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Original Article

Antibody against Distinct Cytoplasmic Antigens in Patients Receiving Anti-Hepatitis C Therapy with Interferon and Ribavirin in Taiwan

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The aim of the present study was to examine the presence of autoantibodies in patients with hepatitis C virus (HCV) infection receiving combination pegylated interferon (Peg-IFN) and ribavirin therapy. Sera were obtained from 46 patients with HCV infection receiving combination Peg-IFN and ribavirin (Peg-IFN/ribavirin) treatment and 152 patients with HCV infection receiving Peg-IFN therapy only at Chung Shan Medical University Hospital, Taiwan. The serum samples were evaluated for the presence of anti-cytoplasmic antibody by indirect immunofluorescence assay (IIF) of HEp-2 cells. HEp-2 cells were also cultured with Peg-IFN (100 U/ml), ribavirin (100 μg/ml) and Peg-IFN/ribavirin. Anti-histone antibody (AHA) was determined by ELISA. The rod and ring index (RRI) was calculated by counting the numbers of rods and rings in 100 cells. Patients with HCV infection treated with Peg-IFN/ribavirin had a higher prevalence of anti-cytoplasmic rod and ring (anti-RR) antibodies than those not treated with Peg-IFN/ribavirin. RRI increased with duration of therapy and decreased after discontinuing therapy. When HEp-2 cells were cultured with Peg-IFN/ribavirin or ribavirin, RRI increased with culture time. Further, AHA increased with increasing anti-RR antibody in patients receiving Peg-IFN/ribavirin therapy. In conclusion, anti-RR antibody is found in HCV patients receiving Peg-IFN/ribavirin therapy and the frequency of anti-RR antibody is correlated with therapy time, suggesting that combination Peg-IFN/ribavirin and ribavirin alone play a role in the production of anti-RR antibodies. Anti-RR antibody should be considered a serological marker for patients receiving Peg-IFN/ribavirin therapy.

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Introduction

Chronic hepatitis C virus (HCV) infection has a global prevalence of about 3%. HCV infection is

not only related to chronic liver diseases such as hepatitis, cirrhosis and hepatocellular carcinoma, but also to autoimmunity, and is the main reason for liver transplantation[1-5]. The current standard therapy for HCV infection is a combination of pegylated interferon (Peg-IFN) and ribavirin[6,7]. Peg-IFN/ribavirin combination therapy produces a number of side effects including fatigue, influenza-like symptoms, systemic influenza-like symptoms, hematologic abnormalities, neuropsychiatric symptoms, psychiatric manifestations, and autoimmune reactions[8,9]. However, these side effects are generally mild. The exacerbation of thyroid autoimmunity and the development of systemic lupus erythematosus due to Peg-IFN/ribavirin combination therapy have been reported [5,10-13]. Previously, we studied the association between HCV infection and autoantibody production and found that the prevalence of autoantibodies was not affected by treatment with interferon-α (IFN-α) alone[3]. The aim of the present study was to examine the presence of autoantibodies in patients with HCV infection receiving a combination of Peg- IFN and ribavirin.

Materials and methods

Patient samples

Serum samples were obtained from 152 patients with HCV infection receiving Peg-IFN therapy and 46 patients with HCV infection receiving Peg-IFN/ribavirin combination therapy. Serum samples from 20 patients with HCV infection not receiving treatment served as the control. The patients were referred to the Department of Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan. The diagnosis of chronic HCV infection was based on the presence of anti-HCV antibodies. All patients had elevated serum ALT levels above the upper limit of the normal value. The quantitative measurements of serum HCV-RNA levels were performed on AmpliSensor Assay (Roche Molecular Systems, Inc; Branchburg, NJ, USA). The requirements for prescription of Peg-IFN/ribavirin combination therapy included: 1) anti-HCV positive; 2) elevated serum ALT levels above the upper limit of the normal value (ALT≥2X); 3. Liver fibrosis class F1 or higher or hepatic changes.

Indirect immunofluorescence (IIF)

IIF was performed on commercial HEp-2 cells (MBL, Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Patient sera were assayed at a dilution of 1:80, incubated for 25 minutes at room temperature (RT), washed with PBS twice for 10 minutes and incubated again for 25 minutes with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulins (MBL) at RT. The slides were washed twice for 10 minutes and observed under fluorescence microscope to determine the presence of anti-cytoplasmic antibodies. Cells were also prepared for in vitro culture study. Briefly, human epidermoid larynx carcinoma HEp-2 (ATCC CCL-23) cells were co-cultured with Peg-IFN (100 U/ml) (Schering-Plough Company, Heist-op-den-Berg, Belgium), ribavirin (100 μg/ml) (Schering-Plough) or Peg-IFN/ribavirin for 1, 4 or 6 hours. Cells were fixed in 2% paraformaldehyde at RT for 15 minutes then 70% methanol at 4oC for 30 minutes. This was followed by incubation with 3% bovine serum albumin at RT for 1 hour. The rod and ring index (RRI) was determined by counting the total numbers of rings and rods in the cytoplasm per 100 cells.

Anti-histone antibody (AHA) enzyme linked immunosorbent assay (ELISA)

AHA was determined using a commercial ELISA kit (Phadia GmbH, Freiburg, Germany) as previously described[14].

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 4.0 software for one-way analysis of variance (ANOVA). Comparisons of two groups were carried out using the T-test. A p value of < 0.05 was considered statistically significant.

Results

Anti-cytoplasmic rings and rods antibodies in HEp-2 cells determined by IIF

The sera of patients with HCV receiving Peg-IFN/ribavirin combination therapy showed unique cytoplasmic staining pattern in HEp-2 cells on IIF.

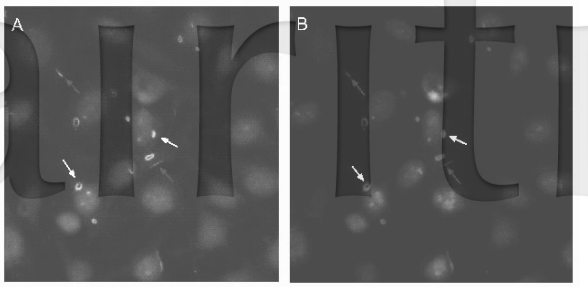

Fig. 1. Detection of anti-cytoplasmic rod and ring (anti-RR) antibodies in HCV patients undergoing Peg-IFN/ribavirin therapy. Patterns of cytoplasmic immunofluorescence in HEp-2 cells observed in patients receiving Peg-IFN/ribavirin therapy for hepatitis C infection. HEp-2 cells stained with human anti-RR serum and incubated with FITC-conjugated goat anti-human IgG antibodies (green) (A) Nuclei were counterstained with Hoechst (blue) (B) The slides were evaluated by fluorescence at ×100 magnification. Two patterns were observed; ring-like staining indicated by white arrows and rod-like staining indicated by red arrows in cytoplasm.

Anti-rings and anti-rods (anti-RR antibodies) have been named for this type of staining pattern (Fig. 1). The anti-RR antibodies appeared at different times during therapy. Twenty of 46 (43.47%) patients with HCV infection receiving Peg-IFN/ribavirin therapy were found to have anti-RR antibodies. Anti-RR antibodies were not present in the sera of 152 patients with HCV infection receiving Peg-IFN only and 20 patients with HCV infection without therapy. Durations of therapy were compared. Patients with anti-RR antibodies had a longer duration of therapy than those without anti-RR antibodies ($P < 0.0001$) (Fig. 2). Figure 3 shows an increase in RRI according to the duration of therapy ($n = 7$). RRI gradually decreased to zero after discontinuing therapy for one year.

Cell cultures with Peg-IFN, ribavirin, and Peg-IFN/ribavirin

To determine the effects of Peg-IFN, ribavirin, and Peg-IFN/ribavirin on RRI, HEp-2 cells were cultured with blank, Peg-IFN (100 U/ml), ribavirin, (100 μg/ml), or Peg-IFN/ribavirin for four hours and stained with a positive anti-RR antibody serum (Fig. 4A). The frequencies of the anti-RR antibodies were determined by counting 100 cells. RRI values were 18, 34, 124 and 155 for the blank, Peg-IFN, ribavirin, and Peg-IFN/ribavirin groups, respectively. There were no statistically significant differences between the Peg-IFN group and the blank group. There were also no statistically

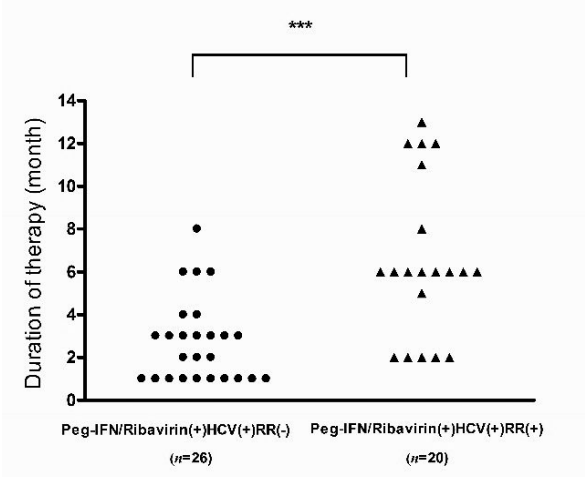

Fig. 2. HCV+ patients undergoing Peg-IFN/Ribavin therapy with anti-RR antibodies had significantly longer duration of therapy than patients without anti-RR antibodies. The duration of therapy was compared with the frequency of anti-RR antibodies. Values represent the means ± SEM. *** $P < 0.0001$. GraphPad Prism 4.0 software. A p value of < 0.05 was considered statistically significant.

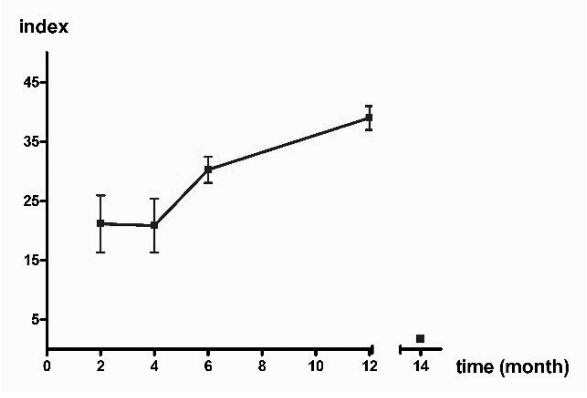

Fig. 3. The relationship between RRI and duration of therapy. RRI was determined by counting the numbers of rings and rods in 100 cells. RRI increased with increasing duration of therapy. Anti-RR in 7 patients was followed at different times. Values represent the means ± SEM. ***$P < 0.0001$. GraphPad Prism 4.0 software. A p value of < 0.05 was considered statistically significant.

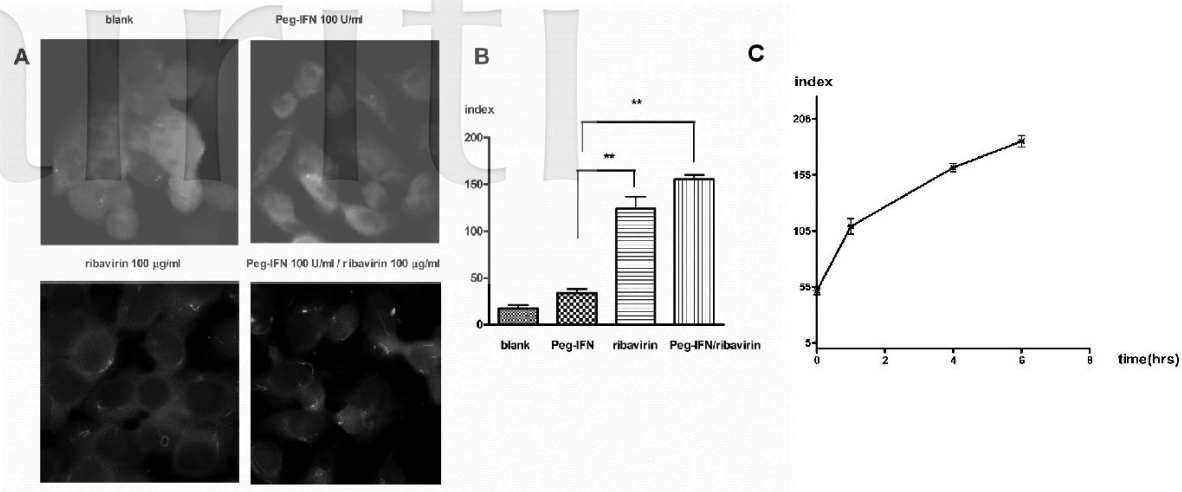

Fig. 4. RRI of HEp-2 cells cultured with Peg-IFN, ribavirin, or Peg-IFN/ribavirin for 4 hours. (A) blank, Peg-IFN, ribavirin, Peg-IFN/ribavirin. Staining of sera positive for anti-RR antibodies for thirty minutes. RRI was calculated for Peg-IFN, ribavirin, and Peg-IFN/ribavirin (B). Values represent the means ± SEM. ** P < 0.01. The relationship between RRI and time course in cells cultured with Peg-IFN/ribavirin. HEp-2 cells were incubated with Peg-IFN/ribavirin for one, four or six hours. RRI increased with increasing culture time. Values represent the means ± SEM. (C).

significant differences between the Peg-IFN/ribavirin and ribavirin groups. Cells cultured with ribavirin or Peg-IFN/ribavirin had higher RRI than those cultured with Peg-IFN or blank (Fig. 4B).

RRI of HEp-2 cells cultured with Peg-IFN/ribavirin based on time course

Figure 4C shows RRI of HEp-2 cells cultured with Peg-IFN/ribavirin. The culture times were one, four and six hours, respectively. The RRI increased after treatment in a time-dependent manner.

Anti-RR antibodies are associated with AHA

Figure 5 shows the correlation between AHA and anti-RR antibody in patients with HCV infection receiving Peg-IFN/ribavirin therapy. The mean concentration of AHA was higher in patients with anti-RR antibody than in those without anti-RR antibody (P < 0.01). The data shows that AHA is present in patients with anti-RR antibody.

Discussion

In the present study, anti-RR antibody was detectable in the sera of HCV patients following Peg-IFN/ribavirin therapy. The presence of this antibody was enhanced by duration of use of the drugs in vivo and incubation time of cell culture with the drugs in vitro. This antibody was restricted to the cytoplasm. The mechanism for the production of this antibody is still unknown, but recently Carcamo et al.[15] demonstrated the

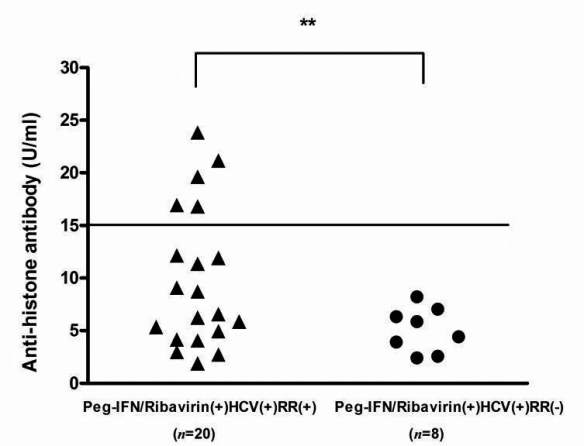

Fig. 5. The relationship between AHA and anti-RR antibody. The concentration of AHA (n = 20) in the group with positive anti-RR antibody was higher than that in the group with negative anti-RR antibody (n = 8). The cut-off of normal value for AHA was 15 U/ml as suggested by the manufacturer. ** P < 0.01.

induction of RR structures by inhibition of the CTP and GTP synthetic pathway in mammalian cells with a molecular weight of 55 kDa on IP-Western analysis. The detection of anti-RR antibody is specific for HCV patients receiving Peg-IFN/ribavirin therapy. Therefore, we recommend that anti-RR antibody be considered a serological marker for HCV patients receiving Peg-IFN/ribavirin therapy.

Patients with HCV infection receiving Peg-IFN only did not possess anti-RR antibodies. In vitro, RR was only induced by ribavirin and Peg-IFN/ribavirin, but not by Peg-IFN. Ribavirin may be a putative agent for inducing the RR structure during anti-HCV therapy. Ribavirin is a nucleoside analogue that can modulate type 1 and type 2 cytokine expressions, T-cell-mediated organ-specific autoimmune diseases, and IL-10 expression by directly inhibiting viral replication and protein synthesis[16,17].

Previous studies have found that anti-RR antibody is associated with HCV patients receiving Peg-IFN/ribavirin therapy[18-20]. Ribavirin-induced autoantibody has been identified as a new cytoplasmic autoantigenic structure in HCV patients following Peg-IFN/ribavirin therapy. This same structure can be induced by ribavirin on in vitro cell culture. The results of this study showed that cells cultured with ribavirin or Peg-IFN/ribavirin have higher RRI than those cultured with Peg-IFN or blank. Ribavirin may play a role in the response to Peg-IFN/ribavirin therapy.

In conclusion, the present study showed that patients with HCV infection treated with Peg-IFN/ribavirin have a higher RRI than patients not treated with Peg-IFN/ribavirin. Patients with anti-RR antibodies had a longer duration of therapy than patients without anti-RR antibodies. RRI increased with increasing duration of therapy. RRI decreased, falling to zero, after discontinuing therapy. This unique pattern makes this antibody a candidate for serological marker for HCV patients receiving Peg-IFN/ribavirin therapy.

Acknowledgements

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