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Carbon tetrachloride-induced liver damage in asialoglycoprotein receptor-deficient mice

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ABSTRACT

The asialoglycoprotein (ASGP) receptor is an abundant hepatocyte-specific receptor involved in receptor-mediated endocytosis. This receptor's abundance and function is decreased by chronic ethanol administration prior to the appearance of pathology such as necrosis or inflammation. Hence, this study aimed to determine if ASGP receptor function is required to protect against liver injury by utilizing a knockout mouse model lacking functional ASGP receptor in the setting of carbon tetrachloride (CCl₄) hepatotoxicity. Briefly, ASGP receptor-deficient (RD) mice and wild-type (WT) mice were injected with 1 ml/kg body weight of CCl₄. In the subsequent week, mice were monitored for liver damage and pathology (aspartate transaminase (AST), alanine transaminase (ALT) and light microscopy). The consequences of CCl₄ injection were examined by measuring α -smooth muscle actin (α -SMA) deposition, contents of malondialdehyde and the percentage of apoptotic hepatocytes. After CCl₄ injection, RD mice showed increased liver pathology together with significantly increased activities of AST and ALT compared to that in WT mice. There were also significantly more apoptotic bodies, lipid peroxidation and deposition of α -SMA in RD mice versus WT mice following CCl₄ injection. Since these two mouse strains only differ in whether or not they have the ASGP receptor, it can be concluded that proper ASGP receptor function exerted a protective effect against CCl₄ toxicity. Thus, receptor-mediated endocytosis by the ASGP receptor could represent a novel molecular mechanism that is responsible for subsequent liver health or injury.

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Abbreviations: ASGP, asialoglycoprotein; CCl₄, carbon tetrachloride; RD, asialoglycoprotein receptor-deficient; WT, wild-type; AST, aspartate transaminase; ALT, alanine transaminase; α -SMA, α -smooth muscle actin.

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1. Introduction

Chronic ethanol consumption causes major health problems by leading to liver injuries such as fatty liver, alcoholic hepatitis and cirrhosis [1,2]. In recent years, many pathways have been studied in search of potential mechanisms involved in the promotion of pathological features associated with alcoholic liver disease. Work from our laboratory has studied how ethanol administration affects key hepatocellular functions involved in protein trafficking, many of which utilize the abundant asialoglycoprotein (ASGP) hepatocyte receptor.

The ASGP receptor is an abundantly expressed hepatocyte-specific receptor [3]. This is a well characterized receptor [4] that has served as an excellent model to study receptor trafficking events such as receptor-mediated endocytosis. During the receptor-mediated endocytosis process, the ASGP receptor binds to its ligand and the receptor–ligand complex is then internalized by a clathrin-coated pathway. Intracellularly, these vesicles lose their clathrin coats and the resulting endosomes are acidified leading to uncoupling of the receptor–ligand complexes. The separated receptors and ligands can then be recycled back to the cell surface or trafficked to the lysosomes for degradation [5]. Ligands recognized by the ASGP receptor include glycoproteins bearing terminal galactose or N-galactosamine residues [3]. Furthermore, it has been determined that the process of apoptosis or programmed cell death, results in the formation of apoptotic bodies, which have an increased amount of desialylated glycoconjugates [6–9]. Importantly, it has been shown that the ASGP receptor recognizes and facilitates the timely removal of apoptotic bodies [6,10,11].

Previous work in our laboratory has shown that many facets of ASGP receptor-mediated endocytosis were impaired in hepatocytes isolated from animals after ethanol administration. These impairments range from decreased binding, internalization, degradation and receptor cycling [12–17]. In addition, chronic ethanol administration also decreases ASGP receptor-specific mRNA and hence the receptor content of the ASGP receptor [18]. Given that the ASGP receptor is required to remove potentially harmful desialylated glycoconjugates, the decreased receptor content caused by ethanol administration could result in increased liver damage. Thus, to determine whether ASGP receptor function is protective against liver injury, a knockout mouse model lacking the ASGP receptor (RD) was employed.

The RD mouse was generated by disrupting the MHL-2 gene that encodes the protein for the minor subunit of the ASGP receptor [19]. In the murine system, the ASGP receptor is comprised of two subunits that are required for a functional receptor [4]. These knockout mice show a complete lack of MHL-2 protein (minor subunit) and a substantial reduction in the expression of the MHL-1 gene (major subunit). In spite of this deletion, RD mice remain viable and fertile, have a normal lifespan and do not display any obvious phenotypic abnormalities [19,20].

In order to determine whether or not ASGP receptor provides protection from liver injury, the animal model was challenged by an acute injection of CCl_4 (1 mg/kg body weight). Carbon tetrachloride is used as a model to study hepatotoxic effects and causes liver damage through a number of

mechanisms. Briefly, CCl_4 can induce liver damage through the formation of reactive free radicals that can bind covalently to cellular macromolecules forming nucleic acid, protein and lipid adducts; through the induction of hypomethylated ribosomal RNA, resulting in inhibition of protein synthesis; and finally, CCl_4 can affect hepatocellular calcium homeostasis [21,22]. Overall, CCl_4 treatment can result in centrilobular steatosis, inflammation, apoptosis and necrosis [21–23]. If the damage exceeds the repair capacity of the liver, the liver will progress to fibrosis and cirrhosis [21,22].

In the present study, we administered an acute dose of CCl_4 to WT and RD mice. The importance of ASGP receptor function was then ascertained by comparing the hepatic responses of these two mouse strains. Using this model, we conclude that the ASGP receptor exerts a protective effect against toxic liver injury.

2. Materials and methods

2.1. Animals

Female WT (C57BL/6 – 129SV) and RD (B6, 129SV-Asgpr2) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in the Animal Research Facility at the Omaha Veterans Affairs Medical Center, which has been approved by the American Association for the Accreditation of Laboratory Animal Care. Mice (20–25 g) were acclimated for 1 week prior to use in experiments and were allowed free access to water and fed a Purina chow diet through the experiment.

2.2. Treatment conditions

Experimental animals were given an intraperitoneal injection of CCl_4 (1.0 ml/kg body weight in olive oil; $\sim 200 \mu\text{l}/\text{mouse}$; CCl_4 from Sigma-Aldrich, St. Louis, MO). Animals were then sacrificed at 12 h, 24 h, 48 h, 72 h, 96 h or 7-day after injection. Control animals were injected with an equivalent amount of olive oil intraperitoneally. Since the injection of olive oil did not affect the results of any assays carried out in this study, the values for the various time points were averaged and the control was presented as a single value in the subsequent results.

2.3. Collection of tissues

Mice were sacrificed by exsanguination after being anaesthetized with sodium pentobarbital (50 ng/g body weight; Ovation Pharmaceuticals Inc., Deerfield, IL). Serum was collected and stored at -70°C until analysis. Following removal, a portion of the liver was saved for histologic analysis and the remaining portion was freeze clamped and stored at -70°C until analysis.

2.4. Histopathological analysis

Haematoxylin–eosin staining, followed by light microscopy analysis, was performed on the liver samples that were formalin fixed at the time of animal death. At least three different sections were examined per liver sample, with the

pathologist blinded to the identity of treatment group when assessing the histology.

2.5. Measurement of activities of AST and ALT

Activities of AST and ALT in the serum were assessed spectrophotometrically using a commercially available kit (Sigma–Aldrich, St. Louis, MO).

2.6. Measurement of malonaldehyde

The content of malondialdehyde (MDA) in the livers was determined by the method of Uchiyama and Mihara [24]. The standard curve (0–12.5 nmol) was produced using malondialdehyde that was a gift from Dr. Geoffrey Thiele (VA Hospital, Omaha, NE). Protein contents were determined using a BCA Protein Reagent Assay Kit (Pierce, Rockford, IL).

2.7. Western blot analysis of α -smooth muscle actin

Liver homogenates (20% w/v in 0.1 M Tris/0.25 M sucrose, pH 7.5) were adjusted to 2–5 mg ml⁻¹ in Laemmli denaturing sample buffer [25]. Aliquots of the suspension were resolved on a 10% SDS-PAGE gel and proteins were transferred to a nitrocellulose membrane. After electrotransfer, the blots were blocked for 1 h at room temperature in blocking buffer containing 0.01 M Tris, 0.1 M NaCl, 0.1% Tween 20 and 5% milk (pH 7.5). The blots were then incubated with a 1:5000 dilution of monoclonal anti- α smooth muscle actin antibody (clone 1A4, Sigma–Aldrich, St. Louis, MO) in blocking buffer (overnight at 4 °C). Following several washes in buffer containing 0.01 M Tris, 0.1 M NaCl and 0.1% Tween 20 (pH 7.5), the blots were incubated in a 1:2500 dilution of alkaline phosphatase-conjugated mouse IgG secondary antibody (Sigma–Aldrich, St. Louis, MO) diluted in blocking buffer (1 h at room temperature). Following several washes in buffer, the immunoreactive proteins were visualized colorimetrically and quantified using a Bio-Rad FluorS-Multi-Imager and Quantity One software (Bio-Rad, Hercules, CA). All other reagents were reagent grade and obtained from commercially available sources.

2.8. Immunohistochemical analysis of apoptotic hepatocytes

The presence of apoptotic hepatocytes was determined qualitatively using the Deadend™ colorimetric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions. An average of 3000 total cells per animal was counted in order to determine the percent of apoptotic hepatocytes.

2.9. Statistical analysis

Results are presented as means \pm standard error of the mean (S.E.M.). Comparisons between the control and various time points were carried out using one-way analysis of variance followed by Bonferroni's multiple range test to evaluate differences between means. Comparisons between WT and RD mice were carried out using independent *t*-tests. Arcsine transformation was applied to all percentage data before

statistical analysis. Differences with $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Injection of CCl₄ increased activities of AST and ALT and increased liver pathology to a greater extent in RD versus WT mice

Activities of AST and ALT showed a similar trend after CCl₄ injection (Fig. 1). AST (Fig. 1A) and ALT (Fig. 1B) activities were significantly increased 48 h after CCl₄ injection for both WT and RD. At this time point, AST activities for WT and RD mice were 63- and 79-fold increased over the control value, respectively. For ALT, activities for WT and RD mice were 311- and 668-fold increased over the control value, respectively. In addition, for activities of AST and ALT 48 h after CCl₄ injection, RD values were significantly greater than WT values by 1.3- and 1.8-fold, respectively.

Liver damage was also assessed by histology. Control livers for WT (Fig. 2A) and RD (Fig. 2D) mice were similar in appearance and showed no pathology. However, by 48 h after CCl₄ injection,

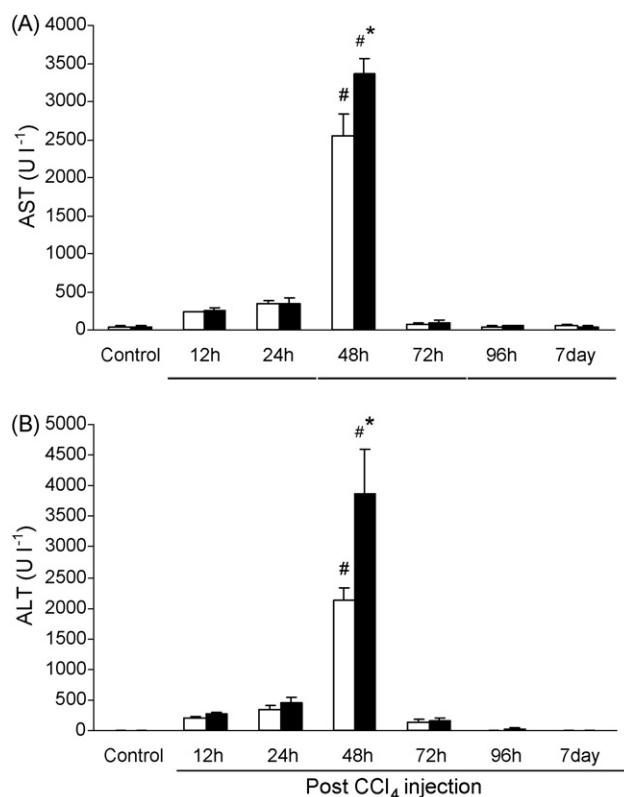


Fig. 1 – Activities (U l⁻¹) of (A) aspartate transaminase (AST) and (B) alanine transaminase (ALT), in the serum of wild-type (WT) and ASGP receptor-deficient (RD) mice after injection with CCl₄. WT values are represented in open bars and RD values in filled bars. Controls were injected with an equivalent amount of olive oil. Values represent means \pm S.E.M. with $N = 3$ –7. *Significantly different from the corresponding WT condition, $P < 0.05$. #Significantly different from the corresponding control condition, $P < 0.05$.

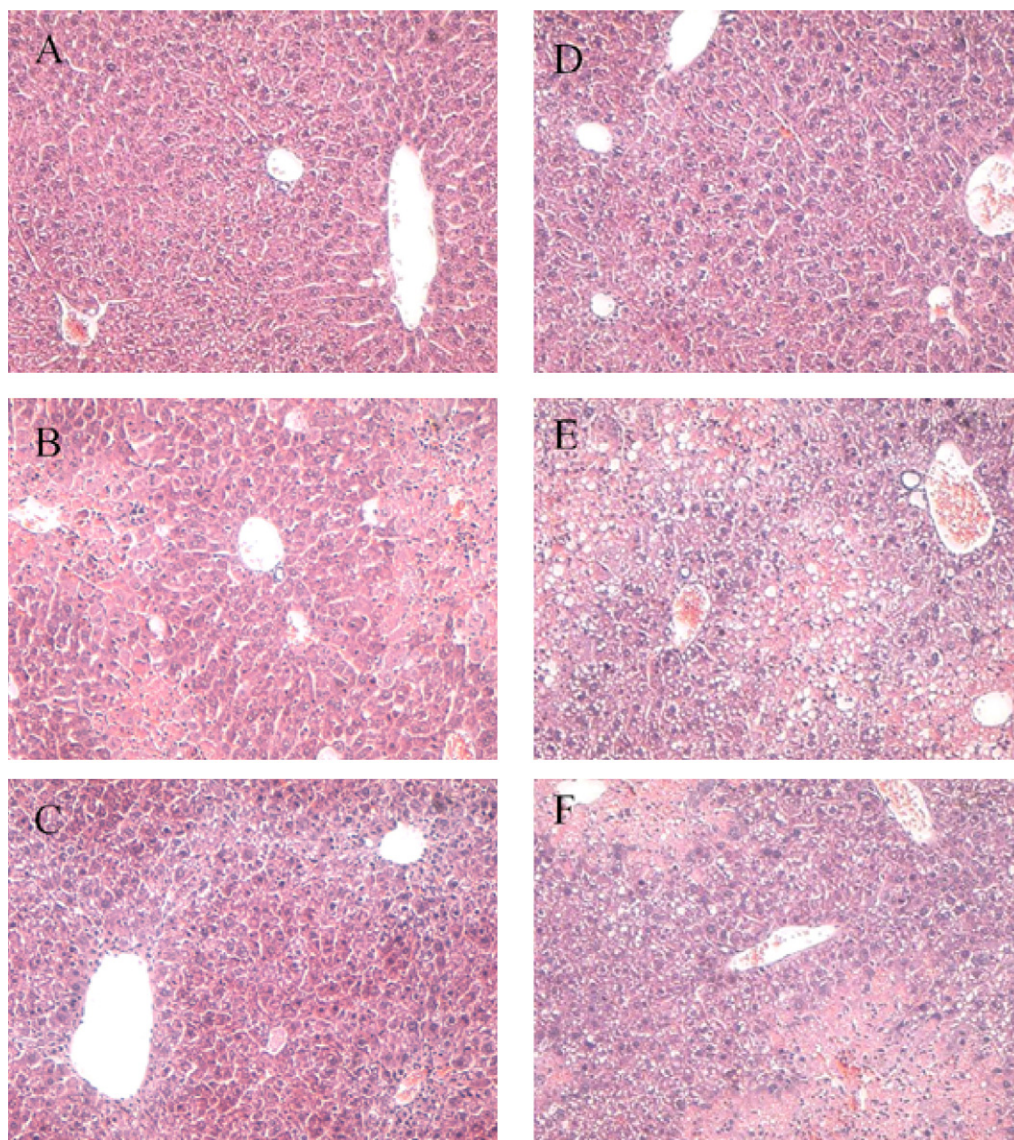


Fig. 2 - Histology of livers from wild-type (WT) and ASGP receptor-deficient (RD) mice after injection with CCl₄. Paraffin embedded sections were prepared and stained with H and E. Controls were injected with an equivalent amount of olive oil. Photomicrographs show representative liver sections at 100 times magnification. (A) WT, control; (B) WT, 48 h after CCl₄ injection; (C) WT, 96 h after CCl₄ injection; (D) RD, control; (E) RD, 48 h after CCl₄ injection; (F) RD, 96 h after CCl₄ injection. Note, the more severe necrosis in the RD (E) versus WT (B) mice at 48 h and even at 96 h after CCl₄ injection (F versus C).

centrilobular liver damage was apparent in both WT (Fig. 2B) and RD (Fig. 2E) mice. The damage was more severe in RD mice, which showed a greater number of neutrophilic inflammatory infiltrates. Although AST and ALT activities returned to normal by 72 h, damage could still be seen histologically up to 96 h. At this point, RD mice still showed more damage than WT mice.

3.2. Lipid peroxidation was increased in RD but not WT livers following injection of CCl₄

The amount of malondialdehyde in liver tissue serves as an indicator of lipid peroxidation, which is a noted occurrence in liver injury due to the generation of reactive oxygen species. The levels of malondialdehyde in WT mice were not significantly different through the course of the experiment

(Fig. 3). However, malondialdehyde in RD mice increased significantly over control values at 24 h and 48 h after CCl₄ injection by 2.5- and 2.6-fold, respectively. These values were also significantly different from that of the WT mice. Subsequently, malondialdehyde decreased by 72 h and continued to decrease till 7 days following CCl₄ injection to reach levels reminiscent of the control condition (Fig. 3).

3.3. α -SMA levels were increased to a greater extent in RD versus WT mice after CCl₄ injection

α -SMA levels were increased significantly in WT and RD mice compared to control (Fig. 4). WT mice had increased levels 72 h after CCl₄ injection (5.1-fold), whilst RD mice had a more prolonged increase at 48 h and 72 h after injection (3.6- and

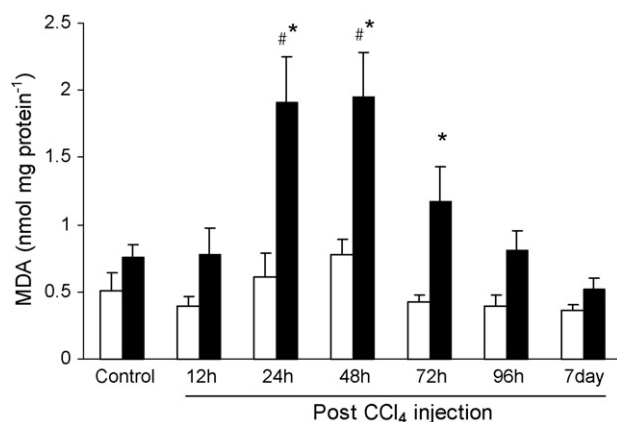


Fig. 3 – Content (nmol mg protein⁻¹) of malondialdehyde (MDA) in the liver of wild-type (WT) and ASGP receptor-deficient (RD) mice after injection with CCl₄. WT values are represented in open bars and RD values in filled bars. Controls were injected with an equivalent amount of olive oil. Values represent means ± S.E.M. with N = 3–7. *Significantly different from the corresponding WT condition, P < 0.05. #Significantly different from the corresponding control condition, P < 0.05.

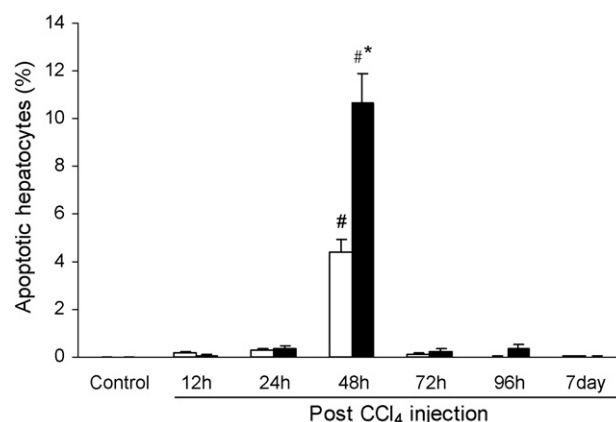


Fig. 5 – Apoptotic hepatocytes (%) in the liver of wild-type (WT) and ASGP receptor-deficient (RD) mice after injection with CCl₄. WT values are represented in open bars and RD values in filled bars. Controls were injected with an equivalent amount of olive oil. Values represent means ± S.E.M. with N = 3–4. *Significantly different from the corresponding WT condition, P < 0.05. #Significantly different from the corresponding control condition, P < 0.05.

3.2-fold, respectively). In addition, α -SMA levels in RD mice were increased significantly over WT mice at 48 h, 96 h and 7-day after injection by 1.9-, 2.1- and 1.7-fold, respectively.

3.4. Injection of CCl₄ increased hepatocyte apoptosis to a greater extent in RD versus WT mice

The presence of apoptotic hepatocytes as detected by TUNEL, was found to be significantly increased in both WT and RD mice at 48 h after CCl₄ injection (Fig. 5). However, mice lacking

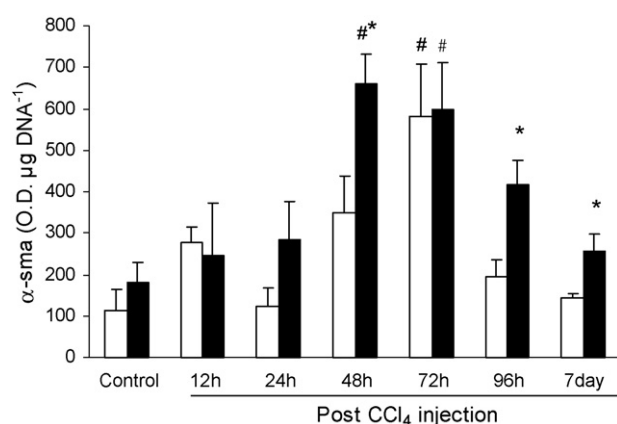


Fig. 4 – Content (O.D. μ g DNA⁻¹) of α -smooth muscle actin (α -SMA) in the liver of wild-type (WT) and ASGP receptor-deficient (RD) mice after injection with CCl₄. WT values are represented in open bars and RD values in filled bars. Controls were injected with an equivalent amount of olive oil. Values represent means ± S.E.M. with N = 3–9. *Significantly different from the corresponding WT condition, P < 0.05. #Significantly different from the corresponding control condition, P < 0.05.

a functional ASGP receptor displayed 2.4-fold more apoptotic hepatocytes than WT mice.

4. Discussion

It is well documented that alcohol perturbs many aspects of liver homeostasis. Clinically, fatty liver is observed in the early stage followed by apoptosis, necrosis, alcoholic hepatitis, fibrosis and cirrhosis in the later stages. Our laboratory has been interested for several years in receptor-mediated endocytosis (particularly that mediated by the asialoglycoprotein receptor) and its role in furthering hepatocellular damage in alcoholic liver disease.

We have found that chronic ethanol administration markedly decreased ASGP receptor mRNA expression and receptor content prior to the appearance of pathology such as necrosis or inflammation [18,26]. This suggests that impairments in ASGP receptor function may initiate a cascade of events leading to subsequent liver injury. In order to examine the ASGP receptor's role in the progression of pathological disease, knockout mice that lack the ASGP receptor (RD) were used in the setting of CCl₄-induced hepatotoxicity. Differences in responses between the mouse strains can be attributed to the loss of ASGP receptor function.

The results of this study demonstrated that RD mice had significantly higher activities of AST and ALT. These results indicate that RD mice sustained greater liver injury than wild-type (WT) mice, since AST and ALT are sensitive indicators of liver cell injury [27]. This was further supported by histological evidence showing that RD mice had larger necrotic foci and more severe neutrophilic inflammatory infiltrates. Since these mice differed only in the presence or absence of the ASGP receptor, it is proposed that proper receptor function may exert a protective effect during toxicant-induced liver damage.

Another aspect that the WT and RD mice differed on was the levels of malondialdehyde. Malondialdehyde is a reactive aldehyde that is cytotoxic [21]. Since it is formed as a by-product of lipid peroxidation, it can also be used as an indicator of the amount of lipid peroxidation. Lipid peroxidation occurs when free radicals produced by the metabolism of CCl_4 attack polyunsaturated fatty acids in the cellular membrane, thereby initiating the subsequent lipid peroxidation and resulting ultimately in a loss in membrane integrity [21,22]. In this study, the earliest significant change observed in RD mice as compared to WT mice was the enhanced level of malondialdehyde content measured at 24 h. Thus, this observation suggests that lipid peroxidation is an early event in liver injury and that the free radicals generated may play a role in subsequent damage, leading to necrosis, apoptosis and extracellular matrix deposition. Presently, evidence is accumulating to suggest that free radicals (1) can cause oxidative mitochondrial damage (that directly causes hepatocyte death or favors alcohol-induced sensitization to the pro-apoptotic action of $\text{TNF-}\alpha$); (2) can regulate gene expression contributing to the development of fibrosis; (3) can perpetuate chronic inflammatory processes [28].

Although the reason(s) behind how the ASGP receptor exerts its protection is unclear, it is recognized that rat hepatocytes undergo apoptosis after injection of CCl_4 [23]. Our results also showed that apoptotic hepatocytes are significantly increased at 48 h and this increase is significantly greater in RD mice compared to WT mice. Increased accumulation of apoptotic cells has also been shown during alcohol-induced injury in a variety of species including humans and is thought to play an important role in the progression of liver injury [29–35]. Since the ASGP receptor has been shown to play a key role in the clearance of apoptotic bodies [11], we speculate that the ASGP receptor exerts a protection through timely removal of apoptotic bodies, preventing secondary necrosis and subsequent leakage of potentially harmful materials. Interestingly, despite the absence of functional ASGP receptor, apoptotic cell numbers in RD mice decrease back to baseline by 72 h as in WT mice. The removal of apoptotic bodies in this case is probably carried out by redundant pathways that recognize exposed terminal sialic acids, externalized phosphatidylserine or modifications that have occurred to membrane lipid asymmetry [36]. Depending on the cell type, the receptors responsible could include several members, such as the scavenger receptor family, the $\alpha_v\beta_3$ vitronectin receptor or CD14 [36,37]. However, it is important to note that these cannot totally compensate for the ASGP receptor's role since there is a significant increase in apoptotic cell number at 48 h in RD compared to WT mice. A further consequence of this impaired uptake in RD mice could be the enhanced phagocytosis of apoptotic bodies by non-parenchymal cells such as kupffer cells or hepatic stellate cells. Uptake of apoptotic bodies by kupffer cells stimulates the generation of death ligands, including Fas ligand and tumor necrosis factor α , leading to increased hepatocyte apoptosis, increased neutrophil infiltration, and markers of stellate cell activation [38]. Uptake of apoptotic bodies by stellate cells induces transforming growth factor β expression and NADPH oxidase activation (which results in the upregulation of procollagen $\alpha 1$) [39]. Thus, uptake of apoptotic cells by non-parenchymal cells such as kupffer cells and stellate cells, could contribute to the progression of liver disease.

Another possible way by which the ASGP receptor may be protective is through its role in regulating the turnover of extracellular matrix. We showed here that RD mice displayed increased α -SMA deposition 24 h earlier than WT and that α -SMA deposition remained increased up to 7 days. This was in contrast to the WT mice, which only showed a spike at 72 h. Thus, RD mice were compromised in their ability to adjust their extracellular matrix turnover, putting them at an increased risk of developing fibrosis. Similarly, it is attractive to speculate that the deposition of other matrix proteins, such as the glycoprotein fibronectin, may also be enhanced as a consequence of altered ASGP receptor function. Particularly, the insoluble form of fibronectin, cellular fibronectin, is deposited as filaments in the extracellular matrix and is one of the first extracellular matrix proteins to accumulate during fibrosis [40–42]. Cellular fibronectin displays a high density of terminal galactose residues, which make it a potential ligand for the ASGP receptor [42,43]. Indeed, studies have shown that the pattern of uptake and degradation of ^{125}I -cellular fibronectin from preloaded liver slices is very similar to that of ^{125}I -asialofetuin (a selective ligand of the ASGP receptor) [42]. Furthermore, the prior infusion of excess asialofetuin or the addition of a terminal sialic acid to the cellular fibronectin inhibits the removal of cellular fibronectin [42,43]. Thus, this suggests that the ASGP receptor is involved in the uptake of cellular fibronectin and as such decreased ASGP receptor function could lead to increased extracellular matrix deposition and hence lead to fibrosis and cirrhosis.

In summary, this study demonstrates that the absence of ASGP receptor led to increased liver pathology and indices of liver damage. Additional consequences of the missing receptor were deposition of α -SMA, increased apoptosis and increased lipid peroxidation. Thus, we conclude that proper ASGP receptor function is essential for protection against liver injury. At present, the exact mechanism by which damage occurs in the absence of ASGP receptor is not known. However, utilizing the knockout mouse model employed in this study may enhance the delineation of potential mechanisms. Overall, understanding how impaired receptor-mediated endocytosis leads to liver injury could represent a novel molecular mechanism through which the ASGP receptor protects against liver injury. This new line of inquiry may provide therapeutic leads for protection and possibly treatment of liver disease.

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