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TOPIC HIGHLIGHT

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Impact of asialoglycoprotein receptor deficiency on the development of liver injury

Serene ML Lee, Carol A Casey, Benita L McVicker

Serene ML Lee, Departments of Internal Medicine and Biochemistry & Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68105, United States
Carol A Casey, Benita L McVicker, Liver Study Unit, Department of Veterans Affairs Medical Center, and Departments of Internal Medicine and Biochemistry & Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68105, United States
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Supported by The National Institute on Alcohol Abuse and Alcoholism and by the Department of Veterans Affairs
Correspondence to: Carol A Casey, PhD, Veterans Affairs Medical Center, Research Service (151), 4101 Woolworth Avenue, Omaha, NE 68105, United States. ccasey@unmc.edu
Telephone: +1-402-9953737 Fax: +1-402-4490604
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Abstract

The asialoglycoprotein (ASGP) receptor is a well-characterized hepatic receptor that is recycled *via* the common cellular process of receptor-mediated endocytosis (RME). The RME process plays an integral part in the proper trafficking and routing of receptors and ligands in the healthy cell. Thus, the mis-sorting or altered transport of proteins during RME is thought to play a role in several diseases associated with hepatocyte and liver dysfunction. Previously, we examined in detail alterations that occur in hepatocellular RME and associated receptor functions as a result of one particular liver injury, alcoholic liver disease (ALD). The studies revealed profound ethanol-mediated impairments to the ASGP receptor and the RME process, indicating the importance of this receptor and the maintenance of proper endocytic events in normal tissue. To further clarify these observations, studies were performed utilizing knockout mice (lacking a functional ASGP receptor) to which were administered several liver toxicants. In addition to alcohol, we examined the effects following administration of anti-Fas (CD95) antibody, carbon tetrachloride (CCl_4) and lipopolysaccharide (LPS)/galactosamine. The results of these studies demonstrated that the knockout mice sustained enhanced liver injury in response to all of the treatments, as shown by increased indices of liver damage, such as enhancement of serum enzyme levels, histopathological scores, as well as hepatocellular death. Overall, the work completed to date suggests a possible link between hepatic receptors and liver injury. In particular, adequate function and content of the ASGP receptor may provide protection against various toxin-mediated liver diseases.

Key words: Asialoglycoprotein receptor; Asialoglycoprotein receptor deficient mice; Receptor-mediated endocytosis; Alcohol; Carbon tetrachloride; Anti-Fas; Lipopolysaccharide/galactosamine; Toxicant-induced liver injury

Peer reviewer: Dr. Robin Hughes, Institute of Liver Studies, King's College London School of Medicine, Bessemer Road, London, SE5 9PJ, United Kingdom

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THE ASIALOGLYCOPROTEIN RECEPTOR AND ITS POTENTIAL ROLE IN LIVER INJURY

The asialoglycoprotein (ASGP) receptor, also termed the hepatic binding protein or the Ashwell receptor, was discovered nearly four decades ago by Ashwell and Morell, and was described as a hepatocellular surface carbohydrate that binds glycoproteins lacking terminal sialic acid residues (asialoglycoproteins)[1,2]. Subsequently, many studies have contributed to the detailed characterization of the ASGP receptor, describing its functional role in the binding, internalization and transport of a wide range of glycoproteins, which have exposed galactose or N-acetylgalactosamine residues, *via* the process of receptor-mediated endocytosis (RME)[3-6]. However, translating altered ASGP receptor function and its altered clearance of serum glycoproteins to disease states remains a topic of current

research efforts. This ongoing interest is fueled by the knowledge that the ASGP receptor can bind a variety of important plasma proteins that include transport proteins (i.e. transferrin)[7], enzymes such as alkaline phosphatase[8], immunoglobulins including IgA[9], apoptotic hepatocytes[10,11], fibronectin[12] and platelets[13]. Additionally, the expression of the ASGP receptor has been clinically correlated to the level of hepatic function that is lost during liver diseases related to cancer, viral hepatitis, and cirrhosis[14,15]. Overall, the quest to identify and understand the physiological role(s) of the ASGP receptor, and the consequences that may result from alterations in the function and/or expression of this abundant hepatocellular binding protein, continues.

In search of the physiological roles of the ASGP receptor, our lab initially concentrated on characterizing the role of the ASGP receptor and RME events during a serious and common form of liver injury, alcoholic liver disease (ALD). Alcoholism, and resultant ALD, are indeed significant biomedical problems. Specifically, recent data has noted that chronic liver disease and cirrhosis was the 12th leading cause of death in the United States in the year 2005, and that out of those deaths, approximately 47% of them were due to ALD[16]. Therefore, defining potential contributing mechanisms (such as altered protein trafficking and impaired hepatic receptor functions) may aid in the elucidation of potential therapeutic treatments for ALD. In that effort, our laboratory has extensively studied the RME process and parameters of the ASGP receptor following the administration of ethanol to rodents.

The ASGP receptor consists of major and minor subunits, which in the rat were identified as rat hepatic lectin (RHL) 1 and RHL 2/3, that have respective molecular weights of 42, 49 and 54 kDa[17]. The selective binding and uptake of terminal galactosyl bearing proteins requires the formation of hetero-oligomers between these major and minor forms, and that binding activity was calcium and pH dependent[2,5,18]. Also, the subcellular distribution of the receptor revealed that approximately one-third of the total ASGP receptor pool was associated with the plasma membrane located on the basolateral surface of the hepatocyte[19]. Additionally, it was shown that the total ASGP receptor population consisted of two functionally distinct receptor populations, designated State 1 and State 2, which were involved in the endocytosis and intracellular processing of ligands by different pathways[20-22].

Utilizing these known properties, we studied the effects of ethanol on the ASGP receptor itself, as well as endocytic processes, using isolated hepatocytes, whole liver sections, and perfused livers obtained from rats voluntarily fed an ethanol containing diet over a time course of administration. In summary, differential effects were observed over the time course of treatment in the ability of ethanol and resultant metabolites to affect the ASGP receptor and RME events. Specifically, after early periods of ethanol feeding (1-2 wk), we found that the observed decrease in ligand binding capacity of the ASGP receptor could be attributed to inactivation and redistribution of the receptor[23]. However, after more chronic ethanol administration (5-8 wk), the functional alterations of the receptor were found to be reflective of reductions in the content, synthesis, and mRNA expression of the receptor[23]. Also, it was determined that ethanol treatment caused equal inactivation of both State 1 and State 2 receptors, suggesting that ethanol may be unique compared to other agents (e.g. monensin, vanadate, and chloroquine) that are known to inflict post-translational modifications, such as acylation, selectively to just the State 2 population[24]. In other studies, it was revealed that the ASGP receptor was hyperphosphorylated over the time course of treatment, which could contribute to the aberrant activity of the receptor by disrupting the phosphorylation/dephosphorylation state associated with normal recycling of the receptor[25]. We were also able to demonstrate that the ASGP receptor is involved in the recognition and uptake of apoptotic cells and that this process was significantly altered in hepatocytes obtained from ethanol fed rats[11]. Overall, the results from these studies revealed that ethanol administration impairs multiple aspects of RME by the hepatic ASGP receptor, such that binding, internalization and degradation of ligands internalized by the receptor were found to be significantly altered. Additionally, it was shown that these defects are associated with alterations in the ASGP receptor's physiologically relevant role of clearing apoptotic cells. Taken together, our findings have important implications for the pathogenesis of alcoholic liver injury and potentially for other forms of liver diseases in which RME is profoundly affected. In more recent studies, a mouse model lacking the ASGP receptor was used to gain a better understanding of the associations that may exist between alterations in receptor function and the generation of pathological liver injury.

THE ASGP RECEPTOR-DEFICIENT MOUSE MODEL

The ASGP receptor in mice is a hetero-oligomeric receptor composed of 2 subunits that are both required for its function. These subunits have been named murine hepatic lectin (MHL), with the major subunit called MHL-1 and the minor subunit called MHL-2[26]. ASGP receptor-deficient (RD) mice have a complete lack of the MHL-2 protein and were generated by homologous recombination with a gene replacement vector in embryonic stem cells[27]. MHL-2 appears to be required for the post-translational stability of MHL-1, as these mice have substantially reduced protein content of MHL-1, even though MHL-1 mRNA expression remains the same[27]. Although the MHL-1 protein is still detected in low levels in the RD mice, these levels are unable to induce a measurable clearance of 125I-labeled asialo-orosomucoid[27]. Despite lacking functional ASGP receptors, these knockout mice remain viable and fertile, and appear to have a normal lifespan. In addition, these mice do not display any obvious phenotypic abnormalities[27,28].

As previously mentioned, we have found that chronic alcohol administration markedly decreased mRNA expression and content of the ASGP receptor

in rats prior to the appearance of pathology such as fibrosis[23,29]. Thus, it was felt that the RD mice might provide a powerful tool to examine the role of the ASGP receptor and help delineate pathways by which liver injury occurs in general, as well as during alcoholic liver injury. Currently, we are utilizing the knockout mouse model to examine the link between ASGP receptor function and liver injury, in the context of various models of toxic liver injury such as alcohol, anti-Fas, carbon tetrachloride (CCl_4) and lipopolysaccharide (LPS)/galactosamine. In this report, we present a brief overview of our findings to date.

MODELS OF LIVER INJURY AND THEIR EFFECTS ON ASGP RECEPTOR-DEFICIENT MICE

Alcohol

Alcohol-induced liver injury has previously been found to be related to several events, including ethanol metabolism (*via* alcohol dehydrogenase[30-33]), generation of reactive oxygen species (*via* cytochrome isoforms such as CYP2E1[34-36]), interaction of other liver products (such as cytokines[37,38]) and the induction of apoptosis through the Fas death receptor system[39]. In the search for cellular signaling and mechanisms resulting as a consequence of these events, studies were performed that examined the effects of ethanol on hepatocellular protein trafficking, particularly the process of RME utilizing the hepatic ASGP receptor.

As mentioned previously, we examined the effects of ethanol administration using a rat model exclusively; the rats showed decreased ligand binding, internalization and degradation of several ligands including asialo-orosomucoid, which are processed by RME[4,23,40-43]. In order to assess the effect of ethanol administration on RME by the ASGP receptor using a mouse model, we obtained wild-type (WT) mice possessing abundant ASGP receptor activity and ASGP receptor-deficient (RD) mice lacking MHL-2 from the Jackson Laboratories (Bar Harbor, ME). The mice were fed a Lieber de-Carli liquid diet (with or without 5% by volume ethanol) for ten days[44]. When hepatocytes from these mice were incubated with 125I-ASOR (a representative ligand for the ASGP receptor), WT mice showed ethanol-induced alterations that were consistent with our observations for rats, with an approximately 50% decrease in ligand binding, internalization and degradation in isolated hepatocytes[4,23,44]. However, binding, internalization and degradation of the ligand by RD hepatocytes was negligible, regardless of diet[44]. In addition, the presence of apoptotic bodies was found to be approximately three-fold higher in the livers of RD mice compared to WT mice, irrespective of diet[44]. As a result of this work, it is hypothesized that a potential consequence of altered ASGP receptor function is impaired clearance of ethanol-generated apoptotic cells, resulting in the observed accumulation of apoptotic bodies. Furthermore, other work has shown that these bodies have the potential to promote a variety of responses within the liver, such as the activation of Kupffer cells and the subsequent release of proinflammatory and profibrogenic substances, leading to the enhanced susceptibility to hepatocellular damage that is observed following ethanol administration[11,45].

Anti-Fas

Anti-Fas is an antibody that specifically recognizes and works as an agonist of the Fas antigen[46]. Fas is a member of the TNF receptor superfamily and is a key mediator of apoptosis[47]. This receptor is found in hepatocytes, cholangiocytes, sinusoidal endothelial cells, stellate cells and Kupffer cells[48]. Ligation of Fas results in the recruitment of adaptor proteins, such as Fas-associated death domain (FADD) and procaspase 8, to form the death-inducing signaling complex (DISC)[47]. Caspase 8 can then either directly or indirectly cleave procaspase 3 to mediate apoptosis[47]. A variety of studies have shown that the injection of anti-Fas into mice causes widespread apoptosis and ultimately results in focal hemorrhage and hepatocyte necrosis, making Fas injection a model for fulminant hepatic failure[46,49,50].

From studies related to Fas-mediated cell death in our laboratory, we have shown that the metabolism of ethanol in WIF-B cells (hepatoma hybrid cells) was involved in enhanced Fas protein localization to the membrane, leading to increased activity of the upstream initiator caspases (caspase 2 and caspase 8) and the subsequent downstream activation of caspase 3[39]. As an extension to these studies, aimed to characterize the role of Fas-mediated death in injured hepatocytes, anti-Fas (0.1 or 0.2 μg/g body weight) was injected intraperitoneally into WT and RD mice, which were monitored for up to 48 h[51]. Receptor-deficient mice showed an enhancement of liver injury with higher aspartate transaminase (AST) and alanine transaminase (ALT) activities in the serum compared to the enzyme levels detected in WT mice[51]. Similarly, pathology showed that the RD mice had increased steatosis, inflammation and necrosis compared to the WT mice[51]. As expected, caspase 3 activities were found to be increased 5- to 6-fold in WT mice at 2 h and 16 h after anti-Fas injection, with caspase activities returning to baseline levels by 24 h[51]. However, the activity of caspase 3 remained elevated in the RD livers at all times following treatment and was significantly enhanced over the WT livers at 24 h and 48 h post anti-Fas injection[51]. Overall, the livers of the RD mice were found to be more susceptible than the livers of the WT mice to anti-Fas injection; showing greater apoptosis and increased ECM deposition of collagen and fibronectin[51].

Carbon tetrachloride

Another agent used to study liver injury is carbon tetrachloride (CCl_4), which can cause liver damage through a number of mechanisms. Carbon tetrachloride is metabolized through the action of the mixed function cytochrome P450 system of the endoplasmic reticulum to form the trichloromethyl free radical (CCl_3•), which can subsequently be converted to the trichloromethyl peroxy radical (CCl_3OO•) in the presence of oxygen[52,53].

These free radicals are highly reactive and can bind covalently to cellular macromolecules forming nucleic acid, protein and lipid adducts. When these radicals attack the polyunsaturated fatty acids of the cellular membranes, the fatty acid free radicals generated initiate autocatalytic lipid peroxidation, ultimately resulting in the loss of membrane integrity[52,53]. Carbon tetrachloride can also induce cellular hypomethylation, leading to inhibition of protein synthesis (possibly through ribosomal RNA hypomethylation) and defects in lipid and lipoprotein metabolism[53]. Finally, CCl_4 also affects hepatocellular calcium homeostasis, either by disrupting membrane integrity or by opening certain membrane calcium channels. High levels of Ca^{2+} in the cell can then activate Ca^{2+}-responsive enzymes such as proteases, endonucleases and phospholipases and lead to cell death *via* apoptosis and necrosis[52,53]. The consequences of CCl_4 toxicity include centrilobular steatosis, inflammation, apoptosis and necrosis[52-54].

In our studies, WT and RD mice were injected with CCl_4 (1 mL/kg body weight) and monitored up to a week after injection[55]. Carbon tetrachloride injection caused greater liver injury in the RD mice, as evidenced by the RD mice having increased AST and ALT activities in the serum, compared to the WT mice 48 h post CCl_4 injection[55]. Histologically, centrilobular liver damage was observed in WT mice by 48 h after injection[55]. At this time point, RD mice had more severe damage, showing a greater number of neutrophilic inflammatory infiltrates[55]. In order to elucidate the mechanisms by which this damage is caused, malondia dehyde (MDA), deposition of α-smooth muscle actin (α-SMA) and the percentage of TUNEL-positive hepatocytes was measured[55]. Levels of MDA in the WT mice were not significantly increased throughout the course of the experiment[55]. In contrast, RD mice showed increased contents of MDA as early as 24 h post CCl_4 injection and these levels were maintained up to 48 h[55]. α-SMA was increased significantly in both the WT and RD mice, with the RD mice having a more prolonged increase (between 48 h to 72 h) than the WT mice (only at 72 h)[55]. In addition, α-SMA content was approximately 2-fold of that in the WT liver at 48 h, 96 h and 7 d following injection[55]. Finally, RD mice had significantly more TUNEL-positive hepatocytes than the WT mice at 48 h post injection (2.4-fold more)[55]. This suggests that the absence of functional ASGP receptor resulted in increased lipid peroxidation, perturbations in ECM turnover and increased apoptosis[55].

LPS/galactosamine

Lipopolysaccharide (endotoxin) and galactosamine can be used either alone or in combination with each other to cause liver injury in mice. The metabolism of galactosamine leads to hepatotoxicity by depleting uridine nucleotides and UDP-hexoses and concurrently increasing UDP-hexosamines, primarily in hepatocytes[56,57]. The depletion of uridine nucleotides (as mentioned above) results in an inhibition of RNA and protein synthesis[58]. It has also been suggested that metabolism of galactosamine results in an impaired biosynthesis of macromolecular cell constituents[56]. This impairment leads to plasma membrane injury, which results in an influx of calcium ions and a commitment to cell death[58]. In rats, galactosamine leads to an inflammatory infiltrate of polymorphonuclear leukocytes and lymphocytes and foci of hepatocellular necrosis, resembling the effects of human viral hepatitis[57,59].

Mice and rats are relatively resistant to the lethal effects of LPS[60]. Thus, LPS is injected in concert with galactosamine for a model of fulminant hepatitis[61]. It is thought that galactosamine-induced suppression of RNA synthesis leads to an increased tumor necrosis factor (TNF) production by macrophages, resulting in an increased susceptibility to LPS[60-62]. Tumor necrosis factor was proposed as the agent responsible for the lethality, because lethality was retained by substituting LPS with TNF and was inhibited by anti-TNF antibody[63,64]. Thus, in LPS/galactosamine injection, apoptosis induced by TNF occurs initially and is followed subsequently by necrosis[65].

In our studies, a sub-lethal dose of LPS (50 μg/kg body weight) combined with galactosamine (350 mg/kg body weight) was injected into WT and RD mice *via* the intraperitoneal route and the mice were monitored up to 4.5 h[66,67]. After LPS/galactosamine injection, WT mice maintained normal liver lobular architecture[66]. However, RD mice showed considerable liver injury with areas of portal inflammation, hepatocellular necrosis, increased inflammatory cell infiltration and hemorrhage[66]. These histological observations were further corroborated by the RD mice having increased serum AST and ALT activities at 4.5 h after LPS/galactosamine injection, which were not observed in the WT mice[66]. Also, RD mice showed increased apoptosis, having significantly enhanced caspase 3 activities and TUNEL-positive cells at 4.5 h post injection, whilst there were no changes measured in WT mice[66]. Additionally, the serum content of the pro-inflammatory cytokine interleukin 6 (IL-6) was increased in RD mice compared to WT mice at 3 and 4.5 h after LPS/galactosamine injection[67]. Overall, the results demonstrate that the RD mice are more susceptible to the development fulminant liver injury as a result of sub-lethal treatment with LPS/galactosamine[66,67]. Given that the induction of apoptosis is a consequence of LPS/galactosamine treatment, the enhanced susceptibility to liver damage observed in the RD mice may be related to the inability of hepatocytes to phagocytose and clear the dying apoptotic cells *via* the ASGP receptor.

THE LINK BETWEEN FUNCTIONAL ASGP RECEPTOR AND LIVER INJURY

After the administration of the four toxicants mentioned above (alcohol, anti-Fas, CCl_4 and LPS/galactosamine), RD mice consistently sustained greater liver injury than WT mice, as evidenced by increased indices of liver damage (serum AST and ALT activities) and worse pathology (light microscopy). These four agents of liver injury cause damage through different biochemical pathways or signaling cascades. Therefore, it appears that proper functioning of the ASGP receptor may provide

universal protection against liver injury from these toxicants and possibly others. Although it is not known exactly how an adequately functioning receptor can protect the hepatocyte, the use of the knockout mice treated with these four toxicants highlight some of the possible mechanisms.

It appears that with all four toxicants, caspase 3 or TUNEL-positive cells are increased. Apoptosis is a highly regulated mode of cell death that helps to maintain tissue homeostasis in a healthy organ[68]. However, it appears that when apoptotic death factors are inappropriately expressed due to the introduction of pathological stimuli, such as the four toxicants, apoptosis becomes one of the common pathways by which liver injury is caused. Increased accumulation of apoptotic cells has been shown to occur during alcohol-induced liver injury in a variety of species, including humans, and is thought to play an important role in the progression of liver injury[69-75]. During the process of forming apoptotic cells, glycoconjugates on the cell surface losing their sialic acid masks the increase[10,76]. Since the ASGP receptor binds to desialylated proteins, the receptor recognizes and binds the altered glycans on apoptotic bodies, resulting in phagocytosis and efficient removal of the dying cells[11]. Thus, we speculate that the ASGP receptor exerts protection by removing apoptotic bodies in a timely fashion.

Another way that the ASGP receptor might be protective is through its role in regulating turnover of ECM. Anti-Fas and CCl_4 treatments lead to increased deposition of collagen, fibronectin or α-SMA in the RD mice in comparison to the WT mice. The ASGP receptor has a direct link to cellular fibronectin clearance because cellular fibronectin displays terminal galactose residues, making it a ligand of the ASGP receptor[12]. Cellular fibronectin is one of the first ECM proteins that accumulates during fibrosis[77-79] and thus impairments of ASGP receptor function could lead to increased ECM deposition and hence lead to fibrosis and cirrhosis.

Two additional ways that liver injury could be mediated is through increased lipid peroxidation (MDA content) or by increased contents of pro-inflammatory cytokines, such as IL-6. At present, it is not known how ASGP receptor function is related to perturbations in levels of these two substances. In addition, there may be dysregulation of other asialoglycoproteins not examined at this point. The total absence of ASGP receptor function does not result in a measurable increase in the steady state concentrations of galactose-terminating glycoproteins in the plasma of knockout mice[28]. Thus, the ASGP receptor is unlikely to be involved in the normal turnover of serum glycoproteins. However, in toxicant-induced injuries, acute surges of asialoglycoproteins may overwhelm the alternative galactose recognition systems[28]. Thus, the ASGP receptor might function to prevent an acute increase in potentially harmful asialoglycoproteins. Altogether, the ASGP receptor knockout mouse model provides an excellent tool to elucidate the relationship between ASGP receptor function and liver injury.

Table 1 Changes in ALT activity, TUNEL positive cells, AST activity, caspase 3 activity, collagen content, IL-6 content, MDA content and α-SMA content in RD mice compared to WT mice

Challenge	RD mice compared to WT mice
Alcohol	Liver TUNEL-positive hepatocytes
Anti-Fas	Liver TUNEL-positive hepatocytes
	Serum ALT
	Serum AST
	Liver caspase 3 activity
	Collagen deposition in extracellular matrix
	Fibronectin deposition in the extracellular matrix
CCl_4	Liver TUNEL-positive hepatocytes
	Serum ALT
	Serum AST
	Liver MDA content
	Liver α-SMA content
LPS/galactosamine	Liver TUNEL-positive hepatocytes
	Serum ALT
	Serum AST
	Liver caspase 3 activity
	Serum IL-6 content

ALT: Alanine transaminase; AST: Aspartate transaminase; IL-6: Interleukin 6; MDA: Malondialdehyde; α-SMA: α-smooth muscle actin; RD: Receptor-deficient; WT: Wild-type. The various conditions the mice were challenged with were alcohol (Lieber de-Carli ethanol diet for 7 d), anti-Fas (0.2 μg/g body weight), CCl_4 (1 mL/kg body weight) and LPS/galactosamine (50 μg LPS/kg body weight and 350 mg galactosamine/kg body weight).

CONCLUSION

The ASGP receptor is an abundant hepatic receptor that recognizes desialylated ligands. After binding to its ligand, the receptor internalizes and facilitates transport of specific ligands by the process of receptor-mediated endocytosis. Previously, studies have shown that ASGP receptor function is impaired in disease states such as alcoholic liver disease. This gave us the impetus to examine if proper ASGP receptor function offers protection against liver injury or if defects in function occurred as a result of liver damage. To examine this, we utilized a knockout mouse model, which lacked functional ASGP receptor in comparison to wild-type animals in the context of various toxic challenges (alcohol, anti-Fas, CCl_4 and LPS/galactosamine). After all four challenges, the receptor-deficient mice consistently showed more liver injury than the wild-type animals (Table 1), proving that ASGP receptor function is protective. Thus, these studies highlight receptor-mediated endocytosis as a novel mechanism that may be involved in the induction of toxin-induced liver injury. At present, the precise nature of the specific ligands involved or the pathways that lead to further injury have not been determined. However, in the studies reviewed here, it is likely that impaired clearance of apoptotic bodies, perturbations in extracellular matrix deposition, oxidative stress, and cytokine dysregulation may play roles in the progression of disease. In the future, further clarification of the pathways by which liver injury occurs (including altered ASGP receptor-mediated endocytosis) will provide new therapeutic leads.

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