Specificities and Clinical Significance of Anti-Cytoskeleton Antibodies in Anti-Smooth Muscle Antibody-Positive Patients with Chronic Liver Disease C

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Abstract

We investigated the specificities and characteristics of anti-cytoskeleton antibodies in 13 anti-smooth muscle antibody (ASMA)-positive patients with chronic liver disease C (CLD-C), and compared them with those in 7 ASMA-positive patients with autoimmune hepatitis (AIH), and 6 ASMA-positive patients with chronic liver disease B (CLD-B). Anti-microfilaments (anti-MF) were found not only in 6/7 AIH patients (85.7%), but also in 8/13 CLD-C patients (61.5%) with a relatively high incidence, when compared with 1/6 CLD-B patients (16.7%), while, there was no significant difference in the incidence of anti-intermediate filaments (anti-IMF), especially anti-IMF IgM, among these patient groups. Among the patients with CLD-C, the mean levels of serum gammaglobulin and IgG in the anti-MF-positive patients were 2.46 +/- 1.03 g/dl and 3277 +/- 1089 mg/dl, respectively, which were higher than those in the anti-MF-negative patients (1.60 +/- 0.53 g/dl, 2245 +/- 610 mg/dl) and those in the patients with CLD-B (1.60 +/- 0.57 g/dl, 2192 +/- 339 mg/dl). Furthermore, 4 of the 8 anti-MF-positive patients with CLD-C satisfied the serological criteria for the diagnosis of AIH. These findings suggest that autoimmune mechanisms might be involved in the pathogenesis of anti-MF-positive CLD-C, and that anti-MF might be used as a marker.

KEYWORDS: chronic liver disease C, autoimmune hepatitis, anti-smooth muscle antibodies, anti-cytoskeleton antibodies, anti-microfilament antibodies
Specificities and Clinical Significance of Anti-Cytoskeleton Antibodies in Anti-Smooth Muscle Antibody-Positive Patients with Chronic Liver Disease C

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We investigated the specificities and characteristics of anti-cytoskeleton antibodies in 13 anti-smooth muscle antibody (ASMA)-positive patients with chronic liver disease C (CLD-C), and compared them with those in 7 ASMA-positive patients with autoimmune hepatitis (AIH), and 6 ASMA-positive patients with chronic liver disease B (CLD-B). Anti-microfilaments (anti-MF) were found not only in 6/7 AIH patients (85.7%), but also in 8/13 CLD-C patients (61.5%) with a relatively high incidence, when compared with 1/6 CLD-B patients (16.7%), while, there was no significant difference in the incidence of anti-intermediate filaments (anti-IMF), especially anti-IMF IgM, among these patient groups. Among the patients with CLD-C, the mean levels of serum gammaglobulin and IgG in the anti-MF-positive patients were 2.46 ± 1.03 g/dl and 3277 ± 1089 mg/dl, respectively, which were higher than those in the anti-MF-negative patients (1.60 ± 0.53 g/dl, 2245 ± 610 mg/dl) and those in the patients with CLD-B (1.60 ± 0.57 g/dl, 2192 ± 339 mg/dl). Furthermore, 4 of the 8 anti-MF-positive patients with CLD-C satisfied the serological criteria for the diagnosis of AIH. These findings suggest that autoimmune mechanisms might be involved in the pathogenesis of anti-MF-positive CLD-C, and that anti-MF might be used as a marker.

Key words: chronic liver disease C, autoimmune hepatitis, anti-smooth muscle antibodies, anti-cytoskeleton antibodies, anti-microfilament antibodies

Anti-smooth muscle antibodies (ASMA) were identified as one of the most characteristic markers for the diagnosis of autoimmune hepatitis (AIH) (1), which is clinically characterized by a high level of serum gammaglobulin and the presence of autoantibodies. Subsequent studies have revealed that ASMA were found not only in AIH but also in viral hepatitis, primary biliary cirrhosis, or other pathological conditions such as acute viral infection, autoimmune diseases and malignant diseases (2, 3). Some of their reactivities can be accounted for by autoantibodies to cytoskeletal components (4), including microfilaments (MF) such as actin, myosin, α-actinin and villin (5), intermediate filaments (IMF) such as vimentin, cytokeratin and desmin (6) and microtubules (MT) such as tubulin and microtubule-associated protein. Several studies on anti-cytoskeleton antibodies in liver diseases have demonstrated a relatively high prevalence of anti-MF in AIH (3, 7-9) and anti-IMF in viral hepatitis (7, 10).

On the other hand, although autoantibodies observed in the clinical course of viral hepatitis are generally weaker and more transiently positive than those observed in AIH, some patients with chronic hepatitis C exhibit autoimmunity similar to AIH, suggesting a relationship between hepatitis C virus (HCV) infection and the pathogenesis of autoimmune responses (11, 12). ASMA with a high titer reactivity are also found in these chronic hepatitis C patients associated with autoimmune phenomena (11).

In this study, we investigated the specificities and characteristics of the anti-cytoskeleton antibodies in sera of ASMA-positive patients with chronic liver disease C (CLD-C), including cases associated with autoimmune phenomena, to assess autoimmunity induced in CLD-C.

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Subjects and Methods

Patients. This study included 13 patients with CLD-C (9 patients with chronic hepatitis, CH, and 4 patients with liver cirrhosis, LC) and 7 patients with AIH (7 with CH and 6 patients with chronic liver disease B (CLD-B) (2 with CH and 4 with LC), who were all positive for ASMA (titer higher than 1:40). The patients were diagnosed on the basis of clinical data and/or typical findings in liver biopsy specimens according to the criteria of an international group (13).

The patients with anti-HCV antibodies and/or HCV-RNA were diagnosed as having CLD-C. Anti-HCV antibodies were tested using a second generation kit utilizing an enzyme-linked immunosorbent assay (ELISA) (Dainabot, Tokyo). HCV-RNA in serum was detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) using the 5’-noncoding region as a primer (14). The patients positive for hepatitis B surface antigen (HBsAg), as determined by ELISA or radioimmunoassay (RIA), were diagnosed as having CLD-B. The patients with AIH all satisfied the serological criteria for the diagnosis of AIH (serum gammaglobulin and/or IgG level ≥ 2.5 g/dl, and the presence of anti-nuclear antibodies, ANA), and were confirmed to be negative for HBsAg and anti-HCV antibody (anti-HCV).

Detection of ANA and ASMA. ANA and ASMA were detected by an indirect immunofluorescence (IF) method using laryngeal carcinoma cells, HEP-2 and cryostat sections of the rat stomach, respectively. The titer was established by sequential twofold dilution of serum diluted 1:10 until an end-point was reached. The titer for positive results in the indirect IF method was 1:20 (20) for ANA, and 1:40 (40) for ASMA.

Detection of anti-cytoskeleton antibodies. Anti-cytoskeleton antibodies were identified by the indirect IF method using rat kangaroo kidney epithelial cells, PtK2 or mouse fibroblast, NIH 3T3 as a substrate. The cells grown on cover-slips for 2 days were treated with 10 μg/ml of vinblastin in minimal essential medium (MEM) for 4 h at 37 °C. After fixation with cold acetone, the cells were allowed to react with 1:10 diluted serum for 1 h at 37 °C (8, 15), followed by five washes with phosphate-buffered saline (PBS). The cells were then incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-human immunoglobulins (DAKO Japan, Tokyo) for 1 h. Following seven additional washes with PBS, the cells were mounted in glycerol-carbonate-bicarbonate buffer. The fluorescence pattern was assessed by a fluorescence microscope. The immunoglobulin classes of anti-cytoskeleton in any positive sera were determined by testing with FITC-labelled goat anti-human IgG or with FITC-labelled goat anti-human IgM, instead of FITC-labelled goat anti-human immunoglobulins.

Detection of anti-actin antibodies by immunoblotting method. Sera positive for anti-MF antibodies by the indirect IF method were tested for their reactivities by an immunoblotting method using actin (Chemicon, Temecula), derived from rabbit skeletal muscle. Aliquots (2 mg/ml) of purified actin were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in 10% gel, and electrophoretically transferred to nitrocellulose membranes for immunoblot analysis according to the method of Towbin et al. (16). The membranes were incubated with positive sera diluted at 1:10 in PBS for 2 h. After being washed, the membranes were then incubated with 1:1000 diluted peroxidase-labelled goat anti-human immunoglobulins. After final washes, the membranes were incubated in 3,3’-diaminobenzidine (DAB) solution (0.2 mg/ml of DAB, 0.006 % of H2O2) until a brown color reaction developed.

Statistical analysis. Student’s t-test was used to determine the significance of differences between means. Differences between incidences were evaluated by the χ²-test.

Results

Antibodies to MF, IMF and MT were identified by the indirect IF technique using vinblastin-treated cultured cells, PtK2 and NIH3T3 as substrate. Fig 1 shows the representative staining patterns of intracellular cytoskeletal filaments of vinblastin-treated PtK2 and NIH3T3, which were obtained by reacting with sera from ASMA-positive patients with AIH or CLD-C.

MF which remain intact after treatment with vinblastin appear as long parallel bundles crossing the cells, "actin cables" or "stress fibers". Sera which stained positively for stress fibers were regarded as having anti-MF (Fig. 1-A, B). IMF of the vimentin type are found in numerous cells, whereas the cytokeratin type are found only in epithelial cells such as PtK2 (17). IMF of the vimentin type form thick "perinuclear coils" in vinblastin-treated cells, whereas IMF of the cytokeratin type, which remain
intact after vinblastin treatment, form a "three-dimensional meshwork". Anti-IMF were judged to be positive when the staining patterns of the perinuclear coils and/or three-dimensional meshwork were observed in these cultured cells (Fig. 1-C, D). MT disrupted by vinblastin treatment appear as tubulin "paracrystals". Sera showing the fluorescence pattern of paracrystals were judged to have anti-MT (Fig. 1-E, F).

Anti-MF-positive sera were estimated to react with actin by the immunoblotting method (representative patterns are shown in Fig. 2). Nine sera obtained from 15 anti-MF-positive patients with AIH or CLD-C were confirmed to react with a 43 kDa band consistent with actin.

Table 1 shows the clinical features and laboratory data of the ASMA-positive patients investigated in this study.
Fig. 2. Immunoblotting for detecting the reactivities of anti-MF-positive sera from patients with autoimmune hepatitis or with chronic liver disease C, with purified actin. Lanes: 1. Molecular weight marker, 2. Coomassie blue staining of purified actin. 3. Staining of purified actin obtained by incubating with anti-actin monoclonal antibody, 4, 5. Staining of purified actin obtained by incubation with anti-MF-positive serum of a patient with chronic liver disease C (4) and with anti-MF-positive serum of a patient with autoimmune hepatitis (5), 6. Serum of a healthy subject as negative control.

Table 1. Clinical profiles and appearances of anti-cytoskeleton antibodies

<table>
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<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
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<th>γ-gl (g/dl)</th>
<th>IgG (mg/dl)</th>
<th>ANA (Titer)</th>
<th>ASMA (Titer)</th>
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<td></td>
<td></td>
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<td>57</td>
<td>1.50</td>
<td>2163</td>
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<td>CLD-B</td>
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<td>F</td>
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<td>33</td>
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<td>2318</td>
<td>-</td>
<td>40</td>
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<td>19</td>
<td>1.12</td>
<td>2031</td>
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<td>40</td>
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</table>

ALT: alanine aminotransferase; γ-gl: gammaglobulin; ANA: anti-nuclear antibody; ASMA: anti-smooth muscle antibody; MF: antimicrofilament antibody; IMF: anti-intermediate filament antibody; MT: anti-microtubule antibody; AlH: autoimmune hepatitis; Ch: chronic hepatitis; CLD-C: chronic liver disease C; LC: liver cirrhosis; CLD-B: chronic liver disease B
An increase in ASMA titer above 320 was observed in 4 of the 7 patients with AIH and 3 of the 13 patients with CLD-C, whereas all titers of ASMA in the patients with CLD-B were 40.

The results of reactivities with each cytoskeletal component in the patients are also shown in Table 1. Table 2 shows the incidence of anti-MF, anti-IMF and anti-MT, and the frequency of their immunoglobulin classes in the patient groups. Anti-MF was found in 6 (85.7%) of the 7 ASMA-positive patients with AIH, but only in 1 (16.7%) of the 6 ASMA-positive patients with CLD-B, and the difference was significant ($P < 0.05$). It was noteworthy that, of the 13 ASMA-positive patients with CLD-C, 8 (61.5%) patients were positive for anti-MF, 7 of whom (87.5%) had anti-MF IgG.

There was no significant difference in the incidences of anti-MF and anti-IMF IgM among the patient groups. However, anti-IMF IgG was detected in 4 (57.1%) patients with AIH, and in 6 (46.1%) patients with CLD-C, but not in the patients with CLD-B. Anti-MT was found in only 3 patients with AIH.

The mean levels of serum gammaglobulin and IgG in the anti-MF-positive patients with CLD-C were 2.46 ± 1.03 g/dl and 3277 ± 1089 mg/dl, respectively, which were higher than those in the CLD-C patients who were anti-MF-negative, 1.60 ± 0.53 g/dl and 2245 ± 545 mg/dl, and those in the patients with CLD-B, 1.60 ± 0.51 g/dl and 2192 ± 339 mg/dl (Table 3-a, b). An increase in gammaglobulin level above 2.5 g/dl or IgG level above 2500 mg/dl was observed in 6 of the anti-MF-positive patients with CLD-C, 4 of whom (CLD-C case nos. 1, 4, 6, 8) satisfied the serological criteria for AIH. Especially, CLD-C case 8, in which anti-MF IgG were detected, was positive for the LE cell reaction, and had a high level of serum gammaglobulin (4.41 g/dl), serum IgG (4.422 mg/dl), and high titers of ANA (1,280) and ASMA (320). These autoimmune phenomena were similar to those observed in the patients with AIH.

**Table 3-a** Clinical profiles of the patients with autoimmune hepatitis, with chronic liver disease C and with chronic liver disease B

<table>
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<th>AIH</th>
<th>CLD-C</th>
<th>CLD-B</th>
</tr>
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<tbody>
<tr>
<td>No. of cases</td>
<td>7</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>1 : 6</td>
<td>7 : 6</td>
<td>5 : 1</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>397 ± 336</td>
<td>86 ± 40</td>
<td>60 ± 44</td>
</tr>
<tr>
<td>γ-gl (g/dl)</td>
<td>2.99 ± 0.61</td>
<td>2.13 ± 0.95</td>
<td>1.60 ± 0.57</td>
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<tr>
<td>IgG (mg/dl)</td>
<td>3703 ± 662</td>
<td>2880 ± 1044</td>
<td>2192 ± 339</td>
</tr>
<tr>
<td>IgG and/or</td>
<td>γ-gl ≥ 2.5 g/dl</td>
<td>7 (100%)</td>
<td>8 (61.5%)</td>
</tr>
<tr>
<td>ANA</td>
<td>7 (100%)</td>
<td>7 (53.8%)</td>
<td>1 (16.7%)</td>
</tr>
<tr>
<td>ANA (+) and</td>
<td>IgG ≥ 2.5 g/dl</td>
<td>7 (100%)</td>
<td>6 (46.2%)</td>
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**Table 3-b** Clinical profiles of patients with chronic liver disease C classified by the presence of anti-MF

<table>
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<th>Anti-MF (+)</th>
<th>Anti-MF (-)</th>
</tr>
</thead>
<tbody>
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<td>No. of cases</td>
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<td>5</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>3 : 5</td>
<td>4 : 1</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>102 ± 42</td>
<td>61 ± 25</td>
</tr>
<tr>
<td>γ-gl (g/dl)</td>
<td>2.46 ± 1.03</td>
<td>1.60 ± 0.53</td>
</tr>
<tr>
<td>IgG (mg/dl)</td>
<td>3277 ± 1089</td>
<td>2245 ± 610</td>
</tr>
<tr>
<td>IgG and/or</td>
<td>γ-gl ≥ 2.5 g/dl</td>
<td>6 (75.0%)</td>
</tr>
<tr>
<td>ANA</td>
<td>4 (50.0%)</td>
<td>3 (60.0%)</td>
</tr>
<tr>
<td>ANA (+) and</td>
<td>IgG ≥ 2.5 g/dl</td>
<td>4 (50.0%)</td>
</tr>
</tbody>
</table>

AIH, CLD-C, CLD-B, ALT, γ-gl, ANA: See Table 1 anti-MF: See Table 2

**Discussion**

In this study, we demonstrated the presence of anti-MF not only in cases of AIH but also at a relatively high incidence in a subset of the ASMA-positive patients with
CLD-C compared to ASMA-positive patients with CLD-B.

ASMA were found in a population of patients with viral hepatitis with lower titer reactivity than that observed in the patients with AIH. In our previous study, ASMA were found in 4 of 6 (66.7 %) patients with AIH, in 9 of 155 (5.8 %) patients with chronic hepatitis C, and 2 of 54 (3.7 %) patients with chronic hepatitis B (11). Abauf et al. (18) found ASMA at a titer higher than 1:40 in 49 (18.0 %) of 272 patients with chronic hepatitis C. In these studies, there was no significant difference in the incidence of ASMA between chronic hepatitis C and chronic hepatitis B.

On the other hand, ASMA react with ubiquitous components that are well expressed in smooth muscle cells, and some of the reactivities of ASMA were confirmed to react with cytoskeletal components (4-6). Several studies of anti-cytoskeleton antibodies in liver diseases revealed a relatively high prevalence of anti-MF (actin) antibodies (3, 7-9) in AIH, the immunoglobulin classes of which were mostly IgG (8). In the present study, anti-MF were found in 6 of the 7 patients with AIH. The incidence of anti-MF in the patients with AIH was higher than that of the patients with CLD-B, indicating a clear difference between ASMA-positive AIH and CLD-B according to the presence of anti-MF (7).

Anti-MF antibodies have been reported to be the major type of ASMA in viral hepatitis (7, 10), and this was observed in our patients with CLD-B as well. Circulating anti-vimentin IgM type IMF has been reported to appear during acute hepatitis type A (10). Although, anti-MF autoantibodies have been noted in some patients with chronic hepatitis B (7), their reactivities are not regarded as specific to hepatitis virus infection, since they have been reported in association with various viral infections, including human immunodeficiency virus-1 (HIV-1) infection where either ASMA or anti-MF were found in 30 % of the patients (19). Also, these autoantibodies have been detected in patients with cytomegalovirus infection (20), and other virus infection (21, 22). These findings support the serological segregation between autoimmune phenomena, as observed in AIH which were characterized by high reactions to several autoantigens including cytoskeletal components, and secondary serological autoimmune reactions as observed in viral infections in which such reactions are restricted and their titers are relatively low. The different profiles of the reactivities of anti-cytoskeleton antibodies in AIH and viral infections including viral hepatitis may contribute to the different immunological abnormalities in these pathological conditions.

Chronic hepatitis C patients appears to include a subset of patients with accompanying autoimmune phenomena similar to those observed in patients with AIH (12) in whom ASMA with high titer reactivity are observed (11). However, few studies have been performed to analyze the anti-cytoskeleton antibodies in patients with chronic hepatitis C, including the cases associated with autoimmune phenomena. A study of non-A, non-B hepatitis, which was conducted before methods for the detection of HCV infection had been developed, demonstrated the presence of anti-MF and anti-IMF in 1 and 3 of 18 patients, respectively (23).

In our ASMA-positive patients with CLD-C, who were confirmed to be positive for anti-HCV and HCV-RNA, the incidence of anti-MF was relatively high compared with that in the patients with CLD-B. Furthermore, the mean levels of serum gammaglobulin and IgG in these anti-MF-positive patients with CLD-C were higher than those in anti-MF-negative CLD-C and CLD-B patients, suggesting that a difference in the degree of stimulation of the immune system exists between anti-MF-positive CLD-C and anti-MF-negative CLD-C or CLD-B patients. Thus, autoimmune mechanisms might be involved in the pathogenesis of anti-MF-positive CLD-C, and anti-MF may be a useful differentiating marker.

In addition, several studies have suggested a major role of defective antigen-specific and/or non-specific T suppressor functions in the pathogenesis of AIH (24, 25). Also, the patients with CLD-C associated with autoimmune phenomena are considered to have decreased suppressor T functions. This impaired immunoregulation may play an important pathogenic role in the expansion of autoimmune responses, resulting in the induction of not only anti-MF but also anti-MF in their clinical courses.

However, the mechanism of the defective suppressor functions and the selectivity of the anti-MF autoantibody responses are largely unknown, and their elucidation will certainly provide further insight into the pathophysiology of autoimmune responses and of AIH.

Acknowledgements. We would like to thank Drs. N. Koide, H. Shimomura for their support and valuable discussions. Also, we would like to thank Professor M. Nambo, of the Department of Cell Biology, Institute of Molecular and Cellular Biology, Okayama University Medical School, for his instructive advice on the culture of fibroblasts. This work was supported

http://escholarship.lib.okayama-u.ac.jp/amo/vol48/iss3/5

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Received February 23, 1994; accepted March 11, 1994.