Research Paper

Presence of Antibodies against Self Human Leukocyte Antigen Class II Molecules in Autoimmune Hepatitis

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Abstract

Autoimmune hepatitis (AIH) can arise de novo after liver transplantation (LT) for non-autoimmune liver diseases. Considering the identical features of de novo AIH after LT and classical AIH, as well as the importance of anti-human leukocyte antigen (HLA) antibodies in graft rejection, we investigated the presence of circulating anti-HLA class II antibodies in the sera of 35 patients with AIH, 30 patients with primary biliary cirrhosis (PBC), and 30 healthy donors using fluorescent dye-impregnated beads bound to HLA molecules. We then investigated the allele specificity of the antibodies and identified the HLA alleles in each patient using DNA-based HLA typing. We also examined HLA class II expression in liver samples using immunohistochemistry. Anti-HLA class II antibodies were detected significantly more frequently in the patients with AIH (88.1%) than in the patients with PBC (33.3%) or in the healthy donors (13.3%) (both P<0.01). We confirmed that the anti-HLA class II antibodies in the AIH patients showed specificity for several HLA class II alleles, including self HLA class II alleles. Moreover, positive reactivity with anti-self HLA class II antibodies was associated with higher serum transaminase levels. In conclusion, we demonstrated, for the first time, that antibodies against self HLA class II alleles were detectable in patients with AIH. Our results suggest that an antibody-mediated immune response against HLA class II molecules on hepatocytes may be involved in the pathogenesis or acceleration of liver injury in AIH.

Key words: HLA; AIH; PBC; anti-HLA antibodies.

Introduction

Autoimmune hepatitis (AIH) is an inflammatory liver disease that is characterized by elevated serum transaminase levels, positive organ-specific and non-specific autoantibodies, elevated serum immunoglobulin (IgG), and histological interface hepatitis in the absence of a known cause of liver disease [1,2]. There are two main forms of AIH, which can be distinguished by the presence of different autoantibodies [3]. AIH type 1 is defined by the presence of smooth muscle antibody (SMA), which is mainly observed in polymeric (filamentous, F) actin pattern, and/or by antinuclear antibody (ANA), which is mainly observed in homogeneous pattern. AIH type 2 is defined by liver/kidney microsomal type 1 antibody (anti-LKMI) and/or liver cytosol type 1 antibodies (anti-LC1) [3]. The molecular targets of SMA and ANA have not been characterized, but the main target of anti-LKMI is the liver cytochrome P4502D6 (CYP2D6), which localizes mainly to the endoplasmic reticulum but also to hepatocyte membranes [4]. Im-
immune reactions against host liver antigens are believed to be the major pathogenic mechanism of AIH, and the disease responds satisfactorily to immunosuppressive treatment. However, the primary cause of AIH remains unknown [5].

Liver transplantation (LT) is the best treatment option for selected patients with end-stage liver disease caused by AIH [6]. However, despite the good outcomes reported, AIH can recur after LT; AIH and autoimmune can also arise de novo after LT for non-autoimmune liver diseases [6-8]. The features of de novo AIH after LT are identical to those of classical AIH, such as hypergammaglobulinemia; positive autoantibodies, including ANA, ASMA, and atypical anti-LKM1; and histological findings of interface hepatitis with an abundance of plasma cells [6,9]. Patients who develop de novo AIH do not exhibit a satisfactory response to standard antirejection regimens, but they do respond to the standard treatment for AIH [6]. However, it remains unclear whether de novo AIH is a distinct entity or a form of atypical hepatitis in individuals who are at risk of autoimmune. Moreover, several reports have suggested that de novo AIH may represent an alloimmune response (i.e., a form of rejection), in which immune-mediated injury is directed toward hepatocytes rather than bile ducts or vascular endothelium [10]. Neil et al. also showed overlapping features of histological findings between rejection and de novo AIH [10].

Allograft rejection was originally believed to be mediated almost exclusively by cellular immune responses, but it has since become clear that humoral responses also play a major role in this process [11,12]. Considering the importance of anti-human leukocyte antigen (HLA) antibodies in graft rejection after LT, we focused on their role in AIH because the involvement of antibodies against self HLA molecules on hepatocytes in the liver injury that is characteristic of AIH remains to be elucidated. In the present study, we investigated the presence of anti-HLA class II antibodies in the sera of patients with AIH and primary biliary cirrhosis (PBC) using a highly sensitive method (Luminex) and evaluated the significance of these antibodies against HLA class II molecule(s) in the sera using fluorescent dye-impregnated beads bound to HLA molecules (Luminex). Screening anti-HLA tests were performed with multiplex technology (LABScreen mixed test, One Lambda, Canoga Park, CA). When the results were positive, the allele specificity of the antibodies was determined using beads bound to a single HLA class II antigen (LABScreen single-antigen test, One Lambda). Published data indicate that the Luminex platform is the most sensitive of the solid phase antibody detection techniques [18]. HLA antibody specificity was considered positive if the normalized mean fluorescence intensity (MFI) value was greater than 1000 [11].

Detection of anti-HLA class II antibodies (Luminex assay)

We investigated the presence of circulating antibodies against HLA class II molecules in the sera using fluorescent dye-impregnated beads bound to HLA molecules (Luminex). Screening anti-HLA tests were performed with multiplex technology (LABScreen mixed test, One Lambda, Canoga Park, CA). When the results were positive, the allele specificity of the antibodies was determined using beads bound to a single HLA class II antigen (LABScreen single-antigen test, One Lambda). Published data indicate that the Luminex platform is the most sensitive of the solid phase antibody detection techniques [18]. HLA antibody specificity was considered positive if the normalized mean fluorescence intensity (MFI) value was greater than 1000 [11].

DNA typing of HLA class II alleles

In cases that were positive for anti-HLA class II antibodies, we then determined the HLA alleles in each patient using DNA-based HLA typing (PCR-Sequencing-Based Typing) (SRL Inc., Tokyo, Japan).

Immunohistochemistry of HLA class II molecules

Tissue sections were first deparaffinized with xylene and then rehydrated through graded alcohol. To retrieve antigenicity, sections were immersed in pH 6.0 citrate buffer. After autoclaving, the sections were then immersed in phosphate-buffered saline (PBS) containing 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. The sections then were incubated with an anti-HLA class II (HLA-DR, HLA-DP, and HLA-DQ) antibody (clone: CR3/43) (GeneTex, Inc., Irvine, CA) at a 1:25 dilution in PBS supplemented with 3% BSA at 4°C overnight. The sections were followed sequentially using a biotinylated secondary antibody and the av-
din-biotin-peroxidase complex method with the Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA). The sections were developed with diaminobenzidine (DAB) substrate (Muto Pure Chemicals, Tokyo, Japan). The specimens then were counterstained with methyl green solution and mounted.

Statistical analysis

The significance of differences was analyzed statistically using Fisher’s exact test, the compared t-test with Welch’s correction, or the Mann-Whitney U test, using SPSS software (Ver.18, SPSS Inc., Chicago, IL). In all cases, the level of significance was set at $P < 0.05$.

Results

Clinical features of the patients

The mean ages at diagnosis of the patients with AIH and PBC were $58.3 \pm 15.2$ and $55.8 \pm 12.6$ years, respectively. The mean alanine aminotransferase (ALT) level in the patients with AIH was $369.7 \pm 406.0$ IU/l, which was significantly higher than in the patients with PBC ($81.5 \pm 103.3$ IU/l, $P < 0.001$) and the healthy donors ($15.7 \pm 5.2$ IU/l, $P < 0.001$). The mean total bilirubin level in the patients with AIH was $1822.7 \pm 725.6$ mg/dl, which was significantly higher than in the patients with PBC ($1822.7 \pm 725.6$ mg/dl, $P = 0.001$).

ANA positivity

We then investigated the expression of HLA class II molecules in the liver using immunohistochemistry. Figure 3 shows representative results from a patient with AIH and a patient with PBC. In the livers of the patients with PBC, in addition to mononuclear cells, including macrophages and B cells, HLA class II molecules were detected around the in-
jured bile ducts, consistent with previous studies [19] (arrow in Figure 3). In contrast, HLA class II mole-
cules were detected around hepatocytes in the livers of the patients with AIH (arrowhead in Figure 3).

Table 2. Comparison between HLA class II alleles defined by DNA typing and the allele specificities of anti-HLA class II antibodies in patients with AIH.

| Age | Gender | DNA typing | DRB1 | DQB1 | DPB1 | Ab (Luminex) | Allele Specificity |
|-----|--------|------------|------|------|------|--------------|-------------------|
| 31  | F      | 04:03      | 15:01| 03:03:02| 06:02:01| DRB1*04:03    |                   |
| 54  | F      | 04:05      | 08:03:02| 03:02:01| 06:01:01| 02:02        | 05:01             |
| 66  | F      | 08:03:02   | 15:02:01| 06:01:01| 02:01:02| DRB1*01:01, DRB1*04:03, DRB1*15:02, DPB1*02:01 |                   |
| 82  | F      | 04:05      | 08:01   | 04:01 | 06:01 | 02:01 | 04:02 | DRB1*04:03, DRB1*02:01, DPB1*04:02, DRB1*04:05 |
| 53  | F      | 04:05      | 08:02   | 04:01:01| 04:02 | 05:01 | DRB1*10:01, DQ81*06:01, DPB1*03:01 |
| 59  | F      | 04:05      | 08:02   | 04:01:01| 04:02 | 05:01 | DRB1*01:01, DRB1*10:01 |
| 67  | F      | 12:02:01   | 12:10  | 03:01:01| 05:01 | DRB1*09:02  |                   |
| 67  | F      | 04:05      | 08:03   | 04:01 | 06:01 | 02:01 | 09:01 | DRB1*04:03 |
| 72  | F      | 04:06      | 12:02:01| 03:01:01| 03:02:01| DPB1*01:01, DPB1*20:01 |                   |

Abbreviations: HLA, human leukocyte antigen; AIH, autoimmune hepatitis; Ab, antibodies; F, female.

Table 3. Comparison of patient characteristics based on the positivity of anti-HLA class II antibodies.

| n   | Anti-self HLA class II antibodies (+) | Anti-self HLA class II antibodies (-) | Anti-HLA antibodies (-) |
|-----|---------------------------------------|---------------------------------------|-------------------------|
| 4   | 5.3 ± 2.15 (31 - 82)                   | 5.3 ± 2.15 (53 - 72)                  | 52.4 ± 17.3 (24 - 72)   |
| 5   | 3.2 ± 0.9     (2.4 - 4.2)               | 3.2 ± 0.9     (2.1 - 4.6)              | 3.7 ± 0.4 (2.8 - 4.1)   |
| 8   | 2271.8 ± 787.2 (1571 - 3381)           | 2381.4 ± 584.3 (1873 - 3343)          | 2809.4 ± 2078.9 (794 - 6578) |
| 8   | 829.5 ± 635.2** (231 - 1728)           | 215.8 ± 218.7 (36 - 587)              | 157.3 ± 84.0 (37 - 272) |
| 8   | 1054.8 ± 671.8*** (208 - 1852)         | 271.4 ± 408.5 (46 - 998)              | 211.3 ± 149.5 (50 - 439) |
| 8   | 11.1 ± 6.3 (3.8 - 18.8)                | 4.3 ± 6.7 (0.8 - 16.3)                | 6.1 ± 7.9 (0.5 - 21.5)  |
| 8   | 16.3 ± 8.6 (8.2 - 26.1)                | 20.5 ± 6.7 (12.3 - 30.9)              | 20.6 ± 8.9 (6.2 - 31.5) |
| 8   | 1.65 ± 0.75 (1.00 - 2.72)              | 1.13 ± 0.19 (0.99 - 1.41)             | 1.39 ± 0.55 (0.95 - 2.40) |
| 8   | 75.0% (3/4cases)                       | 80.0% (4/5cases)                      | 100% (8/8cases)         |

*P<0.05 (compared to HLA negative), **P<0.05 (compared to self-HLA negative). Abbreviations: HLA, human leukocyte antigen; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; T.Bil, total bilirubin; Plt, platelet count; PT, prothrombin time; INR, international normalized ratio.

Figure 2. Comparison of serum transaminase levels between anti-HLA class II antibody-positive and –negative AIH patients at the onset of disease. The presence of anti-HLA class II antibodies in the sera was determined using fluorescent dye-impregnated beads bound to HLA molecules. Among the patients with AIH, positive reactivity with anti-HLA class II antibodies was associated with higher serum transaminase levels.
Figure 3. Immunohistochemical staining of HLA class II molecules in the livers of patients with PBC and AIH. Representative results from immunohistochemical staining (IHC) of HLA class II molecules in the livers of the patients with PBC and AIH are shown. HE staining results are also shown. (High) Higher magnification. In addition to mononuclear cells, including macrophages and B cells, HLA class II molecules are expressed around injured biliary epithelial cells (arrow) in the liver of a patient with PBC and around injured hepatocytes (arrowhead) in the liver of a patient with AIH.

**Discussion**

In the present study, we found that anti-HLA class II antibodies were detected significantly more frequently in patients with AIH than in patients with PBC or healthy donors. The positive reactivity with anti-HLA class II antibodies was associated with significantly higher serum transaminase levels in the patients with AIH. We found that the anti-HLA class II antibodies detected in the patients with AIH showed specificity for several HLA class II alleles. Moreover, we confirmed that anti-HLA class II antibodies with specificity for self HLA class II alleles were detected in a proportion of the patients with AIH. Although HLA class II molecules are expressed on a restricted range of cells, such as antigen-presenting cells, they are also IFN-γ-inducible on other cells, including hepatocytes [13]. We also found that HLA class II molecules were expressed on hepatocytes in the livers of the patients with AIH, consistent with previous reports [20,21]. These results suggest that an antibody-mediated immune response against HLA class II molecules on hepatocytes may be involved in the pathogenesis or acceleration of liver injury in AIH.

Multiple genetic associations with AIH have been described in different ethnic groups [1]. The strongest genetic association in AIH is with genes that encode HLA class II molecules, especially DRB1 alleles [2,22-25]. The alleles that confer susceptibility to AIH-1 are HLA DRB1*03:01 and DRB1*04:01 in European and North American populations, as well as DRB1*04:05 and DRB1*04:04 in Japanese [26], Argentinean, and Mexican populations [24]. It has been speculated that different pathogens or environmental triggers generate diverse epitopes, which are each presented by different disease-associated HLA-DR molecules, inducing the clonal expansion of auto-reactive T cells and ultimately progressing to the same or a very similar form of the clinical disease [24,27]. However, the precise mechanism of the involvement of HLA-DR molecules in the susceptibility and pathogenesis of AIH remains to be elucidated. Interestingly, in the present study, we found that the anti-HLA class II antibodies detected in the patients with AIH showed specificity mainly to DRB1 alleles.
(Table 2). Although the reason why the anti-HLA class II antibodies detected in the patients with AIH showed such biased specificity to DRB1 alleles remains unknown, this may be associated with susceptibility to AIH.

The ability to express major histocompatibility complex (MHC) class II molecules is normally restricted to professional antigen-presenting cells [28]. However, MHC class II molecules have been found on hepatocytes from patients with AIH and primary sclerosing cholangitis, although MHC class I molecules were invariably expressed on hepatocytes [21]. IFN-γ, which is primarily secreted by activated T cells and natural killer cells, is considered the main inducer of the expression of HLA class II molecules on hepatocytes [13,29]. It has been speculated that such aberrant expression of MHC class II molecules and antigen presentation by parenchymal cells may be the cause of autoimmune diseases [28]. Herkel et al. showed that MHC class II-expressing hepatocytes could present antigens and activate CD4 T cells, although the ability of hepatocytes to present antigens on MHC class II molecules did not appear to be sufficient to cause inflammatory autoimmunity or hepatitis [30]. In the present study, we found that HLA class II molecules were expressed on hepatocytes in the livers of the patients with AIH, consistent with previous reports [20,21]. Moreover, we confirmed that anti-HLA class II antibodies with specificity for self HLA class II alleles were detected in patients with AIH. Involvement of an antibody-dependent cell-mediated cytotoxicity (ADCC) in the pathogenesis of autoimmune liver damage has been suggested by the findings that hepatocytes, isolated from patients with AIH, are coated with immunoglobulins and are susceptible to cytotoxicity when autologous Fc-receptor-bearing mononuclear cells [29,31]. Tu et al. showed that complement could play a pivotal role in liver-specific autoantibody-mediated hepatocyte injury in a murine model of AIH [32]. Therefore, our results have led to the hypothesis that such anti-self HLA class II antibodies could be directly involved in the pathogenesis of autoimmune liver damage in AIH. Considering that hepatocytes are the main immune target in AIH, we believe that more attention should be paid to the expression of HLA class II molecules on hepatocytes.

The effects of preoperatively produced anti-HLA antibodies on organ transplantation outcomes have been demonstrated for kidney, heart, and lung transplantation [33]. Although the existence of such effects in LT is controversial, several reports have demonstrated the significance of donor-specific anti-HLA antibodies in rejection and outcome after LT using the Luminex assay and the histological examination of deposition of C4d, a marker of immunoglobulin-dependent activation of complement [33,34]. Previous studies have shown that a substantial proportion of clinical liver biopsies with specific signs of acute cellular and chronic rejection exhibit C4d deposition, strongly suggesting a role for a humoral component in liver allograft failure [35]. Moreover, Castillo-Rama et al. reported that increased rejection episodes were predominantly observed in allografts positive for Luminex-detected anti-HLA class II antibodies [34]. Although we did not find C4d deposition in the livers of the patients with AIH (data not shown), the above findings associated with allograft rejection suggest a possible involvement of antibody-mediated immune responses against HLA class II molecules on hepatocytes, including ADCC, in the pathogenesis of AIH.

In conclusion, we have demonstrated for the first time that anti-HLA class II antibodies were detected significantly more frequently in patients with AIH than in patients with PBC or healthy donors. We found that the anti-HLA class II antibodies detected in the patients with AIH showed specificity for several HLA class II alleles, mainly for DRB1 alleles. Moreover, we confirmed that anti-HLA class II antibodies with specificity for self HLA class II alleles were detected in a proportion of the patients with AIH. Although the present study should be confirmed by studies with larger numbers of patients, our results suggest the possible involvement of an antibody-mediated immune response against HLA class II molecules on hepatocytes in the pathogenesis or acceleration of AIH.

**Abbreviations**

HLA, human leukocyte antigen; MHC, major histocompatibility; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; MFI, mean fluorescence intensity; LT, liver transplantation.

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Competing Interest

The authors have declared that no competing interest exists.

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