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Abstract

In autoimmune chronic active hepatitis (AIH) and primary biliary cirrhosis (PBC), various autoantibodies including anti-asialoglycoprotein receptor (ASGPR) antibodies have been found in patients' sera. We have previously developed a mouse monoclonal antibody against rat and human ASGPR. In this study, we developed a capture enzyme-linked immunosorbent assay (ELISA) for detection of anti-ASGPR antibodies using this monoclonal antibody and investigated the occurrence of anti-ASGPR antibodies in the sera of patients with various liver diseases. Serum samples were obtained from 123 patients with various liver diseases, including 21 patients with AIH and 40 patients with PBC. In this capture ELISA, the target antigen in the crude rat liver membrane extracts was captured on the ELISA wells by the ASGPR-specific mouse monoclonal antibody. Thus, the cumbersome process of antigen purification was rendered unnecessary. Using this capture ELISA, we detected the anti-ASGPR antibody in 67% of the patients with AIH, in 100% of the patients with PBC, and in 57% of the patients with acute hepatitis type A. However, the anti-ASGPR antibody was rarely detected in patients with other liver diseases such as primary sclerosing cholangitis and obstructive jaundice. Our findings suggest that this capture ELISA would be useful for the detection of anti-ASGPR antibodies in autoimmune liver diseases.

KEYWORDS: autoimmue hepatitis, primary biliary cirrhosis, asialoglycoprotein receptor, autoantibodies

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Original Article

Anti-asialoglycoprotein Receptor Autoantibodies, Detected by a Capture-immunoassay, are Associated with Autoimmune Liver Diseases

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In autoimmune chronic active hepatitis (AIH) and primary biliary cirrhosis (PBC), various autoantibodies including anti-asialoglycoprotein receptor (ASGPR) antibodies have been found in patients' sera. We have previously developed a mouse monoclonal antibody against rat and human ASGPR. In this study, we developed a capture enzyme-linked immunosorbent assay (ELISA) for detection of anti-ASGPR antibodies using this monoclonal antibody and investigated the occurrence of anti-ASGPR antibodies in the sera of patients with various liver diseases. Serum samples were obtained from 123 patients with various liver diseases, including 21 patients with AIH and 40 patients with PBC. In this capture ELISA, the target antigen in the crude rat liver membrane extracts was captured on the ELISA wells by the ASGPR-specific mouse monoclonal antibody. Thus, the cumbersome process of antigen purification was rendered unnecessary. Using this capture ELISA, we detected the anti-ASGPR antibody in 67% of the patients with AIH, in 100% of the patients with PBC, and in 57% of the patients with acute hepatitis type A. However, the anti-ASGPR antibody was rarely detected in patients with other liver diseases such as primary sclerosing cholangitis and obstructive jaundice. Our findings suggest that this capture ELISA would be useful for the detection of anti-ASGPR antibodies in autoimmune liver diseases.

Key words: autoimmune hepatitis, primary biliary cirrhosis, asialoglycoprotein receptor, autoantibodies

Autoimmune chronic active hepatitis (AIH) and primary biliary cirrhosis (PBC) are major liver diseases caused by mechanisms related to autoimmune responses. Various autoantibodies to cellular structures such as nucleus, smooth muscle membrane, microsomes, and the asialoglycoprotein receptor (ASGPR) have been found in the sera of patients with AIH and PBC [1-6]. Among them, ASGPR is a transmembrane glycoprotein which mediates binding and internalization of extracellular glycoproteins that have exposed terminal galactose residues [7]. ASGPR is a liver-specific antigen and is present on the sinusoidal surface of hepatocytes, as we have previously shown [8, 9]. A complex antigen preparation referred to as liver-specific protein (LSP) was shown to induce experimental hepatitis in rabbits after long-term immunization [10], and ASGPR has been identified as a component of LSP [11]. The pathogenic relevance of autoimmune responses against ASGPR has

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thus been implicated in the development of AIH and PBC [12]. Thus, an analysis of autoantibody production against ASGPR might facilitate an understanding of the pathogenesis of liver injury in autoimmune-mediated liver diseases.

We have previously developed a mouse monoclonal antibody which recognizes rat and human ASGPR [13–15]. In this study, we developed a capture enzyme-linked immunosorbent assay (ELISA) for the detection of anti-ASGPR antibodies using this monoclonal antibody; we then investigated the occurrence of anti-ASGPR antibodies in serum samples from patients with various liver diseases including AIH and PBC.

Patients and Methods

Patients. Sera were obtained from 123 patients with various liver diseases including 21 patients with AIH and 40 with PBC, 6 patients with various other autoimmune diseases, and 12 healthy volunteers. Profiles of these subjects are summarized in Table 1. AIH was diagnosed according to the international criteria [16]; antinuclear and /or anti-smooth muscle antibody tests were positive in all of the patients with AIH. PBC was diagnosed by standard clinical history, laboratory tests, and histological evaluation of the liver [17, 18]. Antimitochondrial antibody (AMA) tests of the serum of PBC patients, as determined by indirect immunofluorescence, were positive in all of the patients with PBC. Primary sclerosing cholangitis (PSC) was diagnosed by clinical history, laboratory tests, characteristic endoscopic cholangiography and histological evaluation of the liver [19]. Chronic and acute viral hepatitis were diagnosed by clinical history, laboratory tests, viral markers and histological evaluation of the liver. All patients with hepatitis B virus (HBV) were positive for HB surface and e antigens. All patients with hepatitis C virus (HCV) were positive for HCV antibody as assessed by a second-generation enzyme immunoassay and serum HCV RNA by reverse-transcriptase polymerase chain reaction. All patients with acute hepatitis A were positive for IgM antibody to hepatitis A virus. Obstructive jaundice was caused by common bile duct stone (n = 3), pancreas head cancer (n = 2), and bile duct cancer (n = 6). The autoimmune diseases other than liver diseases included Sjogren syndrome (n = 2), systemic lupus erythematosus (n = 3), and Basedow disease (n = 1); all of the patients were positive for anti-nuclear antibodies (speckled patterns) but were negative for AMA. The sera were stored at −20 °C until use. The study was conducted according to the guidelines of the Declaration of Helsinki. The local ethics committee approved the study

Table 1 Patient characteristics

Patient group	No.	Sex (M/F)	Age (yr)		ALT (IU/l)		T Bil (mg/dl)	
			Median	Range	Median	Range	Median	Range
Autoimmune liver diseases								
AIH	21	1/20	54	19–77	129	15–456	1.15	0.7–13.4
PBC	40	0/40	50	35–69	45	10–187	0.8	0.3–16.2
PSC	4	1/3	23	15–29	62	18–112	1.0	0.6–3.2
Viral hepatitis								
AH (A)	7	2/5	30	24–66	419	14–4430	5.9	3.3–22.1
AH (C)	5	2/3	36	26–49	248	72–678	1.4	0.7–6.4
CH (B)	8	1/7	27	11–47	140	41–394	1.1	0.9–1.8
CH (C)	18	8/10	56	34–68	85	37–340	0.9	0.4–1.2
Drug-induced liver injury	9	3/6	44	20–68	81	27–139	0.7	0.3–19.2
Obstructive jaundice	11	3/8	62	53–85	48	15–193	2.8	1.9–20.4
Autoimmune diseases	6	1/5	55	42–69	68	13–168	0.5	0.3–0.9
Healthy controls	12	10/2	31	27–35	23	6–34	0.4	0.2–0.9

AH (A), acute hepatitis type A; AH (C), acute hepatitis type C; AIH, autoimmune hepatitis; ALT, alanine transaminase; CH (B), chronic hepatitis type B; CH (C), chronic hepatitis type C; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; T Bil, total bilirubin.

protocol. Informed consent was obtained from each patient.

Antibodies. A monoclonal antibody to rat ASGPR (clone 8D7, IgG1) was prepared as previously described [13, 14] and purified from ascitic fluids of pristane-treated BALB/C mice bearing 8D7 hybridoma, using protein G-agarose gel affinity chromatography (MAb Trap Kit; Amersham Pharmacia Biotech, Buckinghamshire, England). Preliminary experiments showed that the 8D7 anti-ASGPR antibody reacted with COS-1 cells transfected with the cDNA of rat ASGPR subunit RHL-1 but not with RHL-2/3 (manuscript in preparation). Control normal mouse IgG1 (DAKO Japan, Tokyo, Japan) and horseradish peroxidase (HRP)-conjugated affinity purified goat $F(ab')_2$ anti-human immunoglobulins (absorbed with mouse serum) were purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD, USA).

Capture ELISA procedure. Rat liver membrane extracts (LME) were prepared from homogenates of Sprague-Dawley rats of both sexes weighing 150–250 g, as described [9]. Briefly, rat liver homogenates were centrifuged at 100,000 g for 90 min at 4 °C, and the pellets were treated with cold acetone at −20 °C. The precipitates were solubilized with 1% Triton X-100 at 4 °C. As a control, rat kidney membrane extracts (KME) were prepared from rat kidney acetone powder (Sigma, St. Louis, MO, USA), as described above.

For the detection of anti-ASGPR antibodies, the following assay procedure was used (Fig. 1). Microtiter wells (Immuno Plate II; Nunc, Denmark) were incubated with 100 μl of 8D7 IgG1 monoclonal antibody (10 μg/ml in PBS, pH 7.2) or control mouse IgG1 at 4 °C overnight and were blocked with PBS containing 1% bovine serum albumin (BSA). After washing, 100 μl of LME solution (10 μg/ml in PBS containing 1% BSA and 0.05% Tween 20), KME solution, or buffer alone were incubated in the wells at 4 °C overnight. After washing, 100 μl aliquots of serum diluted 1:10,000 in PBS containing 1% BSA and 0.05% Tween 20 were incubated at 4 °C overnight, followed by washing and incubation with HRP-conjugated goat $F(ab')_2$ anti-human immunoglobulins. After washing, bound peroxidase activity was visualized by reaction with 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and hydrogen peroxide. The optical density (OD) at 415 nm was measured using a microplate reader. Net OD values were calculated by subtraction of the value without LME from the value obtained from wells incubated with LME. The anti-ASGPR index was expressed as follows: (net OD value of sample minus net OD of negative standard) / (net OD of positive standard minus net OD of negative standard). The positive standard was an arbitrarily selected serum sample of a patient with PBC. The negative standard was a serum sample from a healthy control. All samples were tested in duplicate. The data were analyzed by one-way analysis of variance and a post-hoc Scheffe test.

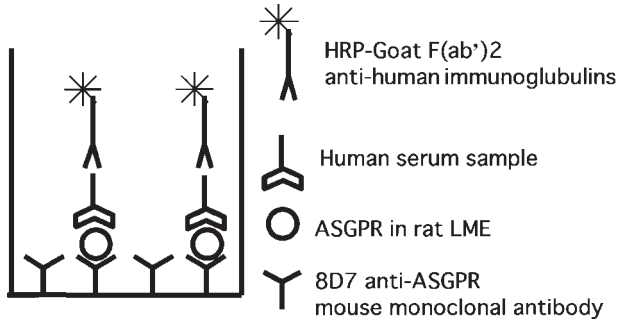

Fig. 1 A capture ELISA for the detection of anti-ASGPR antibody. The graphic depicts the configuration of the assay system.

Immunoblot analysis. LME (10 μg/lane) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were electrophoretically transferred onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was cut into strips. Each strip was blocked with 5% skim milk and then reacted with diluted patients' sera (1:1,000) or 8D7 mouse monoclonal anti-ASGPR antibody at 4 °C overnight. After washing, the strips were developed with a Vectastain ABC Human Kit (Vector Laboratories Inc., Burlingame, CA, USA) for the human sera and with a Mouse IgG Kit for 8D7 monoclonal antibody, according to the manufacturers' protocols.

Results

The distribution of the anti-ASGPR index in patients with various liver diseases is shown in Fig. 2. The anti-ASGPR indices of patients with AIH and PBC were 0.21 ± 0.06 (mean $\pm$ SE) and 0.69 ± 0.05, respectively. The anti-ASGPR indices of patients with PBC were significantly higher than those of patients with other liver diseases as well as those of healthy controls ($P <$

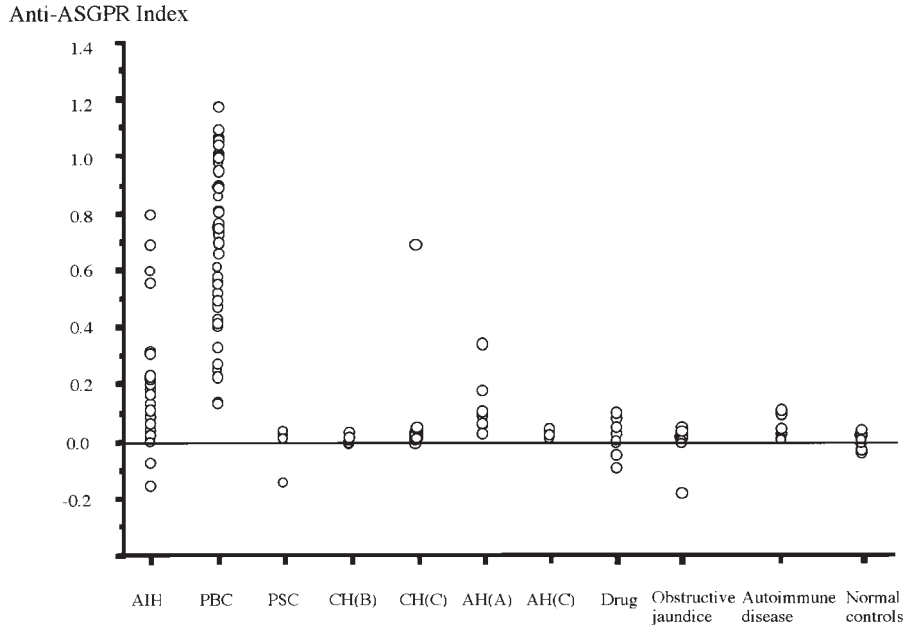

Fig. 2 Distribution of the anti-ASGPR antibody of patients with various liver diseases, autoimmune diseases and healthy controls. Titers of the anti-ASGPR antibody are expressed as the anti-ASGPR index as described in the text. AH (A), acute hepatitis type A; AH (C), acute hepatitis type C; AIH, autoimmune hepatitis; CH (B), chronic hepatitis type B; CH (C), chronic hepatitis type C; Drug, drug-induced liver injury; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

Table 2 Absorbance readings (OD415 nm) obtained from wells incubated with LME or with KME instead of LME

Disease	Crude antigen	Concentrations of LME and KME (μg/ml)			
		10	5	2.5	1.25
AIH					
Patient 1	LME	0.555	0.542	0.320	0.255
	KME	0.234	0.163	0.179	0.189
Patient 2	LME	0.634	0.483	0.355	0.277
	KME	0.188	0.181	0.141	0.177
PBC					
Patient 1	LME	0.927	0.646	0.522	0.306
	KME	0.181	0.175	0.119	0.135
Patient 2	LME	0.638	0.497	0.408	0.302
	KME	0.290	0.204	0.207	0.199
Patient 3	LME	0.678	0.469	0.265	0.168
	KME	0.063	0.063	0.067	0.066

Microtiter wells were coated with 8D7 anti-ASGPR antibody, and 100 μl of LME solution of designated concentrations or KME solution were incubated. The serum samples from 2 patients with AIH and 3 patients with PBC were used. OD values presented are means of duplicate samples.

0.0001). When we used KME instead of LME, the antibody activity was greatly diminished (Table 2). Several immunoblot analysis bands, including a band with an approximate molecular weight of 45 kilodaltons corresponding to rat ASGPR [20, 21], were detected with sera from patients with AIH and PBC (Fig. 3).

When we arbitrarily defined a cut-off value as the mean of the anti-ASGPR index of the healthy controls plus 3 SD, the anti-ASGPR antibody was positive in 67% of the patients with AIH, in 100% of the patients with PBC, and in 57% of the patients with acute hepatitis type A; however, it was rarely detected in the patients with other liver diseases (Table 3). The anti-ASGPR antibody was detected in some patients ($n = 2$) with other autoimmune diseases such as systemic lupus erythematosus.

Discussion

In this study, we developed a capture ELISA to detect anti-ASGPR antibodies. The target antigen in LME was captured on ELISA wells by 8D7 ASGPR-specific mouse monoclonal antibody, thus rendering the cumbersome process of antigen purification unnecessary.

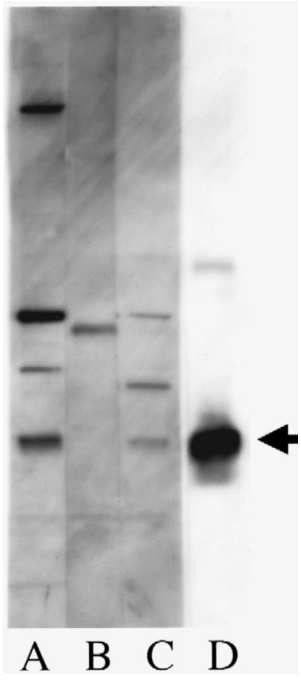

Fig. 3 Representative results of immunoblot analysis of LME against sera from patients with AIH and PBC. LME was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were electrophoretically transferred onto an Immobilon-P membrane. The membrane was reacted with sera from a patient with AIH whose anti-ASGPR index was 0.59 (**A**) and a patient with PBC whose anti-ASGPR index was 1.05 (**C**), serum from a healthy control (**B**), and 8D7 anti-ASGPR antibody (**D**). An arrow indicates a band of ASGPR with an approximate molecular weight of 45 kilodaltons.

Table 3 Occurrence of anti-ASGPR antibody

Patient group	Positive/Total	Occurrence (%)
Autoimmune liver diseases		
AIH	14/21	67
PBC	40/40	100
PSC	0/4	0
Viral hepatitis		
AH (A)	4/7	57
AH (C)	0/5	0
CH (B)	0/8	0
CH (C)	1/18	6
Drug-induced liver injury	2/9	22
Obstructive jaundice	0/11	0
Autoimmune diseases	2/6	33
Healthy controls	0/12	0

A cut-off value was arbitrarily defined as a mean of the anti-ASGPR index of the healthy controls plus 3 SD.

Using this capture ELISA, we observed a high prevalence of the anti-ASGPR antibody in the sera of patients with AIH and PBC. The anti-ASGPR antibody was detected in the patients' sera diluted to 1:10,000 in this study. However, the reported working dilutions of sera for the detection of the anti-ASGPR antibodies are 1:100 to 1:800 [5, 22], which suggests that our capture ELISA might have been more sensitive than the previously reported methods.

In our assay system, we captured rat ASGPR on an ELISA plate and detected antibodies to rat ASGPR. It was shown that autoantibodies against ASGPR in the sera of AIH patients reacted with rat, rabbit, and human ASGPR, but that the reactivity differed among antibodies against these species [5]; anti-human ASGPR antibody was more specific to AIH, and antibodies against rat or rabbit ASGPR were sometimes detected in sera from patients with other liver diseases. Because the protein sequence of ASGPR shows 79% homology between various species [23], there are species-cross-reactive and species-specific epitopes on the ASGPR molecule. It remains unclear which epitope is responsible for the anti-ASGPR antibody detected by the capture ELISA used in the present study.

Prevalence of the anti-ASGPR antibody was very low in most patients with liver diseases induced by hepatitis virus infection, except acute hepatitis A virus infection, as has been reported previously [24]. The anti-ASGPR antibody was detected in two-thirds of the patients with acute hepatitis A. Development of AIH after acute hepatitis A has been previously reported [25, 26], and the detection of the anti-ASGPR antibody may indicate autoimmune responses that were triggered by hepatitis A virus infection.

With our capture ELISA, the prevalence of serum anti-ASGPR antibodies was 67% in our patients with AIH, which was comparable to the prevalences reported in other studies [2, 5, 27]. In contrast, we detected the anti-ASGPR antibody in all of the patients with PBC examined in this study. In previous reports [2, 5], the prevalence of anti-ASGPR antibody ranged from 14 to 33%, which does not correspond to the present findings. When we used KME instead of LME, antibody activity was not detected. By immunoblot analysis, sera from patients with PBC reacted with a protein with a molecular weight similar to that of the ASGPR. These findings support that the antibody detected by this capture ELISA is indeed the anti-ASGPR antibody. However, other

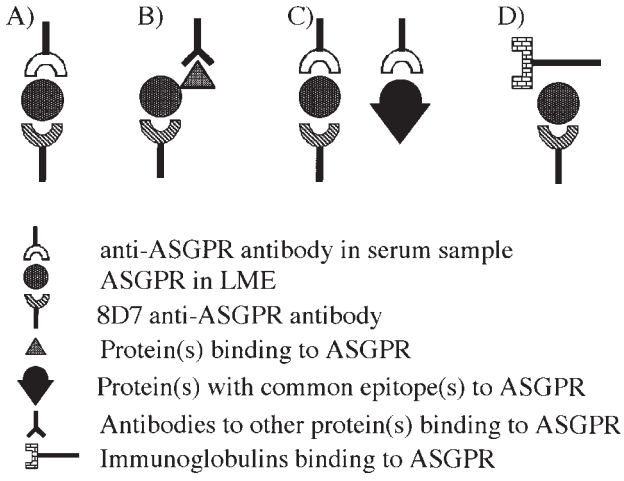

Fig. 4 Possible explanations for the nature of the antibody detected by the capture ELISA. A) Detection of the anti-ASGPR antibody. B) Detection of antibodies to protein(s) in LME, which is in complex with ASGPR. C) Detection of antibodies which cross-react with ASGPR through the molecular mimicry. D) Detection of immunoglobulins binding to ASGPR.

explanations should be considered as well (Fig. 4). An initial explanation asserts that we did indeed detect the anti-ASGPR antibody, as stated above. A second explanation would suggest that the ASGPR was in complex with other protein(s) in the LME, and we might have instead detected the antibody to the bound protein(s). Because we captured the antigen in LME on the ELISA wells by the anti-ASGPR antibody, this could have occurred, in spite of the specificity of the anti-ASGPR antibody. Binding between ASGPR and other protein(s) could take place via the affinity of ASGPR to the galactose residues of desialylated glycoproteins, as well as to the amino-terminal domains of proteins [28]. A third explanation would suggest that a common epitope(s) might be present in the ASGPR and in other protein(s), and that we actually detected antibodies that had cross-reacted with the ASGPR via a molecular mimicry of these proteins. A fourth explanation would suggest that the ASGPR can bind to immunoglobulins in patient sera due to an association of IgA [29] and IgG [30] that has been reported with the ASGPR. We are currently investigating the various explanations for the observed high prevalence of the anti-ASGPR antibody, as detected by our method in patients with PBC.

Even though the full characterization of the antibody in patients with PBC detected by our capture ELISA awaits further study, the high prevalence of the antibody related to the ASGPR suggests an important implication for the mechanisms of hepatocyte injury in patients with PBC. PBC is an autoimmune liver disease characterized by the inflammatory destruction of intrahepatic bile ducts. In addition to bile duct injury, hepatocytes are also destroyed by inflammatory and immune cells; however, the mechanisms for hepatocyte injury remain unknown. Because ASGPR is a highly hepatocyte-specific membrane antigen, the antibody detected by our capture ELISA might prove relevant to the altered immune responses known to contribute to hepatocyte injury in patients with PBC.

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