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) $\times$ 360/(fasting glucose (mg/dL) – 63); insulin resistance (HOMA-IR) = fasting glucose (mg/dL) $\times$ 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.

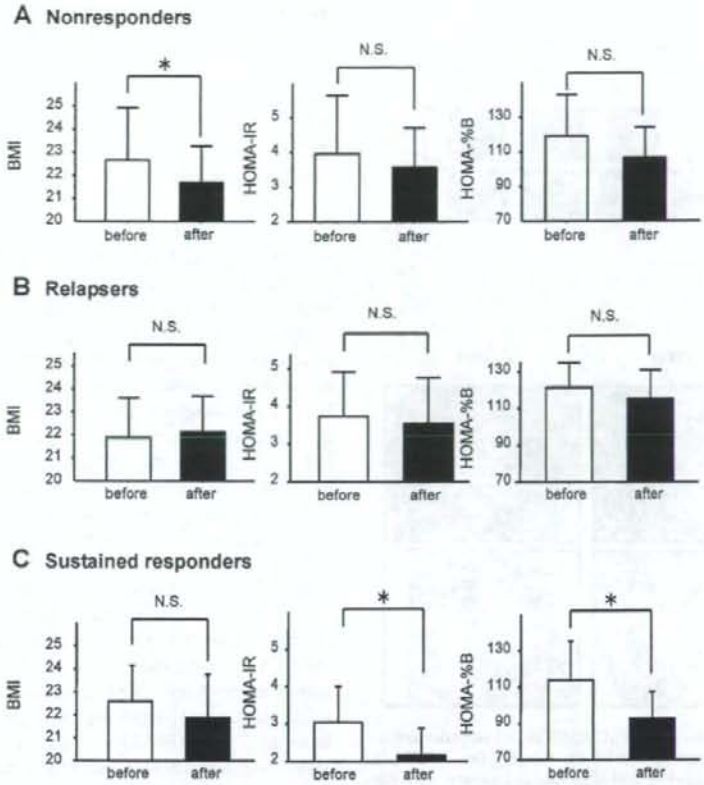

Figure 1. BMI, HOMA-IR, and HOMA-%B before and after antiviral therapy in nonresponders (N = 48; *A*), relapsers (N = 12; *B*), and sustained responders (N = 29; *C*). Data were obtained before antiviral therapy and 6 months after its conclusion. Data are expressed as mean ± SD. $^*P < 0.01$. N.S., not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

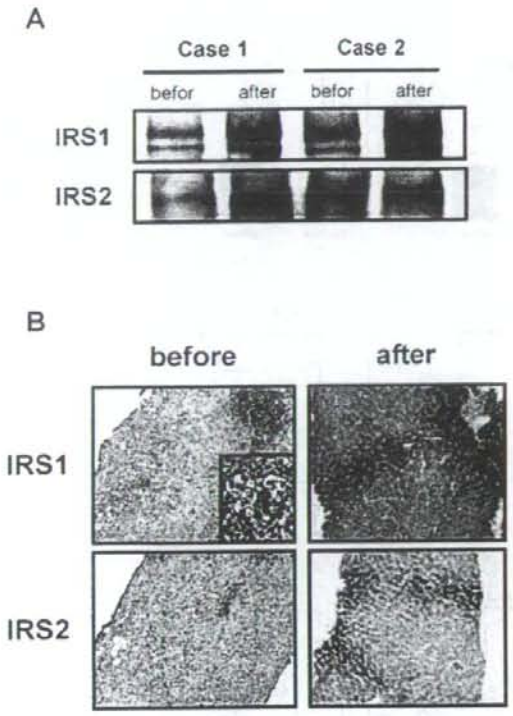

Figure 2. Protein expression levels of IRS1/2 before and after antiviral therapy in sustained responders. Immunoblotting for IRS1/2 (*A*). Proteins in liver extracts before and after antiviral therapy were immunoblotted with anti-IRS1 antibodies (upper panel) or anti-IRS2 antibodies (lower panel). Immunostaining 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 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.

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.

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.

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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.

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CONFLICT OF INTEREST

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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

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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.

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.

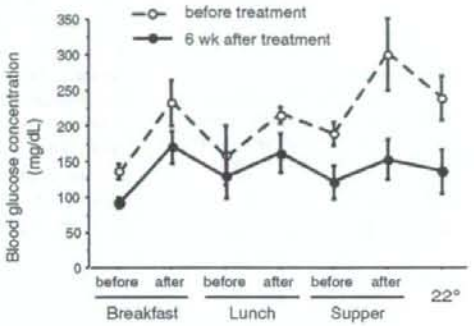

Fig. 1. Effects of nutritional treatment on blood glucose concentration. The patient was treated with diet therapy (31.3 kcal/kg and 1.5 g protein/kg) and two sachets of branched-chain amino acids granules as a late evening snack. Blood glucose concentrations were measured three times a week at the time 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.

Liver International (2007)

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.

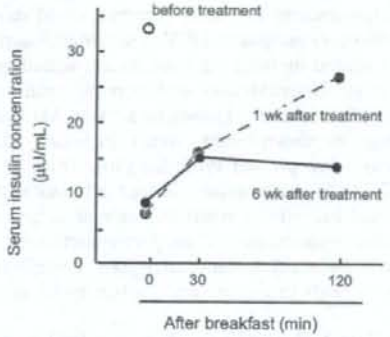

Fig. 2. Effects of nutritional treatment on serum insulin concentration. The patient was treated with diet therapy (31.3 kcal/kg and 1.5 g protein/kg) and two sachets of branched-chain amino acids granules as a late evening snack. Serum insulin concentrations were measured as before, 30 and 120 min after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

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 upregulate 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

Liver International (2007)

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.

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Liver International (2007)

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Impaired Filterability of Erythrocytes from Patients with Chronic Hepatitis C and Effects of Eicosapentaenoic Acid on the Filterability

Ritsuko SEKI[1], Takashi OKAMURA[1], Tatsuya IDE[1], Masayoshi KAGE[2], Michio SATA[1], Nobuhiro UYESAKA[3], and Toru MARUYAMA[4]

[1]Second Department of Internal Medicine, Kurume University School of Medicine, Fukuoka, 830-0011 Japan; [2]Department of Pathology, Kurume University School of Medicine, Fukuoka, 830-0011 Japan; [3]Department of Physiology, Nippon Medical School, Bunkyo-ku, Tokyo, 113-0022 Japan; and [4]Institute of Health Science, Kyushu University, Fukuoka, 816-8580 Japan

Abstract: Although erythrocyte filterability plays a key role in microcirculation, it is unknown whether the filterability of erythrocytes from patients with chronic hepatitis C (CH-C) is impaired. This study aimed to investigate erythrocyte filterability in CH-C patients in relation to medical treatment. The mean erythrocyte filterability (%) for all 24 patients with CH-C (69.2 ± 10.8%) was significantly lower than that for 5 normal controls (80.5 ± 1.7%, $P < 0.03$). In 8 patients, the combination therapy of ribavirin (RBV) and interferon improved liver function but caused anemia. The filterability after treatment (57.8 ± 12.8%) was lower than that before treatment (70.8 ± 9.7%, $P < 0.05$). Decreased filterability showed no correlation with the mean corpuscular volume or mean corpuscular Hb concentration during treatment, suggesting that the decrease in filterability mainly arises from changes in erythrocyte membrane properties. We investigated the protective effects of eicosapentaenoic acid (EPA) on the RBV-induced anemia. Filterability in 7 responders was markedly improved from 68.4 ± 4.6% to 77.4 ± 2.4% ($P < 0.001$), but not in 3 nonresponders. In the responders, the progression of anemia was restrained. In conclusion, we found an obvious impairment of the filterability of erythrocytes from CH-C patients, further impairment of the filterability induced by oxidative membrane damage caused by RBV leading to hemolytic anemia, and amelioration of the filterability caused by the antioxidative effects of EPA.

Key words: chronic hepatitis, erythrocytes, filterability, eicosapentaenoic acid (EPA), oxidative stress.

Hepatitis C virus (HCV) causes one of the most common and serious infections in Japan, affecting almost two million Japanese at present. Approximately 80% of patients with acute HCV infection will subsequently develop chronic infection, and an estimated 20% to 30% will develop cirrhosis and hepatocellular carcinoma [1]. The most effective treatment for chronic hepatitis C (CH-C) is antiviral combination therapy with interferon (IFN) and ribavirin (RBV) [2]. RBV is a water-soluble synthetic guanosine analog that exerts antiviral activity against DNA and RNA viruses after intracellular phosphorylation [3]. Current studies indicate that combination therapy with IFN and RBV (IFN/RBV) is associated with higher rates of sustained virological, biochemical, and histological responses compared to IFN monotherapy [4]. However, one of the major adverse effects of the combination therapy is RBV-induced hemolytic anemia; 67% of treated patients developed anemia [5]. This adverse effect has been ascribed to the accumulation of RBV triphosphate in erythrocytes, which leads to their oxidative injury [5, 6]. It is therefore suspected that the hemolytic anemia stems from impaired erythrocyte deformability. However, there is little data available on the deformability of erythrocytes in patients with CH-C, as well as for the patients treated with IFN/RBV.

The deformability of erythrocytes that pass through the microvascular network is a crucial determinant for the maintenance of physiological blood flow in the microcirculation. Indeed, we have clearly shown that disturbed microcirculation *in vivo* is closely related to impaired erythrocyte filterability (whole cell deformability) [7], using laser Doppler flowmetry [8] and a conventional gravity-based nickel mesh filtration technique that we developed [7, 9, 10]. Moreover, we have quantitatively demonstrated that erythrocyte filterability in cirrhotic patients is impaired in relation to the clinical severity of liver cirrhosis (LC) [11], using a newly developed precision nickel mesh filtration, the driving force of which is a continuously decreasing negative pressure [12]. Although the concept of erythrocyte deformability has no strict definition as a physical quantity, the nickel mesh filtration technique is quite useful to assess an indistinct quantity called erythrocyte deformability, both physiologically and clinically [7, 9–14]. Furthermore, we found that highly purified eicosapentaenoic acid (EPA) has a beneficial effect on patients with RBV-induced anemia [15].

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Correspondence should be addressed to: Toru Maruyama, Institute of Health Science, Kyushu University, Kasuga Kohen 6-1, Kasuga, 816-8580 Japan. Phone: +81-92-583-7685, Fax: +81-92-592-2866, E-mail: maruyama@ihs.kyushu-u.ac.jp

In the present study, we aimed to assess the filterability of erythrocytes from patients with CH-C, in particular those who underwent combination therapy with IFN/RBV, and to investigate the effect of EPA on erythrocyte filterability, using the precision nickel mesh filtration technique. This kind of rheological study will contribute to elucidating the mechanism of RBV-induced anemia.

MATERIAL AND METHODS

Subjects. The present study was performed according to the Declaration of Helsinki. The study population consisted of 24 consecutive Japanese patients with chronic hepatitis C (CH-C) with a mean age of 59.0 ± 6.4 years, ranging from 52 to 75 years. The CH-C patients were hospitalized in Kurume University Hospital or affiliated hospitals from February to December 2003. The CH-C patients underwent routine blood chemical and virological tests and liver biopsy, and they were graded histologically using the criteria of Desmet [16] and colleagues (F0–F4) and of the French METAVIR [17] Cooperative Study Group (A0 to A3), respectively. All patients were positive for HCV antibody, but negative for hepatitis B surface antigen (Hbs-Ag). None of the patients had a significant concurrent systemic illness, gastrointestinal bleeding, history of alcohol abuse, hematological disease, or malignancy. Five healthy volunteers served as normal controls, with a mean age of 30.7 ± 9.1 years ranging from 22 to 50 years. Signed informed consent was obtained from all subjects prior to the commencement of the study.

Blood samples. Venous blood was sampled from the antecubital vein of the subjects in a fasting state for blood cell counting and serum chemistry. Blood cell counting was performed by the autoanalyzer (Sysmex model XE-2100, Sysmex Co., Ltd., Kobe, Japan), concurrently detecting morphological abnormalities of erythrocytes. A microscopic examination was performed to confirm the shape changes of erythrocytes when an autoanalyzer detected morphological abnormalities. Chemical examinations of serum were carried out by using a routine autoanalyzer (Sysmex model K-4500, Sysmex Co., Ltd., Kobe, Japan). For a preparation of the erythrocyte suspension, venous blood was collected into a disposable evacuated syringe with a 21-gauge needle, using a 1/10 volume of 3.3% trisodium citrate as an anticoagulant. After centrifugation at 1,300 × *g* for 10 min, the plasma and buffy coat were carefully removed and replaced with *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) sodium salt (HEPES-Na)-buffered saline solution (HBS: 141 mM of NaCl, 10 mM of HEPES-Na). The osmolality and pH of the HBS were 287 mOsm/kg·H_2O and 7.4, respectively. Erythrocytes were gently washed three times by repeated resuspension with the HBS and centrifuged at 800 × *g*, 600 × *g*, and 500 × *g* for 10 min each, respectively. Residual leukocytes and platelets in the erythrocyte suspension were less than 100/*l* and 10^3/*l*, respectively. The hematocrit (Ht) value of the erythrocyte suspension was adjusted to 3.0% for the filtration experiment.

Erythrocyte filterability. Erythrocyte filtration through a nickel mesh was performed by using a new filtration apparatus [12] (Model NOBU-III, Tsukasa Sokken Co., Ltd., Tokyo, Japan). The system consists of a test unit (a vertical glass tube equipped with a nickel mesh at the bottom end), a negative pressure supply unit, and a measurement control and data analysis unit, as shown in Fig. 1. The vertical tube is connected to an air tank or reservoir through electromagnetic valves inside the negative pressure supply unit. An outline of the operation of the apparatus is as follows: the vertical tube equipped with the nickel mesh is immersed into a sample container filled with a test material. The pore diameter of the nickel mesh filter is 4.10 m. The sample container is surrounded by an isothermal bath that keeps the contents warm. After valves 2 and 3 are closed and valve 1 is opened, a pump evacuates the reservoir until the negative gauge pressure reaches around –200 mmH_2O. After valve 1 is closed, when valve 2 is opened, the test material is sucked into the nickel mesh filter through the action of decreasing negative pressure and rises into the vertical tube; the flow then stops at equilibrium. These operations are automatically performed by measurement software installed on a personal computer (PC). The device continually performs sampling measurement of the pressure (P) during filtration as a time (t) series at 20-ms intervals, performs analog/digital (A/D) conversion, and stores digital data (P-t data) on the PC.

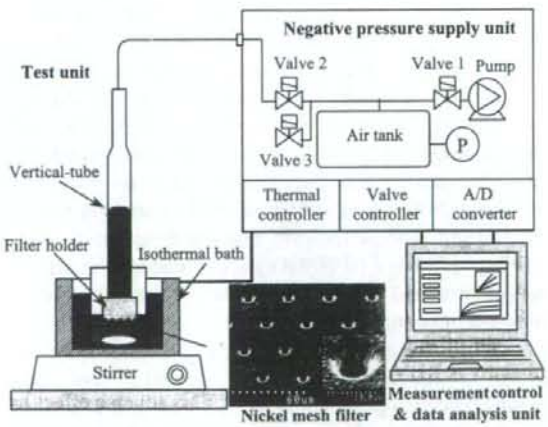

Fig. 1. Schematic illustration of the nickel mesh filtration system (see text), including a scanning electron microscopic photograph of a nickel mesh filter. The filter has an outer diameter of 13 mm, a filtration area diameter of 8 mm, a thickness of about 11 μm, a pore diameter of 4.10 μm, and an interpore distance of 35 μm. The vertical and cylindrical pores are regularly distributed across the filter with no pore coincidence, the pore entrances of which show a round and smooth transition into the pore inside.

Together with the start of the data acquisition process, the software converts the pressure data into the liquid level (height) of the test material and displays ongoing height and measurement time (h-t) as a graph. When the test has been completed, the software automatically utilizes the h-t data to calculate the quantity of flow per unit time; it then displays the pressure and flow rate (P-Q) as a graph [12]. Thus, similar to the gravity-based filtration system [7], the P-Q relation determines the deformability of the cells. A magnetic stirrer was used to prevent the sedimentation of erythrocytes at 100 rpm (Fig. 1). The filtration experiments were carried out at 36°C.

Study protocols. After hospitalization, the patients underwent routine laboratory examinations and liver biopsy because hepatic histological examination is essential for IFN therapy. All patients (n = 24) underwent repetitive subcutaneous injections of human recombinant IFN-α. Simultaneously, RBV was administered orally in 8 patients to complete HCV clearance, depending on the HCV genotype. Serological and hematological tests were repeated every week to confirm the viral clearance and improvement of the liver function, and to prevent the development of anemia, if any, during the course of the combination treatment with IFN and RBV. Empirically, EPA was administered to 10 patients to prevent the progression of anemia. Erythrocyte filterability was evaluated in the pretreatment period and before discharge. The study protocol was approved by the ethical internal committee of Kurume University.

Data analyses. The data were expressed as means ± SD. Data analyses were performed using the paired or non-paired Student's t-test, and practical computation was performed using Microsoft Excel 2000 on Windows 98/XP (Microsoft, Tokyo, Japan). Differences with a P value <0.05 were considered to indicate significance.

RESULTS

Representative pressure–flow rate relationships

Figure 2 shows the representative pressure-flow rate relationships for the HBS (saline) and the several kinds of erythrocyte suspensions used during the continuous filtration experiments. The P-Q relationship for the HBS was linear with the line passing through the origin, showing the Newtonian behavior of the HBS. In contrast, the P-Q relationships for the erythrocyte suspensions, the hematocrit value (Ht) of which was 3%, displayed smooth convex curves along the abscissa over the low-pressure region, revealing non-Newtonian characteristics of the suspensions. The flow rate of the erythrocyte suspension for CH-C patients after IFN/RBV treatment was lower than that before the treatment at any given pressure, indicating an apparent impairment in erythrocyte filterability resulting from the treatment. Hereafter, we use the ratio (%) of the flow rate of the erythrocyte suspension to that of the HBS (suspending medium) at 100 mmH_2O as an index of erythrocyte filterability.

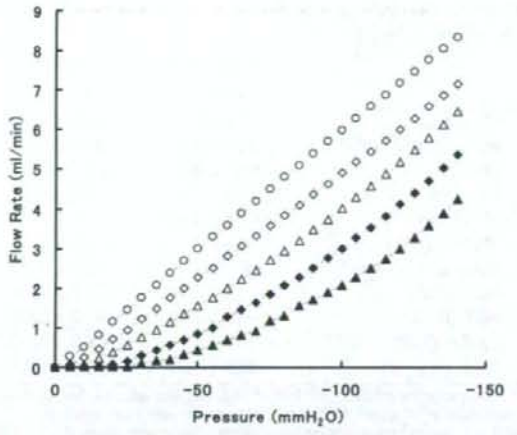

Fig. 2. Representative relationships between the pressure (P; mmH_2O) and flow rate (Q; ml/min) during the continuous filtration experiment utilizing the decreasing negative pressure. The P-Q relationships show HBS (open circles) and 2 representative patients with CH-C before (open diamonds, open triangles) and after (solid diamonds, solid triangles) treatment with IFN/RBV. The hematocrit value of the erythrocyte suspension was 3%.

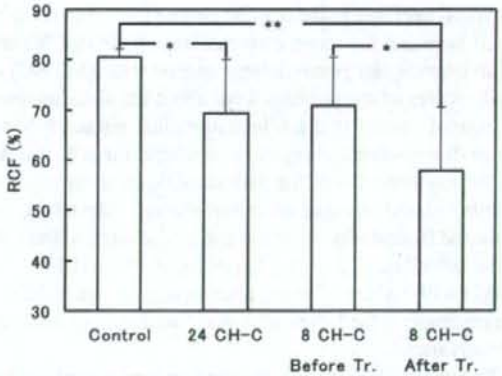

Fig. 3. Filterability of erythrocytes from patients with CH-C. RCF means erythrocyte (red cell) filterability. We used the ratio (%) of the flow rate of the erythrocyte suspension to that of HBS (suspension medium) at 100 mmH_2O as an index of RCF. The column and bar indicate 5 normal controls (Cont), all 24 patients with CH-C (24 CH-C), 8 patients with CH-C before treatment with IFN/RBV (8 CH-C/before Tr.), and those after treatment (8 CH-C/after Tr.). The data are expressed as mean ± SD (*P < 0.05, **P < 0.01).

Filterability of erythrocytes from patients with CH-C

The mean erythrocyte filterability (%) for all 24 patients with CH-C (69.2 ± 10.8%) was significantly lower than that for 5 normal controls (80.5 ± 1.7%: P < 0.03), as

Table 1. Comparisons of controls and patients with chronic hepatitis C (CH-C).

	Control ($n = 5$)	Total CH-C ($n = 24$)
Hb (g/dl)	13.4 ± 0.8	13.8 ± 1.4
RBC ($\times 10^4$/mm^3)	422 ± 39	441 ± 53
Ht (%)	39.8 ± 2.9	41.1 ± 3.6
MCV (m^3)	93.5 ± 3.4	93.5 ± 4.9
MCH (pg)	32.2 ± 1.8	32.3 ± 1.7
MCHC (g/dl)	34.2 ± 1.3	32.9 ± 1.6
Platelet (10^4/mm^3)	19.0 ± 1.5	12.4 ± 5.2**
AST (IU/l)	– (13 – 33)	73.7 ± 35.5
ALT (IU/l)	– (6 – 27)	96.7 ± 53.9
γ GTP (IU/l)	– (10 – 47)	72.9 ± 42.0
RCF (%)	80.5 ± 1.7	69.2 ± 10.8*

Hb: hemoglobin; RBC: red blood cell; Ht: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; AST: aspartate aminotransferase; ALT: alanine aminotransferase; γGTP: γ-glutamyl transpeptidase; RCF: erythrocyte (red cell) filterability. We used the ratio (%) of the flow rate of the erythrocyte suspension to that of HBS (suspending medium) at 100 mmH_2O as an index of RCF. Values are expressed as the mean ± SD (*$P < 0.05$, **$P < 0.01$). The values enclosed in parentheses are standard values for normal controls.

Table 2. Influences of IFN/RBV therapy in CH-C patients.

	Pretreatment ($n = 8$)	Posttreatment ($n = 8$)
Hb (g/dl)	13.9 ± 1.2	11.1 ± 1.8***
RBC ($\times 10^4$/mm^3)	454 ± 43	353 ± 69**
Ht (%)	41.9 ± 2.1	34.3 ± 4.6***
MCV (m^3)	92.6 ± 6.5	98.1 ± 6.6
MCH (pg)	31.4 ± 2.1	31.6 ± 1.4
MCHC (g/dl)	33.4 ± 1.9	32.3 ± 1.2
Platelet (10^4/mm^3)	11.2 ± 6.4	12.9 ± 6.7
AST (IU/l)	67.8 ± 33.2	30.5 ± 12.4**
ALT (IU/l)	94.6 ± 65.3	26.0 ± 13.1*
γ GTP (IU/l)	71.3 ± 45.5	26.3 ± 12.8*
RCF (%)	70.8 ± 9.7	57.8 ± 12.8*

Hb: hemoglobin; RBC: red blood cell; Ht: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GTP: γ-glutamyl transpeptidase; RCF: erythrocyte (red cell) filterability. We used the ratio (%) of the flow rate of the erythrocyte suspension to that of HBS (suspending medium) at 100 mmH_2O as an index of RCF. Values are expressed as the mean ± SD (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

shown in Fig. 3 and Table 1. The result of hematological examinations for the CH-C patients before treatment was within the normal range except for thrombocytopenia, i.e., a marked decrease in the platelet count (Table 1). It is to be noted here that the mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) of erythrocytes of the patients were about the same as those in normal controls; these hematological parameters are major determinants of erythrocyte filterability [9, 10]. Also, the erythrocytes of the patients did not show any discernible shape changes (data not shown). Moreover, the impaired filterability (x) in the patients showed a weak inverse correlation ($y = -1.973x + 233.2$, $R = -0.396$, $P = 0.057$) with values of alanine aminotransferase (ALT; y), suggesting that the filterability decreased as liver dysfunction advanced.

To evaluate the relation between erythrocyte filterability and the histological index (F0, F1, F2, F3, and F4), which reflects the severity of histological fibrosis in the liver, we divided the 24 patients with CH-C, who received liver biopsy into two groups (F0-3 and F4). The difference between the erythrocyte filterability for the F0-3 group and for the F4 group was not significant. Furthermore, we investigated the relation between erythrocyte filterability and the histological activity classification (A0, A1, and A3). The difference between the erythrocyte filterability for the A0-2 group and that for the A3 group was also not significant.

Effects of IFN/RBV therapy on erythrocyte filterability in CH-C patients

Erythrocyte filterability in the 8 patients after combination treatment with IFN and RBV (57.8 ± 12.8%) significantly decreased compared with that before treatment (70.8 ± 9.7%, $P < 0.05$), as shown in Fig. 3 and Table 2. The MCV and MCHC of erythrocytes of these patients were about the same as those in normal controls (Tables 1 and 2). Treatment improved the liver function of patients; the values of asparatate aminotransferase (AST) dropped from 67.8 ± 33.2 to 30.5 ± 12.4 ($P < 0.01$), those of ALT from 94.6 ± 65.3 to 26.0 ± 13.1 ($P < 0.03$), and those of γ-glutamyl transpeptidase (γ-GTP) from 71.3 ± 45.5 to 26.3 ± 12.8 ($P < 0.03$). However, the treatment induced anemia in patients (Table 2), which is a well-known adverse effect of the treatment [5, 6]; the hemoglobin content of blood (Hb) decreased from 13.9 ± 1.2 g/dl to 11.1 ± 1.8 g/dl ($P < 0.001$), and the Ht value decreased from 41.9 ± 2.1% to 34.3 ± 4.6% ($P < 0.001$). Furthermore, erythrocytes from IFN/RBV-treated patients showed no discernible shape changes, and there were no signs of Heinz body formation (data not shown).

Effects of EPA on erythrocyte filterability in IFN/RBV/EPA therapy

We investigated the effects of EPA on the erythrocyte filterability of 10 patients with CH-C who underwent combination therapy of IFN, RBV, and EPA (IFN/RBV/EPA). The decreased filterability of erythrocytes in patients before the treatment (65.3 ± 10.0%) was significantly improved compared with that after treatment (71.0 ± 11.9%, $P < 0.04$). However, the Hb content decreased from 14.1 ± 1.1 g/dl to 11.8 ± 1.4 g/dl ($P < 0.001$), and the Ht value decreased from 42.1 ± 2.3% to 36.3 ± 3.8% ($P < 0.001$). The degree of anemia (Hb: 11.8 ± 1.4 g/dl, Ht: 36.3 ± 3.8%) after this treatment was lower compared with that after conventional treatment with IFN/RBV (Hb: 11.1 ± 1.8 g/dl, Ht: 34.3 ± 4.6%), although a significant

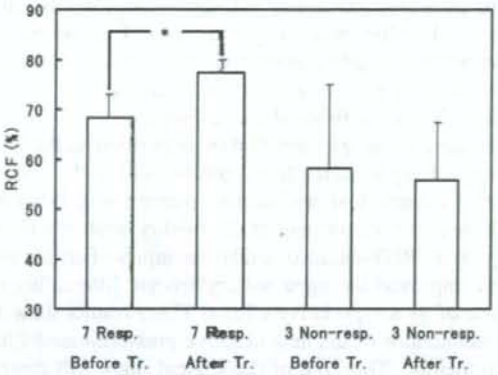

Fig. 4. Effects of EPA on erythrocyte filterability in IFN/RBV/EPA therapy. RCF means erythrocyte (red cell) filterability. We used the ratio (%) of the flow rate of the erythrocyte suspension to that of HBS (suspension medium) at 100 mmH_2O as an index of RCF. The column and bar indicate erythrocyte filterability in 7 patients with CH-C, who belong to responders, before IFN/RBV/EPA treatment (7 resp./before Tr.) and after treatment (7 resp./after Tr.), and erythrocyte filterability in 3 patients with CH-C who belong to nonresponders, before INF/RBV/EPA treatment (3 non-resp./before Tr.) and after treatment (3 non-resp./after Tr.). The data are expressed as means ± SD (*$P < 0.05$).

difference was not observed between the two. Note clinically that Hb < 12 g/dl and Ht < 37% indicate anemia. Furthermore, we found that among the 10 patients, 7 were responders and the other 3 were nonresponders. Filterability in responders markedly improved *via* treatment, from 68.4 ± 4.6% to 77.4 ± 2.4% ($P < 0.001$), whereas the nonresponders revealed no improvement from 58.2 ± 9.6% to 55.9 ± 6.7%, as shown in Fig. 4. There were no hematological or histological differences between responders and nonresponders to EPA, except for erythrocyte filterability.

DISCUSSION

We clearly showed that erythrocyte filterability is impaired in patients with CH-C (Fig. 3 and Table 1). To our knowledge, this is the first quantitative study to show impaired filterability in these patients. Erythrocyte filterability is mainly determined by the membrane properties, cellular internal viscosity reflected in the MCHC, and the geometric factors of erythrocytes that are reflected in the surface area to volume ratio, such as the MCV and changes in shape [9, 10]. In the present study, the MCHC of erythrocytes of patients with CH-C was about the same as that of the normal controls (Table 1), suggesting that the impaired filterability in the subjects is not attributable to an increase in the cellular internal viscosity. Also, the MCV of the subjects was about the same as that of the normal controls (Table 1), and no discernible shape changes were observed, suggesting that the impaired filterability is not caused by changes in geometric factors. These results suggest that the impaired filterability in this study mainly arose from changes in membrane properties. In relation to this, we quantitatively demonstrated that the erythrocyte filterability in patients with LC, which is subsequently developed from CH-C, is impaired in relation to the clinical severity of LC [11]. The abnormal lipid composition of erythrocyte membranes in LC because of a high cholesterol/phospholipid ratio is considered to cause a reduced membrane fluidity [18], resulting in impaired erythrocyte filterability [11]. It is well known that unlike LC, fibrosis in CH-C is not severe; in fact, there was no relation between erythrocyte filterability and the severity of histological fibrosis (graded F1–F4). It is therefore unlikely that changes in the membrane properties in CH-C arose from mechanical stress, which was strongly suggested in LC [11].

Hemolytic anemia is a nearly universal event associated with RVB combination therapy, although the extent of anemia can vary considerably between individuals. Indeed, anemia occurred in our patients, though their liver function was improved (Table 2). Anemia may result in fatigue, loss of functional capacity, and cognitive impairment, which are associated with the quality of life and could lead to treatment noncompliance [19]. The mechanism of RBV-induced anemia has been recently described [6]. RBV is taken up by erythrocytes and converted to RBV triphosphate that cannot be metabolized by erythrocytes; thus it accumulates within erythrocytes to levels 60-fold greater than plasma concentrations. This results in a depletion of erythrocyte adenosine triphosphate, impairing antioxidant defenses and inducing erythrocyte membrane oxidative damage. The erythrocyte is therefore rendered highly susceptible to oxidative stress by the reticuloendothelial system, resulting in extravascular hemolysis. Moreover, RBV treatment induced an apparent increase in erythrocyte methemoglobin (met-Hb) levels [6]. It is well known that the formation of met-Hb generates various reactive oxygen species, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyradical [20, 21]; accordingly, these reactive oxygen species exert further oxidative stress on intact erythrocytes and facilitate additional impairment of their membranes. In fact, we confirmed that several kinds of oxidative stresses remarkably impair erythrocyte filterability, using O_2^- [22], phenylhidrazine [10], and *tert*-butyl hydroperoxide (tBHP) [23]. Thus it is very likely that the marked decrease in filterability induced by the combination therapy (Figs. 2 and 3 and Table 2) was due to the oxidative injury of erythrocytes, including membrane lipid peroxidation and the segmentation of cytoskeletal proteins. It is noteworthy here that the oxidative stress induced by RBV treatment was not as severe as that generated by a strong oxidant such as phenylhydrazine, because phenylhydrazine induces Heinz body-forming

erythrocytes [10], but RBV treatment did not.

Oxidative injury is reported to play an important role in the progression from CH-C to LC, and antioxidative treatment with vitamin E is known to effectively prevent this progression [24]. This line of evidence is compatible with the results of this study, showing the favorable effects of EPA. The wide range of EPA biological effects include benefits on lipoprotein metabolism, platelet function, endothelial function, vascular reactivity, inflammatory markers, cytokine production, coagulation, and fibrinolysis, many effects of which are related to a potent antioxidative effect [25–28]. Therefore we investigated the effects of EPA on erythrocyte filterability with IFN/RBV/EPA treatment. It is interesting that among the 10 patients treated with EPA, we found 7 who showed a marked improvement in filterability (68.4 ± 4.6% to 77.4 ± 2.4%; $P < 0.001$), but the other 3 patients did not (i.e., nonresponders: 58.2 ± 9.6% to 55.9 ± 6.7%). It can therefore be concluded that the improvement observed in the 7 responders was attributable to the antioxidative effect of EPA. Concerning the 3 nonresponders, it is to be noted here that besides the hemolysis mentioned above, RVB suppresses erythropoiesis, probably as a result of the down-regulation of erythropoietin receptors [5, 19]. Also, IFN induces anemia via various mechanisms, including the suppression of hematopoietic progenitor cell proliferation, activation of apoptosis in erythroid progenitor cells, initiation of immune hemolysis, and impairment of renal function [19, 29]. The existence of nonresponders suggests that the impaired filterability may be due to causes other than oxidative injury; in particular, the suppression of erythropoiesis could be suspected. It is well known that the use of recombinant human erythropoietin to treat patients receiving IFN/RBV combination therapy is potentially beneficial for reasons [19, 30]. Therefore we would have treated the nonresponders with erythropoietin if it had been available through governmental medical insurance in Japan.

Finally, we must refer to the predominance of the new, negative pressure-based filtration method [11, 12], which revealed clear quantitative results that are shown in Figs. 2–4 and Tables 1 and 2. The pressure-flow rate relationships obtained by the new method (Fig. 2) were similar to those of gravity-based filtration [7]. The performance of the gravity-based method requires great care to avoid contamination from emboli formed, such as when filling test materials into the vertical tube and installing the nickel mesh filter in the holder; however, in the new method there are no such handling problems. Many methods have been involved in the sucking of test cells into a filter by using a constant pressure or constant rate as a driving force. In these methods, the test cells are continuously sucked even when they plug the filter, thereby sometimes leading to cell breakage. The driving force used in our new method does not lead to such cell rupture, causing only physiological and/or pathophysiological cell plugging in the filter, much as gravity does. Thus we believe that our new, highly quantitative, and convenient method will contribute to the evaluation of erythrocyte deformability for related fields of medical science.

In conclusion, we identified an impairment in the filterability of erythrocytes from patients with CH-C. Moreover, we found that combination therapy with IFN/RBV enhanced the impairment of filterability, probably resulting from RBV-induced oxidative injury. Furthermore, EPA improved the impaired erythrocyte filterability because of its antioxidative effects. These results show the predominance of the new negative pressure-based filtration method. This kind of rheological study will contribute to related fields of medical science, as well as to the elucidation of the mechanism leading to the development of RBV-induced anemia.

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HEPATOLOGY

Insulin resistance and lichen planus in patients with HCV-infectious liver diseases

Yumiko Nagao,* Katsuya Kawasaki† and Michio Sata*‡

*Department of Digestive Disease Information & Research, †Clinical Laboratory and ‡Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Asahi-machi, Kurume, Fukuoka, Japan

Key words
diabetes mellitus, extrahepatic manifestations, hepatitis C virus, insulin resistance, lichen planus.

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Correspondence
Dr Yumiko Nagao, Department of Digestive Disease Information & Research, Kurume University School of Medicine, 67 Asahimachi, Kurume 830-0011, Japan.
Email: nagao@med.kurume-u.ac.jp

Abstract

Background and Aim: Hepatitis C virus (HCV) causes liver diseases and extrahepatic manifestations, and also contributes to insulin resistance and type 2 diabetes mellitus (DM). The aims of the present study were to examine the incidence of extrahepatic manifestations including lichen planus in HCV-infected patients and to evaluate the relationship between lichen planus and insulin resistance.

Methods: Of 9396 patients with liver diseases presenting to the study hospital, 87 patients (mean age 60.0 ± 11.5 years) with HCV-related liver diseases were identified and examined for the incidence of extrahepatic manifestations. Insulin resistance and the presence of *Helicobacter pylori* antibodies were also measured.

Results: The prevalence of DM was 21.8% (19/87), hypertension was 28.7% (25/87), thyroid dysfunction was 20.7% (18/87), and extrahepatic malignant tumor was 9.2% (8/87). The prevalence of lichen planus at oral, cutaneous, pharyngeal, and/or vulval locations was 19.5% (17/87). Characteristics of 17 patients with lichen planus (group A) were compared with 70 patients without lichen planus (group B). Prevalence of smoking history, presence of hypertension, extrahepatic malignant tumor, and insulin resistance (HOMA-IR) were significantly higher in group A than in group B. Significant differences were not observed for age, sex, body mass index, diagnosis of liver disease, alcohol consumption, presence of DM, thyroid dysfunction, liver function tests, or presence of *H. pylori* infection between the two groups.

Conclusions: Infection with HCV induces insulin resistance and may cause lichen planus. It is necessary for an HCV-infected patient to be assayed for insulin resistance, and to be checked for different extrahepatic manifestations of this infection, particularly lichen planus.

Introduction

The number of fatalities due to hepatocellular carcinoma (HCC) in Japan continues to increase, and it is estimated that this tendency will continue at least until 2015. Of the HCC cases in Japan, approximately 16% are caused by hepatitis B virus (HBV) infection and approximately 80% by hepatitis C virus (HCV) infection.[1] The average prevalence of HCV carriers in Japan is about 2%, with the absolute number estimated at 2 million.[2] The increase in HCC in Japan depends on the spread of HCV infection.[2]

Infection with HCV induces various extrahepatic manifestations as well as chronic liver diseases.[3,4] HCV infects cells or organs except hepatocytes and multiplies. Representative extrahepatic manifestations of HCV infection include lichen planus, diabetes mellitus (DM), malignant lymphoma, Sjögren's syndrome, cryoglobulinemia, and membranoproliferative glomerulonephritis. It has been reported that combined therapy using interferon and ribavirin is effective for different extrahepatic manifestations that are apt to be overlooked.[5,6]

At present, it has been shown that HCV multiplies in skin and oral mucosa leading to HCV-related lichen planus,[7,8] and that the risk of malignant transformation is higher in lichen planus with HCV infection than in lichen planus without HCV.[9] However, a mechanism for these extrahepatic manifestations has not been elucidated. Recently it was reported that there is a significant correlation between lichen planus and HCV and DM in southern Taiwan, particularly in HCV patients with elevated serum alanine aminotransferase (ALT) levels and atrophic-erosive oral lichen planus (OLP).[10] In our previous report, patients with lichen planus having DM were all found to be HCV-infected.[11]

In addition, it has been reported that DM is a risk factor for HCV-related hepatocarcinogenesis[12] and for decreased survival

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