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Autoantibodies to asialoglycoprotein receptor (ASGPR) measured by a novel ELISA—Revival of a disease-activity marker in autoimmune hepatitis

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ABSTRACT

Background: The liver-specific ASGPR is an autoantigen in autoimmune hepatitis (AIH) patients. Anti-ASGPR antibody correlates with disease activity, however, only in-house assays have been reported so far.
Methods: Rabbit ASGPR was purified by affinity chromatography on galactose-Sepharose and used for standardised detection of anti-ASGPR by ELISA. Anti-ASGPR IgG was measured in sera from 45 patients with AIH, PBC ($n=43$), alcoholic liver disease ($n=13$), HBV infection ($n=35$), HCV infection ($n=53$), and 118 blood donors. Anti-ASGPR was correlated with biochemical parameters of disease activity in 22 AIH patients with consecutive samples.
Results: Twenty-one of 30 untreated (70%) and five of 15 treated AIH patients (30%) showed elevated anti-ASGPR at first presentation. Only one blood donor demonstrated anti-ASGPR. ALD and PBC patients were all negative. ROC curve analysis of AIH and disease-control patients revealed a sensitivity of 77.8% and a specificity of 99.4%. Three (8.6%) of 35 HBV and 7 (13.2%) of 53 HCV patients demonstrated elevated anti-ASGPR. In AIH patients, anti-ASPGR correlated with liver-transaminases levels. In 22 follow-up patients, elevation of anti-ASPGR preceded liver-transaminases increase.
Conclusions: The novel anti-ASGPR ELISA is a readily available and specific diagnostic tool for anti-ASGPR detection in AIH. Quantification of anti-ASGPR is helpful in monitoring disease activity.

1. Introduction

Autoimmune hepatitis is a chronic and progressive inflammatory liver disease of unknown aetiology. The autoimmune response is driven by autoreactive T- and B-cells with production of disease-related autoantibodies [1,2]. Common clinical characteristics of AIH are portal plasma cell infiltration and interface hepatitis on histological examination. The mean incidence of AIH among North European Caucasians is 1.9 per 100,000 and women are affected more frequently than men with a gender ratio of 3.6/1, respectively [3,4]. However, men appear to have a higher relapse rate and younger age of disease onset [5].

Three variants of AIH have been proposed according to the presence of autoantibodies to intracellular targets. Type 1 AIH is associated particularly with anti-nuclear (ANA) and/or anti-smooth muscle antibodies (SMA) reacting with actin, type 2 AIH with anti-liver-kidney microsomal type 1 (LKM1) and/or liver-cytosol type 1 (LC1) antibodies, and type 3 AIH with antibodies to soluble liver/liver-pancreas antigen (SLA/LP) [6-8]. However, in the American literature only two types of AIH are suggested. Patients with Anti-SLA/LP antibodies have been added to the AIH type 1 group [9]. However, like autoantibodies used for the serological diagnosis of other autoimmune liver disorders such as PBC or primary sclerosing cholangitis [10,11], all these antibodies are non-organ or only partially organ-specific.

Abbreviations: ASGPR, asialoglycoprotein receptor; AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; ANA, anti-nuclear antibody; AST, asparagine aminotransferase; CHAPS, 3[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate; BD, blood donors; CI, confidence interval; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; FAS, functional assay sensitivity; HBV, hepatitis B virus; HCV, hepatitis C virus; LC1, liver-cytosol type 1 antibody; LKM1, liver-kidney microsomal type 1 antibody; LSP, liver-specific protein; LMA, liver membrane antigen; MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; OD, optical density; PBC, primary biliary cirrhosis; ROC, receiver operating characteristics; rho, Spearman's rank coefficient of correlation; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SLA, soluble liver/liver-pancreas antigen; SMA, anti-smooth muscle antibody.

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Searching for pathogenesis-related organ-specific autoantibodies, antibodies to liver-specific protein (LSP) or liver membrane antigens (LMA) have been described [12]. ASGPR was identified as the main transmembrane glycoprotein within the LSP fraction [13] to be recognised by T-cells and autoantibodies in AIH patients [14,15]. Anti-ASGPR levels have been shown to correlate with biochemical evidence and histological activity of disease [16]. Furthermore, patients with AIH have demonstrated higher titres of anti-ASGPR before the beginning of immunosuppressive therapy and elevated titres have been found in patients with a higher frequency of relapse after its withdrawal [17]. In a rat infusion model anti-ASGPR bound mainly to periportal hepatocytes supporting a possible pathogenic role of such antibodies [18].

Despite several successful attempts to obtain purified native or recombinant human ASGPR it has not been possible to develop a sensitive and specific commercially available solid-phase assay for the detection of anti-ASGPR antibodies yet [19,20]. The complex structure of the receptor consisting of two different glycosylated subunits and its insufficient autoantigenic stability after purification requiring an intact mature hetero-oligomer might explain the problems to detect anti-ASGPR reproducibly in human serum [21]. In this study, purification and stabilization steps for ASGPR were significantly optimized. By maintaining the antigenic properties of the receptor, a standardised enzyme-linked immunosorbent assay (ELISA) was established providing an excellent diagnostic performance. This novel and commercially available assay for detection of antibodies against ASGPR is a useful diagnostic tool in autoimmune hepatitis and might further improve disease monitoring.

2. Patients and methods

2.1. Patients with AIH

Forty-five patients with AIH (36 females, 9 males, median age 47 years, range 24–85 years) were included into the study. Diagnosis of AIH had been established by typical clinical, biochemical, histological and serological criteria according to the criteria of the International Autoimmune Hepatitis Group [22]. All patients fulfilled the revised classification criteria and had an aggregated score of more than 15.

Thirty-two patients had AIH type 1, five had AIH type 2 and eight had AIH type 3. Sera from these patients were analysed at first presentation at the Department of Internal Medicine I, Tuebingen. At that time, 30 patients were still untreated, and 15 have been already treated with steroids and/or azathioprine.

From 22 of the 45 AIH patients at least 4 consecutive serum samples had been collected over a minimum treatment period of 5 years. Complete clinical, biochemical [alanine aminotransferase (ALT), asparagine aminotransferase (AST)] and serological follow-up data of these patients were available. A total of 195 serum samples were analysed. Thirteen of the 22 patients were untreated at first presentation, and 9 were treated. All patients received steroids and/or azathioprine in the follow-up.

2.2. Patients with other hepatic disorders and healthy individuals

As disease controls sera of 43 patients with primary biliary cirrhosis (PBC) were included (40 females, 3 males; median age 63 years, range 27–80 years). Diagnosis was established by typical clinical and laboratory parameters. All 43 patients had PBC-specific antibodies like anti-M2 or anti-sp100 detected by ELISA and Western blotting.

Furthermore, sera from 18 patients suffering from alcoholic liver disease (ALD) were analysed (5 females, 13 males; median age 61 years, range 33–88 years).

Complete physical examinations and standard laboratory tests of liver inflammation and function had been performed in all patients with hepatic disorders by one investigator (C.B.).

As negative controls, 118 sera from healthy anonymous donors were used (66 females, 52 males; median age 34 years, range 24–60 years).

For the investigation of patients with viral hepatitis, 35 patients suffering from hepatitis B virus (HBV) infection (16 females, 19 males; median age 33 years, range 5–56 years) and 53 patients with hepatitis C virus (HCV) infection (16 females, 37 males; median age 48 years, range 6–71 years) were included into the study. Diagnosis was established by typical clinical, serological and laboratory parameters.

The study was approved by the local ethics committee and conducted in accordance with the Helsinki declaration. Written informed consent was obtained from each individual.

All sera had been stored at −20 °C.

2.3. Animals

Rabbits (2000–3000 g; Animal Facility, Seramun, Heidesee, Germany) and rats (200–250 g; Animal Facility, Charité Hospital, Berlin, Germany) used in the study were handled according to German regulations concerning the protection of animals. The animals were killed by asphyxiation with CO_2 and exsanguinations, and the liver was excised, immediately frozen, and stored at −20 °C.

2.4. Purification of ASGPR

Frozen rat or rabbit liver was meshed by a chilled homogenisator (Bühler, Tübingen, Germany), homogenised in 10 mmol/L Tris–HCl buffer containing 0.4 mol/L KCl, 50 mmol/L $CaCl_2$, 0.5% Triton X-100, pH 7.8 (loading buffer), and subsequently clarified by centrifugation and filtration. The soluble liver fraction was subjected to affinity chromatography using galactose-Sepharose 6B as the affinity resin according to Halberg et al. [23].

After application of soluble liver fraction, the galactose-Sepharose column was rinsed with loading buffer and eluted with 30 mmol/L ammonium acetate containing 0.4 mmol/L KCl and 0.1% Triton X-100, pH 6.0 (elution buffer).

Fractions containing ASGPR were pooled and applied to a galactose-Sepharose column equilibrated with loading buffer containing 0.1% 3[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) instead of Triton X-100. Subsequently, the column was washed with loading buffer until the photometer baseline stability of the effluent. After elution with elution buffer containing 0.1% CHAPS, ASGPR containing fractions were pooled, the pH was adjusted to 7.8, and protein was precipitated by 65% saturation with ammonium sulphate. The precipitate was dissolved in 10 mmol/L Tris–HCl, pH 7.0, dialysed against 1 mmol/L Tris–HCl, pH 7.0, and the obtained ASGPR solution stored at −20 °C until further use.

2.5. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Purified ASGPR was examined by SDS-PAGE using the Mini-Vertical Gel electrophoresis Unit SE250 (Hoefer Pharmacia Biotech, San Francisco, CA, USA) with 12% polyacrylamide gels and Coomassie Brilliant Blue G250 for protein staining and Schiff's reagent for carbohydrate staining.

For immunoblotting separated polypeptides were transferred to nitrocellulose membranes (Schleicher und Schüll, Dassel, Germany) by semi-dry blotting, blocked with skimmed milk and probed with goat polyclonal anti-ASGPR (Seramun, Heidesee, Germany) followed by anti-goat immunoglobulin conjugated to alkaline phosphatase.