors, Liver, Endocytosis, Rat Diet, Alcoholic Liver Injury
INTRODUCTION

We have 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 (1-6). A majority of these findings were demonstrated 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-acetylgalactosamine residues and removes these potentially harmful desialylated glycoproteins from the circulation (7). 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 (9), 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) (10). 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 four weeks 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 one week, with decreases in ligand binding to the receptor observed without a corresponding change in receptor protein content. Upon prolonged ethanol feeding (5-7 weeks),
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 (12). Since 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) are included in the diet, the toxicity of ethanol is noted by the presence of fatty liver, inflammation, necrosis, and fibrosis (13,14). 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 fish oil 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 if 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 g and 300 g were fed a liquid diet by means of continuous intragastric infusion through permanently implanted gastric tubes for one month as previously described (15). The rats were given their total nutrient intake by intragastric infusion in which the percentage of calories derived from fat was 35% of total calories. Vitamins and minerals were given as described previously (16). The amount of ethanol
administered to the animals started at 10 g/kg/day and was gradually increased over an 8 to 10 day period to 16 g/kg/day (full ethanol dose) as the animals developed tolerance. In addition the ethanol concentration was modified throughout to maintain high levels of blood ethanol (150 to 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 weeks of full dose ethanol administration, the animals were sacrificed, 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, and 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. Casey’s laboratory to be used for Western and Northern blot analysis. The samples obtained for analysis were procured from animals fed medium-chain triglyceride plus dextrose (MCTD) or medium-chain triglyceride plus ethanol (MCTE) and fish oil plus dextrose (FD) or fish oil 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 formalin-fixed at the time of animal sacrifice. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat) was scored 1+ when less than 25% of the cells contained fat, 2+ when 26 to 50% of the cells contained fat, 3+ when 51 to 75% of the cells contained fat, and 4+ when greater than 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 using an alcohol dehydrogenase kit from Sigma Chemical Company (St. Louis, MO). The level of serum alanine aminotransferase (ALT: 2.6.1.2) activity was determined 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 using TriReagent (Molecular Research Center, Inc. Cincinnati, OH). Twenty to 30 µg of the liver RNA and 3 µg of a 0.24-9.5 kilobase RNA ladder (Life Technologies, Inc) 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 hours using the TurboBlotter assembly (Schleicher & Schuell). Once transferred, the membranes were hybridized (NorthernMax Kit, Ambion) using random primer (Ready-To-Go DNA Labeling Beads, Amersham Biosciences) probes for the major subunit (RHL-1) of the rat liver ASGP-R or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization as previously described (12).

Western blot analysis. Frozen liver pieces (50-100 mg) were homogenized in 0.25 M sucrose containing protease inhibitors (Sigma Protease Cocktail, catalog #P-2714) and solubilized in Laemmli denaturing sample buffer (17). Aliquots of the suspension (10-25 µg protein) were resolved on a 10% SDS-polyacrylamide gel using the Mini-Protean II Cell (Bio-Rad). After electrophoresis, the proteins were transferred at 10 volts for 30 min onto 0.45-µm
nitrocellulose using the TransBlot Semi-dry Transfer Cell (Bio-Rad). After electro-transfer, immunochemical detection of the various proteins of interest was performed as described here. Nitrocellulose blots were incubated overnight (4°C) or for 11 hours 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 hour at room temperature) in the same Tris/milk buffer that contained either non-immune serum or the following antisera to the liver proteins of interest: polyclonal antiserum against ASGP-R (12), 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 x 10^5 cpm/ml of 125I protein A or a 1:2500 dilution of anti-rabbit IgG HRP conjugate (Promega, catalog #W4011). After multiple washings, the blots were dried and exposed to KODAK X-OMAT film for 24 hours at –70°C (for iodinated Protein A) or detected by ECL (for HRP-conjugated antibody probed blots). Quantification of the autoradiograms or the ECL exposed blots was performed 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 (E) or dextrose (D)-containing diets, with either fish oil (FO) or medium chain triglycerides (MCT) as the source of fat, for a period of one month. 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 using the Lieber-DeCarli liquid diet has been reported to be in the range of 100-150 mg/dl (18). No significant difference in weight gain was observed between 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 presence of fish oil. In contrast, the MCTE group showed minimal fatty liver damage and no necrosis or inflammation. The control groups (dextrose-fed rats) showed no pathology (Table 1). In comparison, when rats are orally fed the standard Lieber-DeCarli ethanol-containing liquid diet for at least four weeks, 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 fish oil-ethanol (FE) fed animals compared to 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 (greater than 5 weeks) 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 (12). In this study we wanted to examine whether intragastric ethanol feeding, with its resultant pathologic 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, FD, 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 (Figure 1). The decrease was significantly different between the two ethanol-fed groups and correlated with the increased presence of pathologic changes in the FE group. These data confirm that receptor content is decreased after ethanol feeding in both the Lieber-DeCarli ad lib model, as well as the intragastric model; and these changes were further potentiated when the animals were fed ethanol with fish oil. The latter feeding, as pointed out previously, resulted in increased pathology in the liver while 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 in order 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 Figure 2 demonstrate that the most severe liver damage resulting from intragastric infusion of ethanol in the presence of fish oil correlated with alterations in ASGP-R content, while the other proteins remained unaffected. Significant changes in the level of Fas-ligand and clathrin were observed in the MCTE-fed animals when compared to those fed MCT-dextrose, 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. Figure 3 shows a typical Northern blot for ASGP-R mRNA that was extracted from the livers of animals fed liquid diet with dextrose or ethanol as the major source of carbohydrate and with MCT or fish oil as the source of fat. When the results of 2 to 4 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 as compared to the dextrose-fed controls (Figure 4).

DISCUSSION

We have previously shown that chronic ethanol administration alters multiple aspects of the hepatic RME pathway (1-6, 12). Specifically, it has been demonstrated that ethanol administration to rats 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 (12). 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. We have recently shown that the ASGP-R is involved in the in vitro clearance of apoptotic bodies and that the oral administration of ethanol using the Lieber-DeCarli diet significantly impaired the ASGP-R mediated uptake of apoptotic cells (9).
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 pro-inflammatory mediators, the introduction of auto-immune 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 intragastrically administered fish oil-ethanol 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 when compared to other models of ethanol administration, additional impairments to the ASGP-R may include further alterations in apoptotic cell clearance that may be involved in the accumulation of apoptotic cells in the liver. Indeed, it has been demonstrated in animals intragastrically administered ethanol that the number of apoptotic cells detected in the liver correlated to the development of ethanol-induced pathological liver injury (19, 20). Thus, in animals that exhibit more significant pathological liver changes (i.e. fish oil-ethanol treated rats), the ASGP-R was found to be down-regulated 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 fibrosin, a lymphokine whose expression has been shown to be increased in fish oil-ethanol fed rats (21). 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 (22). 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 down-regulation of functioning ASGP receptors may be involved in the expression or activity of specific non-parenchymal 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.
ACKNOWLEDGMENTS

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REFERENCES


Figure 1. Immunochemical detection of total ASGP-R content in livers of rats fed ethanol intragastrically.  A. Representative immunoblot of solubilized liver fractions from control animals: medium-chain triglyceride plus dextrose, MCTD (lane 1), and fish oil plus dextrose, FD (lane 3), and ethanol-fed rats: medium-chain triglyceride plus ethanol, MCTE (lane 2) and fish oil plus ethanol, FE (lane 4), that were loaded onto gels and resolved by SDS-PAGE followed by transblot analysis as described under “Materials and Methods”.  The three bands represent the subunits of ASGP-R (RHL-1, 2, and 3).  B. Densitometric analysis of blots prepared with liver samples obtained from four pairs of intragastrically fed rats.  Western blot analysis was performed on liver extractions containing 10-25ug of protein obtained from dextrose and ethanol containing MCT and FO-fed animals.  Quantification of the resultant blots was achieved as the densitometric values obtained were normalized to the amount of protein loaded on the gel and are represented as a percentage of the value obtained for the corresponding dextrose-fed control animal.  Those values that are significantly different from the dextrose-fed controls are indicated (*, p<0.05).  Significance between the two ethanol-fed groups, MCTE and FE, is indicated (#, p<0.05).

Figure 2. Comparison of protein content levels in intragastrically fed rats.  Liver samples from animals intragastrically fed liquid diets containing ethanol (E) or isocaloric dextrose (D) in the presence of either saturated fat (MCT) or fish oil (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 ± S.E. for four pairs of animals in each treatment group.  Values that are significantly different from dextrose-fed controls are
indicated (*, p<0.05). Significance between the two ethanol-fed groups, MCTE and FE, is indicated (#, p<0.05).

**Figure 3: Northern blot analysis of ASGP-R mRNA in livers of rats fed ethanol intragastrically for four weeks.** mRNA was obtained from livers of animals fed a dextrose-containing control diet (MCTD or FD) or ethanol diet (MCTE or FE) in the presence of saturated fat (MCT) or fish oil (F) for four weeks. The extracted mRNA was fractionated on agarose gels, transferred to nylon membranes, and hybridized with the random primer cDNA probe as described under “Materials and Methods”. The expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also performed (data not shown) to indicate uniform loading of RNA samples into the gel. The results shown are one representative Northern blot from three 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).

**Figure 4. Determination of ASGP-R mRNA levels after intragastric ethanol feeding.** RNA from liver specimens obtained from the various experimental groups: animals intragastrically infused with diets containing medium-chain triglyceride in the presence of dextrose (MCTD) or ethanol (MCTE) or diets containing fish oil with dextrose (FD) or ethanol (FE), were subjected to Northern blot analysis as described in “Materials and Methods”. Results represent densitometric analysis of the blots and are expressed as the amount of RNA detected in the ethanol-fed animals as a percent of their dextrose-fed controls. Values that are significantly different from dextrose-fed controls are indicated (*, p<0.05).
Table 1. Observed pathological changes in different experimental treatment groups of intragastric feeding.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Fatty Liver</th>
<th>Necrosis</th>
<th>Inflammation</th>
<th>ALT (U/L)</th>
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<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>
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<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 ± 4</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>

Abbreviations: MCT – medium-chain triglyceride. *, p<0.01 versus dextrose-fed controls and MCT-ethanol.
A. 

B. 

% of Dextrose-fed

MCT+D  MCT+E  FishOil+D  FishOil+E

B. 

% of Dextrose-fed

MCT+D  MCT+E  FishOil+D  FishOil+E

1 2 3 4
120% of Dextrose-fed

% of Dextrose-fed

MCT+D  MCT+E  F+D  F+E

*