Med Microbiol Immunol (2007) 196:157–164
DOI 10.1007/s00430-007-0038-1

ORIGINAL INVESTIGATION

Serum levels of IgG to the peptide of HCV1b core at positions 35–44 correlated with persistent infection, while levels of IgG to the peptide of NS5A at positions 2132–2140 correlated with better prognosis in HCV-infected patients

Yukari Takao · Akira Yamada · Shigeru Yutani ·
Takeharu Ono · Yumiko Nagao · Eiji Ando ·
Tatsuya Ide · Kyogo Itoh · Michio Sata

Received: 27 September 2006 / Published online: 16 February 2007
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Abstract We previously reported that two IgG Abs to the hepatitis C virus (HCV), anti-core 35–44 (C35) and anti-NS5A 2132–2140 (NS5A2132), existed in the sera of the majority of patients with HCV infection. This study investigated if measuring the two Abs would facilitate the prediction of a patient's prognosis. The serum levels of anti-C35 were found to correlate with persistent infection, while those of anti-NS5A2132 correlated with a better prognosis in HCV-infected patients. These results suggest that sequential measurement of the two Abs together may provide new information for the prediction of prognosis.

Keywords HCV · Peptide · Antibody · Prognosis · CTL-epitope

Y. Takao · S. Yutani · K. Itoh
Department of Immunology,
Kurume University School of Medicine,
Kurume, Fukuoka 830-0011, Japan

Y. Takao · Y. Nagao · E. Ando · T. Ide · M. Sata
Second Department of Medicine,
Kurume University School of Medicine,
Kurume, Fukuoka 830-0011, Japan

Y. Takao · A. Yamada (✉) · T. Ono · K. Itoh
Cancer Vaccine Development Division,
Kurume University Research Center for
Innovative Cancer Therapy, 67 Asahi-machi,
Kurume, Fukuoka 830-0011, Japan
e-mail: akiymd@med.kurume-u.ac.jp

A. Yamada · K. Itoh · M. Sata
Center of the Innovative Cancer Therapy, 21st Century COE program for Medical Science, Kurume 830-0011, Japan

Introduction

The hepatitis C virus (HCV) infection is one of the most serious health problems worldwide, as 170 million people around the world are persistently infected with HCV and are thus at high risk of developing liver cirrhosis (LC) and hepatocellular carcinoma (HCC) at later stages of the disease [5]. Although many studies of the immunological mechanisms involved in patient prognosis have been conducted during the past 2 decades, no suitable immunological monitoring system to predict the prognosis of each patient is available at the present time [1, 6, 7, 13, 14, 16, 18, 19]. In acute resolving hepatitis, HCV RNA is cleared in some individuals, whereas the majority (>50%) do not clear the virus, but instead develop chronic hepatitis (CH). Some CH patients respond to interferon (IFN)-therapy with a disappearance of virus, but others develop further progressive diseases such as LC and HCC. Immunity is thought to be largely involved in these processes, but there is no suitable laboratory marker to arrive at a reliable prognosis. This lack of useful findings might in part be due to an insufficient understanding of the biological roles of antibodies (Abs) that are specific to HCV. There are several lines of evidence suggesting the involvement of cytotoxic T-lymphocyte (CTL) and T helper cell responses in viral eradication [1, 6, 7, 13, 14, 16, 18, 19]. However, it is difficult to carry out CTL and T helper cell assays, which in any case are not sensitive enough to be utilized as laboratory markers.

We recently found two unique Abs specific to HCV1b-derived peptides; one of these Abs was specific to the human leukocyte antigen (HLA)-A2-restricted

Table 1 Diagnoses of residents in 1995 and 2002

1995	n	2002	n	Gender (M/F)	Age ± SD
CH	17	CH	12	3/9	67.7 ± 11.9
		LC	3	2/1	59.0 ± 7.9
		HCC with CH	1	0/1	82
		HCC with LC	1	1/0	65
LC	1	LC	1	0/1	69
ASC	4	CH	1	0/1	75
		ASC	1	0/1	76
		Past history of HCV infection	2	0/2	57.7 ± 6.36
Past history HCV infection	11	Past history HCV infection	11	2/9	68.8 ± 10.71
Total	33		33	8/25	67.1 ± 10.6

CH chronic hepatitis, *LC* liver cirrhosis, *HCC* hepatocellular carcinoma, *ASC* asymptomatic healthy carrier

CTL epitope peptide of the HCV1b core protein at positions 35–44 (anti-C35 Ab), and the other Ab was specific to the HLA-A24-restricted CTL epitope peptide of the NS5A protein at positions 2132–2140 (anti-NS5A2132 Ab) [17]. These two Abs were detected in a majority of a sample of patients with HCV$^+$, regardless of differences in terms of their disease conditions, but the biological activities of these Abs remain unclear at the present time. To gain a better understanding of their biological features, we investigated in the present study whether or not the measurement of these Abs would facilitate the prediction of a patient's prognosis. Our approach primarily involved the analysis of sera from patients at various stages of disease, including patients from a cohort study of HCV individuals evaluated from 1995 to 2002. That cohort study has been ongoing since 1990 in H town of Fukuoka prefecture in Japan, where the prevalence of HCV infection is the highest in the country [10, 11]. Our present results showed that the levels of anti-C35 Ab correlated with persistent infection, while those of anti-NS5A2132 correlated with a better prognosis in the sera of HCV$^+$ patients.

Materials and methods

Subjects

Informed written consent was obtained from all subjects whose serum samples were used in this study. The samples were obtained from 33 inhabitants with HCV-related diseases as part of a cohort study conducted from 1995 to 2002. The results of our follow-up survey of 33 inhabitants are given in Table 1. Serum samples from patients who were not participants in the cohort study, but who were treated at Kurume University Hospital, were also provided for the analyses; these patients were diagnosed with CH ($n = 68$), LC ($n = 43$), and HCC ($n = 52$). Anti-HCV Ab was measured by a chemiluminescent enzyme immunoassay (CLEIA) kit (Lumipulse II HCV, Fujirebio Inc., Tokyo, Japan). A second- or third-generation enzyme immunoassay was performed by a clinical lab company, SRL, Tokyo, Japan. Measurement of HCV-RNA and genotyping was performed by SRL using RT-PCR and direct sequencing of HCV in the sera of patients. Serum samples were also obtained from subjects with diseases other than HCV infection, including 17 cases of hepatitis B virus (HBV) infection (positive for HBV-surface antigen), 3 patients with human immunodeficiency virus (HIV), and 10 cases with human T cell leukemia virus type I (HTLV-1) infection. All of the subjects used in this study did not contain multi-infected patients. As a negative control, the sera from 37 healthy donors (HDs) with no history of either viral hepatitis or HBV vaccination, and who also had normal liver function test results, were used for this study.

Peptides

The following two peptides with a purity of >90% were purchased from Bio Synthesis (Lewisville, TX, USA); HCV1b core protein at positions 35–44 (YLLPRRGPRL), HCV1b NS5A protein at positions 2132–2140 (RYAPACKPL). HIV derived-peptide with an HLA-A2 binding motif (SLYNTVATL) and an HLA-A24 binding motif (RYLRDQQLLGI) were used as negative controls.

Measurement of peptide-reactive Abs

The levels of peptide-specific IgG were measured by means of an enzyme-linked immunosorbent assay (ELISA) that was carried out according to a previously reported method [10]. In brief, serum samples were diluted at 1:100, 1:200, and 1:400 with 0.05% Tween20-Block Ace, and 100 μl/well of the sample was added to each well that was pre-coated with a peptide. After incubation at 37°C for 2 h, the plates were washed with 0.05% Tween20-PBS (PBST) three times and were incubated at 37°C for 2h with 100 μl/well of 1:1,000-diluted

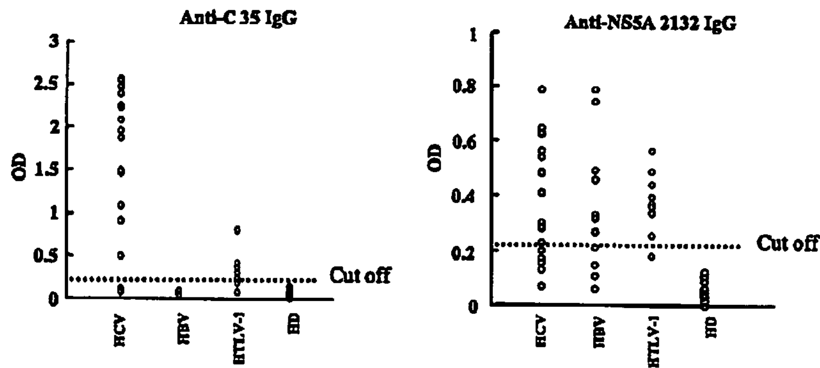

Fig. 1 Detection of anti-C35 and -NS5A2132 Abs in serum samples from patients with various diseases. Sera from patients with various viral infection-related diseases (HCV-related CH, HBV-related diseases, HTLV-1-related adult T-cell leukemia) along with HDs were measured for their reactivity to C35 and NS5A2132 peptides. The optical density (*OD*) values of each sample were assayed in serially diluted serum samples in order to estimate peptide-reactive IgG levels by ELISA. Representative OD results at a serum dilution of 100:1 are shown. The cut-off values were set as the mean + 2SD of the OD value of HDs (0.23 and 0.22 for anti-C35 and anti-NS5A2132, respectively). Statistical analyses were performed using the χ^2 test. Values of $P < 0.05$ were considered statistically significant

rabbit anti-human IgG (γ-chain-specific) (DAKO, Glostrup, Denmark). After the plates were washed three additional times, 100 μl/well of 1:100-diluted anti-rabbit IgG-conjugated horseradish peroxidase-dextran polymer (En Vision, DAKO) was added to each well, and the plates were incubated at room temperature for 40 min prior to stopping the reaction.

Statistics

The statistical analyses were performed using the χ^2 test. Values of $P < 0.05$ were considered statistically significant.

Results

Cross-reactivity, HLA-restriction, and genotype-specificity

We first investigated the cross-reactivity of the two Abs. Serum samples of 22 patients with HCV$^+$ CH were provided to test their reactivity to the HCV core protein at positions 35–44 (C35) and the NS5A protein at positions 2132–2140 (NS5A2132). Serum samples were also obtained from 17 patients with HBV infection and 10 patients with HTLV-1$^+$ adult T-cell leukemia. Sera from 37 HDs served as negative controls. As shown in Fig. 1, significant levels of anti-C35 IgG were detectable in 20 of 22 (90.9%) patients with HCV$^+$. Significant, but low, levels of anti-C35 IgG were also found in 8 of 10 (80%) patients with HTLV-1$^+$. In contrast, none of the patients with HBV$^+$ and none of the healthy donors possessed positive levels of anti-C35 Ab in their sera. Anti-NS5A2132 IgG was more widely found in patients with HCV, HBV, HTLV-1, but not in HDs.

We studied the relationship between the HLA-class I A-locus subtypes and serum levels of the two Abs, since the C35 and NS5A2132 peptides have been originally defined as HLA-class I A-restricted CTL-epitope peptides. Each of the Abs was detectable in the majority of HCV-infected patients, regardless of differences among the HLA-class IA phenotypes (Fig. 2a). We then examined the serum levels of anti-C35 Ab of patients with HCV1b ($n = 29$), HCV2a ($n = 16$), and HCV2b ($n = 3$) infection to determine the HCV-genotype specificity of anti-C35 Ab; as a result, we detected this Ab in 26 of 29, 16 of 16, and 3 of 3 patients, respectively. The results from all samples at a serum dilution of 1:100 are shown in Fig. 2b. Similarly, anti-NS5A2132 Ab was detectable in the sera from 23 of 29 patients infected with HVC1b, 8 of 16 patients with HCV2a, and all 3 HCV2b patients (Fig. 2b). These results suggest that both anti-C35 and -NS5A2132 Abs are detectable in HCV$^+$ patients, regardless of differences in HLA-class IA subtypes, and also with respect to differences in the genotypes of HCV.

Serum Ab levels and clinical stages of HCV infection

We next examined the sera of HCV-related CH ($n = 24$), LC ($n = 22$), HCC ($n = 26$), and HCV$^-$ HD ($n = 9$) subjects, and measured the levels of anti-C35 and anti-NS5A 2132 IgG in each of these groups (Fig. 3a). No significant difference was observed

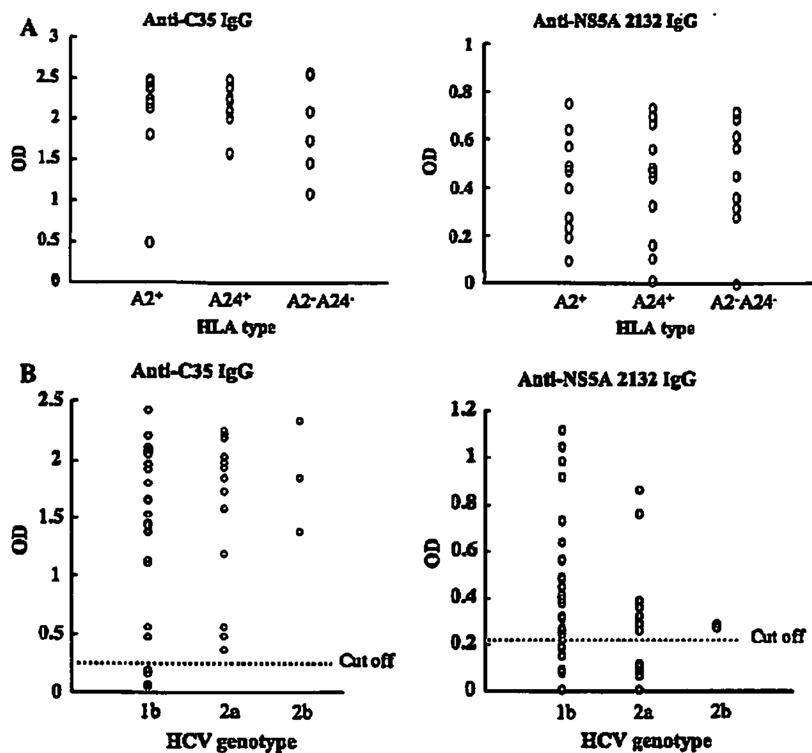

Fig. 2 No restriction of anti-peptide Abs with HLA or HCV genotypes. **a** The relationship between HLA-class IA phenotypes and the levels of anti-C35 or -NS5A2132 IgG were studied in 29 patients with HCV-related diseases. HLA-class IA phenotypes were determined by standard serological methods, and 9 patients were HLA-A2$^+$, 13 patients were HLA-A24$^+$, and the remaining 7 patients were HLA-A2$^-$/A24$^-$. Anti-C35 and -NA5A2132 were measured by standard ELISA as described above, and the OD value of each patient at a serum dilution of 1:100 was plotted. **b** The relationship between HCV genotypes and the levels of anti-C35 or -NS5A2132 IgG was studied in a double-blind manner using sera from patients with HCV1b (n = 29), HCV-2a (n = 16), and HCV-2b infection (n = 3). HCV genotypes were determined by the direct sequencing of HCV in the sera of HCV-infected patients. The results obtained from the sera of all subjects at a dilution of 1:100 are shown. Anti-C35 and -NA5A2132 Abs were measured by standard ELISA as described above, and the OD value of each patient's serum sample at a dilution of 1:100 was plotted. The cut-off value was set at 0.23 and 0.22 for anti-C35 and anti-NS5A2132, respectively

between the anti-C35 Ab levels of these three groups. In contrast, the anti-NS5A 2132 Ab levels of CH were higher than those of LC or HCC, i.e., the mean ± SD values of the Ab were 0.51 ± 0.24, 0.27 ± 0.20, 0.26 ± 0.23, and 0.08 ± 0.07, respectively. The levels of anti-NS5A 2132 Ab in the LC and HCC patients were significantly ($P < 0.05$) lower than those of the CH patients, but still higher than those of the HDs. Statistic analysis among the subgroups classified by disease severity, such as inflammatory activity or inactivity, suggested no difference between the subgroups of each clinical stages (data not shown). The levels of anti-HCV Ab measured by the third-generation assay did not significantly differ between the three groups (CH, 10 ± 2.7; LC, 11 ± 1.4; HCC, 11 ± 1.1) (Fig. 3b). The HCV RNA levels of the HCC patients (360 ± 269.6) were lower ($P < 0.05$) than those of the CH patients (610 ± 347.3) and the LC patients (610 ± 246.4) (Fig. 3c). However, there was no apparent correlation between the levels of anti-NS5A Ab and the HCV RNA levels (Fig. 3c).

Results of the cohort study

To further investigate the individual levels of correlation between anti-NS5A2132 Ab and the prognosis of HCV$^+$ patients, we investigated the serum levels of the two Abs in the samples from a cohort study that was conducted in H town in Fukuoka prefecture, where HCV-infected residents received an annual health screening from 1990 to 2002 [10, 11]. For this study, a total of 66 serum samples from 33 residents were used; here, the samples were harvested twice from the same individual, once in 1995 and once in 2002. The diagnosis of these 33 residents in 1995 were CH (n = 17), LC (n = 1), asymptomatic carrier (ASC) (n = 4), and a past

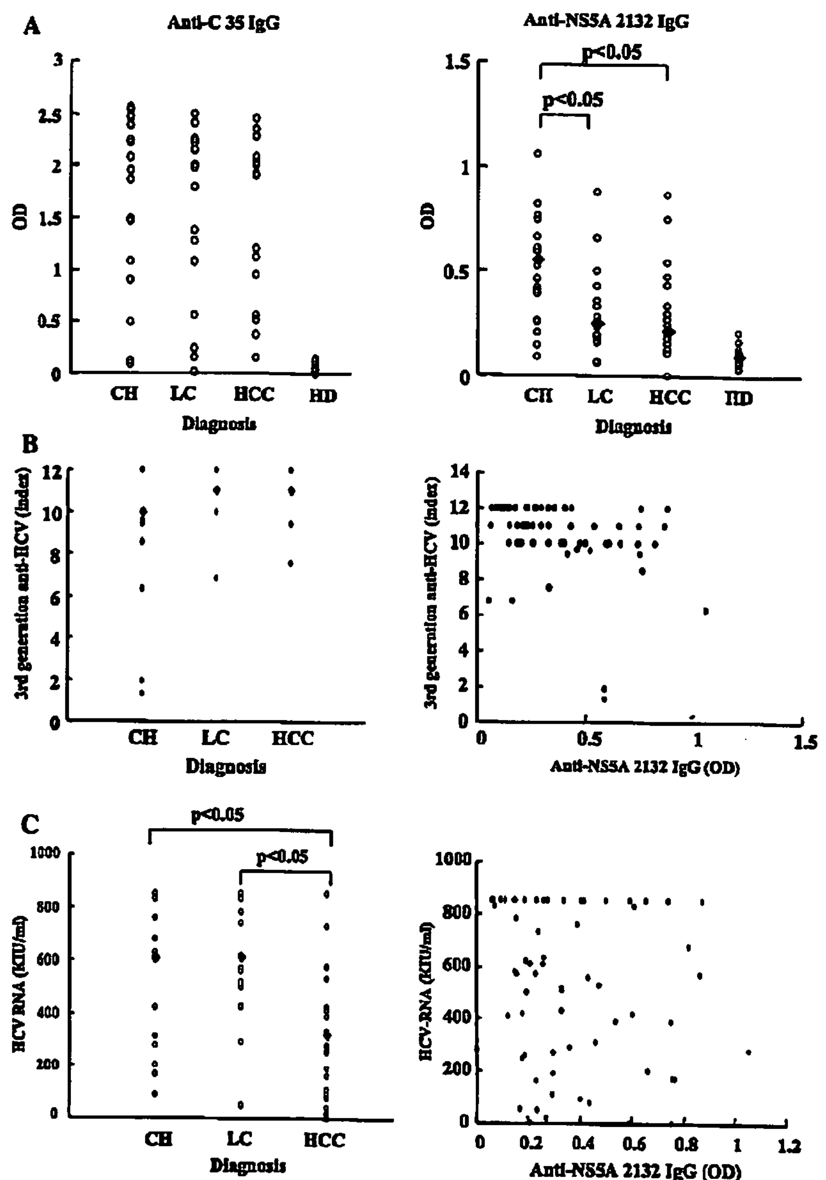

Fig. 3 Measurement of anti-NS5A 2132 Ab. **a** The sera of patients with CH ($n = 24$), LC ($n = 22$), HCC ($n = 26$) and that of HDs ($n = 8$) were provided for the measurement of the anti-C35 and anti-NS5A 2132 IgG levels. The levels of anti-NS5A 2132 Ab in the LC and HCC patients were significantly ($P < 005$) lower than those of the CH patients, but were still higher than those of the HDs. **b** All of the current serum samples were used for the measurement of the anti-HCV Ab levels by means of a commercially available kit (third-generation assay, SRL). These levels were given as the index (*left column*). The relationship between the levels of anti-HCV, as shown in the Index, and those of anti-NS5A2132 are given in the *right column*. **c** All of the present serum samples were also used for the measurement of the HCV RNA levels by means of a commercially available kit (Roche). The HCV RNA levels in the HCC patients (360 ± 269.6) were lower ($P < 0.05$) than those of the CH patients (610 ± 347.3) and the LC patients (610 ± 246.4). The relationship between the levels of HCV-RNA and those of anti-NS5A2132 is given in the *right column*

history of HCV infection (spontaneously recovered individuals; anti-HCV Ab^+ and HCV RNA^-) ($n = 11$), whereas in 2002, the diagnoses were as follows: CH ($n = 13$), LC ($n = 4$), HCC ($n = 2$), asymptomatic carrier (ASC) ($n = 1$), and a past history of HCV infection ($n = 13$). Namely, disease progression was not observed in 26 inhabitants, but was observed in the remaining 7 inhabitants during the 7 years (Table 1). As expected, the levels of anti-C35 Ab measured in the year 1995 were for the most part equal to those measured in 2002 in the majority of these 33 cases, regardless of the condition of the disease (Fig. 4a). In contrast, the levels of anti-NS5A Ab measured in the year 1995 had decreased in all seven residents with disease progression when they were measured again in the year 2002, whereas these levels were for the most part equal to those measured in the year 2002 in the majority of the 25 subjects who had no disease progression (Fig. 4a). The mean ± SD of anti-NS5A 2132 Ab (0.67 ± 0.13) in 1995 in the sera of the seven patients who had disease progression was significantly ($P < 0.05$) higher than that (0.27 ± 0.11) measured in 2002.

These 33 subjects were then subdivided into five different groups (CH, LC, HCC, past history of HCV-infection recovered individuals, and ASC), and the levels of the two Abs in the year 2002, measured at a serum dilution of 1:100, are plotted in Fig. 4b. The levels of anti-C35 Ab were consistently high in CH, LC, and HCC residents, and became very low or were

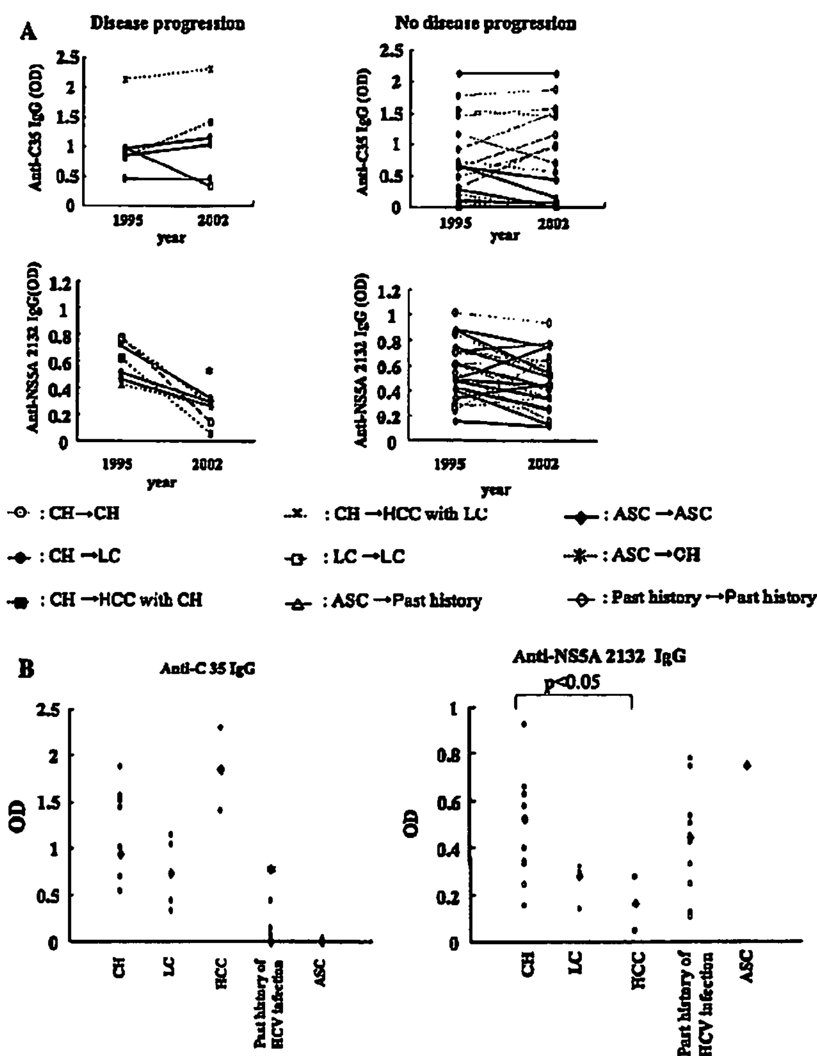

Fig. 4 Detection of anti-C35 and -NS5A2132 Abs in the cohort study. a A total of 66 serum samples from 33 inhabitants (harvested twice, once in 1995 and once in 2002) were simultaneously tested for their levels of anti-C35 and -NS5A2132 IgG. The diagnoses of these 33 patients in 1995 and 2002 are shown in Table 1. The patients were subdivided into two groups as follows: no disease progression (25 inhabitants) and disease progression ($n = 8$) groups. The measurement was repeated twice with consistent results, and the representative results of one experiment are shown. The OD values of anti-C35 Ab and anti-NS5A2132 IgG measured in 1995 and again in 2002 at a serum dilution of 1:100 are plotted. b Thirty-three residents were then subdivided into five different groups (CH, LC, HCC, past history of HCV-infection cured individuals, and ASC), and the results of the two tests of Ab levels measured in the year 2002 at a dilution of 1:100 are plotted. Statistical analyses were performed using the χ^2 test. Values of $P < 0.05$ were considered statistically significant

undetectable in cured individuals, whereas the levels of anti-NS5A 2132 Ab were high in CH residents, recovered individuals, and ASC residents, moderate in LC patients, and lowest ($P < 0.05$ vs. CH) in the HCC patients.

Discussion

The cross-reactivity of anti-C35 IgG was observed in the sera of HTLV-1⁺ patients, which could in part be due to the fact that the HTLV-1 envelope protein also possesses the same previously reported fine epitomic sequence (LPRR) as the C35 peptide at positions 37–40 [17]. The HIV tat protein also shares five amino acids (PRRGP) with the C35 peptide at positions 38–42; furthermore, the HIV envelope protein shares five amino acids (RRGPR) with the C35 peptide at positions 39–43. We therefore used the sera from three HIV⁺ subjects to examine the cross-reactivity to the C35 peptide. As a result, significant levels of anti-C35 IgG were found in the sera from all three subjects (data not shown). However, it should be noted that this cross-reactivity needs to be confirmed by the analysis of a greater number of samples from HIV⁺ individuals. HBV core protein, as well as HBV envelope protein, also shares four amino acids (PRRG) with the C35 peptide at positions 38–41, and thus we used the sera of 24 HBV⁺ subjects to investigate the cross-reactivity to the C35 peptide; here, it was observed that none of the serum samples tested had detectable levels of anti-C35 Ab. These results suggest that the sequence homology with the C35 peptide alone cannot account for this type of cross-reactivity. Further studies will therefore be conducted in order to resolve this issue.

In contrast to anti-C35 Ab, anti-NS5A2132 Ab was detected in patients with other diseases, including HBV⁺ individuals and HTLV-1⁺ subjects, whereas it

was rarely detected in the HDs. This wide range of cross-reactivity could be in part due to the fact that four of ten amino acids of the NS5A2132 peptide at positions 2133–2136 (YAPA) are identical to those of oligodendrocyte-related protein, which is expressed in normal cells [3]. Four of ten amino acids of the NS5A2132 peptide at positions 2134–2137 (APAC) are also shared with the HBV surface protein.

Both anti-C35 and -NS5A Abs were observed in HCV-infected individuals, regardless of the HCV-genotypes tested. This finding could be due at least partly to the fact that the C35 sequence of HCV1b is 100% conserved in both HCV2a and HCV2b. Similarly, the NS5A2132 sequence is largely conserved in HCV2a (five of nine amino acids matched). The cross-reactivity to the serum samples of HCV1a patients, along with that to the HCV3, HCV4 and the other HCV genotypes will be examined, but the sera from patients infected with these HCV genotypes are rarely available in Japan [2, 8].

The cohort study revealed that the levels of anti-C35 Ab were consistently high at all stages of disease in patients with CH, LC, and HCC, and these levels were very low or undetectable in recovered individuals; however, the levels of anti-NS5A2132 were high in recovered individuals and CH inhabitants, modest in LC inhabitants, and lowest in HCC patients. All of the subjects of the cohort study had been treated during the study term. Therefore, the Ab levels, as well as prognoses, of the subjects might be affected by the treatments. Using sera other than the samples from the cohort study, we also demonstrated here that the levels of anti-NS5A 2132 Ab in the LC and HCC patients were significantly ($P < 0.05$) lower than those of the CH patients, but still higher than the levels observed in HDs. Collectively, these results demonstrated that the serum levels of anti-C35 correlated with persistent infection, while those of anti-NS5A2132 correlated with better prognosis in HCV-infected patients. Anti-C35 Ab was detectable in HCV, HTLV-1, and is most likely also detectable in HIV-infected individuals, but not in patients with the other diseases tested, whereas anti-NS5A Ab was detectable in the sera from patients with variable diseases. The third-generation assay utilizes four recombinant HCV proteins from the core, NS3, NS4, and NS5 regions as a source of antigens, and yet this type of assay cannot provide conclusive information with regards to either a patient's prognosis or to immunological responses to CTL epitope peptides. Therefore, from a clinical perspective, the sequential measurement of these two Abs together may provide new information for predicting a patient's prognosis, although follow-up studies with a larger number of samples are needed.

However, if one considers the possible mechanisms responsible for the different patterns of these two Abs, no information is available at the present time. Anti-C35 IgG might be required, either directly or indirectly, for the elimination of HCV, primarily because it has been shown to decrease in recovered individuals. A gradual decrease in the Ab to HCV core protein in the sera of recovered individuals was reported, although the mechanisms of this decrease remain obscure [6]. In contrast to anti-C35 IgG, anti-NS5A2132 IgG might be produced by long-lasting memory B cells, because it is associated with good prognosis. Long-lasting memory CTL and T helper cells reactive to certain recombinant HCV proteins besides the NS5A protein at positions 2003–2267 appear to be present in persons who were accidentally exposed to HCV1b, but not in CH patients [6]. It would be of interest to study T helper activity to the NS5A protein in the vicinity of the NS5A 2132–2140 peptides. It might also be of interest to study the relationship between these two Abs, and in particular, anti-NS5A2132 and autoimmune hepatitis, since a variety of antibodies reactive to nuclei, smooth muscle cells and liver/kidney microsomes type 1 (LKM-1) have been found in patients with HCV-related diseases [4].

Although the present results do not provide any clear information for elucidating the biological roles of anti-C35 and -NS5A Abs, the results may encourage further studies of Abs reactive to CTL epitope peptides from HCV.

Acknowledgments This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (no. 12213134 to K.I. and the 21st Century COE Program for Medical Science to K.I. and A.Y.) and from the Ministry of Health, Labor and Welfare, Japan (no. H14-trans-002 11-16 to K.I.).

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American Journal of Gastroenterology
© 2007 by Am. Coll. of Gastroenterology
Published by Blackwell Publishing

ISSN 0002-9270
doi: 10.1111/j.1572-0241.2006.01038.x

Clearance of HCV Improves Insulin Resistance, Beta-Cell Function, and Hepatic Expression of Insulin Receptor Substrate 1 and 2

Takumi Kawaguchi, M.D., Ph.D.,[1,2] Tatsuya Ide, M.D., Ph.D.,[1,2] Eitaro Taniguchi, M.D., Ph.D.,[2] Eiichi Hirano, Ph.D.,[1] Minoru Itou, M.D.,[2] Shuji Sumie, M.D.,[2] Yumiko Nagao, M.D., DDS.,[1,2] Chikatoshi Yanagimoto, M.D.,[2] Shinichiro Hanada, M.D., Ph.D.,[2] Hironori Koga, M.D.,[2] and Michio Sata, M.D.[1,2]

[1]*Department of Digestive Disease Information and Research,* [2]*Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Japan*

OBJECTIVES: Hepatitis C virus (HCV) infection is linked to greater insulin resistance. Although HCV itself is a candidate for the development of insulin resistance, the effects of antiviral treatment on impaired glucose metabolism remain unclear. The aim of this study is to examine the effects of clearance of HCV on insulin resistance, beta-cell function, and hepatic expression of insulin receptor substrate (IRS)1/2, central molecules for insulin signaling.

METHODS: We analyzed 89 biopsy-proven patients with chronic HCV infection. Patients received interferon-α or interferon-α plus ribavirin for 6 months and were classified into three groups at 6 months after the conclusion of antiviral therapy according to their response to antiviral therapy: sustained responders ($N = 29$), relapsers ($N = 12$), and nonresponders ($N = 48$). Insulin resistance and beta-cell function were assessed by the homeostasis model assessment method (HOMA-IR and HOMA-%B, respectively). Hepatic expression of IRS1/2 was evaluated by immunoblotting and immunostaining in 14 sustained responders.

RESULTS: In nonresponders and relapsers, there were no significant changes in HOMA-IR and HOMA-%B values after antiviral therapy. On the other hand, in sustained responders, HOMA-IR values significantly decreased to 1.7 ± 0.8 from 3.1 ± 1.1 ($P < 0.05$) after antiviral therapy. Similarly, HOMA-%B values significantly decreased to 90.6 ± 10.0 from 113.7 ± 15.3 ($P < 0.05$). Immunoblotting showed a threefold increase in IRS1/2 expression after clearance of HCV. Immunostaining revealed that greater IRS1/2 expression was seen in hepatocytes.

CONCLUSIONS: We showed that clearance of HCV improves insulin resistance, beta-cell function, and hepatic IRS1/2 expression.

(Am J Gastroenterol 2007;102:1–7)

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is associated with a greater risk for the development of insulin resistance (1). Greater insulin resistance is more prevalent among patients with HCV infection compared with those with other liver diseases and with the general population (2). In patients with HCV infection, insulin resistance is involved in progression of hepatic fibrosis (3), the development of hepatocellular carcinoma (4, 5), extrahepatic manifestations (6), and prognosis (7). Thus, insulin resistance plays a crucial role in patients with HCV infection.

Insulin resistance can be caused by many factors. In general, obesity, inflammation, and various kinds of metabolic disorders are common factors for the development of insulin resistance. Similarly, body mass index (BMI), serum tumor necrosis factor-α (TNF-α) and hepatic iron concentrations, and hepatic steatosis are reported to be possible causative factors for the development of insulin resistance in patients with HCV infection (8–11). In addition to these factors, HCV itself is also known to have a variety of biological effects (12).

In HCV core transgenic mice, the development of insulin resistance is seen by 1 month of age, in the absence of either overt liver injury or excessive body weight gain (12, 13). Furthermore, even if liver function is restored by transplantation, postliver transplantation diabetes mellitus occurs more frequently among patients who undergo transplantation for HCV than for other conditions (14). Although precise mechanisms for HCV-associated insulin resistance have not been fully elucidated, we recently demonstrated the involvement of insulin receptor substrate 1 and 2 (IRS1/2), central molecules in insulin signaling. Downregulation of IRS1/2 is seen in

livers from HCV core transgenic mice as well as in patients with HCV infection (15). Thus, HCV itself is a candidate risk factor for the development of insulin resistance.

If HCV is a causal factor, then clearance of HCV might decrease insulin resistance just as histologic improvement of fibrosis and reduction in the risk of hepatocellular carcinoma are seen in patients with hepatitis C who have sustained response to interferon therapy (16, 17). The ability of antiviral therapy to improve glucose metabolism would support the notion that HCV causes insulin resistance in patients with HCV infection. Accordingly, we studied the effects of HCV clearance on insulin resistance, beta-cell function, and hepatic expression of IRS1/2.

MATERIALS AND METHODS

Materials

All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated.

Patients

We analyzed 89 patients with HCV infection. The diagnosis was based on elevated serum aminotransferase level, histological examination, consistent detection of anti-HCV, and HCV-RNA. Patients who coincided with other causes of liver disease such as chronic hepatitis B, autoimmune hepatitis, or alcoholic liver disease (greater than 80 g alcohol per day for at least 1-month duration prior to the onset of illness) were excluded, as were those who had been taking corticosteroids or those with a history of, or evidence of, pancreatitis or a pancreatic tumor. Clinical data collected before antiviral therapy included age, sex, and alcohol use. BMI was calculated as body weight in kilograms divided by the square of height in meters (kg/m^2). Informed consent for participation in the study was obtained from each subject. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in prior approval by the Ethics Committee of the Kurume University School of Medicine. None of the subjects was institutionalized.

Laboratory Determinations

Venous blood samples were taken in the morning after a 12-h overnight fast. Plasma glucose, serum aspartate aminotransferase, alanine aminotransferase, albumin, total bilirubin, and immunoreactive insulin (IRI) levels were measured by using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital). Beta-cell function and insulin resistance were calculated on the basis of fasting levels of plasma glucose and IRI, according to the homeostasis model assessment (HOMA) method (18). The formulas for the HOMA model are as follows: beta-cell function (HOMA-%B) = fasting IRI (μU/mL) × 360/(fasting glucose (mg/dL) − 63); insulin resistance (HOMA-IR) = fasting glucose (mg/dL) × fasting IRI (μU/mL)/405. HCV genotyping was performed according to Okamoto's method (19) and genotypes were classified according to Simmonds's classification system (20). An Amplicor-HCV-Monitor 1.0 (Roche Diagnostics K.K., Tokyo, Japan) was used to quantify HCV-RNA levels.

Liver Biopsy

Liver tissue was obtained by percutaneous ultrasound image-guided liver biopsy. The biopsies were performed by two staff gastroenterologists using a Pro-Mag™ Biopsy Needle (Medical Device Technologies Inc., Gainesville, FL), which has a biopsy specimen notch of 20.00 mm in width and 2.05 mm in diameter. More than 95% (vol/vol) of liver tissue was used for histological and immunostaining analyses. Less than 5% (vol/vol) of liver tissue was homogenized and 80 μg of protein was used for immunoblotting analysis.

Histological Data

For each patient, a liver biopsy specimen was fixed in 10% formalin buffer and stained with hematoxylin-eosin. Liver biopsy specimens were evaluated by a single, experienced pathologist who was unaware of the patients' clinical and laboratory data. The specimens were scored according to the METAVIR scoring system (21), which is suited for evaluation of chronic hepatitis C.

Treatment Outcome

All patients were treated with 3 to 10 million U of interferon-α (interferon-α2a, Nippon Roche K.K., Tokyo, Japan; interferon-α2b, Schering-Plough K.K., Osaka, Japan; or natural interferon-α, Dainippon Sumitomo Pharma Co., Osaka, Japan) by subcutaneous injection three times per week, or 6 to 10 million U of interferon-α2b plus ribavirin (600 to 1,000 mg daily, Schering-Plough Co) for 6 months. Patients were followed up until 6 months after the conclusion of antiviral therapy and classified into three groups: sustained responders (N = 29), who had undetectable serum HCV-RNA; relapsers (N = 12), who had undetectable HCV-RNA at the end of antiviral therapy but HCV-RNA relapse during follow-up; and nonresponders (N = 48), who had detectable HCV-RNA during and after treatment.

Immunoblotting

Liver tissue was homogenized on ice in 1 mmol/L $NaHCO_3$ containing protease inhibitors, stored at −80°C as previously described (22, 23). Equal amounts of protein (40 μg) from liver homogenates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% acrylamide gel. The resolved proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (Amersham International, Buckinghamshire, UK). The membranes were incubated with an antihuman IRS1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or an antihuman IRS2 polyclonal antibody (Santa Cruz Biotechnology), and were subsequently incubated with an HRP-conjugated goat antirabbit IgG (Amersham International). The membranes were then incubated with chemiluminescence reagents (ECL kit, Amersham International) and immediately exposed on

radiograph film. Immunoblotting intensities were determined using NIH-Image J (developed at the National Institutes of Health and available from the Internet by an anonymous FTP from http://rsb.info.nih.gov/ij/download.html) as previously described (22, 23).

Immunohistochemistry

In 14 sustained responders, liver biopsy was performed before and after conclusion of antiviral therapy. Paraffin-embedded liver sections from patients with HCV infection were deparaffinized and subjected to immunohistochemical staining using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with an antihuman IRS1 polyclonal antibody (Santa Cruz Biotechnology) or an antihuman IRS2 polyclonal antibody (Santa Cruz Biotechnology), and developed with 3,3'-diaminobenzidine (DAB). The primary antibodies for IRS1/2 were used at a 1:100 dilution. The specificity of IRS1/2 staining was confirmed by immunization using an excess amount of the N-terminal peptide of IRS1/2.

Statistical Analysis

All data are expressed as mean ± SD. The Wilcoxon's single-rank test was employed for analysis of paired samples. Statistical comparisons among multiple groups were performed by analysis of variance followed by Scheffe's *post hoc* test. P values < 0.05 were considered significant.

RESULTS

Characteristics of the Patients

Characteristics of the patients before antiviral therapy are summarized in Table 1. There was no significant difference in age or sex distribution among the groups. In sustained responders, higher infection rates of genotype 2 (62.0%) were seen compared with nonresponders (12.5%) or relapsers (16.7%). Although HCV viral load, hepatic fibrosis, and HOMA-IR were lower in sustained responders, BMI and hepatic necroinflammatory activity were not significantly different among the groups.

Changes in BMI, Insulin Resistance, and Beta-Cell Function After Antiviral Therapy

Changes in BMI, insulin resistance, and beta-cell function after antiviral therapy are summarized in Figure 1. In nonresponders (N = 48), BMI significantly decreased to 21.7 ± 1.6 kg/m^2 from 22.7 ± 2.3 kg/m^2 ($P < 0.01$) at the end of follow-up. However, there were no significant changes in HOMA-IR and HOMA-%B values at the end of follow-up compared with those before antiviral therapy (HOMA-IR 4.0 ± 1.7 *vs* 3.6 ± 1.2, $P = 0.11$, HOMA-%B 120.0 ± 26.1 *vs* 112.4 ± 24.1, $P = 0.09$) (Fig. 1A). In relapsers (N = 12) no significant differences were seen in BMI (21.8 ± 1.7 kg/m^2 *vs* 22.1 ± 1.6 kg/m^2, $P = 0.70$), HOMA-IR values (3.7 ± 1.2 *vs* 3.6 ± 1.2, $P = 0.69$), and HOMA-%B values (121.5 ± 13.3 *vs* 117.4 ± 17.4, $P = 0.24$) at the end of follow-up compared with those before antiviral therapy (Fig. 1B). In sustained responders (N = 29), there was no significant difference in BMI at the end of follow-up (22.6 ± 1.6 kg/m^2 *vs* 21.9 ± 1.9 kg/m^2, $P = 0.07$). On the other hand, HOMA-IR values significantly decreased to 2.2 ± 0.7 from 3.1 ± 1.0 ($P < 0.01$) by the end of follow-up. Similarly, HOMA-%B values significantly decreased to 92.6 ± 14.0 from 113.7 ± 21.3 ($P < 0.01$) (Fig. 1C).

Changes in Hepatic Expression of IRS1/2 in Sustained Responders

Immunoblotting demonstrated a significant increase in expression of IRS1/2 after antiviral therapy in livers from sustained responders (Fig. 2A). After antiviral therapy, mean IRS1 and IRS2 intensities showed a two- and threefold increase, respectively, compared with intensities before antiviral therapy (Table 2). In immunostaining, IRS1 occurred mainly in lymphocytes (Fig. 2B, left upper panel) before antiviral therapy, but occurred in hepatocytes after antiviral therapy (Fig. 2B, right upper panel). On the other hand, IRS2

Table 1. Characteristics of the Patients

	Nonresponders	Relapsers	Sustained Responders	*P* Value
N	48	12	29	
Age (yr)	61.7 ± 7.7	63.2 ± 6.1	58.5 ± 8.6	N.S.
Male/female	27/21	8/4	19/10	N.S.
BMI	22.7 ± 2.3	21.9 ± 1.7	22.6 ± 1.6	N.S.
Aspartate aminotransferase (U/L)	68.1 ± 36.3	75.7 ± 24.4	64.2 ± 30.4	N.S.
Alanine aminotransferase (U/L)	92.5 ± 35.1	86.3 ± 24.8	88.7 ± 30.4	N.S.
γ-glutamyltranspeptidase (U/L)	97.9 ± 44.6	88.0 ± 37.1	94.0 ± 34.9	N.S.
Total bilirubin (mg/dL)	0.84 ± 0.11	0.81 ± 0.20	0.85 ± 0.15	N.S.
Albumin (g/dL)	3.79 ± 0.26	3.71 ± 0.29	3.87 ± 0.28	N.S.
Genotype 1/2	42/6	10/2	11/18	0.008
Viral load (× 10^3 copies)	485 ± 299	534 ± 254	309 ± 212	0.024
Necroinflammatory activity	2.04 ± 0.71	2.00 ± 0.74	1.90 ± 0.78	N.S.
Fibrosis	2.29 ± 0.74	2.25 ± 0.87	1.82 ± 0.81	0.046
HOMA-IR	3.95 ± 1.69	3.73 ± 1.21	3.07 ± 0.95	0.01

Data are expressed as mean ± SD or as number of patients.
BMI = body mass index; HOMA-IR = homeostasis model assessment for insulin resistance.

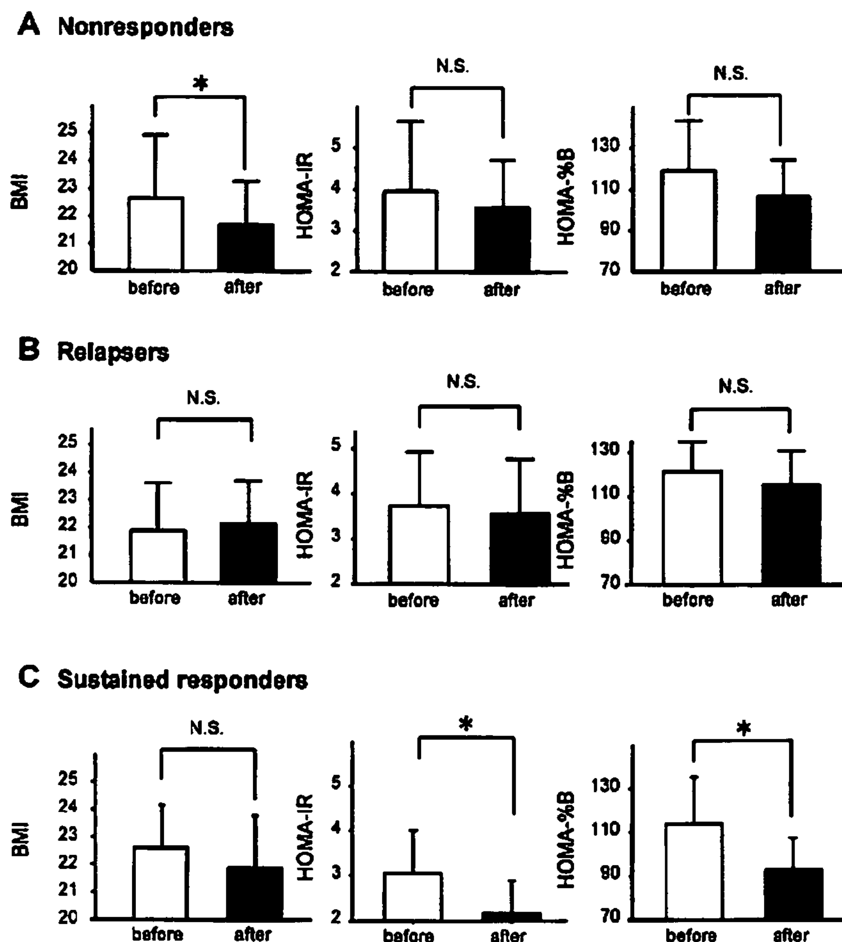

Figure 1. BMI, HOMA-IR, and HOMA-%B before and after antiviral therapy in nonresponders (N = 48; *A*), relapsers (N = 12; *B*), and sustained responders (N = 29; *C*). Data were obtained before antiviral therapy and 6 months after its conclusion. Data are expressed as mean ± SD. *$P < 0.01$. N.S., not significant.

occurred in hepatocytes both before and after antiviral therapy (Fig. 2B, lower panels). After antiviral therapy, expression of IRS2 was upregulated mainly in periportal hepatocytes (Fig. 2B, right lower panel).

DISCUSSION

The present study demonstrates that clearance of HCV improves HOMA-IR, HOMA-%B, and hepatic expression of IRS1/2. These findings indicate that HCV itself is involved in the development of insulin resistance.

Insulin resistance can be caused by many factors. Obesity is a common factor for the development of insulin resistance (24). Although greater insulin resistance was seen in patients with chronic hepatitis C, BMI values were within normal limits in this study. Improved HOMA-IR was only seen in sustained responders and HOMA-IR remained unchanged in nonresponders, despite a decrease in BMI after antiviral therapy. In an epidemiologic study, Bahtiyar *et al.* reported that obesity is not associated with the development of insulin resistance in patients with HCV infection (25). In addition, the development of insulin resistance is seen by 1 month of age, in the absence of either overt liver injury or excessive body weight gain in HCV core transgenic mice (13) and serum HCV core protein levels are associated with HOMA-IR values in patients with chronic hepatitis C (15). Moreover, a significant increase in the incidence of diabetes was seen in subjects with high titers of HCV core compared with subjects with low titers of HCV core or anti-HCV-negative subjects at the population level during 7 yr of follow-up (26). Taken together, these findings suggest that HCV itself causes insulin resistance.

Interferon is known to induce insulin resistance. However, our results showed that interferon leads to a reduction in insulin resistance in sustained responders. Even in nonresponders or relapsers, interferon did not worsen insulin resistance. Interferon-induced insulin resistance is observed only in the early phase of treatment (27). Indeed, after 3 months of treatment, interferon-induced insulin resistance disappears (28).

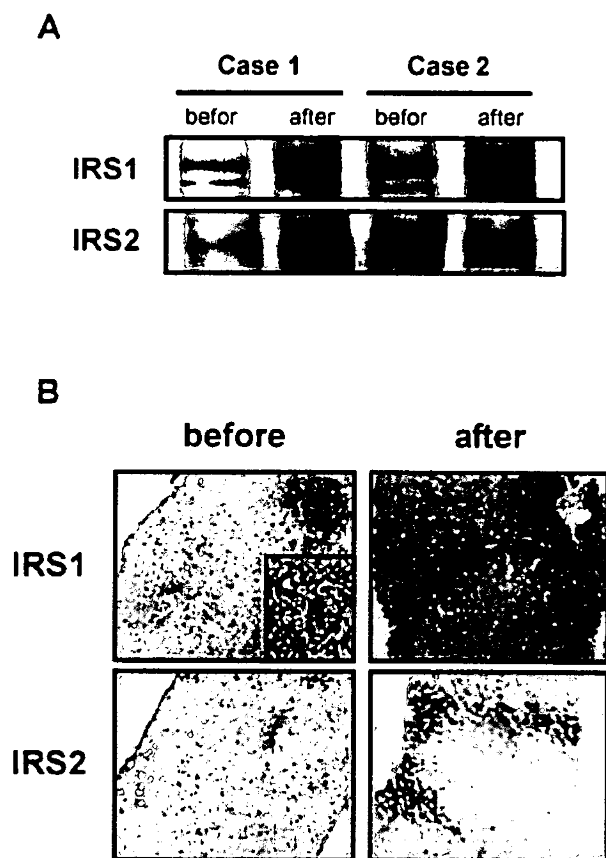

Figure 2. Protein expression levels of IRS1/2 before and after antiviral therapy in sustained responders. Immunoblotting for IRS1/2 (*A*). Proteins in liver extracts before and after antiviral therapy were immunoblotted with anti-IRS1 antibodies (upper panel) or anti-IRS2 antibodies (lower panel). Immunostaining for IRS1/2 (*B*). Liver sections before and after antiviral therapy were immunostained with anti-IRS1 antibodies (upper panels) or anti-IRS2 antibodies (lower panels). Expression of IRS1 and IRS2 were visualized by 3,3′-diaminobenzidine (brown). Expression of IRS1 in lymphocytes was shown. Original magnification ×400. Protein expression levels of IRS1/2 were examined in 14 sustained responders and representative immunoblotting and immunostaining images are shown.

Romero-Gomez *et al.* reported that improved insulin resistance during and after interferon therapy is correlated with a positive response to antiviral therapy (29), which is in good agreement with our findings. These finding also suggest the involvement of HCV in the development of insulin resistance.

Pancreatic beta-cells play a crucial role in maintaining glucose homeostasis. Although HCV infects not only liver but also pancreas (30), our results demonstrated that beta-cell function, especially the ability to secrete insulin, was preserved in patients with chronic hepatitis C. Because HOMA-%B was significantly decreased after antiviral therapy in sustained responders, increase in HOMA-%B seems to be an adaptation against greater insulin resistance. On the other hand, Narita *et al.* reported that beta-cell function is significantly decreased in patients with HCV infection (31). Although the reasons for this discrepancy are not clear, it

Table 2. Hepatic Expression Levels of IRS1/2 Before and After Antiviral Therapy in Sustained Responders

	N	Before (Arbitrary Units)	After (Arbitrary Units)	*P*
IRS1	14	83.3 ± 47.9	156.8 ± 47.5	0.002
IRS2	14	31.7 ± 16.4	88.0 ± 33.8	0.001

Data are expressed as mean ± SD.

could be explained by following reasons: First, BMI in the previous study is higher than that in our study. Second, patients who consumed alcohol were enrolled in the previous study, while we excluded the patients who had >80 g/day of alcohol. Obesity and alcohol consumption lead to a decrease in early-phase insulin secretion (32, 33). In addition, HCV core-transgenic mice exhibited a significant increase in early-phase insulin secretion compared with control mice (13). Thus, dysfunction of beta-cells does not seem to be responsible for HCV-associated insulin resistance.

TNF-α is a causative factor for greater insulin resistance. However, there was no significant difference in serum TNF-α level between HCV patients with insulin resistance and without insulin resistance (34). Impairment of insulin receptor can cause insulin resistance. However, there was no significant change in hepatic insulin receptor between controls and HCV core-transgenic mice (15). Recently, we identified a molecular mechanism for HCV-associated insulin resistance. HCV core downregulates hepatic expression of IRS1/2 (15). Because IRS1 and IRS2 are central molecules in intracellular insulin signaling, downregulation of these molecules should decrease downstream insulin effects such as glucose uptake, thereby contributing to insulin resistance. In this study, we first demonstrated increases in hepatic expression of IRS1/2 after antiviral therapy in sustained responders. These findings support our proposed molecular mechanism that HCV directly downregulates hepatic expression of IRS1/2.

In conclusion, we showed that clearance of HCV improves HOMA-IR, HOMA-%B, and hepatic expression of IRS1/2. These findings indicate that HCV itself is involved in the development of insulin resistance in patients with HCV infection.

ACKNOWLEDGMENTS

The authors thank Yumi Ogo and Mari Hagihara for technical assistance. This study was supported, in part, by a Grant-in-Aid for Scientific Research (C) (No. 16590648 to M.S.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Vehicle Racing Commemorative Foundation, and the 21st Century COE Program for Medical Science, Kurume University. A part of this study was presented at the 54th Annual Meeting of the American Association for the Study of Liver Diseases (Boston, MA) in October 2005 and published in abstract form (Hepatology 2005:42:540A).

STUDY HIGHLIGHTS

What Is Current Knowledge

- Greater insulin resistance and hyperinsulinemia are seen in patients with hepatitis C virus (HCV) infection.
- Insulin receptor substrate 1 and 2 (IRS1/2), central molecules in insulin signaling, are downregulated in livers from patients with HCV infection.

What Is New Here

- Clearance of HCV reduced insulin resistance and improved hyperinsulinemia.
- Hepatic expression of IRS1/2 was increased by clearance of HCV.

Reprint requests and correspondence: Takumi Kawaguchi, M.D., Ph.D., Department of Digestive Disease Information and Research, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan.

Received September 8, 2006; accepted October 20, 2006.

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CONFLICT OF INTEREST

Guarantor of the article: Takumi Kawaguchi, M.D., Ph.D.
Financial support:. This study was supported, in part, by a Grant in Aid for Scientific Research (C) (No. 16590648 to M.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Vehicle Racing Commemorative Foundation, and the 21st Century COE Program for Medical Science, Kurume University.
Potential competing interests: None

Liver International ISSN 1478-3223

CASE REPORT

Branched-chain amino acids improve insulin resistance in patients with hepatitis C virus-related liver disease: report of two cases

Takumi Kawaguchi[1,2], Eitaro Taniguchi[2], Minoru Itou[2], Shuji Sumie[2], Tetsuharu Oriishi[2], Hisako Matsuoka[1], Yumiko Nagao[1,2] and Michio Sata[1,2]

1 Department of Digestive Disease Information & Research, Kurume University School of Medicine, Kurume, Japan
2 Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Japan

Keywords
branched-chain amino acids – hepatitis C virus – insulin resistance – lipid metabolism – nutritional support – protein-energy malnutrition

Correspondence
Takumi Kawaguchi, MD, PhD, Department of Digestive Disease Information & Research, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan.
Tel: +81 942 31 7902
Fax: +81 942 31 7747
e-mail: takumi@med.kurume-u.ac.jp

Received 21 March 2007
accepted 6 June 2007

DOI:10.1111/j.1478-3231.2007.01559.x

Abstract
Hepatitis C virus (HCV) infection causes insulin resistance. Because increased insulin resistance is a risk factor for development of hepatocellular carcinoma and reduced long-term survival, insulin resistance is a therapeutic target in patients with HCV infection. Branched-chain amino acids (BCAAs) are not only structural constituents of proteins but they are also considered as regulators of insulin signalling. We first describe two cases suggesting that administration of BCAAs improves insulin resistance associated with HCV-related liver disease. Although there were no changes in body weight, plasma glucose concentration and haemoglobin A1c (HbA1c) value were decreased. Moreover, BCAAs caused a decrease in both fasting insulin concentration and the value of homeostasis model assessment for insulin resistance. Thus, BCAAs are a potential therapeutic agent for improving insulin resistance in patients with HCV-related liver disease.

Patients with hepatitis C virus (HCV) infection frequently develop insulin resistance (1,2). Increased insulin resistance and postprandial hyperinsulinaemia are risk factors for development of hepatocellular carcinoma (HCC) (3,4) as well as reduced long-term survival in patients with liver cirrhosis (5). Thus, the occurrence of insulin resistance has an impact on the prognosis of patients with chronic liver disease and therefore, improvement of insulin resistance is a therapeutic target.

Although biguanides and thiazolidinediones are known to improve insulin resistance, neither is always recommended for patients with liver cirrhosis. Biguanides predispose the cirrhotic patients to lactic acidosis (6). Thiazolidinediones cause overproduction of hydrogen peroxide, leading to severe hepatotoxicity (7). Although lactic acidosis and severe hepatotoxicity are rare adverse effects, they are life-threatening complications in patients with liver cirrhosis.

Branched-chain amino acids (BCAAs) are not just structural constituents of proteins, but have some relevant pharmacological properties. BCAAs modulate the metabolism of neuroactive mediators and are used for the treatment of hepatic encephalopathy (8). In addition, BCAAs are known to modulate insulin signalling: BCAAs cause glucose uptake in skeletal muscle, adipocytes and hepatocytes of rodents (9–11); in a rat model of liver cirrhosis, BCAAs improved glucose metabolism (12). These previous reports imply that BCAAs could be important in the treatment of insulin resistance associated with chronic liver disease, although it has never been examined in human subjects.

In this report, we first present two cases showing that administration of BCAAs improved insulin resistance associated with HCV-related liver disease. BCAA supplementation may be considered as a complementary treatment for insulin resistance associated with HCV-related liver disease.

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Table 1. Effects of nutritional treatment on metabolism in Case 1

	Normal range	Before treatment	6 weeks after treatment
BMI	21.8±3.7*	21.6	21.4
Arm muscle circumference (cm)	20.2±2.7*	18.4	18.8
Triceps skinfold thickness (cm)	17.1±6.8*	16	16
Glucose (mg/dL)	80–109	145	75
HbA1c (%)	4.3–5.8	7.9	5.2
Insulin (μU/mL)	0.61–1.04	32.7	8.4
HOMA-IR	< 3	11.7	1.6
Albumin (g/dL)	4–5	2.85	3.12
Total cholesterol (mg/dL)	128–220	159	127
Ammonia (μg/dL)	12–66	39	34

*Normal ranges for BMI, arm muscle circumference and triceps skinfold thickness were taken from Japanese anthropometric reference data (13).
BMI, body mass index; HbA1c, haemoglobin A1c; HOMA-IR, homeostasis model assessment for insulin resistance.

Case reports

Case 1

A 73-year-old Japanese woman was referred to Kurume University Hospital for management of HCV-related liver cirrhosis. Chronic hepatitis C and type 2 diabetes mellitus were diagnosed when the patient was 58 and 68 years old respectively. A physical examination on admission showed that the patient had a height of 144 cm, a weight of 44.8 kg and a body mass index (BMI) of 21.6. The patient did not have a past history of endocrine or pancreatic diseases, alcohol consumption or a family history of diabetes mellitus. Abdominal ultrasound and computed tomography examinations showed no steatosis in the liver. Her type 2 diabetes mellitus had been treated with diet therapy and glibenclamide (5 mg/day). However, laboratory data showed severe insulin resistance and a marked increase in postprandial blood glucose concentration, indicating poor glycaemic control (Table 1 and Fig. 1).

Although her Child–Turcotte–Pugh classification was grade A, her serum albumin concentration was markedly decreased (2.85 g/dL). In order to improve protein-energy malnutrition, we increased the daily caloric intake from 1000 kcal (22.3 kcal/kg) to 1400 kcal (31.3 kcal/kg) and the daily protein intake from 33 g (0.7 g/kg) to 65 g (1.5 g/kg) based on European Society for Parenteral and Enteral Nutrition guidelines (14). In addition, we prescribed two daily sachets of BCAA granules (L-isoleucine 0.952 g, L-leucine 1.904 g, L-valine 1.144 g per sachet; Livact® granules; Ajinomoto Co. Inc., Tokyo, Japan) orally at bedtime.

As expected, the serum albumin concentration gradually increased, reaching 3.12 g/dL 6 weeks after the start of nutritional treatment. Unexpectedly, the patient showed hypoglycaemia repeatedly 2 weeks after the beginning of nutritional treatment, although there was no change in body weight or in her life style including eating habits and physical activity. Glibenclamide was withdrawn 3 weeks after implementation of nutritional treatment. Despite an increase in daily caloric intake and withdrawal of glibenclamide, laboratory data showed a reduction of postprandial blood glucose concentration. The area under the curve for blood glucose concentration showed about a 30% decrease 6 weeks after nutritional treatment compared with that before nutritional treatment (Fig. 1). Homeostasis model assessment for insulin resistance (HOMA-IR) value also decreased from 11.7 to 1.6.

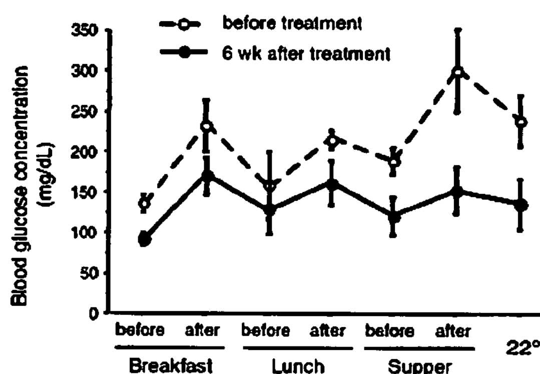

Fig. 1. Effects of nutritional treatment on blood glucose concentration. The patient was treated with diet therapy (31.3 kcal/kg and 1.5 g protein/kg) and two sachets of branched-chain amino acids granules as a late evening snack. Blood glucose concentrations were measured three times a week at the time of day indicated in the figure using the glucose dehydrogenase pyrroloquinolinequinone method three times a week. The white circle and black circles show blood glucose concentration before ($n = 3$) and 6 weeks after ($n = 3$) nutritional treatment respectively. Values are expressed as mean ± standard deviation.

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Moreover, prolonged postprandial insulin secretion was reduced (Fig. 2) and haemoglobin A1c (HbA1c) was reduced to 5.2% (Table 1). Thus, diet therapy and BCAA granules markedly improved the malnutrition as well as the insulin resistance.

Case 2

A 58-year-old Japanese man had been attending an outpatient clinic of our hospital for 3 years where he received treatment for chronic hepatitis C and for type 2 diabetes mellitus. A physical examination showed that he had a height of 169.5 cm, a weight of 59.4 kg and a BMI of 20.7. The patient did not have a past history of endocrine or pancreatic diseases, alcohol consumption or a family history of diabetes mellitus. An abdominal ultrasound examination showed no steatosis in the liver. The chronic hepatitis C and the type 2 diabetes mellitus were treated with ursodeoxycholic acid and diet therapy respectively. Despite treatment with interferon, HCV was not eradicated and laboratory data showed a continuous increase in serum ALT concentration. In order to reduce the occurrence of various complications of chronic liver disease, one sachet of BCAA supplement (L-isoleucine 0.8 g, L-leucine 1.6 g, L-valine 0.8 g per sachet; Aminofeel®; Seikatsu Bunkasya Co. Inc., Chiba, Japan) was administered orally twice a day.

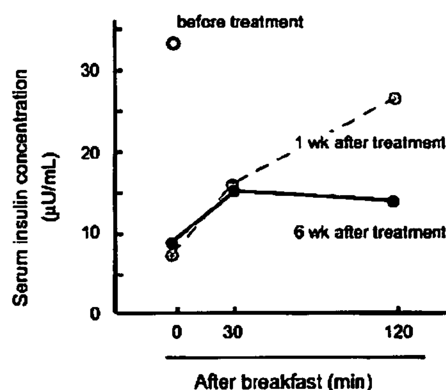

Fig. 2. Effects of nutritional treatment on serum insulin concentration. The patient was treated with diet therapy (31.3 kcal/kg and 1.5 g protein/kg) and two sachets of branched-chain amino acids granules as a late evening snack. Serum insulin concentrations were measured as before, 30 and 120 min after breakfast. The white, grey and black circles show serum insulin concentration before, 1 week and 6 weeks after nutritional treatment respectively.

Although there were no changes in body weight or in his life style including eating habits and physical activity compared with before and after BCAA administration, routine laboratory tests showed decreases in fasting blood glucose concentration and serum HbA1c value 4 weeks after BCAA administration. In addition, the fasting serum insulin concentration and HOMA-IR value were decreased (Table 2). BCAAs also caused a decrease in triglyceride concentration (Table 2). To investigate the effects of BCAAs on lipid metabolism, we examined the fatty acid profile. Although there were no compositional changes in saturated fatty acids and monounsaturated fatty acids, the percentage of linoleic acid decreased and the percentage of

Table 2. Effects of branched-chain amino acid on metabolism in Case 2

	Normal range	Before treatment	4 weeks after treatment
BMI	22.8 ± 2.9*	20.7	20.7
Alanine transaminase (U/L)	8–42	70	77
Total bilirubin (mg/dL)	0.3–1.5	0.85	1.14
Prothrombin time (INR)	N/A	1.11	1.16
Albumin (g/dL)	4–5	4.21	4.08
BCAA (μmol/L)	344–713	544.0	941.8
Tyr (μmol/L)	51–98	82.1	91.8
Ferritin (ng/mL)	23–183	73.6	87.3
Ammonia (μg/dL)	12–66	38	37
Fasting glucose (mg/dL)	80–109	112	103
HbA1c (%)	4.3–5.8	6.2	5.7
Insulin (μU/mL)	3.0–16.9	14.8	10.5
HOMA-IR	< 3	4.1	2.7
Total cholesterol (mg/dL)	128–220	134	124
Triglyceride (mg/dL)	38–207	109	74
Visceral fat area (cm^2)	N/A	82	77.6

*Normal ranges for BMI were taken from Japanese anthropometric reference data (13). Visceral fat area was estimated by the direct segmental multifrequency-bioelectrical impedance analysis method (InBody720, Biospace, Tokyo, Japan).
BMI, body mass index; BCAA, branched-chain amino acid; HOMA-IR, homeostasis model assessment for insulin resistance; N/A, not applicable.

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Table 3. Effects of branched-chain amino acid on fatty acid profiles in Case 2

	Before treatment	4 weeks after treatment
Fatty acid profiles		
∑SAFA (%)	34.90	34.32
∑MUFA (%)	22.47	23.86
∑n-6 PUFA (%)	35.73	32.69
Linoleic acid (%)	28.30	24.40
∑n-3 PUFA (%)	6.85	8.99
Eicosapentaenoic acid (%)	1.57	2.24
Docosahexaenoic acid (%)	0.57	0.70
n-3/n-6 ratio	0.19	0.28

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SAFA, saturated fatty acids.

eicosapentaenoic acid increased, leading to an increase in the n-3/n-6 polyunsaturated fatty acids (PUFA) ratio (Table 3). Thus, BCAA supplement improved both insulin resistance and lipid metabolism.

Discussion

We presented two cases suggesting that BCAAs improve insulin resistance associated with HCV-related liver disease. Both cases showed an improvement in insulin resistance without changes in body weight. BCAAs also increased the serum albumin concentration and the n-3/n-6 PUFA ratio. Although it is unclear how BCAAs improved insulin resistance, these findings suggest cross-talk between insulin signalling and protein or lipid metabolism and that BCAAs may improve insulin resistance through regulation of protein or lipid metabolism.

Insulin resistance can be caused by many factors. Obesity, hepatic inflammation and hepatic steatosis are reported to be possible causative factors for the development of insulin resistance in patients with HCV infection (15). However, these factors were not seen in our cases.

In Case 1, an improvement in insulin resistance was accompanied by an increase in serum albumin concentration. Although it is unclear why insulin resistance and protein metabolism improved simultaneously, there are two likely possibilities. Cirrhotic patients frequently show protein-energy malnutrition with a catabolic state in the early morning. Catabolic states decrease albumin synthesis and increase insulin resistance (16). In fact, her daily caloric and protein intakes were insufficient and she did not have any late evening snack. Thus, one possibility is that proper diet therapy, including a late evening snack, might have improved her protein-energy malnutrition and catabolic state, leading to an increase in serum albumin concentration and a decrease in insulin resistance simultaneously. Alternatively, not only fasting serum insulin concentration but also postprandial insulin secretion was markedly decreased. In this case, plasma glucose concentration was also decreased. Therefore, reduction in fasting and postprandial insulin secretion indicated increased insulin sensitivity. Moreover, discontinuance of glibenclamide suggested increased insulin sensitivity. Because insulin resistance and postprandial hyperinsulinaemia are associated with HCC development (4) and decreased long-term survival (5), reduction of insulin secretion could substantially improve prognosis. HCV core protein inactivates Akt, a central molecule in both insulin signalling and protein synthesis cascades, and increases insulin resistance (17). Leucine is known to activate Akt and up-regulate its downstream events including glucose transport and protein synthesis (10). Thus, another possibility is that leucine-induced activation of Akt increased her insulin sensitivity as well as her serum albumin concentration. Thus, proper diet therapy and BCAAs, especially leucine, may have contributed to improvements in glucose and protein metabolisms in Case 1.

In Case 2, there was no change in BMI or his life style including eating habits between before and after administration of BCAAs. However, the fasting plasma glucose concentration and HbA1c value were decreased. Because the fasting serum insulin concentration and the HOMA-IR value also decreased, BCAAs appeared to have improved the insulin resistance. Unexpectedly, a decrease in triglyceride concentration and an increase in the n-3/n-6 PUFA ratio were also seen following BCAA administration. Although it is unclear why insulin resistance and lipid metabolism improved simultaneously, one would think the involvement of peroxisome proliferator-activated receptor (PPAR)-α in BCAA-induced metabolic changes. Not only glucocorticoids and bile acids but also BCAAs are known to activate PPAR-α (18). Thus, a reason for the decreased triglyceride concentration could be activation of PPAR-α. In addition, PPAR-α up-regulates delta-6 desaturase, the rate-limiting enzyme for PUFA synthesis, and increases the n-3/n-6 PUFA ratio, leading to improvement in insulin sensitivity (19,20). In fact, his n-3/n-6 PUFA ratio increased and his HOMA-IR value decreased. There were no marked changes in factors related to insulin resistance such as hepatic inflammation, liver function and iron accumulation, supporting our hypothesis.

Our results indicate the effects of BCAAs on insulin resistance. However, we must be cautious about this interpretation, because some previous studies have

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reported different results. Tabaru *et al.* (21) reported that BCAAs causes hyperglycaemia in mild cirrhotic patients. Rossi-Fanelli *et al.* (22) also reported that BCAAs do not affect serum insulin concentrations in patients with chronic liver failure. Although the reasons for this discrepancy are not clear, it might be explained as follows: in our cases, the aetiology of liver disease was HCV, while patients with various aetiologies were enrolled in the previous studies. It is known that insulin resistance is more severe in patients with HCV infection than patients with other hepatobiliary disorders (17). Thus, differences in the aetiology of liver disease could be a possible reason for the discrepancy. In addition, the amino acids administered in previous studies contained not only BCAAs but also other essential and non-essential amino acids, while we administered only BCAAs. Other amino acids might affect insulin resistance (23). Thus, the different composition of the administrated amino acids could be another possible reason for the discrepancy. In order to establish the exact role of BCAAs in the treatment of insulin resistance, randomized-controlled trials are required.

In conclusion, we have reported two cases here in which BCAAs improved insulin resistance that was accompanied by impairment of protein or lipid metabolisms in HCV-related liver disease. BCAAs could potentially become a therapeutic agent for improving insulin resistance in patients with HCV-related liver disease.

Acknowledgements

We thank Eri Tsukagawa, Momoka Otsuka, Shoko Iwasaki, Tokiko Matsuda, Ryoko Ibi, Michiko Muto, Satomi Shiraishi, Shoko Tanaka, Yuko Saruwatari and Machiko Takakura for collecting clinical data. We also thank Noriko Kamimura (Seikatsu Bunkasya Co. Inc., Chiba, Japan) and Masaharu Ito (Livence Co. Inc., Tokyo, Japan) for providing the BCAA supplement (Aminofeel®).

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