Asialoglycoprotein Receptor Deficiency in Mice Lacking the Major Receptor Subunit

ITS OBLIGATE REQUIREMENT FOR THE STABLE EXPRESSION OF OLIGOMERIC RECEPTOR*

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The asialoglycoprotein receptor is an abundant hetero-oligomeric endocytic receptor that is predominantly expressed on the sinusoidal surface of the hepatocytes. A number of physiological and pathophysiological functions have been ascribed to this hepatic lectin (HL), the removal of desialylated serum glycoproteins and apoptotic cells, clearance of lipoproteins, and the sites of entry for hepatotropic viruses. The assembly of two homologous subunits, HL-1 and HL-2, is required to form functional, high affinity receptors on the cell surface. However, the importance of the individual subunits for receptor transport to the cell surface is controversial. We have previously generated HL-2-deficient mice and showed that the expression of HL-1 was significantly reduced, and the functional activity as the asialoglycoprotein receptor was virtually eliminated. However, we failed to detect phenotypic abnormalities. To explore the significance of the major HL-1 subunit for receptor expression and function in vivo, we have disrupted the HL-1 gene in mice. Homozygous HL-1-deficient animals are superficially normal. HL-2 expression in the liver is virtually abrogated, indicating that HL-1 is strictly required for the stable expression of HL-2. Although these mice are almost unable to clear asialo-orosomucoid, a high affinity ligand for asialoglycoprotein receptor, they do not accumulate desialylated glycoproteins or lipoproteins in the plasma.

The asialoglycoprotein receptor (ASGPR)† was originally identified by Ashwell and Morell as a hepatic receptor that mediates the rapid clearance of serum glycoproteins containing terminal galactose residues from the circulation (see Refs. 1–3 for review). ASGPR is abundantly expressed on the sinusoidal surface of the parenchymal cells of the liver. Its primary physiological function has been considered to be the removal and degradation of desialylated circulating proteins.

Nonreducing terminal of oligosaccharide moieties of glycoproteins are usually capped by sialic acid residues. When the terminal sialic acid residues are removed by neuraminidases, penultimate galactose residues are exposed and recognized by ASGPR. High affinity binding requires the receptor to be assembled as a hetero-oligomer consisting of two highly homologous subunits termed hepatic lectin (HL) 1 and 2 (4). Both subunits contain an N-terminal cytoplasmic domain, a single transmembrane segment, a stalk domain, and a C-terminal carbohydrate recognition domain (5). ASGPR belongs to C-type animal lectins because of the requirement of Ca²⁺ for ligand binding and disulfide bonds in carbohydrate recognition domains (6).

A number of diverse physiological roles have been proposed for ASGPR over the years. Among them, hepatic clearance of the desialylated and senescent serum proteins was most originally proposed (1). ASGPR was also postulated to account for the low density lipoprotein (LDL) receptor-independent clearance of lipoproteins including chylomicron remnants (7, 8). Recently, immunoglobulin A (9) and fibronectin (10) have emerged as likely candidates of natural ligands for ASGPR. The clearance of apoptotic cells or a subpopulation of lymphocytes in the liver has also attributed to ASGPR (See Ref. 3 for review). It is particularly noteworthy that ASGPR has also been proposed to be utilized as entry sites into hepatocytes by several hepatotropic viruses including hepatitis B virus (11), Marburg virus (12), and hepatitis A virus (13).

As an attempt to elucidate the bona fide functions of ASGPR, we have previously generated mice lacking a minor subunit of mouse ASGPR (MHL-2) (14). As a result of disruption of MHL-2, the expression of MHL-1 was severely reduced, and the plasma clearance of asialo-orosomucoid was almost completely abrogated in the MHL-2/− mice. However, the MHL-2/− mice were apparently normal and showed no detectable abnormalities even in the metabolism of remnant lipoproteins. Because MHL-2/− liver expresses small but significant amounts of MHL-1 (14, 15), it is still possible that the residual MHL-1 is sufficient to execute the primary task of ASGPR as suggested by in vitro transfection experiments (16, 17). In the current study we have generated mice lacking the major sub-
unit (MHL-1) of ASGPR in mice and analyzed the resulting phenotypes.

**EXPERIMENTAL PROCEDURES**

**Generation of MHL-1 Knockout Mice**—The MHL-1 gene was cloned from the 129/Sv mouse genomic library. The genomic organization of the MHL-1 gene was essentially the same as recently reported by Soukharev et al. (18). A replacement-type targeting vector was constructed so that the genomic fragment containing exons 2–3, which encoded the ATG initiation codon and transmembrane domain, was replaced by the pol2neo cassette (19). The short arm containing a 0.8-kb StuI/BamHI fragment containing exon 2 and the long arm containing a 9-kb XhoI/StuI fragment spanning exons 3–9 were inserted into the XhoI and NotI sites, respectively, of the vector pPol2short-neobpA-HSVTK as described previously (14, 20).

After linearization by digestion with SalI, the vector was electroporated into JH-1 embryonic stem (ES) cells (a gift from Dr. Herz at the University of Texas Southwestern Medical Center). Targeted clones, which had been selected in the presence of G418 and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5 iodouracil, were identified by polymerase chain reaction using the following primers: 5'-CTGGTCA-GGCATGC-3' and 5'-GATTGGGAAGACAATAGCA-GCCATGC-3' (see Fig. 1). Homologous recombination was verified by Southern blot analysis after digesting the genomic DNA with EcoRI using a 0.7-kbp StuI fragment as a probe (see Fig. 1). Targeted ES clones were injected into the C57BL/6 blastocysts, yielding 14 lines of chimeric mice that transmitted the disrupted allele through the germ line from four independent ES cell clones. All experiments reported here were performed with 129/Sv-C57BL6 hybrid descendants (F1 and subsequent generations) of these animals.

**Northern Blot Analysis**—Total RNA was isolated from the liver by TrizolTM reagent (Life Technologies, Inc.). 20 μg of total RNA was subjected to electrophoresis in an agarose gel and transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech). The probe for Southern blot analysis is indicated by the shaded box (see Fig. 1). Analyses were performed using specific rabbit polyclonal anti-peptide antibodies for MHL-1 and MHL-2 (14). The antibodies were visualized by peroxidase-conjugated anti-rabbit IgG and with an ECL chemiluminescence detection kit (Amersham Pharmacia Biotech).

**Plasma Turnover Experiments**—Asialo-orosomucoid (ASOR) was prepared by incubating 100 mg of orosomucoid (Sigma) at 37 °C in 10 ml of sodium acetate buffer containing 2 μM CaCl₂, pH 5, together with 1 unit of neuraminidase-type XA (Clostridium perfringens) attached to agarose beads (Sigma). After 4 h another unit of enzyme was added, and the incubation was continued overnight. Asialofetuin (Sigma) and ASOR were labeled with 125I using the IODO-GEN procedure (Pierce). Specific activities of 125I-asialofetuin and 125I-ASOR were 257 and 502 cpm/μg, respectively. 10 μg of iodinated protein in 200 μl of saline containing 2 mg/ml bovine serum albumin were injected intravenously into the jugular vein of anesthetized male mice (n = 3) that were wild-type and homozygous for MHL-1 gene disruption. Blood was collected at the indicated intervals from the retroorbital venous plexus. After the labeled proteins in 20 μl of plasma were precipitated with trichloroacetic acid, their radioactivities were determined.

**Analyses of Mouse Plasma Lipoprotein Profile**—Plasma lipoprotein analyses were performed as previously described (21). Briefly, after mice were bled from the retroorbital venous plexus, the blood was collected into tubes containing EDTA. Total cholesterol and triglyceride levels in the plasma were determined enzymatically using kits (Determiner TC555 and Determiner TG555; Kyowa Medex). 5 μl of plasma was diluted to 100 μl with saline and subjected to high performance liquid chromatography (HPLC) analyses using four columns of TSK gel Lipopropak XL (TOSOH, Tokyo, Japan) connected in tandem.

**Lectin Blot Analyses of Plasma Protein**—1 μl of plasma was separated by 3–15% SDS polyacrylamide gel electrophoresis under a reducing condition and transferred to polyvinylidene difluoride membranes. Lectin blotting was done using Maackia amurensis agglutinin (MAA;
Northern blot analysis showed that MHL-1/− mice lacked 1.3-kb wild-type MHL-1 transcript completely (Fig. 2A). MHL-1/+ mice expressed an intermediate amount of the MHL-1 transcript. A reduced amount of truncated MHL-1 transcript was expressed in mice having the disrupted MHL-1 allele (MHL-1+/− or MHL-1/−). On the other hand, there were no differences in the size and amounts of MHL-2 mRNA. The disruption of the MHL-1 gene resulted in complete absence of the 40-kDa-encoded protein in MHL-1/−/− animals (Fig. 2B). MHL-1/+− mice showed reduced expression of MHL-1 protein as compared with the wild-type mice, indicating the gene dosage effect on the expressed protein levels of MHL-1. In wild-type and MHL-1+/− mice, four distinct bands that were immunoreactive with the anti-MHL-2 antibody were observed (36, 47, 80, and 192 kDa). Because the bands with higher molecular mass were not observed under a reducing condition (data not shown), three of these multiple bands may represent oligomers of MHL-2 protein in different degrees (monomer for 36 kDa, dimer for 80 kDa, and tetramer for 192 kDa). No bands immunoreactive with the anti-MHL-2 antibody were visible in MHL-1−/− mice.

To examine whether the disruption of the MHL-1 gene resulted in the impairment of the hepatic clearance of asialoglycoproteins, we injected the iodinated asialofetuin or ASOR into +/+, +/−, and −/− mice and compared the radioactivities remaining in the plasma (Fig. 3). The plasma clearance of 125I-asialofetuin was severely, albeit not completely, impaired in MHL-1−/− mice (Fig. 3A). At 30 min after the injection, plasma 125I-asialofetuin was decreased to 4, 8, and 40% of the initial dose in +/+, +/−, and −/− mice, respectively. Heterozygous mice cleared 125I-asialofetuin with only partially reduced efficiency. The plasma clearance of 125I-ASOR was more severely impaired in MHL-1−/− mice (Fig. 3B). At 30 min after the injection, plasma 125I-ASOR was decreased to 3, 24, and 65% of the initial dose in +/+, +/−, and −/− mice, respectively. Heterozygous mice cleared 125I-ASOR with intermediate efficiency.

To examine the expression of the sialic acid- and galactose-terminated serum glycoproteins, plasma from the wild-type and MHL-1−/− mice were subjected to lectin blot analysis (Fig. 4). The following lectins were used to detect terminal sugars: MAA, a lectin specific for NeuNacα2–3Gal on N-linked carbohydrate; SNA, a lectin specific for NeuNacα2–6Gal on N-linked carbohydrate; and RCA120, a lectin specific for β-galactose. There were no consistent differences either in the pattern of the bands or in their intensities between MHL-1+/+ and MHL-1−/− mice.

To test whether ASGPR is involved in chylomicron remnant clearance by the liver, we analyzed the plasma lipid levels of MHL-1−/− mice and MHL-1−/− mice that also lacked the LDL receptor (Table I). The plasma lipid levels of the MHL-1−/− mice were indistinguishable from those of control animals expressing wild-type MHL-1, in the absence or presence of functional LDL receptors. The lipoprotein profiles evaluated by HPLC analyses failed to reveal any differences in lipoprotein fractions between wild-type and MHL-1−/− mice (Table II).

**RESULTS**

The MHL-1 gene was cloned by hybridization screening of a mouse genomic library using a mouse cDNA probe. A gene replacement vector was constructed so that the initiation codon and transmembrane domain were interrupted by the pol2neo cassette (Fig. 1A). Following electroporation of the linearized targeting vector into JH-1 ES cells, targeted clones were obtained. Chimeric mice were generated from six independently targeted clones using a standard procedure. Four independent ES cell clonal yielded total of 14 germ line chimeric males. They were bred to wild-type female C57BL/6 mice. Heterozygous offspring (F1 generation) were crossed with each other and gave rise to mice wild-type (+/+), heterozygous (+/−), or homozygous (−/−) for the disrupted MHL-1 allele in accordance with Mendelian law (+/+; +/−; −/− = 29:65:43; χ2 = 1.51; p = 0.47) (Fig. 1B). Heterozygous MHL-1-deficient mice were viable and displayed no obvious phenotype under laboratory housing conditions. The animals appeared to have a normal life span.
mice were virtually null for the MHL-1 gene. In MHL-1+/− mice, the amounts of both mRNA and protein of MHL-1 were reduced 2-fold, indicating the gene-dosage effects of the inactivation. This reduction was accompanied by the reduction in the amounts of MHL-2 protein, even if the mRNA levels were not affected. In MHL-1−/− mice, MHL-2 protein was undetectable. These results strongly indicate that MHL-1 is obligatorily required for the stable expression of MHL-2. This is consistent with the in vitro results in transfected cells that the minor subunit is unstable in the absence of coexpression of the major subunit (22–24). Without HL-1, HL-2 may succumb to degradation within endoplasmic reticulum. It is interesting to compare this to MHL-2−/− mice in which substantially reduced but still significant amounts of MHL-1 were expressed. Together these results indicate that both subunits are required for the stable expression of oligomeric receptor and that HL-1 is more strictly required than HL-2.

As was observed in MHL-2−/− mice, the plasma clearance of asialoglycoproteins was severely impaired in MHL-1−/− mice (Fig. 3). It is interesting to note that the impairment of the clearance of ASOR appeared milder than that of asialofetuin in the heterozygotes. This is probably because some other pathway also contributes to the plasma clearance of asialofetuin but not to that of ASOR. For example, a macrophage lectin that is conceivably expressed in the liver (25, 26). The plasma clearance curve of ASOR was indistinguishable from that of orosomucoid (data not shown), suggesting that MHL-1−/− mice clear ASOR via a pathway(s) that is irrelevant to the galactose-recognition system. Although ASGPR function was severely impaired in MHL-1−/− mice, the plasma glycoproteins levels were not significantly increased (Fig. 4). Together with our previous observations in MHL-2 knockout mice, ASGPR is unlikely to be essential for the homeostasis of the major plasma glycoproteins as has been frequently discussed. In support of this, Kido et al. (27) have recently reported that serum glycoprotein levels were maintained in mice lacking β-1,4-galactosyltransferase I. Although nearly 90% of the serum glycoproteins lacked β-1,4-galactose in the knockout mice, their serum protein concentrations were similar to those in wild-type mice. This does not necessarily rule out the possible role of ASGPR in the regulation of minor serum glycoproteins (28). In this context, Rotundo et al. (10) have proposed that ASGPR is responsible for the disposal of cellular fibronectin from the plasma and/or in the
liver. Cellular fibronectin contains large amounts of terminal galactose residues, and intravenous infusion of excess asialofetuin caused retention of labeled cellular fibronectin in the liver (29). However, in multiple experiments not shown here, by Western blot analyses in the liver membrane we failed to find the abnormal accumulation of fibronectin in the liver of MHL-1−/− mice. Furthermore, we failed to find significant morphological changes in the liver of MHL-1−/− mice. With regard to IgA metabolism, Rifai et al. (9) have recently reported the impaired clearance of the IgA2 isofrom in MHL-2−/− mice. However, its physiological relevance is currently unknown.

Windler et al. (7) proposed a possible function of ASGPR in the hepatic lipoproteins metabolism. ApoB-100 and apo(a) are heavily glycosylated giant proteins. Therefore it is reasonable to consider that the lipoproteins containing these apolipoproteins is cleared by ASGPR when the LDL receptor system is dysfunctional. To test this hypothesis, we crossed MHL-1−/− mice to the LDL receptor-deficient mice to generate mice lacking both ASGPR and the LDL receptor. As was the case in the mice lacking both MHL-2 and the LDL receptor, we failed to detect the elevation of plasma lipoproteins as compared with the LDL receptor knockout mice (Tables I and II). Accumulating evidence indicates that LDL receptor-related protein is involved in the plasma clearance of apoE-rich remnant lipoproteins (30). Therefore, the role of ASGPR in the plasma clearance of lipoproteins is minimal if present.

ASGPR is a member of animal C-type lectins (6). Because most of C-type lectins appear to be involved in host defense, ASGPR may have evolved as a molecule to protect mammals from viral or bacterial insults (31), probably because exposed galactose residues may be harmful to the vascular system (32). Avian and reptiles have a similar hepatic lectin that binds to -acetylgalactosamine. The ASGPR system may protect vertebrates from pathogenic organisms that take advantage of neuraminidase to invade the hosts. A significant decrease in the expression of ASGPRaccompanied by accumulation of serum asialoglycoproteins is observed in the patients with advanced liver diseases such as liver cirrhosis (33, 34). They frequently develop serious infectious diseases such as spontaneous bacterial peritonitis and sepsis. It is tempting to speculate that ASGPR is relevant to these complications associated with liver diseases. Besides hepatocytes, macrophages express a lectin specific for both galactose and N-acetylgalactosamine as mentioned above (25, 26). Its presence may have masked the phenotype in ASGPR-deficient animals. Further studies are absolutely needed to prove these possibilities. In this context, it is intriguing that some hepatotropic virus infects the hepatocytes through ASGPR (11–13).

In summary, ASGPR functions were more completely abrogated in MHL-1-deficient mice as compared with in MHL-2-deficient mice. Probably, requirement of HL-1 for stable expression of functional ASGPR is stricter than that of HL-2. Despite such absolute deficiency of ASGPR function, we failed to detect physiological evidence for several postulated functions that have been ascribed to ASGPR. These MHL-1-deficient mice should provide the basis for understanding the physiology of this receptor.

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