14 Swart GR, Zillikens MC, van Vuure JK, van den Berg JW. Effect of a late evening meal on nitrogen balance in patients with cirrhosis of the liver. *BMJ* 1989; **299**: 1202–3.
15 Gores GJ, Wiesner RH, Dickson ER, Zinsmeister AR, Jorgensen RA, Langworthy A. Prospective evaluation of esophageal varices in primary biliary cirrhosis: development, natural history, and influence on survival. *Gastroenterology* 1989; **96**: 1552–9.
16 Tong MJ, el-Farra NS, Reikes AR, Co RL. Clinical outcomes after transfusion-associated hepatitis C. *N Engl J Med* 1995; **332**: 1463–6.
17 Lai CL, Yuen MF. The natural history and treatment of chronic hepatitis B: a critical evaluation of standard treatment criteria and end points. *Ann Intern Med* 2007; **147**: 58–61.
18 Barrett BJ, Parfrey PS, Vavasour HM, O'Dea F, Kent G, Stone E. A comparison of nonionic, low-osmolality radiocontrast agents with ionic, high-osmolality agents during cardiac catheterization. *N Engl J Med* 1992; **326**: 431–6.
19 Rolls BJ, Rolls ET, Rowe EA, Sweeney K. Sensory specific satiety in man. *Physiol Behav* 1981; **27**: 137–42.
20 Kawaguchi T, Taniguchi E, Itou M *et al.* Appearance-specific satiety increases appetite and quality of life in patients with metastatic liver tumor: a case report. *Kurume Med J* 2006; **53**: 41–6.
21 Rolls BJ, Rowe EA, Rolls ET, Kingston B, Megson A, Gunary R. Variety in a meal enhances food intake in man. *Physiol Behav* 1981; **26**: 215–21.
22 Shimoyama Y, Kusano M, Kuwamura O *et al.* High-viscosity liquid meal accelerates gastric emptying. *Neurogastroenterol Motil* 2007; 879–86.
23 Nakaya Y, Okita K, Suzuki K *et al.* BCAA-enriched snack improves nutritional state of cirrhosis. *Nutrition* 2007; **23**: 113–20.
24 Reibel DK, Greeson JM, Brainard GC, Rosenzweig S. Mindfulness-based stress reduction and health-related quality of life in a heterogeneous patient population. *Gen Hosp Psychiatry* 2001; **23**: 183–92.
25 Greco CM, Rudy TE, Manzi S. Effects of a stress-reduction program on psychological function, pain, and physical function of systemic lupus erythematosus patients: a randomized controlled trial. *Arthritis Rheum* 2004; **51**: 625–34.
26 Fernstrom JD, Fernstrom MH. Exercise, serum free tryptophan, and central fatigue. *J Nutr* 2006; **136**: 553S–9S.
27 Davis JM, Alderson NL, Welsh RS. Serotonin and central nervous system fatigue: nutritional considerations. *Am J Clin Nutr* 2000; **72**: 573S–8S.
28 Martins C, Morgan LM, Bloom SR, Robertson MD. Effects of exercise on gut peptides, energy intake and appetite. *J Endocrinol* 2007; **193**: 251–8.
29 Kojima M, Kangawa K. Drug insight: the functions of ghrelin and its potential as a multitherapeutic hormone. *Nat Clin Pract Endocrinol Metab* 2006; **2**: 80–8.
30 Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646–9.
31 Kamath PS, Wiesner RH, Malinchoc M *et al.* A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; **33**: 464–70.
32 Gunsar F, Raimondo ML, Jones S *et al.* Nutritional status and prognosis in cirrhotic patients. *Aliment Pharmacol Ther* 2006; **24**: 563–72.
33 Swart GR, van den Berg JW, Wattimena JL, Rietveld T, van Vuure JK, Frenkel M. Elevated protein requirements in cirrhosis of the liver investigated by whole body protein turnover studies. *Clin Sci (Lond.)* 1988; **75**: 101–7.
34 Ijichi C, Matsumura T, Tsuji T, Eto Y. Branched-chain amino acids promote albumin synthesis in rat primary hepatocytes through the mTOR signal transduction system. *Biochem Biophys Res Commun* 2003; **303**: 59–64.
35 Muller MJ, Bottcher J, Selberg O *et al.* Hypermetabolism in clinically stable patients with liver cirrhosis. *Am J Clin Nutr* 1999; **69**: 1194–201.
36 Kawaguchi T, Yoshida T, Harada M *et al.* Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. *Am J Pathol* 2004; **165**: 1499–508.
37 Itou M, Kawaguchi T, Taniguchi E *et al.* Altered Expression of Glucagon-Like Peptide-1 and Dipeptidyl Peptidase IV in Patients with HCV-related Glucose Intolerance. *J Gastroenterol Hepatol* 2008; **23**: 224–51.

doi:10.1111/j.1440-1746.2007.05183.x

HEPATOLOGY

Altered expression of glucagon-like peptide-1 and dipeptidyl peptidase IV in patients with HCV-related glucose intolerance

Minoru Itou,* Takumi Kawaguchi,*†‡ Eitaro Taniguchi,*‡ Shuji Sumie,* Tetsuharu Oriishi,* Keiichi Mitsuyama,* Osamu Tsuruta,* Takato Ueno*‡ and Michio Sata*†‡

Departments of *Medicine and †Digestive Disease Information and Research, Kurume University School of Medicine and ‡Research Center for Innovative Cancer Therapy, and the 21st Century Center of Excellence Program for Medical Science, Kurume University, Kurume, Japan

Key words
dipeptidyl peptidase IV, glucagon-like peptide-1, glucose intolerance, hepatitis C virus, insulin resistance.

Accepted for publication 5 June 2007.

Correspondence
Dr Takumi Kawaguchi, Department of Digestive Disease Information and Research, and Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, 830-0011, Japan.
Email: takumi@med.kurume-u.ac.jp

Abstract

Background and Aim: The pathogenesis of hepatitis C virus (HCV)-associated glucose intolerance remains unclear. Glucagon-like peptide-1 (GLP-1), a gut hormone, synthesizes hepatic glycogen and is inactivated by dipeptidyl peptidase IV (DPPIV). The aims of this study were to investigate the alterations in the expression of GLP-1 and DPPIV in HCV-associated glucose intolerance.

Methods: We enrolled patients with HCV- or hepatitis B virus (HBV)-related liver disease ($n = 94$ and 37, respectively), patients with inflammatory bowel disease (IBD; $n = 14$) as disease controls, and healthy controls ($n = 48$). The serum or tissue GLP-1 and DPPIV expression levels were determined by enzyme immunoassay, immunoblotting, or immunostaining. The hepatic glycogen content was assayed by periodic acid–Schiff staining.

Results: The serum GLP-1 levels were significantly decreased in the HCV group (4.9 ± 0.3 ng/mL) than those in the controls (7.5 ± 0.6 ng/mL), the HBV group (7.0 ± 0.5 ng/mL), or the IBD group (10.8 ± 1.0 ng/mL, $P < 0.01$). Although the ileum GLP-1 expression was not significantly different between the controls and the HCV group, the DPPIV expression was significantly increased in the ileum, liver, and serum in the HCV group. Hepatic glycogen content was decreased to a greater extent in the HCV group than that in the HBV group (127.5 ± 5.3 *vs* 187.7 ± 6.6 arbitrary units; $n = 19$, $P < 0.01$).

Conclusion: We demonstrated the altered expressions of GLP-1 and DPPIV in patients with HCV-associated glucose intolerance. Since hepatic glycogen synthesis, a GLP-1 action, was impaired, the altered expressions of GLP-1 and DPPIV may be involved in the development of HCV-associated glucose intolerance.

Introduction

The liver plays crucial roles in glucose metabolism,[1] and chronic liver diseases are associated with glucose intolerance.[2] Several epidemiological studies have revealed an association between hepatitis C virus (HCV) infection and impaired glucose metabolism.[3–5] In patients with HCV infection, glucose intolerance decreases the sustained response rate to antivirus therapy and is an important risk factor for the development of hepatocellular carcinoma as well as long-term survival in patients with cirrhosis.[6–8] Therefore, it is important to investigate the mechanisms for HCV-related glucose intolerance. Increased body mass index (BMI) and decreased insulin secretion are typical characteristics of type 2 diabetes mellitus.[9] In patients with HCV infection, however, glucose intolerance is independent of BMI, and insulin secretion is increased in patients with HCV infection.[3,10] These findings suggest that other factors mediate HCV-related glucose intolerance.

In patients with HCV infection, the homeostasis model assessment method for insulin resistance (HOMA–IR) value is significantly higher than in other hepatobiliary disorders.[1] Recently, we found that the HCV core directly downregulates the intracellular insulin signaling cascade in hepatocytes.[11] Thus, increased hepatic insulin resistance is one of the pathogenesis for HCV-associated glucose intolerance.

The gut has currently been recognized as an endocrine system that contributes to glucose metabolism.[12] Among several gut hormones, glucagon-like peptide-1 (GLP-1) is well known to be involved in glucose metabolism. GLP-1 is secreted from L-cells in the ileum and exerts a direct insulinotropic effect on the pancreatic β-cell.[13,14] GLP-1 activates adenylate cyclase and subsequently enhances insulin secretion from pancreatic β-cells.[15] GLP-1 also has insulin-independent effects on glucose disposal in extra-pancreatic tissues including liver.[16] In hepatocytes, GLP-1 activates glycogen synthesis through activation of glycogen

Journal of Gastroenterology and Hepatology **23** (2008) 244–251

synthesis α.[17] The presence of GLP-1 receptors on the hepatocytes is consistent with the insulin-independent effect of GLP-1.[16] Thus, GLP-1 increases insulin secretion and hepatic glycogen production.

GLP-1 has an extremely short duration of action due to rapid inactivation by the enzyme dipeptidyl peptidase IV (DPPIV, enzyme code number 3.4.14.5).[13,14,18] DPPIV is a membrane-associated peptidase and is widely distributed in numerous tissues. Increased serum DPPIV activity has been reported in diabetic mice and patients with type 2 diabetes mellitus.[19,20] Thus, DPPIV is involved in glucose metabolism through the regulation of GLP-1 activity.

The aim of this study was to investigate alterations of GLP-1 and DPPIV expressions in HCV-associated glucose intolerance.

Materials and methods

Materials

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

Patients

In total, 145 patients with liver or bowel disease, HCV-related liver disease (HCV; $n = 94$), hepatitis B virus-related liver disease (HBV; $n = 37$), or inflammatory bowel disease (IBD; $n = 14$), and 48 healthy controls (CON) were enrolled. All of the patients and healthy controls were Asian men. All of the diagnoses were based on clinical, serological, and/or histological evidence. Demographic data were collected on the same day as collecting blood. The BMI was calculated as body weight in kilograms divided by the square of height in meters (kg/m^2). To reduce the selection bias, age, BMI, aspartate aminotransferase (AST), alanine aminotransaminase (ALT), albumin, and total bilirubin were not matched among the groups. Since it is possible that treatments for diabetes mellitus affect GLP-1 levels, patients who had been taking oral hypoglycemic agents or insulin injections were excluded. In addition, patients with a history of and evidence of pancreatitis or a pancreatic tumor were excluded, as were those with other causes of liver disease, in particular those known to be involved in the pathogenesis of diabetes mellitus, such as hemochromatosis or alcoholic liver disease.

The study protocol was approved by the institutional review board, and informed consent for participation in the study was obtained from each patient. None of the patients was institutionalized. The study protocol was approved by the Ethics Committee of the Kurume University School of Medicine. All of the experiments were carried out in accordance with the Declaration of Helsinki.

Laboratory determinations

Venous blood samples were taken in the morning after a 12-h overnight fast. Plasma glucose, serum aspartate, AST, ALT, albumin, total bilirubin, and immunoreactive insulin (IRI) levels were measured using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital, Kurume, Japan). Insulin resistance and pancreatic β-cell function were calculated on the basis of the fasting levels of plasma glucose and IRI, according to the HOMA equation: Insulin resistance (HOMA–IR) = fasting glucose (mg/dL) × fasting IRI (μU/mL)/405; pancreatic β-cell function (HOMA-β) = fasting IRI (μU/mL) × 360/(fasting glucose (mg/dL) − 63).[21] The stage of liver fibrosis was assessed by AST to the platelet ratio index (APRI): the serum AST level (U/L)/upper limit of normal AST (33 U/L) × 100/platelet count (×10^9/L).[22] APRI is one of the models for predicting the stage of liver fibrosis. Patients with APRI values of 0.5 or less were diagnosed as chronic hepatitis (CH) patients, and patients with APRI values above 0.5 were diagnosed as having liver cirrhosis.[22]

The degree of liver cirrhosis was categorized based on Child–Turcotte criteria.[23]

GLP-1 assay

The quantification of GLP-1 in the serum samples was accomplished by a GLP-1 (7–36) amide enzyme immunoassay (EIA) kit (YK 160; Yanaihara, Fujinomiya, Japan) that specifically quantifies the biologically active form of GLP-1, GLP-1 (7–36) amide.[24] This EIA kit is based on a competitive enzyme immunoassay using a combination of highly specific antibodies to GLP-1 (7–36) amide with a biotin-avidin affinity system. The absorbance (492 nm) of each well was then measured with a Bio-Rad Model 550 microplate reader (Bio-Rad, Hercules, CA, USA). Each serum sample was assayed in duplicate and the values were averaged.

Immunohistochemistry

Frozen samples of the ileum and paraffin-embedded liver sections were deparaffinized and subjected to immunochemical staining using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) with an antihuman GLP-1 antibody (Santa Cruz, Santa Cruz, CA, USA) or an antihuman DPPIV antibody (Santa Cruz, USA). The primary antibodies for GLP-1 or DPPIV were used at a 1:100 dilution. After washing, the sections were incubated with a fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin M (Cappel, Aurora, OH, USA) or tetramethylrhodamine isomer R-conjugated swine antirabbit immunoglobulin (Dako, Kyoto, Japan) diluted at 1:100 in phosphate-buffered saline (PBS). Subsequently, the sections were washed with PBS and mounted in VECTASHIELD (Vector Laboratories, USA).

Immunoblotting liver or ileum specimens

The immunoblotting of DPPIV was performed as previously described,[25] The liver and ileum tissues were dissolved in a minimum volume of radioimmunoprecipitation buffer and were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% acrylamide gel. The resolved proteins were transferred electrophoretically to polyvinylidene difluoride membranes (FLUOROTRANS; Pall Life Sciences, Ann Arbor, MI, USA). The membranes were incubated with an anti-DPPIV polyclonal rabbit antibody (Santa Cruz, USA) or an anti-actin rabbit antibody (Sigma, St Louis, MO, USA), and then incubated with horseradish peroxidase-conjugated goat antirabbit immunoglobulin G (Vector Laboratories, USA). The membranes were then incubated with chemiluminescent reagents (ECL kit; GE Health-

Table 1 Baseline characteristics of patients

	CON	HBV	HCV	IBD
No. patients	48	37	94	14
Age (years)	60.1 ± 2.2	54.6 ± 1.4	61.2 ± 1.1*	41.1 ± 3.9
Body mass index (kg/m^2)	22.3 ± 0.3	22.8 ± 0.4	23.1 ± 0.3**	20.2 ± 0.7
Aspartate aminotransferase (U/L)	24.2 ± 1.7	55.6 ± 9.2	69.1 ± 4.0***	21.3 ± 2.8
Alanine aminotransaminase (U/L)	21.1 ± 1.4	56.9 ± 11.2	72.1 ± 4.7***	22.4 ± 3.3
Albumin (g/dL)	4.1 ± 0.1	3.8 ± 0.1	3.7 ± 0.1****	3.9 ± 0.1
Total bilirubin (mg/dL)	0.7 ± 0.1	1.3 ± 0.2	1.1 ± 0.1***	0.6 ± 0.1
Glucose (mg/dL)	89.6 ± 3.9	82.1 ± 2.8	92.4 ± 3.9	82.5 ± 4.2
Immunoreactive insulin (μU/mL)	7.8 ± 1.1	8.3 ± 0.7	17.1 ± 3.4****	9.8 ± 2.5
HOMA–IR	1.7 ± 0.3	1.7 ± 0.2	2.9 ± 0.4*****	2.1 ± 0.6

Note: Data are expressed as mean ± SEM or number of patients. All of the patients were Asian men. *$P < 0.05$ compared to that of hepatitis B virus (HBV) or inflammatory bowel disease (IBD) group; **$P < 0.05$ compared to that of IBD group; ***$P < 0.05$ compared to that of the healthy controls (CON) or IBD groups; ****$P < 0.05$ compared to that of CON group; *****$P < 0.05$ compared to that of the CON or HBV groups. HCV, hepatitis C virus; HOMA–IR, homeostasis model assessment method for insulin resistance. Statistical comparisons among multiple groups were performed by ANOVA followed by Fisher's protected least significant difference.

care, Little Chalfont, Buckinghamshire, UK) and immediately exposed in a LAS-1000 plus lumino-image analyzer (Fuji Photo Film, Tokyo, Japan).

Quantitation

The immunoblotting intensities were determined using NIH-Image J (developed at the National Institutes of Health [NIH], Bethesda, MA, USA) available on the Internet at http://rsb.info.nih.gov/ij/download.html). In preliminary studies, the band intensity showed a linear correlation with the sample concentration over the range analyzed.

Serum DPPIV assay

The quantification of DPPIV in the serum samples was accomplished using a soluble human DPPIV ELISA according to the manufacturer's instructions (Chemicon, Temecula, CA, USA). The absorbance (492 nm) of each well was then measured with a Bio-Rad Model 550 microplate reader (Bio-Rad, USA). Each serum sample was assayed in duplicate and the values were averaged.

Oral glucose tolerance test and insulinogenic index

Twenty-one patients with HCV infection underwent oral glucose tolerance tests (OGTT) with 75 g of glucose according to the recommendations of the National Diabetes Data Group of National Institutes of Health.[26] After overnight fasting, blood samples were drawn for the determination of glucose and IRI at 0, 30, and 120 min after glucose loading. Pancreatic β-cell function was evaluated by the insulinogenic index, which represents the capacity of insulin secretion. The formula is as follows: Insulinogenic index = (30-min IRI – fasting IRI [μU/mL])/(30-min glucose – fasting plasma glucose [mg/dL]).[21] Pancreatic β-cell function was also evaluated by HOMA-β.[21]

Hepatic glycogen content

The liver specimens were stained with periodic acid–Schiff reagent to evaluate the hepatic glycogen content.[27] The hepatic glycogen content was quantitated by the public domain NIH Image-J program by an investigator without any information.

Statistical analysis

All data are expressed as mean ± SEM. Differences between groups were analyzed by the Mann–Whitney *U*-test and the Spearman correlation test. Statistical comparisons among multiple groups were performed by ANOVA followed by Fisher's protected least significant difference using StatView Power PC version for Macintosh (version 5.1; SAS, Cary, NC, USA). *P*-values < 0.05 were considered significant.

Results

Baseline characteristics of all patients

Baseline clinical and laboratory data for all patients and healthy controls are summarized in Table 1. There were no significant differences in age and BMI between the control and HCV groups. Although the HBV group was significantly younger than the HCV group ($P < 0.05$), there were no significant differences in BMI, aspartate aminotransferase, alanine aminotransferase, albumin, and total bilirubin levels between the HBV and HCV groups. Fasting glucose levels were within the normal range in all of the groups and there were no significant differences among the groups (Table 1). However, the fasting insulin levels were significantly increased in the HCV group compared to those of the controls, but were not significantly different compared to the HBV or the IBD groups (Table 1). The HOMA–IR values in the HCV group were significantly higher than those in the controls ($P < 0.05$) or the HBV group ($P < 0.05$) (Table 1).

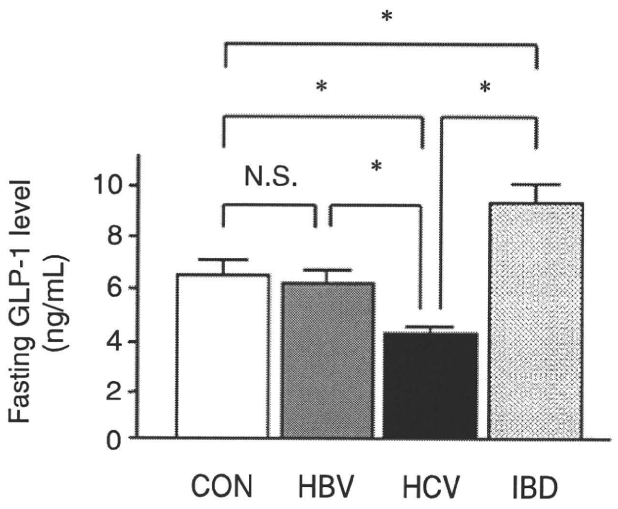

Figure 1 Fasting glucagon-like peptide-1 (GLP-1) levels in healthy controls (CON group, $n = 48$), patients with hepatitis B virus-related liver disease (HBV group, $n = 37$), patients with hepatitis C virus-related liver disease (HCV group, $n = 94$), and patients with inflammatory bowel disease (IBD; $n = 14$). Values are expressed as mean ± SEM. Comparisons between the groups were performed by ANOVA followed by Fisher's protected least significant difference. N.S., not significant. $^*P < 0.01$.

Changes in fasting GLP-1 levels

The fasting serum GLP-1 levels were significantly decreased in the HCV group compared to those in the controls ($P < 0.01$), the HBV group ($P < 0.01$), or the IBD group, an intestinal disease control ($P < 0.01$) (Fig. 1).

GLP-1 content of the ileum in patients with HCV-related chronic liver disease

The GLP-1 content of the ileum was not significantly different between the control and the HCV groups (Fig. 2a). Immunostaining demonstrated that the GLP-1 intensity of L-cells was not significantly different between the controls and the HCV group (Fig. 2b).

Association between liver function and fasting GLP-1 levels in patients with HCV-related liver disease

In the HCV group, no significant difference was seen in the fasting GLP-1 levels among CH ($n = 9$), Child–Turcotte criterion A ($n = 54$), B ($n = 22$), and C ($n = 9$). No significant association was also seen between the fasting GLP-1 levels and albumin levels ($P = 0.08$, $r^2 = 0.001$), total bilirubin levels ($P = 0.07$, $r^2 = 0.04$), or platelet count in the HCV group ($P = 0.77$, $r^2 = 0.001$). Moreover, no significant correlations were found between the fasting GLP-1 levels and APRI, an indicator of liver fibrosis ($P = 0.82$, $r^2 = 0.001$) or serum IRI levels ($P = 0.09$, $r^2 = 0.003$).

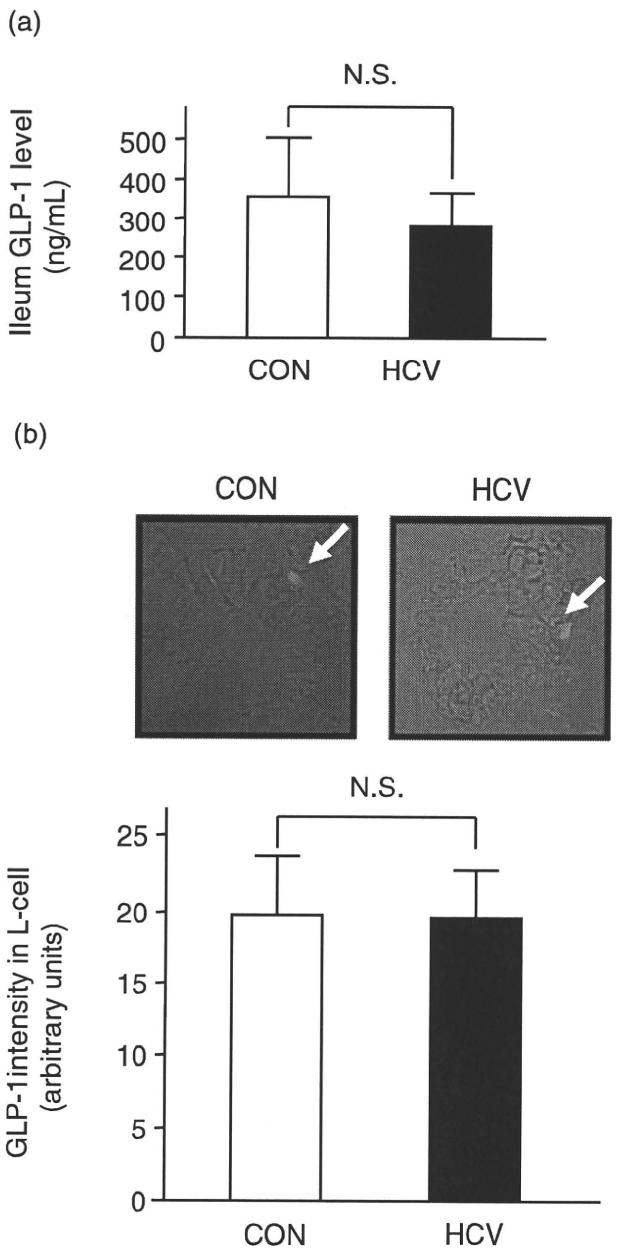

Figure 2 Ileum glucagon-like peptide-1 (GLP-1) content. Ileum GLP-1 content was evaluated by (a) enzyme immunoassay (control [CON] group, $n = 4$; HCV group, $n = 4$) and (b) immunostaining (CON group, $n = 4$; hepatitis C virus [HCV] group, $n = 4$). Representative photographs show GLP-1 expression of immunostaining in ileal tissue. Arrows show GLP-1 staining in endocrine L-cell of the ileum. Values are expressed as mean ± SEM. Comparisons between groups were made using the Mann–Whitney *U*-test. N.S., not significant. $^*P < 0.05$.

DPPIV expression in the ileum, liver, and serum

In the ileum tissue, immunostaining showed that the DPPIV expression levels were increased in the HCV group compared to

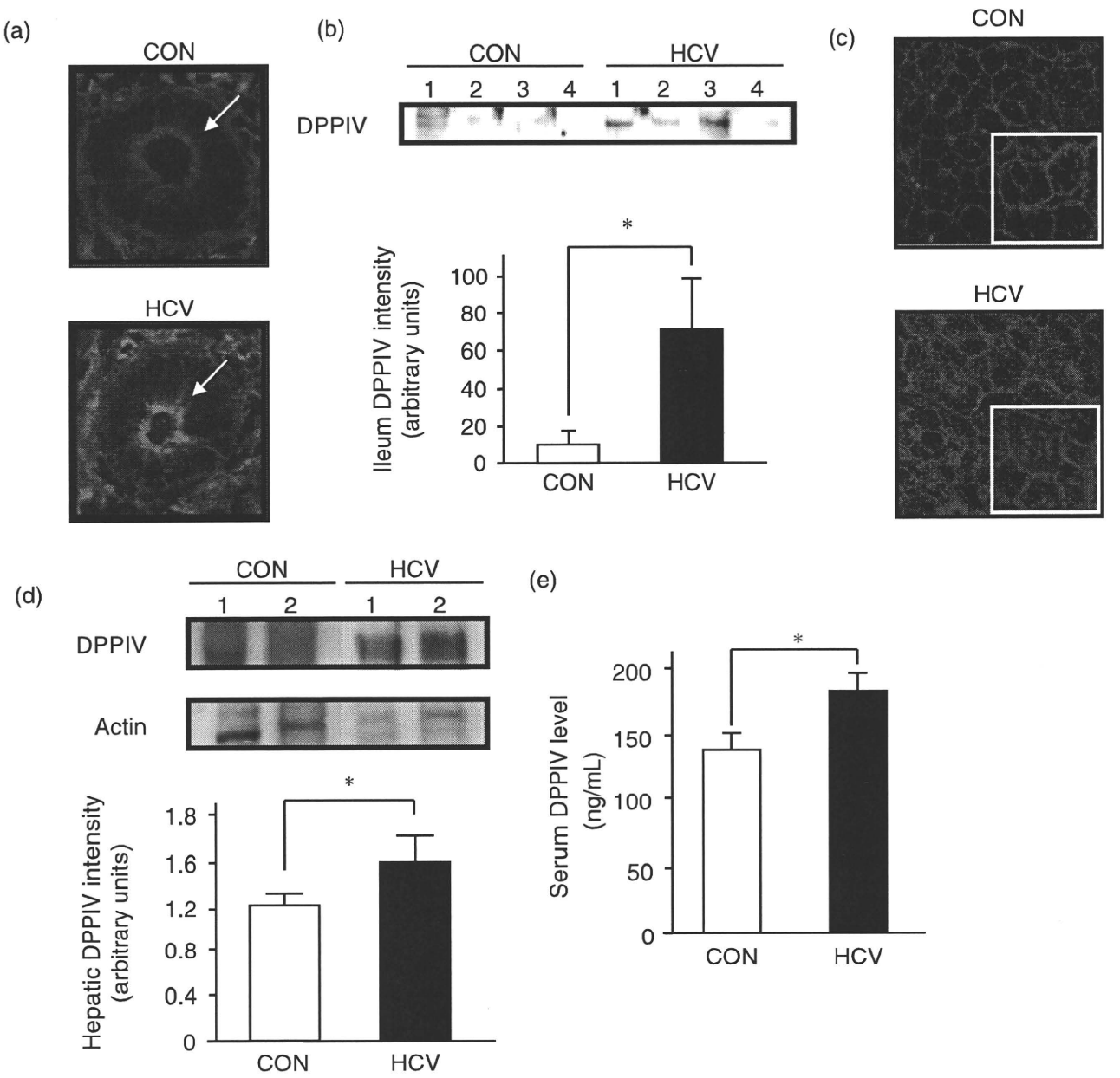

Figure 3 Dipeptidyl peptidase IV (DPPIV) expression in the ileum, hepatocytes, and serum. Ileum DPPIV expression was evaluated by (a) immunostaining and (b) immunoblotting (control [CON] group, $n = 4$; hepatitis C virus [HCV] group, $n = 4$). DPPIV expression in hepatocytes was evaluated by (c) immunostaining and (d) immunoblotting (CON group, $n = 5$; HCV group, $n = 4$). Representative photographs show the DPPIV expression of immunostaining and immunoblotting in the ileum or hepatocytes. (e) Serum DPPIV levels was measured by ELISA (CON group, $n = 10$; HCV group, $n = 19$). Values are expressed as mean ± SEM. Comparisons between groups were made using the Mann–Whitney *U*-test. $^*P < 0.05$.

that in the controls ($P < 0.05$)(Fig. 3a). Immunoblotting also showed a significant increase of DPPIV expression levels in the HCV group ($P < 0.05$) compared to that in the controls ($P < 0.05$) (Fig. 3b). In the hepatocytes, immunostaining showed that DPPIV expression levels were increased in the HCV group ($P < 0.05$) compared to the controls ($P < 0.05$) (Fig. 3c). Immunoblotting also revealed that the DPPIV expression levels were significantly increased in the HCV group than those in the controls ($P < 0.05$) (Fig. 3d). The serum DPPIV levels were significantly increased in the HCV group than that in the controls ($P < 0.05$) (Fig. 3e).

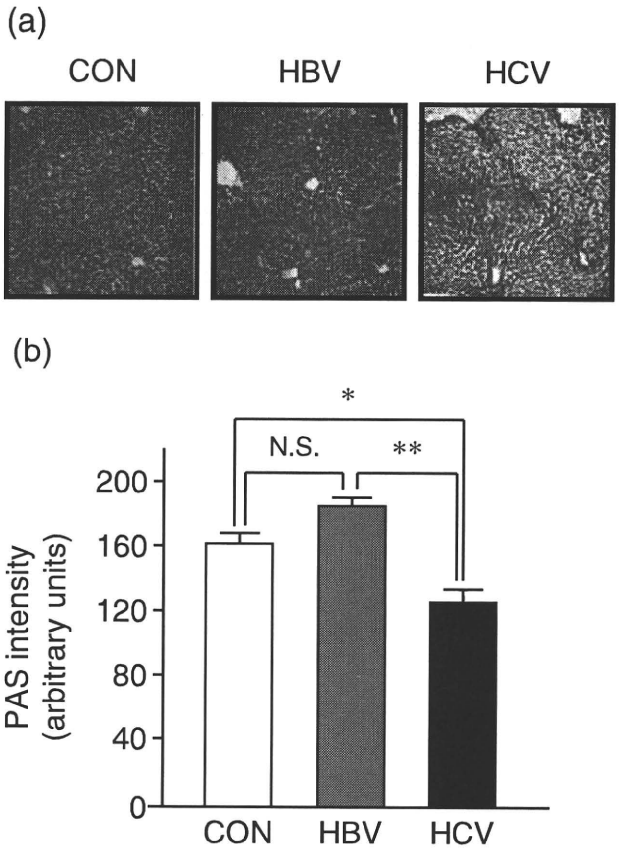

Figure 4 (a) Hepatic glycogen content was examined by periodic acid–Schiff (PAS) staining in healthy controls (CON; $n = 5$), patients with hepatitis B virus-related liver disease (HBV group, $n = 19$), and patients with hepatitis C virus-related liver disease (HCV group, $n = 20$). (b) PAS staining intensity was evaluated by measuring pixel intensities using NIH Image-J. Values are expressed as mean ± SEM. Comparisons between the groups were performed by ANOVA followed by Fisher's protected least significant difference. N.S., not significant. $*P < 0.05$; $**P < 0.01$.

Ability of glucose uptake and insulin secretion in patients with HCV-related chronic liver disease

The ability of glucose uptake and insulin secretion was examined by an OGTT. The HCV group ($n = 21$) showed persistent increases in glucose (208.5 ± 25.2 mg/dL) and insulin levels (63.2 ± 7.4 μU/dL) at 120 min, although Asian men with normal glucose tolerance showed decreases in glucose at 120 min.[26] The HOMA-β-values were within the normal range in the HCV group (97 ± 15.3; $n = 21$). Similarly, the insulinogenic index in the HCV group was within the normal range (1.0 ± 0.3; $n = 21$).

Glycogen accumulation levels in the liver

The glycogen accumulation levels of the liver specimens were significantly decreased in the HCV group than that in the controls ($P < 0.05$) or the HBV group ($P < 0.01$) (Fig. 4a,b).

Discussion

In this study, we demonstrated decreased serum GLP-1 levels and increased DPPIV expression in the liver, ileum, and serum in patients with HCV infection. We also showed impaired hepatic glycogen storage in patients with HCV infection.

Insulin resistance was significantly increased in the HCV group, which is in accordance with previous reports.[11,28] HCV genotype 3 is associated with insulin resistance.[1,2] Although we could not examined the HCV genotype for all of the enrolled patients, it was reported that the prevalence of HCV genotype 3 is only found in 1.3% of Japanese (5/379 patients).[4] In addition, there were no patients with HCV genotype 3 infection among the 158 patients with HCV infection in our previous investigation, which was conducted in an area similar to the one in the current study.[5] Thus, it seems that genotype is not a factor responsible for insulin resistance in this study. Insulin resistance can be also caused by the dysfunction of several organs. The gut is involved in the development of insulin resistance through the regulation of gut hormones, such as GLP-1.[13,14] In this study, we first demonstrated the decrease in the active form of serum GLP-1 levels in patients with HCV-associated insulin resistance. In contrast, increases in both insulin resistance and the active form of serum GLP-1 levels are seen in patients with type 2 diabetes mellitus and alcoholic liver disease.[29,30] This discrepancy suggests that HCV is involved in the downregulation of GLP-1.

GLP-1 mainly occurs in the terminal ileum.[13,14] In both immunostaining and ELISA, there was no significant difference in ileum GLP-1 expression levels between the controls and the HCV group. Thus, in patients with HCV infection, the downregulation of serum GLP-1 levels was not caused by a decrease in GLP-1 expression in the ileum. However, the liver is considered a major site for the regulation of serum GLP-1 levels and more than 40% of circulating GLP-1 is degraded in the liver.[18] Therefore, we investigated a relationship between serum GLP-1 levels and liver function. There was no correlation between the serum GLP-1 levels and the indicators of liver function. Thus, liver function also does not seem to be a responsible factor for the downregulation of serum GLP-1 in patients with HCV infection. It would be reasonable to assume that the downregulation of serum GLP-1 levels reflects negative feedback resulting from the increased insulin resistance in HCV-infected patients. However, there was no significant correlation between fasting serum GLP-1 levels and serum IRI levels. In addition, a previous study demonstrated that hyperinsulinemia does not inhibit GLP-1 secretion.[31] Thus, negative feedback does not seem to directly downregulate serum GLP-1 levels in HCV-infected patients.

DPPIV rapidly inactivates active GLP-1.[32] DPPIV localizes at intestinal brush-borders and membrane of hepatocytes.[33,34] In addition, the circulating soluble form of DPPIV exhibits enzymatic activity.[35] In this study, we found increased DPPIV expression of the ileum, liver, and serum in patients with HCV infection. Although we could not assess tissue DPPIV activity, DPPIV expression levels are positively correlated with its peptidase activity.[35] It seems therefore that the upregulation of DPPIV increases its peptidase activity, which subsequently causes a decrease in serum GLP-1 levels. The reason for the increased DPPIV expression in patients with HCV infection is unclear. However, DPPIV is also known as CD26, an immune-regulating molecule expressed

on T cells.[36] A significant increase in DPPIV/CD26 expression is seen in a hepatoma cell line transfected with a HCV non-structural genome region.[37] HCV infects not only liver, but also extrahepatic tissues, including the intestine and lymphocytes.[38] Thus, it is possible that HCV directly upregulates DPPIV expression in the ileum, liver, and serum.

GLP-1 plays an important role in the early phase of insulin secretion.[39] Unexpectedly, in the HCV group, the HOMA-β-value, an indictor of basal insulin secretion, and the insulinogenic index, an indicator for early-phase insulin secretion, were equal to those of healthy Asian patients.[26] Although the serum GLP-1 levels were decreased, the downregulation of GLP-1 in patients with HCV infection did not seem to have a significant influence on the early phase of insulin secretion. Insulin secretion might be compensated by other stimuli of insulin secretion, including glucose-dependent insulinotropic polypeptide.

GLP-1 increases glycogen synthesis in hepatocytes by stimulating glycogen synthase α.[17,40] It is well known that hepatic glycogen storage is depleted in patients with cirrhosis. However, etiological differences in glycogen storage have never been investigated. Here, we demonstrated that glycogen content was significantly decreased in the HCV group than in the controls and the HBV group. The depletion of glycogen storage leads to an increase in free fatty acid (FFA) levels.[41] Serum FFA levels were increased in HCV-related chronic liver disease.[42] Increased FFA impairs intracellular insulin signaling, insulin receptor substrate-1 tyrosine phosphorylation, and results in increased insulin resistance.[41,43] These findings suggest that the downregulation of GLP-1 is involved in increased insulin resistance through the depletion of glycogen storage in hepatocytes.

The results of the ileum tissue experiments are limited, because the data were obtained from a small subgroup. Although all of the cases showed a similar trend, these patients may not be representative of the whole group. Furthermore, the significance of the DPPIV–GLP-1 regulatory pathways in HCV-related glucose intolerance is unclear. Thus, the comparison of the DPPIV–GLP-1 regulatory pathway with other factors linked to glucose intolerance, such as BMI, is required for further elucidation. Another limitation is patient selection. The majority of enrolled patients were in the early stage of liver cirrhosis (Child–Turcotte A). Moreover, patients who had been taking oral hypoglycemic agents or insulin injections were excluded from this study. Thus, we have to be cautious in the interpretation of data from a relatively selected population of HCV infection.

In conclusion, this study showed decreased serum GLP-1 levels and increased DPPIV levels in the ileum, liver, and serum in patients with HCV infection. Decreased GLP-1 levels may be due to an upregulation of DPPIV expression. A GLP-1 action, hepatic glycogen synthesis, was impaired in patients with HCV infection. Thus, the altered expression of GLP-1 and DPPIV may be responsible for HCV-associated glucose intolerance.

Acknowledgments

The authors thank Eiichi Hirano, Yumi Ogo, Mari Hagihara, Yuko Fujimoto, Syunichi Hattori and Hidetoshi Itoya (Ortho–Clinical Diagnostics, Kabushiki Kaisha, Tokyo, Japan) for technical assistance.

This study was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 16590648 to M.S.) and a Grant-in-Aid for Young Scientists (B) (No. 19790643 to T.K.) from the Ministry from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Vehicle Racing Commemorative Foundation, a grant from the Ishibashi Foundation for the Promotion of Science, and a grant from the 21st Century Centre of Excellence Program for Medical Science, Kurume University.

References

1 Blendis L, Brill S, Oren R. Hepatogenous diabetes: reduced insulin sensitivity and increased awareness. *Gastroenterology* 2000; **119**: 1800–2.
2 Megyesi C, Samols E, Marks V. Glucose tolerance and diabetes in chronic liver disease. *Lancet* 1967; **2**: 1051–6.
3 Fartoux L, Poujol-Robert A, Guechot J, Wendum D, Poupon R, Serfaty L. Insulin resistance is a cause of steatosis and fibrosis progression in chronic hepatitis C. *Gut* 2005; **54**: 1003–8.
4 Zekry A, McHutchison JG, Diehl AM. Insulin resistance and steatosis in hepatitis C virus infection. *Gut* 2005; **54**: 903–6.
5 Koike K. Hepatitis C as a metabolic disease. Implication for the pathogenesis of NASH. *Hepatol. Res.* 2005; **2**: 145–50.
6 Romero-Gomez M, Del Mar Viloria M, Andrade RJ *et al.* Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology* 2005; **128**: 636–41.
7 Hassan MM, Hwang LY, Hatten CJ *et al.* Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; **36**: 1206–13.
8 Bianchi G, Marchesini G, Zoli M, Bugianesi E, Fabbri A, Pisi E. Prognostic significance of diabetes in patients with cirrhosis. *Hepatology* 1994; **20**: 119–25.
9 Sone H, Yoshimura Y, Ito H, Ohashi Y, Yamada N. Energy intake and obesity in Japanese patients with type 2 diabetes. *Lancet* 2004; **363**: 248–9.
10 Furutani M, Nakashima T, Sumida Y *et al.* Insulin resistance/beta-cell function and serum ferritin level in non-diabetic patients with hepatitis C virus infection. *Liver Int.* 2003; **23**: 294–9.
11 Kawaguchi T, Yoshida T, Harada M *et al.* Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. *Am. J. Pathol.* 2004; **165**: 1499–508.
12 Seltzer HS, Allen EW, Herron AL Jr, Brennan MT. Insulin secretion in response to glycemic stimulus: relation of delayed initial release to carbohydrate intolerance in mild diabetes mellitus. *J. Clin. Invest.* 1967; **46**: 323–35.
13 Holst JJ. Glucagon-like peptide-1: from extract to agent. The Claude Bernard Lecture, 2005. *Diabetologia* 2006; **49**: 253–60.
14 Drucker DJ. The biology of incretin hormones. *Cell Metab.* 2006; **3**: 153–65.
15 Miura Y, Matsui H. Glucagon-like peptide-1 induces a cAMP-dependent increase of [Na+]i associated with insulin secretion in pancreatic beta-cells. *Am. J. Physiol. Endocrinol. Metab.* 2003; **285**: E1001–9.
16 Ahren B. GLP-1 and extra-islet effects. *Horm. Metab. Res.* 2004; **36**: 842–5.
17 Redondo A, Trigo MV, Acitores A, Valverde I, Villanueva-Penacarrillo ML. Cell signalling of the GLP-1 action in rat liver. *Mol. Cell. Endocrinol.* 2003; **204**: 43–50.
18 Deacon CF, Pridal L, Klarskov L, Olesen M, Holst JJ. Glucagon-like peptide 1 undergoes differential tissue-specific metabolism in the anesthetized pig. *Am. J. Physiol.* 1996; **271**: E458–64.

19 Nagakura T, Yasuda N, Yamazaki K, Ikuta H, Tanaka I. Enteroinsular axis of db/db mice and efficacy of dipeptidyl peptidase IV inhibition. *Metabolism* 2003; **52**: 81–6.
20 Mannucci E, Pala L, Ciani S *et al.* Hyperglycaemia increases dipeptidyl peptidase IV activity in diabetes mellitus. *Diabetologia* 2005; **48**: 1168–72.
21 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–19.
22 Wai CT, Greenson JK. Fontana RJ *et al.* A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 2003, 38, 518–26.
23 Christensen E, Schlichting P, Fauerholdt L *et al.* Prognostic value of Child–Turcotte criteria in medically treated cirrhosis. *Hepatology* 1984; **4**: 430–5.
24 Hirasawa A, Tsumaya K, Awaji T *et al.* Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat. Med.* 2005; **11**: 90–4.
25 Kawaguchi T, Sakisaka S, Sata M, Mori M, Tanikawa K. Different lobular distributions of altered hepatocyte tight junctions in rat models of intrahepatic and extrahepatic cholestasis. *Hepatology* 1999; **29**: 205–16.
26 Tanaka Y, Atsumi Y, Asahina T *et al.* Usefulness of revised fasting plasma glucose criterion and characteristics of the insulin response to an oral glucose load in newly diagnosed Japanese diabetic subjects. *Diabetes Care* 1998; **21**: 1133–7.
27 Kudryavtseva MV, Sakuta GA, Skorina AD, Stein GI, Emelyanov AV, Kudryavtsev BN. Quantitative analysis of glycogen content in hepatocytes of portal and central lobule zones of normal human liver and in patients with chronic hepatitis of different etiology. *Tissue Cell* 1996; **28**: 279–85.
28 Nagao Y, Kawaguchi T, Tanaka K, Kumashiro R, Sata M. Extrahepatic manifestations and insulin resistance in an HCV hyperendemic area. *Int. J. Mol. Med.* 2005; **16**: 291–6.
29 Orskov C, Jeppesen J, Madsbad S, Holst JJ. Proglucagon products in plasma of noninsulin-dependent diabetics and nondiabetic controls in the fasting state and after oral glucose and intravenous arginine. *J. Clin. Invest.* 1991; **87**: 415–23.
30 Kruszynska YT, Ghatei MA, Bloom SR, McIntyre N. Insulin secretion and plasma levels of glucose-dependent insulinotropic peptide and glucagon-like peptide 1 [7–36 amide] after oral glucose in cirrhosis. *Hepatology* 1995; **21**: 933–41.
31 Takahashi H, Manaka H, Suda K *et al.* Hyperglycaemia but not hyperinsulinaemia prevents the secretion of glucagon-like peptide-1 (7–36 amide) stimulated by fat ingestion. *Scand. J. Clin. Lab. Invest.* 1991; **51**: 499–507.
32 Hansen L, Deacon CF, Orskov C, Holst JJ. Glucagon-like peptide-1-(7–36) amide is transformed to glucagon-like peptide-1-(9–36) amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology* 1999; **140**: 5356–63.
33 Thum A, Hupe-Sodmann K, Goke R, Voigt K, Goke B, McGregor GP. Endoproteolysis by isolated membrane peptidases reveal metabolic stability of glucagon-like peptide-1 analogs, exendins-3 and -4. *Exp. Clin. Endocrinol. Diabetes* 2002; **110**: 113–18.
34 Deacon CF. Circulation and degradation of GIP and GLP-1. *Horm. Metab. Res.* 2004; **36**: 761–5.
35 Kobayashi H, Hosono O, Mimori T *et al.* Reduction of serum soluble CD26/dipeptidyl peptidase IV enzyme activity and its correlation with disease activity in systemic lupus erythematosus. *J. Rheumatol.* 2002; **29**: 1858–66.
36 Ohnuma K, Yamochi T, Hosono O, Morimoto C. CD26 T cells in the pathogenesis of asthma. *Clin. Exp. Immunol.* 2005; **139**: 13–16.
37 Harada T, Kim DW, Sagawa K *et al.* Characterization of an established human hepatoma cell line constitutively expressing non-structural proteins of hepatitis C virus by transfection of viral cDNA. *J. Gen. Virol.* 1995; **76**: 1215–21.
38 Yan FM, Chen AS, Hao F *et al.* Hepatitis C virus may infect extrahepatic tissues in patients with hepatitis C. *World J. Gastroenterol.* 2000; **6**: 805–11.
39 Rask E, Olsson T, Soderberg S *et al.* Insulin secretion and incretin hormones after oral glucose in non-obese subjects with impaired glucose tolerance. *Metabolism* 2004; **53**: 624–31.
40 Mommsen TP, Andrews PC, Plisetskaya EM. Glucagon-like peptides activate hepatic gluconeogenesis. *FEBS Lett.* 1987; **219**: 227–32.
41 Boden G. Free fatty acids and insulin secretion in humans. *Curr. Diab. Rep.* 2005; **5**: 167–70.
42 Konrad T, Vicini P, Zeuzem S *et al.* Interferon-alpha improves glucose tolerance in diabetic and non-diabetic patients with HCV-induced liver disease. *Exp. Clin. Endocrinol. Diabetes* 1999; **107**: 343–9.
43 Hirosumi J, Tuncman G, Chang L *et al.* A central role for JNK in obesity and insulin resistance. *Nature* 2002; **420**: 333–6.

Hepatology Research 2008; 38: 683–688 doi: 10.1111/j.1872-034X.2008.00319.x

Original Article

Suppressive effect of oral administration of branched-chain amino acid granules on oxidative stress and inflammation in HCV-positive patients with liver cirrhosis

Tomoyoshi Ohno,[1] Yasuhito Tanaka,[2] Fuminaka Sugauchi,[3] Etsuro Orito,[3] Izumi Hasegawa,[1] Haruhiko Nukaya,[1] Atsunaga Kato,[1] Seijiro Matunaga,[1] Masayuki Endo,[1] Yoshito Tanaka,[1] Kenji Sakakibara[1] and Masashi Mizokami[2]

[1]*Department of Gastroenterology, Social Insurance Chukyo Hospital,* [2]*Department of Clinical Molecular Informative Medicine, and* [3]*Internal Medicine and Bioregulation, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*

Aim: In chronic hepatitis C virus (HCV) infection, it is thought that both chronic persistent inflammation and oxidative stress contribute to the development of hepatocellular carcinoma (HCC), and it has been reported that long-term oral supplementation with branched-chain amino acid (BCAA) granules could inhibit liver carcinogenesis. However, the extent of the involvement of these factors remains obscure.

Methods: To clarify the involvement of inflammation and oxidative stress in the inhibition of liver carcinogenesis, we evaluated the effect of oral administration of BCAA granules on oxidative stress and inflammation in HCV-positive patients with liver cirrhosis.

Results: Twenty-seven patients were enrolled in the study: 18 of the patients were treated with BCAA granules (administered group) and nine were observed without BCAA granules (non-administered group). In the non-administered group, the production of oxidative stress, as indicated by urine 8-hydroxydeoxyguanosine (8-OHdG) and 15-F2t-Isoprostane (8-IsoPs), significantly increased with time, while in the administered group the levels of ferritin and 8-OHdG decreased significantly. Comparison of the two groups demonstrated that highly sensitive CRP, ferritin, 8-OHdG and 8-IsoPs were significantly reduced by taking BCAA granules. The time-course analysis showed that ferritin and highly sensitive CRP seemed to decrease first, followed by a decrease of 8-OHdG and 8-IsoPs.

Conclusion: These findings indicated that the administration of BCAA granules influenced microinflammation and the metabolism of iron in HCV-positive patients with liver cirrhosis, and subsequently seemed to reduce the production of oxidative stress, possibly leading to a decrease in the occurrence of HCC.

Key words: branched-chain amino acid, hepatitis C virus, liver cirrhosis, oxidative stress

INTRODUCTION

THERE ARE APPROXIMATELY 170 million people infected with hepatitis C virus (HCV) worldwide.[1] HCV is a major causative agent of liver disease, including chronic hepatitis, liver cirrhosis (LC) and hepatocellular carcinoma (HCC). It is estimated that 70–80% of HCV infections evolve into chronic infection. Once HCV develops into cirrhosis, HCC develops at an annual rate of 5–7%.[2] Nevertheless, the precise mechanism underlying HCV-associated HCC is not completely understood. HCV may contribute to the development of HCC by facilitating the accumulation of genetic damage as a result of continuous cell death followed by regeneration in the course of chronic hepatitis. If this is the case, HCV would only be associated indirectly with hepatocarcinogenesis. Another possibility is the direct involvement of HCV in hepatocarcinogenesis, whereby some products of the virus may be oncogenic and involved in cell transformation. Recently, the core protein of HCV has been shown to induce HCC in a transgenic mouse model and has been suggested to play a central role in the development of HCC in chronic

Correspondence: Masashi Mizokami, Department of Clinical Molecular Informative Medicine, Nagoya, City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. Email: mizokami@med.nagoya-cu.ac.jp
Received 23 July 2007; revision 1 November 2007; accepted 28 November 2007.

hepatitis C.[3] However, it still remains unclear how the core protein influences the development of HCC. An additional possibility is the involvement of oxidative stress. Recently, numerous studies have shown that HCV infection itself causes oxidative stress to a greater degree than other inflammatory liver disease.[4,5]

Oxidative stress is defined as a disturbance of the normal balance between oxidants and antioxidants in the body. Under oxidative stress, reactive oxygen species (ROS) can modify macromolecules such as lipids, proteins and nucleic acids (DNA). If ROS-mediated DNA damage is not repaired, it leads to the production of genetic mutations as well as gross chromosomal alterations and contributes to cancer development in multistep carcinogenesis.[6]

Clinically, when long-term supplementation with branched-chain amino acids (BCAA) is given to patients with compensated or decompensated cirrhosis, their nutritional status, as indicated by hypoalbuminemia, ammonia metabolism and glucose metabolism, is improved.[7] Additionally, a recent study has shown that long-term oral supplementation with BCAA can inhibit liver carcinogenesis, especially in overweight or obese patients.[8]

Against this background, we hypothesized that when BCAA granules are administered orally to patients with LC, the improvement of hypoalbuminemia, glucose and ammonia metabolism, as well as the inhibitory effect on hepatocarcinogenesis, might be due to a reduction of oxidative stress by the BCAA supplementation. In the present study, we investigated the effects on oxidative stress of administering BCAA granules orally to patients with LC.

METHODS

Subjects, design and protocol

THE SUBJECTS INCLUDED in the present study were outpatients with compensated cirrhosis who had hypoalbuminemia despite adequate food intake.

A total of 27 subjects, 18 of whom were given a 4 g BCAA preparation (LIVACT Granules; Ajinomoto, Tokyo, Japan) administered orally three times daily after meals (administered group), and nine of whom were followed up without the BCAA granules as a control (non-administered group), were enrolled in the present study. All of subjects were confirmed to be HCV-positive and HBV-negative patients with liver cirrhosis and without other liver diseases, including HCC as shown by image techniques, and not to be have received any medication with BCAA granules, intravenous albumin administration, or enteral nutrition for hepatic insufficiency within 8 weeks before enrollment. Patients were followed up for at least 6 months and blood and urine samples were collected at treatment initiation (0 M) and at 1 month (1 M), 2 M, 3 M, 4 M and 6 M for as long as possible. Routine laboratory parameters as shown in Table 1, highly sensitive CRP, ferritin and oxidative stress markers (urine 8-hydroxydeoxyguanosine [8-OHdG] and urine 15-F2t-Isoprostane [8-IsoPs]) were evaluated.[9] Routine laboratory parameters were measured on every visit to hospital. Blood and urine samples were stored at −70°C until highly sensitive CRP, ferritin, urine 8-OHdG and urine 8-IsoPs were centrally measured at SRL MediSearch (Tokyo, Japan). The raw values (nanogram: ng/mL) of urine 8-OHdG and urine 8-IsoPs were conventionally corrected by urinary creatinine (mg/dL) to make it possible to compare them with each other.[9]

It was confirmed that the patients' medications had not been changed within 8 weeks before enrollment or

Table 1 Characteristics of HCV patients with LC

	P	C
Number of patients	18	9
Age	56–80	52–79
Median	70.2	66.8
Sex (male/female)	9/9	4/5
Routine parameters		
Uric acid (mg/dL)	5.44	5.8
NH3 (μg/dL)	64.44	68.56
T-protein (g/dL)	7.59	7.53
Alb (g/dL)	3.84	3.68
AST (IU/L)	68.39	67.44
ALT (IU/L)	49.94	46
LDH (IU/L)	228.29	230.44
ALP (IU/L)	338.12	325.67
T-chol (mg/dL)	153.94	168.56
FBS (mg/dL)	138.56	119.44
PT-INR	1.1	1.13
AFP (ng/mL)	33.39	16.06
Centrally measured parameters		
Ferritin (ng/mL)	65.93	45.23
Highly sensitive CRP (ng/mL)	1043.56	428.11
8-OHdG (ng/mL)	11.07	9.35
8-IsoPs (ng/mL)	373.96	338.57
Hepatocellular carcinoma	0	0

ALT, alanine transaminase; C, control patients; HCV, hepatitis C virus; LC, liver cirrhosis; P, patients who were orally administered branched-chain amino acid (BCAA) preparation thrice a day.

during the administration of BCAA. The eligibility and exclusion criteria were made according to the manufacturer's instructions.

This study was a post-marketing clinical trial and was performed with the approval of the review board of Social Insurance Chukyo Hospital. Written informed consent to participate in this trial was obtained from all subjects.

Statistical analysis

The primary end point was the amount of change in oxidative stress markers. The *t* statistic was used to evaluate whether the study drug was useful for reducing oxidative stress. Data were further analyzed by the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

THE CHARACTERISTICS OF the two groups of patients were comparable in terms of age, sex and underlying liver disease severity, as shown in Table 1.

To assess how the evaluated variables altered during the observation, the data from before (0 M) and at 6 months (6 M) were compared in each group (Table 2). The results showed that total cholesterol (T-chol) was significantly decreased and PT-INR was significantly prolonged, and also that urine 8-OHdG and 8-IsoPs were significantly increased at 6 M in the non-administered group. The decrease in T-chol and the prolongation of PT-INR might mean that the liver diseases were becoming more severe and that oxidative stress was increasing in the patients over time. In contrast, in administered group, urine 8-OHdG and serum ferritin at 6 M significantly decreased, suggesting that administering BCAA granules regularly might reduce oxidative stress. Considering that highly sensitive CRP was slightly, although not significantly, increased in the non-administered group and slightly decreased in the administered group during the observation period, it was considered necessary to compare the alterations between the non-administered group and the administered group. Therefore, the time-dependent alterations (Δp or $\Delta c = value_{6M} - value_{0M}$) of all parameters were compared between the two groups using the Mann–Whitney *U*-test. The results showed that ferritin, highly sensitive CRP, urine 8-OHdG and urine 8-IsoPs were significantly decreased by BCAA administration. Considering that ferritin reflects the body's iron storage[10] and that highly sensitive CRP can detect changes in microinflammation, our data suggested that taking

Table 2 Results of statistic analysis

	Uric acid	NH3	T-protein	Alb	AST	ALT	LDH	T-chol	FBS	PT-INR	AFP	Ferritin	H-CRP	8-OHdG	8-IsoPs
P (0 M)	5.44	64.44	7.59	3.84	68.39	49.94	228.29	154.94	138.56	1.10	33.29	65.03	1043.56	11.07	373.96
P (6 M)	5.70	75.22	7.63	3.87	63.44	48.56	213.92	160.33	122.89	1.11	34.88	53.15	351.28	0.03	337.89
P-value*	0.284	0.334	0.877	0.62	0.602	0.619	0.238	0.664	0.236	0.454	0.758	0.024	0.095	0.0003	0.518
C (0 M)	5.80	68.56	7.53	3.68	67.44	46.00	230.44	168.56	119.44	1.13	16.06	45.23	428.11	9.35	338.57
C (6 M)	5.61	80.89	7.29	3.59	59.33	41.11	211.89	158.00	120.56	1.16	26.21	44.87	591.44	10.64	512.41
P-value*	0.433	0.439	0.086	0.303	0.181	0.359	0.055	0.021	0.865	0.004	0.445	0.881	0.311	0.042	0.047
Mann–Whitney**	0.245	> 0.10	0.291	0.194	0.857	>0.10	0.483	$P > 0.010$	0.799	$P > 0.05$	$P > 0.10$	0.037	0.041	0.00038	0.025

ALT, alanine transaminase; AST, aspartate aminotransferase; C, control patients; H-CRP, highly sensitive CRP; P, patients who were orally administered branched-chain amino acid (BCAA) preparation thrice a day.
*$P < 0.05$ versus data of 0 M for data of 6 M (*T*-test). **$P < 0.05$ versus data of ΔP ($P_{6M} - P_{0M}$) for ΔC ($C_{6M} - C_{0M}$) (Mann–Whitney *U*-test).
Data depict mean values.

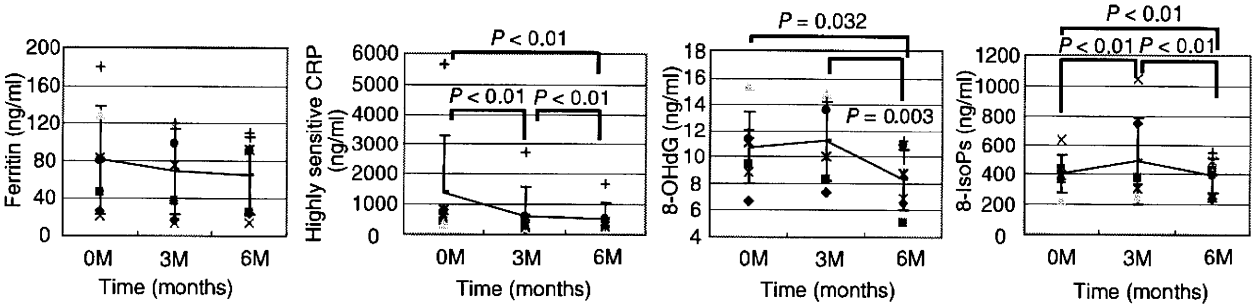

Figure 1 Time-course analysis of ferritin, highly sensitive CRP, 8-OHdG and 8-IsoPs. In seven patients of the administered group, markers of oxidative stress (8-OHdG and 8-IsoPs), ferritin and highly sensitive CRP were measured at 0 M, 3 M and 6 M. Each point represents the mean ± SD. Ferritin decreased slightly, although not significantly, and highly sensitive CRP significantly decreased over time. The values of 8-OHdG and 8-IsoPs did not decrease, but rather increased at 3 M and then significantly decreased at 6 M.

BCAA granules regularly reduced iron storage, microinflammation and oxidative stress.

Next, we evaluated how these parameters that were reduced by BCAA administration altered over time in seven patients of the administered group. As shown in Figure 1, ferritin decreased slightly although not significantly, while highly sensitive CRP significantly decreased with time. In contrast, urine 8-OHdG and 8-IsoPs were increased at 3 M and then significantly decreased at 6 M, compared to their levels before BCAA administration. Considering that it has been reported that hepatic iron accumulation was present in patients with chronic hepatitis C, the decrease of ferritin might indicate the improvement of not only hepatic iron accumulation, but also iron metabolism.[10] Therefore, these data may indicate that the reduction of oxidative stress was followed by an improvement of the iron metabolism and microinflammation.

DISCUSSION

THE AIM OF this study was to analyze the effects on oxidative stress of the oral administration of BCAA granules to HCV-positive patients with LC. In terms of the usefulness of BCAA granules, in the present study the level of albumin did not change significantly, which was probably due to the number of enrolled patients, although some papers have described BCAA granules as improving nutritional disorders associated with abnormal protein and amino acid metabolism in patients with LC.[4,11] Additionally, one *in vitro* study has shown that an increase in the molar ratio of BCAA to aromatic amino acids reduced the growth of HepG2 cells;[12] and recently Muto *et al.* reported that long-term supplementation with BCAA decreased the risk for HCC in patients with LC. These authors pointed out that hyperinsulinemia and peripheral insulin resistance are important mechanisms, although these mechanisms are not yet fully understood.[8] Under these circumstances, if BCAA granules did suppress the production of oxidative stress in HCV-positive patients with LC, it would be another potential mechanism by which the incidence of HCC in HCV-LC patients could be reduced. Therefore, in this study, which we believe is the first report on this topic, we focused on the effect of BCAA on oxidative stress. However, the present study was not a control study but a preliminary study; in the future a larger scale study will be required.

The risk of developing HCC is significantly increased in patients with chronic hepatitis. Hepatocarcinogenesis is considered a multi-step process, in a similar way to the carcinogenesis of other cancers, and is thought to involve at least two factors. One is HCV viral protein, especially the core protein that has been shown to alter the oxidant–antioxidant status of the liver,[4] and another is chronic inflammation. Regarding the former, several studies have investigated the possible role of oxidative injury in the pathogenesis of hepatitis C.[13,14] In particular, oxidative damage is evident in both HCV-infected patients and in HCV-transgenic mice in the absence of necroinflammatory changes or transaminoferase release.[4,15] Moreover, a direct relationship between HCV core protein and HCC has been reported recently.[3,16] Certainly, it is easily to imagine that when DNA damage caused by oxidative stress is not repaired, the accumulation of genetic damage leads to the development of hepatic neoplasm. It has actually been demonstrated that antioxidant treatment improved the liver injury of chronic hepatitis C patients in a prospective study.[17] Clinically, with oral administration of BCAA, we expe-

rienced not only that the nutritional status of such patients improved, but also that the incidence of HCC was reduced.[8] These findings prompted us to evaluate the effects of BCAA granules on the production of oxidative stress. The results, as expected, showed that urine 8-OHdG and 8-IsoPs were significantly reduced compared with those patients followed up without BCAA administration.

In addition to the substantial role of oxidative stress in HCV-associated hepatocarcinogenesis, chronic inflammation is also important. In asymptomatic patients who show normal alanine transaminase (ALT) levels despite having HCV-RNA, the possibility of progression of the disease to LC and HCC has been reported, but HCV-positive HCC is rarely observed in healthy carriers, and is mainly observed in patients with LC.[18] This means that chronic inflammation, creating a cycle of repeated hepatocyte destruction and regeneration, is very important to the development of HCC. On the other hand, chronic hepatitis in the absence of hepatitis virus infection, such as autoimmune hepatitis, carries a low risk for the development of HCC.[19] Based on these findings, it is thought that both HCV proteins and chronic inflammation contribute to the development of HCC additively or synergistically. In the present study, the level of ALT and aspartate aminotransferase (AST) was not significantly changed, but microinflammation, as indicated by highly sensitive CRP, not usual CRP, was found to be first reduced, followed by the reduction of oxidative stress by oral administration of BCAA granules.

However, oxidative stress is reported to be influenced by various things: alcohol consumption, supplementations such as vitamin A tablets, other inflammation and malignancy complications. In considering the mechanism by which the production of oxidative stress was reduced by the oral administration of BCAA granules, we experienced one interesting patient. This patient took iron supplementation for anemia from before entry into this study until 6 M after the enrollment. Despite administrating BCAA granules, the production of oxidative stress was not decreased in this patient, but rather gradually increased. When the patient stopped taking the iron supplements, the production of oxidative stress gradually decreased (data not shown). In the present study ferritin, which is considered to reflect the body's iron store, was shown to be significantly decreased, along with oxidative stress, in the administered group. There are some papers in the literature reporting that iron stimulates the production of ROS and that hepatic iron overload induces HCC.[20] These findings may suggest that BCAA is able to reduce the production of ROS by involving with the iron metabolism.

BCAA granules certainly seemed to reduce the production of oxidative stress and microinflammation, which could possibly lead to a decrease in the occurrence of HCC with long-term supplementation, but as the number of enrolled patients in this study was limited, in the future large-scale studies in which patients with LC, including patients without HCV infection, are administered BCAA granules, will be necessary.

ACKNOWLEDGMENTS

THIS STUDY IS supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology and a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1 Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 2005; 3: 507–20.
2 Ikeda K, Saitou S, Suzuki Y *et al.* Disease progression and hepatocellular carcinogenesis in patients with chronic hepatitis: a prospective observation of 2215 patients. *J Hepatol* 1998; 28: 930–8.
3 Moriya K, Fujie H, Shintani Y *et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; 4: 1065–7.
4 Moriya K, Nakagawa K, Santa T *et al.* Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001; 61: 4365–70.
5 Korenaga M, Wang T, Li Y *et al.* Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005; 280: 37481–8.
6 Cerutti PA. Oxy-radicals and cancer. *Lancet* 1994; 344: 862–3.
7 Sato S, Watanabe A, Muto Y *et al.* Clinical comparison of branched-chain amino acid (1-Leucine, 1-Isoleucine, 1-Valine) granules and oral nutrition for hepatic insufficiency in patients with decompensated liver cirrhosis (LIV-EN study). *Hepatol Res* 2005; 31: 232–40.
8 Muto Y, Sato S, Watanabe A *et al.* Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. *Heptol Res* 2006; 35: 204–214.

9 Nakano M, Kawanishi Y, Kamohara S *et al.* Oxidative DNA damage (8-hydroxydeoxyguanosine) and body iron status: a study on 2507 healthy people. *Free Radic Biol Methods* 2003; **35**: 826–32.
10 Farinati F, Cardin R, De Maria N *et al.* Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. *J Hepatol* 1995; **22**: 449–56.
11 Fan ST, Lo CM, Lai EC, Chu KM, Liu CL, Wong J. Perioperative nutritional support inpatients undergoing hepatectomy for hepatocellular carcinoma. *N Engl J Med* 1994; **331**: 1547–52.
12 Saito Y, Saito H, Nakamura M *et al.* Effect of the major ratio of branched-chain to aromatic acids on growth and albumin m RNA expression of human liver cancer cell lines in a serum-free medium. *Nutr Cancer* 2001; **39**: 126–31.
13 De Maria N, Colantoni A, Fagiuoli S *et al.* Association between reactive oxygen species and disease activity in chronic hepatitis C. *Free Radic Biol Med* 1996; **21**: 291–5.
14 Cardin R, Saccoccio G, Masutti F, Bellentani S, Farinati F, Tiribelli C. DNA oxidative damage in leukocytes with the severity of HCV-related liver disease: validation in an open population study. *J Heptol* 2001; **35**: 587–92.
15 Barbaro G, Di Lorenzo G, Asti A *et al.* Hepatocellular mitochondrial alterations in patients with chronic hepatitis C: ultrastructural and biochemical findings. *Am J Gastroenterol* 1999; **94**: 2198–205.
16 Kato T, Miyamoto M, Date T *et al.* Repeated hepatocyte injury promotes hepatic tumorigenesis in hepatitis C virus transgenic mice. *Cancer Sci* 2003; **64**: 679–85.
17 Houglum K, Venkataramani A, Lyche K, Chojkier M. A pilot study of the effects of d-alpha-tocopherol on hepatic stellate cell activation in chronic hepatitis C. *Gastroenterology* 1997; **113**: 1069–73.
18 Di Bisceglie AM, Goodman ZD, Ishak KG, Hoofnagle JH, Melpolder JJ, Alter HJ. Long-term clinical and histological follow-up of chronic post-transfusion hepatitis. *Hepatology* 1991; **14**: 969–74.
19 Ryder SD, Koskinas J, Rizzi PM *et al.* Hepatocellular carcinoma complicating autoimmune hepatitis: role of hepatitis C virus. *Hepatology* 1995; **22**: 718–22.
20 Furutani T, Hino K, Okuda M *et al.* Hepatic iron overload induces hepatocellular carcinoma transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2006; **130**: 2087–98.

VIRAL IMMUNOLOGY
Volume 21, Number 3, 2008

Pp. 365–369
DOI: 10.1089/vim.2008.0020

Detection of IgE Antibody Specific to a Hepatitis C Virus–Derived Peptide Being Recognized by Cellular Immunity in Patients with HCV Infection

Shojiro Gohara,[1,2] Shigeki Shichijo,[1,3] Nobukazu Komatsu,[1] Seiya Okuda,[2] Shigeru Yutani,[2] and Kyogo Itoh[1,3]

Abstract

The determination of immunogenic peptides of hepatitis C virus (HCV) is pivotal for vaccine development. We previously reported that the majority of patients infected with HCV have significant levels of IgG specific to an HCV-derived peptide at positions 35–44 of core protein (C35–44), a major epitope recognized by cellular immunity. This study addresses whether or not the other subclasses of immunoglobulins to this peptide exist. As a result, IgE, but not IgM or IgA, specific to this peptide is consistently detectable in the majority of patients with HCV infection, regardless of the different HLA types and disease conditions. These results provide additional information on this immunogenic peptide with new insights that contribute to a better understanding of host responses to HCV.

Introduction

ONE HUNDRED AND SEVENTY MILLION PEOPLE AROUND THE WORLD are persistently infected by hepatitis C virus (HCV) with its associated high risk of liver cirrhosis (4) or cancer. The determination of immunogenic peptides of HCV is pivotal for vaccine development, a long-awaited treatment modality. Thus, extensive studies were carried out in the past decade to find immunogenic HCV peptides capable of inducing immune responses (2,9,10,14,15–19). As a result, an HCV-derived peptide at positions 35–44 of core protein (C35–44) was determined to be highly immunogenic because of its ability to induce cytotoxic T lymphocytes in HLA-A2-positive HCV-positive patients (2,15,16). We recently reported that IgG response to this C35–44 peptide was observed in the majority of HCV-positive patients without apparent HLA class I restriction (16). There is strong evidence that both cellular and humoral immune responses play major roles in the control of HCV infection. Furthermore, the region around C35–44 was very stable in the sequence of virus DNA, with only rare mutations. Therefore, C35–44 peptide could be an appropriate molecule for specific immunotherapy toward HCV. Immune responses to HCV, however, are suggested to be partly responsible for damage to hepatocytes and oral epithelial cells (13). These results indicate that detailed studies of immune responses are needed in order to develop a clinically effective HCV vaccine with minimal adverse effects. This study extends our previous studies and investigates the other classes of immunoglobulins that are detectable in these patients.

Materials and Methods

Subjects

Sera from 50 HCV-positive patients, who were positive for anti-HCV antibodies (Abs) as determined by a second- or third-generation immunoassay test, were used in this study. The diagnoses of these patients as determined by biochemical and histological findings, ultrasound-sonography, and computed tomography were chronic hepatitis (CH, n = 25), liver cirrhosis (LC, n = 12), and hepatocellular carcinoma (HCC, n = 13) at the first serum samplings. Sera from two patients with human immunodeficiency virus-1 (HIV-1) infection and 34 healthy donors (HDs) with no history of either viral hepatitis or vaccination for HBV and normal liver function were also used as negative controls.

[1]Department of Immunology, Kurume University School of Medicine, Kurume, Japan.
[2]Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan.
[3]Kurume University Research Center for Innovative Cancer Therapy, Kurume, Japan.

Peptide and measurement of Abs against peptides

The peptide C35–44, a synthetic peptide that corresponds to the aa sequence (residues 35–44) of the HCV core region, and an HIV-derived peptide with an HLA-A2 binding motif as a negative control were purchased from Bio-Synthesis (Lewisville, TX). Desalted grade materials were used for the screening of peptides reactive to serum IgG, while >90% purified materials were used for further studies as assessed by reverse-phase high-pressure liquid chromatography. Each peptide was dissolved in dimethylsulfoxide and then stored at −20°C.

Preparation of xMAP beads

The xMAP carboxylate beads and Luminex® system platform were obtained from Luminex Corp. (Austin, TX) as reported previously (6). The 96-well filter plates (MABVN12) and vacuum manifold apparatus (MAVM 09601) were from Millipore Corp. (Bedford, MA). Goat anti-human IgE was purchased from Biosource International (Camarillo, CA). Streptavidin-PE (S-866) was purchased from Molecular Probes (Eugene, OR). 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC 22980) was obtained from Pierce (Rockford, IL). Peptides were coupled to xMAP beads according to the modified manufacturer's instructions as reported previously. In brief, 100 μL of xMAP beads were washed with 0.1 M MES buffer, pH 7.0, followed by mixing with 100 μL of peptide (1 mg/mL in 0.1 M MES buffer, pH 7.0). The peptide-loaded beads were incubated with EDC (1 mg/mL) at room temperature for 30 min in darkness, and then incubated twice more under the same conditions, and the beads were washed with 0.05% Tween 20-PBS. Finally, the beads were treated with 2-aminoethanol for 15 min at room temperature in darkness, then washed twice and resuspended with 1 mL of 0.05% NaN_3 in Block-Ace.

Anti-peptide antibody measurement by flowmetry assay

Peptide-specific IgE levels in plasma were measured by flowmetry assay using the Luminex system as reported previously (6). In brief, plasma or serum samples were diluted to 1:50 with 0.05% Tween 20-Block-Ace, and 100 μL/well of sample was added to each well. Plasma was incubated with 5 μL each of the peptide-coated beads for 2 h at 37°C in a 96-well filter plate on a plate shaker. After incubation, the plate was washed using a vacuum manifold apparatus and incubated with 100 μL of 1:100 diluted goat anti-human IgE for 1 h at 37°C on a plate shaker. The plate was then washed, 100 μL of 1:200 diluted streptavidin-PE was added to the wells, and the plate was incubated for 30 min at 37°C on a plate shaker. The bound beads were washed three times followed by the addition of 100 μL of Tween 20-PBS into each well, and the plate was placed for 3 min on a plate shaker.

TABLE 1. THE LEVELS OF IGG, IGA, IGM, AND IGE ANTIBODIES AGAINST C35-44 PEPTIDE IN PATIENTS WITH HCV

Serum Ig classes	*IgM*	*IgA*	*IgG*	*IgE*
HD1	0	2	0	0
HD2	276	11	0	2
HD3	333	11	0	0
HCV1	1649	100	10280	613
HCV2	0	79	25525	466
HCV3	0	19	8481	60
HIV1	429	43	0	5
HIV2	291	26	0	6

The levels of IgG, IgA, IgM, and IgE antibodies against C35-44 peptide were assayed in sera (100-fold dilution) from HDs (n = 3) and patients with HCV (n = 3) and HIV (n = 2) by flowmetric analysis with Luminex. The representative results of net value of the levels of IgG, IgA, IgM, and IgE antibodies as indicated in units of mean fluorescent intensity units (FIU) are shown in the table.

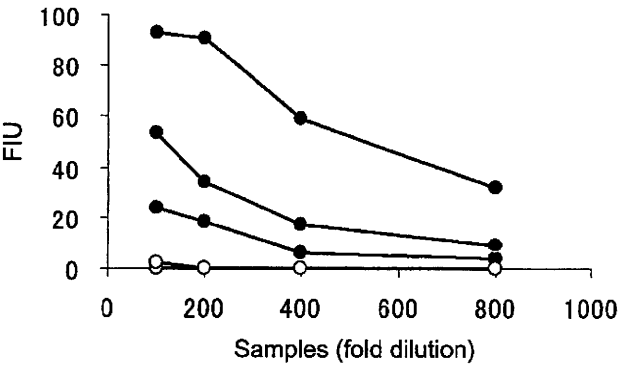

FIG. 1. Dose dependency. Anti-C35–44 peptide IgE antibody was measured by flowmetry analysis with Luminex at four different serum dilutions (100-, 200-, 400-, and 800-fold). Representative results of the dose-dependent curves are shown in this figure. The levels of IgE are shown in terms of FIU and the results are shown as closed circles (three patients) and as open circles (three HDs).

The antibody absorption test

To prepare the peptide-immobilized ELISA plate for the antibody absorption test, peptides were diluted in 0.1 M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (DSS) (Pierce), as reported previously (6). ELISA plates were coated overnight at 4°C with the target peptides (20 μg/well). The wells were rinsed three times with 0.05% Tween 20-PBS (PBST), and the plates were blocked overnight at 4°C with Block-Ace. To test the specificity of anti-peptide IgE in serum samples, 100 μL/well of serum samples (1:1000 dilution with 0.05% PBST) were absorbed with the immobilized peptide (20 μg/well) in wells kept for 2 h at room temperature. The absorption was repeated three times, and then the level of peptide-specific IgE in the resultant supernatant was measured by flowmetric analysis.

Statistical analysis

Data are presented as the median with 95% confidence intervals (CIs), and distributions were compared using nonparametric (Mann-Whitney U test) analysis. The results of the absorption test were analyzed by a two-tailed Student's *t*-test. All tests of significance were two-sided.

Results

First, we measured the levels of IgM, IgA, IgG, and IgE antibodies against C35–44 in sera from three patients infected

with HCV by means of flowmetric analysis with Luminex. Sera from patients with HIV (n = 2) and HDs (n = 3) served as negative controls. As expected based on the previous results (16), the levels of IgG antibodies as indicated in mean fluorescent intensity units (FIU) were very high (19,280, 25,525, and 8481 FIU) in all three HCV-infected patients. The levels of IgE antibodies were also high (613, 466, and 60 FIU) in all three HCV-infected patients (Table 1). The higher titer of IgG compared to that of IgE was expected since the magnitude of serum IgG antibodies is genetically 10^6 times higher than that of IgE. In contrast to the sera of HCV patients, none of the sera from the negative controls showed such IgG or IgE responses to this C35–44 peptide (Table 1), suggesting that the response is specific. In contrast to IgE, low levels of IgM responses to C35–44 peptide were observed in certain samples from HDs, and those infected with HCV and HIV, suggesting that the IgM response is non-specific. A similar non-specific trend was observed in the IgA responses. Dose-dependent reactions of the IgE responses in three different patients with different levels of antibodies (Table 1) are shown in Fig. 1. The specificity of anti-C35–44 IgE antibodies was confirmed with an absorption experiment. Peptide-specific IgE was absorbed with the addition of C35–44, but not with an irrelevant control peptide (NS5A2132); representative results are shown in Fig. 2. These results suggest that the IgE class of anti-C35–44 antibody, but neither the IgM nor IgA class, is detectable in sera from patients with active HCV infection.

Next, we extended the study and measured the levels of IgE reactive to C35–44 peptide in sera from 50 HCV-positive patients and 34 HDs with no history of either viral hepatitis or HBV vaccination and normal liver function. The level of anti-C35–44 IgE antibody in HCV patients was significantly ($p < 0.001$; Mann-Whitney analysis) higher than that of HDs (Fig. 3). The results indicated that anti-C35–44 IgE antibody was specifically detected in HCV-infected patients. When the cut-off value for the FIU of sera from HDs taken as a negative control was calculated as 22 (the mean ± 2 SD), there was one false-positive case among 34 HDs (specificity 98%), while there were two false-negative cases among 50 HCV-infected patients (sensitivity 96%).

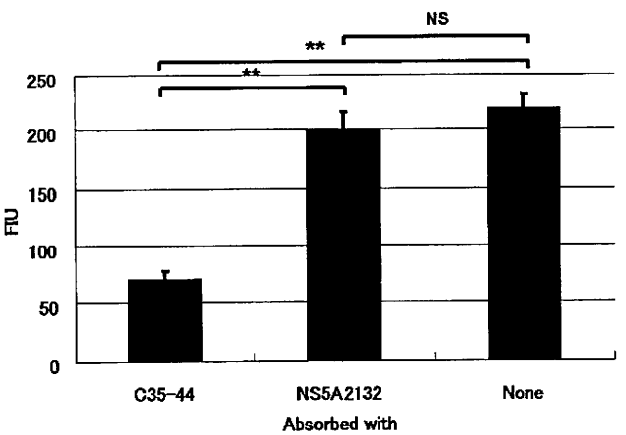

FIG. 2. Absorption test. The IgE antibody activity to C35–44 peptide was absorbed three times with an immobilized corresponding peptide (C35–44) and an irrelevant control peptide (NS5A2132). Serum at 100-fold dilution was used for this experiment. Values are given as the means ± SD of FIU. A two-tailed Student's t-test was employed for statistical analyses. $^{**}p < 0.001$. NS, not significant.

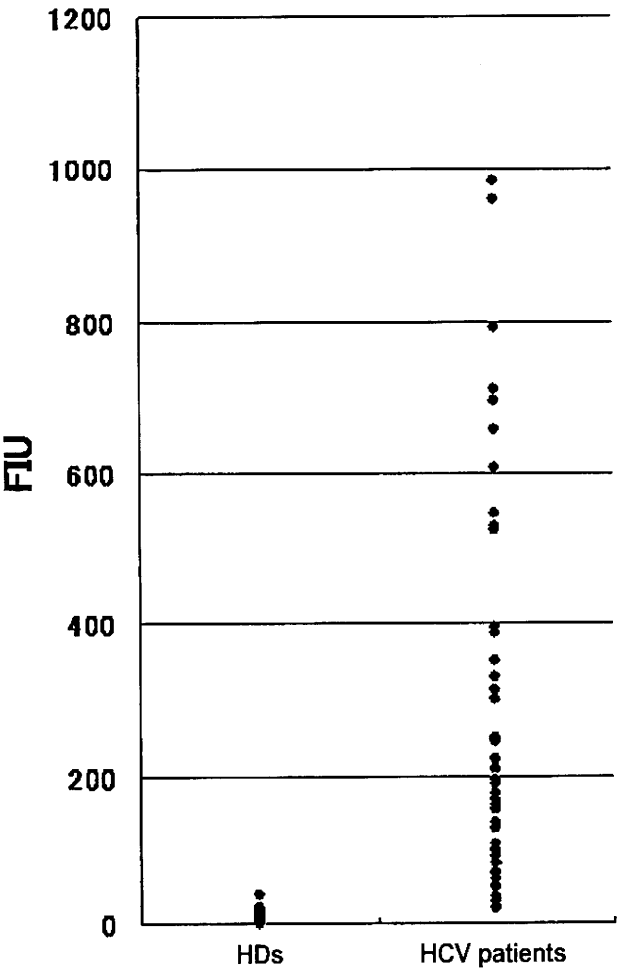

FIG. 3. Screening of patient sera. The levels of anti-C35–44 IgE antibody of the 50 HCV-infected patients were compared to those of the 34 HDs by means of flowmetry analysis with Luminex. The patient sera showed significantly higher levels of IgE ($p < 0.001$; Mann-Whitney U-test) than the means of the FIU scores from the sera of the HDs (100-fold dilution).

We then studied sera from subjects (n = 4) with a history of HCV infection with no detectable levels of HCV-RNA (subjects with HCV serological conversion). IgG anti-C35–44 antibodies could be detected in all four (100, 142,000, 6200, and 10,000 FIU), and IgE anti-C35–44 antibodies could be detected in three (400, 190, and 200 FIU) of four HCV-RNA-negative patients. These results suggest that both the IgG and IgE classes of anti-C35–44 peptide are detectable in sera not only from patients with active HCV infection, but also in subjects with a history of HCV infection. Neither IgM nor IgA was detectable in those subjects (n = 4) with a history of HCV infection.

Finally, we addressed whether the levels of anti-C35–44 antibody were different among the different stages of HCV

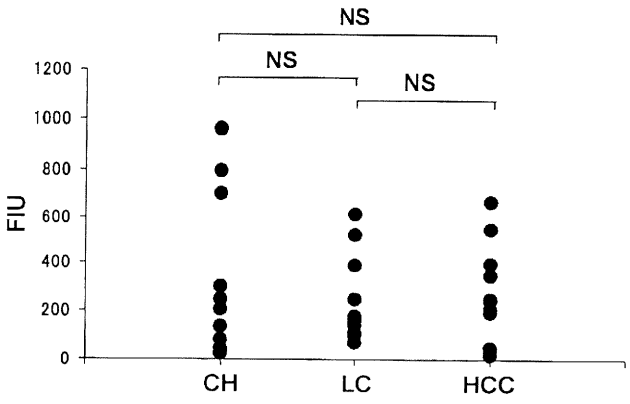

FIG. 4. The level of IgE among those with different stages of disease. Sera at 100-fold dilution from 11 patients of each group (chronic hepatitis, CH; liver cirrhosis, LC; hepatocellular carcinoma, HCC) were assayed, and estimation was done using the Mann-Whitney U-test. There were no significant differences (NS) in the levels of IgE antibody against C35–44 among those patients with different stages of liver disease with HCV infection.

infection. Namely, IgE levels were measured in sera from 11 patients of each group (CH, LC, and HCC). However, there was no statistical difference among these patients with different stages of liver disease with HCV infection (Fig. 4).

Discussion

This study extended the previous studies and reported that significant levels of IgE specific to the C35–44 peptide, but not IgM or IgA, were detectable in the majority of HCV-infected individuals without HLA-A2 restriction. It have been reported that HIV infection seemed to be associated with a marked decrease in the Th-1 cytokines IL-12 and IFN-γ, along with a marked increase of the Th-2 cytokines IL-4 and IL-5 (1,5,7,8,11,18). Th-2 cytokines promote IgE production (5), suggesting that an increase in IgE level in sera of HCV-infected patients is a risk factor for progression of the disease, such as to LC or HCC. Thus, in order to study the possible involvement of IgE antibody against C35–44 peptide in disease progression, the correlation of the level of IgE to C35–44 peptide and the stages of liver disease was analyzed. However, there was no difference in anti-C35–44 IgE antibody among the CH, LC, and HCC groups, suggesting that the level of anti-C35–44 IgE antibody was not a risk factor for disease progression.

HCV infection is a serious health problem, with 170 million people worldwide persistently infected with HCV and at high risk of LC and HCC at later stages. The presently available diagnostic and treatment modalities, such as third-generation enzyme immunoassay/immunoradiometric assays and interferon, are too expensive for patients in underdeveloped countries. Therefore the development of new, less expensive diagnostic or treatment modalities is needed. One way to achieve these goals would be to determine highly immunodominant HCV peptides recognized by both cellular and humoral immunity, and utilize them for such development. We reported that IgG and IgE specific to C35–44 peptide were detectable in sera of at least 90% of patients with HCV infection, regardless of differing HLA types or differing stages of disease (16). Therefore, this C35–44 peptide, as well as proteins containing this sequence, could be a novel target for development of new diagnostic and therapeutic tools.

Of particular advantage to laboratories in developing countries, the IgE test can be rapidly performed without complex laboratory equipment, and can be run at room temperature, and the same technology may be used for both children and adults (12). Furthermore, IgE is one of the antibodies that does not cross the placenta, making it potentially valuable for viral detection in fetuses or neonates. In addition, the IgE response is rapid and reaches a peak earlier than the IgG antibody response (3), suggesting that an IgE-based assay may detect seroconversion earlier than conventional methods (3).

This C35–44 peptide, as well as proteins containing this sequence, could also be a novel target for development of specific immunotherapy for HCV infection to prevent disease progression, primarily because of higher immunogenicity. The translational clinical study of C35–44 peptide vaccination for HCV-positive patients who failed standard therapies is currently in progress at Kurume University Hospital.

The biological roles of anti-C35–44 IgE are unclear at the present time. We previously reported that the administration of CTL-epitope peptides to which IgE was detectable might not elicit a systemic reaction in animal models (19). Indeed, its subcutaneous administration did not elicit it in the translational clinical study (data not shown). Further studies are needed to elucidate the biological roles of anti-C35–44 IgE.

Conclusion

In conclusion, we can report the presence of anti-C35–44 peptide IgE antibody in HCV-infected patients. This information may provide better understanding of the host response against HCV.

Acknowledgment

This study was supported in part by Grant-in-Aids from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Research Center of Innovative Center Therapy of 21st COE Program for Medical Science (to K.I. and S.S.), and Toshi area program (to K.I. and S.S.).

References

1. Autran B, Legac E, Blanc C, and DebrÇ P: A Th0/Th2-like function of CD4+CD7– T helper cells from normal donors and HIV-infected patients. J Immunol 1995;154:1408–1417.
2. Cemy A, McHutchison JG, Pasquinelli C, et al.: Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. J Clin Invest 1995;95:521–530.
3. Fletcher M, Miguez-Burbano MJ, Shor-Posner G, Lopez V, Lai H, and Baum MK: Diagnosis of human immunodeficiency virus infection using an immunoglobulin E-based assay. Clin Diagn Lab Immunol 2000;7:55–57.
4. Franceschini B, Ceva-Grimaldi G, Russo C, Dioguardi N, and Grizzi F: The complex functions of mast cells in chronic human liver disease. Dig Dis Sci 2006;51:2248–2256.

5. Klein SA, Dobmeyer JM, Dobmeyer TS, et al.: Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1 infection using cytoplasmic cytokine detection on single cell level by flow cytometry. AIDS 1997;11:1111–1118.
6. Komatsu N, Shichijo S, Nakagawa M, and Itoh K: New multiplexed flow cytometric assay to measure anti-peptide antibody: A novel tool for monitoring immune responses to peptides used for immunization. Scand J Clin Lab Invest 2004;64:535–546.
7. Lebman DA, and Coffman RL. Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. J Exp Med 1988;168:853–862.
8. Maggi E, Giudizi MG, Biagiotti R, et al.: Th2-like CD8+ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. J Exp Med 1994;180:489–495.
9. Mashiba T, Udaka K, Hirachi Y, et al.: Identification of CTL epitopes in hepatitis C virus by a genome-wide computational scanning and a rational design of peptide vaccine. Immunogenetics 2007;59:197–209.
10. Matsueda S, Yamada A, Takao Y, et al.: A new epitope peptide derived from hepatitis C virus 1b possessing the capacity to induce cytotoxic T-lymphocytes in HCV1b-infected patients with HLA-A11, -A31, and -A33. Cancer Immunol Immunother 2007;56:1359–1366.
11. Meroni L, Trabattoni D, Balotta C, et al.: Evidence for type 2 cytokine production and lymphocyte activation in the early phases of HIV-1 infection. AIDS 1996;10:23–30.
12. Miguez-Burbano MJ, Hutto C, Shor-Posner G, et al.: An IgE-based assay for early diagnosis of HIV infection in infants. Lancet 1997;350:1635.
13. Nagao Y, Kawasaki K, and Sata M: Insulin resistance and lichen planus in patients with HCV-infectious liver diseases. J Gastroenterol Hepatol 2008;23:580–585.
14. Nakamoto Y, Kaneko S, Takizawa H, Kikumoto Y, Takano M, Himeda Y, and Kobayashi K: Analysis of the CD8-positive T cell response in Japanese patients with chronic hepatitis C using HLA-A*2402 peptide tetramers. J Med Virol 2003;70:51–61.
15. Shirai M, Okada H, Nishioka M, et al.: An epitope in hepatitis C virus core region recognized by cytotoxic T cells in mice and humans. J Virol 1994;68:3334–3342.
16. Takao Y, Yamada A, Yutani S, Sata M, and Itoh K: Antibody reactive to a hepatitis C virus (HCV)-derived peptide capable of inducing HLA-A2 restricted cytotoxic T lymphocytes is detectable in a majority of HCV-infected individuals without HLA-A2 restriction. Microbiol Immunol 2004;48:507–517.
17. Takao Y, Yamada A, Yutani S, et al.:. Identification of new immunogenic peptides in conserved region of hepatitis C virus (HCV) 1b with the potentiality to generate cytotoxic T lymphocytes in HCV1b+ HLA-A24+ patients. Hepatol Res 2007;37:186–195.
18. Wasik TJ, Jagodzinski PP, Hyjek EM, Wustner J, Trinchieri G, Lischner HW, Kozbor D: Diminished HIV-specific CTL activity is associated with lower type 1 and enhanced type 2 responses to HIV-specific peptides during perinatal HIV infection. J Immunol 1997;158:6029–6036.
19. Yamada A, Yano H, Takao Y, Ono T, Matsumoto T, and Itoh K: Nonmutated self-antigen-derived cancer vaccine peptides elicit an IgE-independent but mast cell-dependent immediate-type skin reaction without systemic anaphylaxis. J Immunol 2006;176:857–863.

Address reprint requests to:
Dr. Shigeki Shichijo
Department of Immunology
Kurume University School of Medicine
67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan

E-mail: shichijo@med.kurume-u.ac.jp

Received February 26, 2008; accepted June 2, 2008.