Table 4 Univariate analysis to identify the significantly different factors between SVR and non-SVR (201 patients received over 80% adherences of both PEG-IFN and RBV)

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	111 (55.2%)	90	
Gender			
Male	93 (62.8%)	55	0.00037
Female	18 (34.0%)	35	
Age			
Median (range)	51 (18–70)	56 (23–74)	0.00025
<60 years	91 (60.3%)	60	0.014
60 years≤	20 (40.0%)	30	
Age: <60 years			
Male	79 (66.4%)	40	0.0042
Female	12 (37.5%)	20	
Age: 60 years≤			
Male	14 (48.3%)	15	0.243
Female	6 (28.6%)	15	
F stage			
F0–2	103 (60.9%)	67	0.0012
F3–4	8 (25.8%)	23	
Grade (A factor)			
A0–1	80 (59.3%)	55	0.189
A2–3	31 (47.0%)	35	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (110–5000<)	1280 (130–5000<)	0.351
ALT 0 week (IU/L)			
Median (range)	74 (16–268)	67.5 (19–504)	0.752
BMI			
Median (range)	23.1 (17.3–31.0)	23.6 (16.1–33.9)	0.626
Alb (g/dL)			
Median (range)	3.95 (3.3–5.2)	3.9 (3.0–4.8)	0.079
LDL-C (mg/dL)			
Median (range)	96 (31–185)	97.5 (30–182)	0.865
T-Chol (mg/dL)			
Median (range)	170 (85–248)	170 (105–273)	0.624
PLT count ($\times 10^4$/mm^3)			
Median (range)	18.9 (8.7–30.9)	15.55 (7.2–28.4)	0.00003
<15	23 (35.9%)	41	0.00024
15≤	88 (64.2%)	49	
Amino acid mutation of ISDR			
0–1	84 (52.5%)	76	0.159
2≤	27 (65.9%)	14	
Amino acid substitution of core 70			
Wild	91 (61.5%)	57	0.0037
Mutant	20 (37.7%)	33	
Amino acid substitution of core 91			
Wild	73 (60.3%)	48	0.083
Mutant	38 (47.5%)	42	

Virological responses and aa substitution

The rates of RVR, cEVR, LVR, ETR and SVR in males and females were 12.5 versus 11.3% ($p = 1.000$), 59.6 versus 43.4% ($p = 0.053$), 74.3 versus 50.0% ($p = 0.0018$), 76.2 versus 66.7% ($p = 0.198$), and 62.8 versus 34.0% ($p = 0.00037$), respectively (data not shown). The backgrounds and characteristics of SVR and non SVR patients are shown in Table 4. There were significant differences in gender (male vs. female; $p = 0.00037$), age (<60 years vs. ≥60 years; $p = 0.014$), F stage (F0-2 vs. F3,4; $p = 0.0012$), PLT count ($<15 \times 10^4/mm^3$ vs. $15 \times 10^4/mm^3 \leq$; $p = 0.00024$), and substitution of core aa 70 (wild type vs. mutant, p = 0.0037) between SVR and non-SVR patients. The distribution of fatty change in liver tissue (≤10% vs. 11–33% vs. 34%≤; $p = 0.046$) and the grade of HOMA-IR (1.7 vs. 3.9, $p = 0.0018$) were significantly different between SVR and non-SVR (data not described in Table 4).

Factors affecting SVR by multivariate logistic regression analysis

Male gender ($p = 0.0006$), mild fibrosis stage ($p = 0.027$), and wild type of core aa 70 ($p = 0.043$) were independent predictors of SVR.

Valuable markers for predictions of sustained virological response to peginterferon and ribavirin therapy

Two or more aa mutations in the ISDR, wild type core aa 70, $\geq 15 \times 10^4/mm^3$ of PLT count, and male gender were selected statistically as independent predictors of SVR. We show here SVR rates of the patients having over 80% adherences to both PEG-IFN and RBV (Fig. 2b). In males having no or one aa substitution in the ISRD and PLT count of $<15 \times 10^4/mm^3$, wild type core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). In females, the SVR rate was very low in those who had substitution of core aa 70, but there was no significant difference between patients with wild type and substitution of core aa 70. The number of female patients was too small to provide a definite conclusion.

Discussion

The present multivariate logistic regression analysis revealed that male gender, low HCV RNA load, high PLT count, and two or more aa mutations in the ISDR and wild type core aa 70 were independent predictors for SVR. PLT count significantly decreased corresponding to the progression to the stage of liver fibrosis in CHC [9, 30, 31].

It has been considered that the low adherence level to PEG-IFN/RBV is a major cause of a significantly lower SVR rate in females and older patients [32]. The percentage of patients having over 80% adherences to both PEG-IFN and RBV was significantly lower in females than males, however, differences in the adherence to PEG-IFN/RBV between males and females were not independent predictive factors of non-SVR.

A recent report from Japan showed six or more mutations in the variable region 3 (V3) of nonstructural protein 5A (NS5A) plus upstream flanking region NS5A (aa 2334–2379), referred to as the IFN/RBV resistance determining region (IRRDR), was a useful marker for predicting SVR, but the ISDR sequence was not valuable for predicting SVR [33]. However, the number of subjects in that study was too small ($n = 45$) to reach an acceptable conclusion.

To elucidate the factors affecting low SVR rate in older female patients, we performed a multivariate logistic regression analysis using patients who achieved ≥80% adherence to both PEG-IFN and RBV. Male gender, stage of mild liver fibrosis, and wild type core aa 70 were independent predictors of SVR. In this study, blood concentration of RBV was determined in fewer than 50% of cases during treatment. Thus we cannot exclude the possibility of the effect of the blood concentration of RBV during treatment on the low SVR rate in females and older patients.

From the present analysis, it was clear that ALT, BMI, Alb, T. Chol, and adherence to RBV differed significantly between males and females, however, these factors were not independent predictors of SVR. There is a report that steatosis is an important cofactor that reduces the SVR rate in genotype 1 infected patients [34], however, such an effect was not seen in this study. Thus we could not identify the factors associated with a significantly lower SVR rate in females than males.

In the present multivariate logistic regression analyses, patients having wild type core aa 91 had significantly higher rates of RVR and cEVR, but not SVR, and patients with wild type core aa 70 had significantly higher rates of cEVR and SVR, but not RVR. Patients having two or more aa substitutions in the ISDR had significantly higher rates of RVR, cEVR, and SVR. Although several possibilities have been considered concerning the effects of aa substitutions of core protein on SVR in PEG-IFN/RBV therapy for CHC patients, the exact mechanisms have not yet been elucidated.

Recent reports have indicated that low serum IP-10 (interferon-γ inducible protein 10 kDa) [35], a higher HCV-specific CD8 cell proliferation potential [36], and a high ratio of Th1/Th2 [37] are good predictors of SVR to

PEG-IFN/RBV therapy. These results indicate the importance of immunological status and immunological response to treatment in patients difficult to treat with PEG-IFN/RBV therapy for CHC.

The present univariate analyses revealed that there were many factors relating to RVR, cEVR, and SVR including LDL-C, HOMA-IR, fatty change in liver tissue, and hyaluronic acid, however some of these factors had not been examined in some participating institutes. We consider that we must perform a prospective mass study using many factors including immunological aspects, viral factors, disease status, and therapeutic aspects to elucidate the reason that older female patients are resistant to a combination of PEG-IFN and RBV therapy in CHC with a high viral load genotype 1b.

In conclusion, our results demonstrated that wild type core aa 70, two or more aa mutations in the ISDR, low viral load, high PLT counts, and male gender are useful markers for predicting SVR.

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Mutations in the Interferon Sensitivity-Determining Region of Hepatitis C Virus Genotype 2a Correlate With Response to Pegylated-Interferon-Alpha 2a Monotherapy

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The interferon sensitivity-determining region (ISDR) is thought to be inhibited by the double-stranded RNA-dependent protein kinase (PKR). Several studies have reported a relationship between the ISDR and interferon (IFN) responsiveness. However, this relationship is controversial. The aim of this study was to investigate whether genomic heterogeneity of the ISDR among patients with hepatitis C virus (HCV) genotype 2a affects the response to pegylated-IFN-alpha 2a monotherapy. Eighty patients (47 men, 33 women; mean age: 54.2 ± 12.9 years) infected with HCV genotype 2a were evaluated. HCV viral loads were determined by real-time PCR. The ISDR (amino acids 2193–2228) was examined by direct sequencing. Thirty-one patients received subcutaneous injections of pegylated-IFN-alpha 2a (180 μg) once weekly for 24 weeks, and 35 patients received injections for 48 weeks. Fourteen patients withdrew from treatment. Of the remaining 66 patients, 51 (77.3%) showed a sustained virologic response. Factors related to sustained virologic response on multivariate analysis were rapid virologic response (negative HCV at 4 weeks; odds ratio: 0.033; 95% confidence interval (95% CI) 0.003–0.363; $P = 0.0052$) and the number of mutations in the ISDR (odds ratio: 0.025; 95% CI 0.001–0.476; $P = 0.0141$). There were no significant differences in other factors, including sex, age, aspartate aminotransferase, alanine aminotransferase, platelet count, duration of treatment, and HCV viral load. Rapid virologic response and the ISDR sequence variations are significantly associated with response to pegylated-IFN-alpha 2a monotherapy in Japanese patients with HCV genotype 2a. ***J. Med. Virol. 81:459–466, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: sustained virologic response; rapid virologic response; chronic hepatitis C

INTRODUCTION

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family and causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma (HCC) [Seeff, 2002]. HCV infection is a significant global health problem, affecting 170 million individuals worldwide. HCV consists of three structural proteins (core, envelope 1, and envelope 2) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). HCV NS5A protein was reported to have a domain associated with interferon (IFN) response. This domain, located in the NS5A region of HCV, is closely associated with response to IFN therapy and is known as the IFN sensitivity-determining region (ISDR) [Enomoto et al., 1996; Murakami et al., 1999; Nakano et al., 1999; Pascu et al., 2004]. There are several modes of IFN action

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against HCV infection, and the final mode is still under debate. However, one mechanism of IFN action involves inhibition of viral replication by inducing the double-stranded RNA-dependent protein kinase (PKR). The ISDR is located in the 5′ end of the PKR-binding domain and is inhibited by PKR in vitro [Gale et al., 1998]. Therefore, ISDR heterogeneity is an important factor that may affect response to IFN. The utility of ISDR sequences for predicting IFN responsiveness has been investigated for HCV genotype 1b, as well as for genotypes 2 and 3, because HCV genotypes, which vary in prevalence around the world, influence IFN responsiveness [Manns et al., 2001; Fried et al., 2002; Simmonds et al., 2005]. HCV genotype 2a is relatively common in Japan [Enomoto et al., 1990; Hayashi et al., 2003]. However, there are few reports regarding the ISDR and IFN responsiveness in HCV genotype 2a [Murakami et al., 1999; Kobayashi et al., 2002; Akuta et al., 2005], and the association of mutations in the ISDR and response to IFN therapy among patients with HCV genotype 2a is not well understood. The aim of the present study was to determine whether genomic heterogeneity of the ISDR among patients with HCV genotype 2a affects the response to pegylated-IFN-alpha 2a monotherapy.

MATERIALS AND METHODS

This prospective analysis involved 80 patients with chronic hepatitis C who received pegylated-IFN-alpha 2a monotherapy between January 2004 and December 2005. Patients who were previously treated with IFN were excluded. All patients were positive for serum anti-HCV antibody on a commercial enzyme-linked immunosorbent assay (Dinabot, Tokyo, Japan) and for HCV-RNA on a commercial polymerase chain reaction (PCR) test (Roche Diagnostic Systems, Tokyo, Japan). No patients had hepatitis B surface antigen, coinfection with human immunodeficiency virus, autoimmune disease, or chronic alcohol abuse.

Schedule of IFN Therapy

Patients received pegylated-IFN-alpha 2a (Pegasys Roche, Tokyo, Japan) at a dose of 180 μg injected subcutaneously once weekly for 24 or 48 weeks. The patients were allocated, at the discretion of the physician in charge, to a protocol lasting either 24 or 48 weeks. Laboratory tests and evaluation of adverse events were performed once weekly during treatment. The pegylated-IFN-alpha 2a dose was dropped to 90 μg when clinically significant adverse events or laboratory abnormalities such as neutropenia (<750 cells/mm^3) or thrombocytopenia (<50,000 cells/mm^3) occurred. Pegylated-IFN-alpha 2a was discontinued when neutropenia (<250 cells/mm^3) or a platelet count below 25,000 cells/mm^3 was observed. Patients who did not receive 80% of the ideal total dose of IFN were defined as the reduced-dose group. Serum HCV-RNA levels were examined at 4, 12 weeks, at the end of IFN therapy, and 6 months after the end of treatment. Serum was stored at −80°C for virologic examination. Patients who were persistently negative for serum HCV-RNA and who had a normal serum alanine aminotransferase (ALT) level 24 weeks after withdrawal of IFN treatment were considered to have a sustained virologic response. Patients who were HCV-negative at the end of the treatment but returned to HCV-positive status after withdrawal of IFN were defined as virologic relapsers. Patients who did not become HCV-negative with IFN therapy were defined as virologic non-responders. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virologic Tests

The HCV-RNA quantitative viremia load was determined using real-time PCR [Takeuchi et al., 1999]. HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions, as described previously [Otagiri et al., 2002; Hayashi et al., 2003]. The genotypes were classified according to the nomenclature proposed by a previous report [Simmonds et al., 2005]. Direct sequencing of the ISDR region was performed using serum samples taken within 2 days before the first administration of pegylated-IFN-alpha 2a. In brief, RNA was extracted from 140 μl of sera with a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA) and dissolved in 50 μl of diethylpyrocarbonate-treated water. Ten nanograms of the RNA was used for reverse transcription using the oligo and random hexamer primers of a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). The ISDR was amplified by hemi-nested PCR. In brief, each 50-μl PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μl of GeneAmp 10× PCR buffer, 2 μl of dNTPs, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Primer sequences were sense, 5′-ACGTCCATGCTAACAGACCC-3′ and antisense, 5′-GGGAATCTCTTCTTGGGGAG-3′. Amplification conditions consisted of 10 min at 94°C followed by 40 cycles of 94°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was done in the same reaction buffer with the first-round PCR product as the template, the sense primer from the first-round PCR, and a new antisense primer, 5′-CGAGAGAGTC-CAGAACGACC-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers using a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). The neighbor-joining method was used for phylogenetic analysis of the ISDR (amino acids 2193–2228) in the NS5A region [Saitou and Nei, 1987], and bootstrap analysis (1,000 replicates) was performed [Felsenstein, 1985].

Statistical Analysis

The data are expressed as mean ± standard deviation (SD). The paired *t*-test was used to analyze differences in the variables. A *P*-value of <0.05 was considered statistically significant. Multiple logistic regression models were used to identify factors predictive of sustained virologic response. Statview 5.0 software (SAS Institute Inc., Cary, NC) was used for all analyses.

RESULTS

The patients' clinical characteristics are summarized in Table I. All patients were infected with HCV genotype 2a, and 27 of 80 (33.8%) patients had a serum HCV-RNA level higher than 1 million copies/ml. Eighty patients were initially entered, but 14 patients withdrew from IFN therapy, and 4 of these 14 patients could not be followed-up. The remaining 66 patients were followed-up for 6 months after the end of treatment. The completion rate was 82.5% (66/80). Thirty-one patients were treated with pegylated-IFN-alpha 2a for 24 weeks, and 35 patients were treated for 48 weeks. Virologic response is shown in Table II. The rapid virologic response rate, which was defined as negativity for HCV after 4 weeks of treatment, was 74.2% (49/66). The early virologic response rate, which was characterized by undetectable HCV at 12 weeks, was 92.4% (61/66). The virologic response rate at the end of the treatment was 97.0% (64/66). Finally, 51 of 66 (77.3%) patients achieved sustained virologic response. There were no significant differences in clinical characteristics and virologic response between patients treated for 24 weeks and those treated for 48 weeks. ISDR sequences were obtained in 62 patients, and the sequence alignments of the ISDR according to virologic response are shown in Figure 1. The mean number of ISDR mutations in patients with non-sustained virologic response was 1.2 ± 0.6, and that in patients with sustained virologic response was 2.8 ± 2.1. Patients with sustained virologic response had a significantly higher number of mutations in the ISDR than did patients with non-sustained virologic response ($P = 0.0090$). Codon 2205 was frequently changed. The association of this single mutation with sustained virologic response was examined; however, there was no significant relationship between a single mutation at codon 2205 and sustained virologic response. Sequences of the HCJ6 strain and the HCJ6 strain with all nucleotide substitutions in codon 2205 were defined as the wild type, and ISDR sequences that deviated from these strains were defined as mutant type. A rapid virologic response was achieved in 7 of 33 patients with wild-type ISDR and 5 of 41 patients with mutant-type ISDR. There were no correlations between rapid virologic response and ISDR sequence. Mutant-type ISDR was detected more frequently in sustained virologic response patients (66.7%) than in non-SVR patients (28.6%) (odds ratio: 0.200; 95% confidence interval (95% CI) 0.054–0.738; $P = 0.015$). Phylogenetic analyses of the ISDR (amino acids 2193–2228) of the 62 patients were performed, and the results are shown in Figure 2. There were differences in distinctive clustering between the wild type and the mutant type defined by counting the number of substitutions in the ISDR, but no distinctive clustering was observed in wild types with A2205 and with T2205 and with V2205. The phylogenetic analyses did not show a significant relationship between the ISDR sequences and sustained virologic response. The clinical characteristics of the patients who achieved sustained virologic response are compared to those without sustained virologic response in Table III. There were significant differences in four factors (age, HCV-RNA level, the number of mutations in the ISDR, and rapid virologic response) between the sustained virologic response group and the non-sustained virologic response group on univariate analysis. The results of the multivariate analyses of factors predictive of sustained virologic response are shown in Table IV. The variables were recorded categorically as ordinal data. The background factors were: age (<60 years vs. ≥60 years); sex (male vs. female); platelet count ($<15 \times 10^4/mm^3$ vs. $\geq 15 \times 10^4/mm^3$); HCV-RNA level ($<10^6$ copies/ml vs. $\geq 10^6$ copies/ml); ALT levels (<70 IU/L vs. ≥70 IU/L); AST levels (<60 IU/L vs. ≥60 IU/L); length of IFN therapy (24 weeks vs. 48 weeks); reduction of IFN dose (yes or no); ISDR (wild type vs. mutant type); and rapid virologic response (yes or no). Rapid virologic response at 4 weeks was the most influential factor ($P = 0.0052$), followed by mutations in the ISDR ($P = 0.0141$). No other factors achieved statistical significance. Analysis of rapid virologic response in combination with the ISDR revealed that 28 of 29 patients with mutant-type ISDR and rapid virologic response achieved sustained virologic response. The positive predictive value for sustained virologic response was 96.6% (28/29). IFN therapy was withdrawn from 14 patients. The reasons for discontinuing therapy, length of IFN therapy, ISDR sequences, rapid virologic response, and outcomes are shown in Figure 3. Ten patients discontinued therapy within 16 weeks, but 4 of the 10 patients achieved sustained virologic response. All sustained virologic response patients who withdrew from therapy within 16 weeks had at least three ISDR mutations.

TABLE I. Clinical Characteristics

	N = 80
Age (y.o.)	54.2 ± 12.9
Sex: male/female	47/33
AST (IU/L)	57.9 ± 37.5
ALT (IU/L)	81.1 ± 65.3
Platelet count ($10^4/\mu l$)	20.7 ± 22.2
HCV-RNA level (copies/ml)	360,000 (540–63,000,000)
Body weight (kg)	60.8 ± 9.8

Data are expressed as mean ± standard deviation. HCV-RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

TABLE II. Virologic Response Rates

	All (n = 66)	24 weeks (n = 31)	48 weeks (n = 35)
Rapid virologic response	74.2% (n = 49)	77.4% (n = 24)	71.4% (n = 25)
Early virologic response	92.4% (n = 61)	96.8% (n = 30)	88.6% (n = 31)
End of treatment response	97.0% (n = 64)	96.8% (n = 30)	97.1% (n = 34)
Sustained virologic response	77.3% (n = 51)	77.4% (n = 24)	77.1% (n = 27)

Rapid virologic response as HCV-negative at 4 weeks. Early virologic response as HCV-negative at 12 weeks. End of treatment response as HCV-negative at the end of the treatment. Sustained virologic response as HCV-negative at 24 weeks after withdrawn of treatment.

DISCUSSION

HCV genotype is one of the most important factors that predict response to IFN therapy. Genotypes 1 and 4 respond poorly to IFN therapy, whereas genotypes 2 and 3 show a sustained virologic response to IFN therapy. However, patients infected with HCV genotype 2 respond differently to IFN therapy, suggesting that an additional viral factor associated with resistance to IFN exists. The ISDR sequence in the HCV NS5A region may influence the IFN response of patients with HCV genotype 1b [Enomoto et al., 1996; Nakano et al., 1999; Pascu et al., 2004]. The influence of the ISDR sequence in response to IFN has been investigated in patients with HCV genotypes 2a and 2b [Murakami et al., 1999; Kobayashi et al., 2002; Akuta et al., 2005]. In the present study, it was hypothesized that the amino acid variations in ISDR would explain differences in IFN resistance in patients infected with HCV genotype 2a. Multivariate analyses showed that mutation of the ISDR is one of the most influential factors for sustained virologic response (odds ratio: 0.025; 95% CI 0.001–0.476; $P = 0.0141$). The sustained virologic response rate of patients with more than three mutations in the ISDR was 100% (23/23) in the present study. The results confirmed that the number of mutations in the ISDR is an important determinant of the effectiveness of pegylated-IFN-alpha 2a monotherapy in patients with

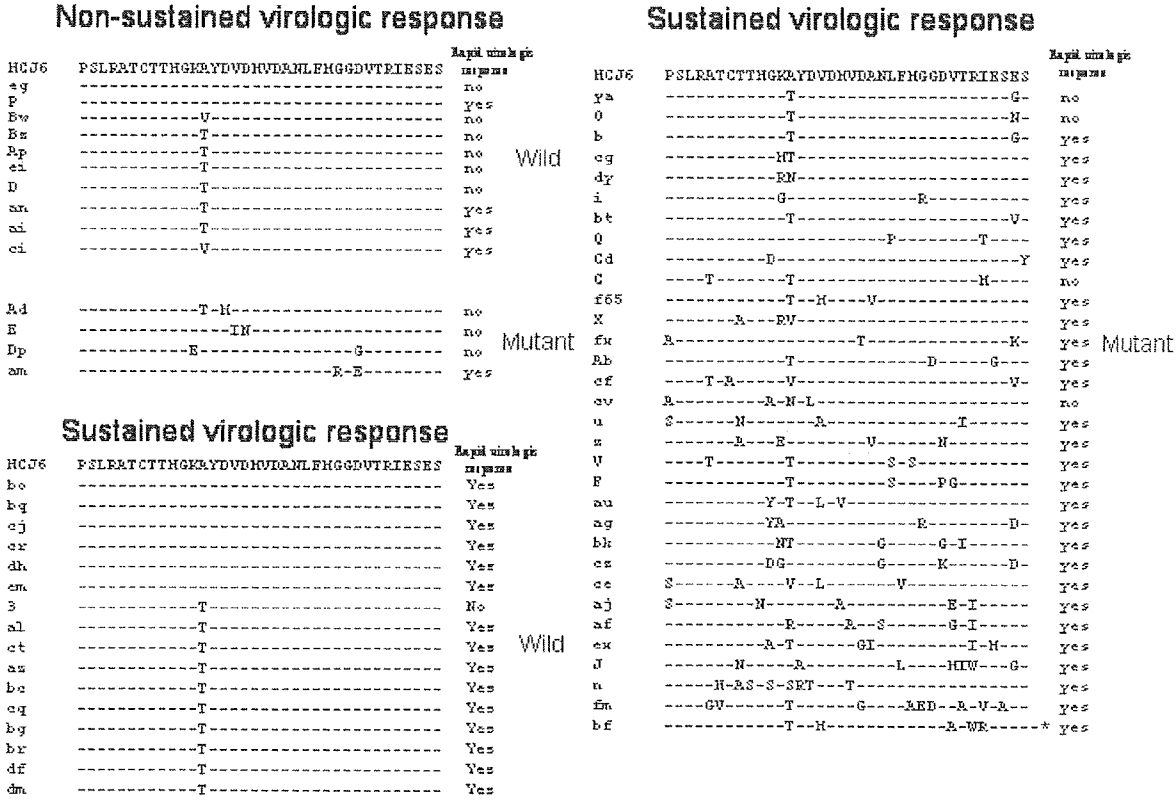

Fig. 1. Alignment of the amino acid sequence of the ISDR and response to pegylated-interferon-alpha 2a therapy. In the sequence alignment, dashes indicate amino acids identical to consensus sequence HCJ6. Sequences of the HCJ6 strain and the HCJ6 strain with all nucleotide substitutions in codon 2205 were defined as wild-type ISDR, and the other strains were defined as mutant-type ISDR. The strain marked with an asterisk had an insertion mutation. ISDR, interferon sensitivity-determining region.

Fig. 2. Results of phylogenetic analysis of 62 sequences from the interferon sensitivity-determining region (amino acids 2193–2228) and relationship with the response to pegylated-interferon-alpha 2a therapy. Phylogenetic analysis was performed by the neighbor-joining method. HCVJ, which is the prototype of genotype 1b, was used as the outer group. The scale bar indicates genetic distance. Each strain from the present study is shown with original code followed by the virologic response. All strains without description of virologic response were rapid virologic response and sustained virologic response. Definition of wild type was counting the number of substitution in the ISDR.

TABLE III. Clinical Characteristics of Patients With or Without Sustained Virologic Response

Factors	Sustained virologic response (n=51)	Non-sustained virologic response (n=15)	*P*-value
Age (y.o.)	52.7 ± 13.1	60.3 ± 6.8	0.0356
Gender: male/female	33/18	6/9	0.1346
ALT (IU/L)	75.6 ± 57.7	66.8 ± 14.1	0.6002
AST (IU/L)	51.8 ± 29.4	56.5 ± 40.1	0.6218
PLT ($\times 10^4$/mm^3)	18.5 ± 6.0	15.5 ± 5.4	0.0866
HCV-RNA level (copies/ml)	340,000 (2,600–63,000,000)	1,400,000 (50,000–22,000,000)	0.0067
Reduction: yes/no	8/43	6/9	0.0691
Duration: 24 weeks/48 weeks	24/27	7/8	0.9999
Mutations in the ISDR	2.8 ± 2.1	1.2 ± 0.6	0.0090
Rapid virologic response: yes/no	44/7	5/10	0.0001

Data are expressed as mean ± standard deviation. HCV-RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region.

HCV genotype 2a. In addition, 10 patients discontinued IFN therapy within 16 weeks in the present study; 4 of these 10 patients achieved sustained virologic response. All sustained virologic response patients who discontinued IFN therapy were infected with mutant-type ISDR. Thus, the mutant-type ISDR appears to be associated with good response to IFN. The ISDR sequence variation of HCV genotype 2a may also play an important role as a predictor of IFN responsiveness. However, most Western reports have not confirmed the clinical usefulness of ISDR analysis for predicting response to IFN therapy [Zeuzem et al., 1997; Chung et al., 1999; Squadrito et al., 2002]. Bias relating to the IFN therapy regimens, racial differences, and HCV strains may have produced this conflicting result. To investigate the role of the ISDR while avoiding bias, all of the patients in the present study were infected with genotype 2a and received pegylated-IFN-alpha 2a monotherapy. Most studies that did not find ISDR analysis useful had a lower dose of IFN than those that reported that ISDR analysis was useful (3 million units vs. 6–10 million units). A low IFN dose was associated with a low sustained virological response rate. The present study and the studies that confirmed the usefulness of ISDR analysis had a higher sustained virological response rate (mean 50.5%) than those that did not confirm the usefulness of ISDR analysis (mean 9.6%) [Enomoto et al., 1996; Zeuzem et al., 1997; Chung et al., 1999; Murakami et al., 1999; Nakano et al., 1999; Squadrito et al., 2002]. The low sustained virological response rate, as well as the low IFN dose, would not favor the use of ISDR analysis for predicting IFN responsiveness. The number of substitutions in the ISDR in reports with negative results was significantly smaller than in studies that confirmed the correlation between ISDR mutations and IFN responsiveness [Herion and Hoofnagle, 1997]. The present study and other studies that confirmed the association between ISDR mutations and IFN sensitivity frequently found that the patients had ISDR mutant type [Saiz et al., 1988; Murakami et al., 1999; Nakano et al., 1999]. The prevalence of patients infected with ISDR mutant type would affect the association between ISDR sequence and IFN responsiveness. Thus, a study including a large number of patients with two or more amino acid substitutions in the ISDR would be suitable for using the ISDR system to predict sustained virologic response. The original classification for the ISDR sequence of genotype 1b included three categories (wild, intermediate, and mutant) according to the number of amino acid substitutions compared to the HCVJ strain. In the present study, sequences of the HCJ6 strain and the HCJ6 strain with all amino acid substitutions in codon 2205 were defined as the wild type, and the other strains were mutant type. The classification for the ISDR sequence was minimally modified for ease of analysis

TABLE IV. Multivariate Analysis: Factors Predictive of Sustained Virologic Response

Factors	*P*-value	Risk ratio	95% CI	
Age: <60 years	0.0554	8.306	0.952	72.486
Sex: male	0.8270	1.228	0.194	7.778
ALT: <70 IU/L	0.5065	0.227	0.003	17.976
AST: <60 IU/L	0.9923	1.020	0.018	58.089
PLT: $<15 \times 10^4$/mm^3	0.1528	0.154	0.012	2.001
HCV-RNA level: $<10^6$ copies/ml	0.4830	0.437	0.043	4.425
Reduction: yes	0.2242	0.187	0.013	2.790
Duration: 48 weeks	0.1016	8.100	0.662	99.135
ISDR: wild	0.0141	0.025	0.001	0.476
Rapid virologic response: no	0.0052	0.033	0.003	0.363

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region.

Characteristics of the 14 patients withdrawing the treatment

length	reasons	HCJ6	PSLRATCTTHGKAYDVDMVDANLFMGGDVTRIESES	Rapid virologic response	effect
3wks	economic	none	////////////////////////////////////	no	relapse
4wks	fatigue	none	////////////////////////////////////	yes	Sustained virologic response
5wks	economic	ah	----------T----------	no	No virologic response
9wks	rash	bu	----------D----------R----W-----	yes	Sustained virologic response
10wks	ALT elevate	G	S------A----T----LT----------I------	yes	Sustained virologic response
10wks	moving	ca	----------	yes	dropout
11wks	ALT elevate	ec	----------R----------	no	relapse
12wks	unknown	fv	----------	yes	dropout
13wks	fatigue	aq	----------M----S--S-------G-	yes	Sustained virologic response
15wks	ineffective	Y	----T-------T----------	no	No virologic response
20wks	unknown	cs	----T--A--------A----------I------	yes	dropout
27wks	depression	L	A----------V----------G-V------	yes	Sustained virologic response
28wks	moving	h	----------YC----------	yes	dropout
40wks	pneumonia	r	--------YG---H-M----------	yes	Sustained virologic response

Fig. 3. Clinical characteristics of the 14 patients who withdrew from pegylated-interferon-alpha 2a therapy. Reasons for discontinuing therapy, length of therapy, alignment of the amino acid sequence of the ISDR, rapid virologic response, and response to IFN therapy are shown. ISDR, interferon sensitivity-determining region.

and adjusted for genotype and IFN protocol. Adjustment for racial differences, diversity between the HCV strains with respect to genotype and ISDR sequence, and IFN regimen would be needed to use the ISDR as a simple diagnostic tool to predict sustained virologic response. Nevertheless, the present study had a few limitations. Only the correlation between mutations within the ISDR and sustained virologic response was analyzed, although other parts of NS5A have been reported to be associated with IFN response [Nousbaum et al., 2000; Murphy et al., 2002]. The approach of counting the number of mutations to the chosen consensus sequence in the ISDR, originally reported by Enomoto, was used for the present analysis; however, this method may not be the best way to measure sequence variation. Phylogenetic analyses of the ISDR were used to evaluate the diversity of the ISDR sequence, but distinctive clustering was not found in the wild types with A2205 and with T2205 and with V2205. The ISDR interacts with PKR and inactivates replication of HCV in vitro [Gale et al., 1998]. However, some reports have not confirmed the interaction between PKR and NS5A [Podevin et al., 2001; Tan and Katze, 2001]. PKR-independent effects of NS5A have been reported [Polyak et al., 2001; Evans et al., 2004]. Although the effect of amino acid substitutions of the ISDR was unclear, the ISDR system could be used clinically as a simple diagnostic tool to predict sustained virologic response in patients infected with genotype 2a who received pegylated-IFN-alpha 2a monotherapy.

The current recommended therapy for patients with HCV genotype 2 is a combination of pegylated-IFN and ribavirin for 24 weeks [Strader et al., 2004]. However, pegylated-IFN-alpha 2a monotherapy in patients with HCV genotype 2a resulted in a high sustained virologic response rate (77.3%). Most reports dealing with pegylated-IFN-alpha and ribavirin combination therapy did not differentiate between HCV genotypes 2 and 3 or did not classify subgenotypes 2a and 2b [Zeuzem et al., 2004; Mangia et al., 2005; von Wagner et al., 2005; Shiffman et al., 2007]. There is also limited information regarding sustained virologic response in patients with HCV genotype 2a treated with pegylated-IFN-alpha and ribavirin combination therapy. Thus, it is difficult to compare the present results to those obtained with pegylated-IFN-alpha and ribavirin combination therapy. Large, randomized, prospective studies of pegylated-IFN-alpha with or without ribavirin for patients with genotype 2a, especially ISDR mutant, are needed to clarify these issues. The present study combined two predictive factors: rapid virologic response and the amino acid variations in ISDR compared to the reference sequence. Rapid virologic response is considered to be a strong indicator of progression to sustained virologic response for patients with HCV genotype 2a. Knowledge of both the ISDR sequence and rapid virologic response would be useful for individualization of IFN regimens for chronic hepatitis C patients, but rapid virologic response cannot be assessed before treatment. In the present study, there were no predictive factors associated with rapid virologic response on multivariate analyses (data not shown). Thus, it is impossible to predict which patients will be rapid virologic responders before IFN therapy. With respect to assessment before starting treatment, the number of mutations in the ISDR is a better predictor than rapid virologic response.

In conclusion, the present results indicate that pegylated-IFN-alpha 2a monotherapy is effective for achieving sustained virologic response in Japanese patients with HCV genotype 2a, particularly in those with rapid virologic response and mutant-type ISDR.

The ISDR sequence variation of HCV genotype 2a is useful for predicting IFN responsiveness.

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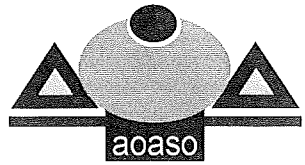

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ORIGINAL ARTICLE

Heterozygosity for leptin receptor (fa) accelerates hepatic triglyceride accumulation without hyperphagia in Zucker rats

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KEYWORDS
Leptin receptor;
Zucker fatty rat;
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Fatty acid

Summary Leptin, *ob* gene product, and its receptors are involved in the regulation of peripheral lipid and glucose metabolism. The present study sought to clarify the functional role of peripheral leptin receptors in hepatic lipid metabolism through analysis of Zucker rats (fa/fa, +/fa), as complete or partial leptin receptor insufficiency models, respectively. In Zucker fa/fa rats, calorie intake, body weight, liver weight, hepatic triglyceride content and serum insulin, triglycerides, FFA, and leptin were elevated compared to lean littermates (+/+ rats). In contrast, Zucker +/fa rats showed no remarkable changes in calorie intake, body weight and serum FFA compared with +/+ rats. Nevertheless, hepatic triglyceride content, liver weight and other serum parameters such as insulin, triglyceride and leptin were higher than in +/+ rats. In the representation of fatty acids component in the liver, there were no changes in +/fa rats relative to +/+ rats. Thus, in Zucker +/fa rats, fatty liver may develop in the absence of hyperphagia, obesity or changes in hepatic fatty acid metabolism. These results indicate that partial insufficiency of leptin receptor rather than changes in serum insulin, triglyceride and leptin may contribute to the increase in hepatic triglyceride content observed in +/fa rats.

Introduction

Reflecting its wide range of physiological functions, receptors for leptin are distributed widely throughout the body. Notably, leptin receptors have been shown to be expressed at relatively high levels in the hypothalamus, the choroid plexus, the lung and

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the kidney, and at lower levels in the liver, adipose tissue, skeletal muscle and the pancreas [1]. The abundance of leptin receptors in the brain and the dramatic effects of centrally administered leptin on feeding behavior and body weight indicate that the hypothalamus is the major target of leptin [2–4]. On the other hand, *in vivo* and *in vitro* studies have demonstrated functional significance of peripheral adipocytokines action including leptin in the regulation of metabolic disorder [5–7]. Especially in the liver, leptin has been shown to inhibit intracellular lipid concentration by reducing synthesis of triglyceride and concomitantly increasing β-oxidation of fatty acids [7]. This indicates that leptin receptor insufficiency induced by genetic defect or acquired leptin resistance, even at the peripheral level, may induce abnormal peripheral lipid metabolism, such as hyperlipidemia and fatty liver, independent of a distortion of central leptin action, which would lead to hyperphagia.

Zucker fatty (fa/fa) rats, which are leptin receptor defective animals, are useful for analyzing various metabolic disorders induced by disruption of leptin signaling [8]. In fact, hypertriglyceridemia, hyperinsulinemia, hyperleptinemia and severe fatty liver have been observed in this obese animal. In Zucker fa/fa rats, however, it is difficult to distinguish the relative contributions of peripheral and central leptin signaling because disruption of hypothalamic leptin signaling induces hyperphagia, which in turn affects peripheral lipid metabolism. Furthermore, inactivation of efferent sympathetic outflow in Zucker fa/fa rats [9], which is normally activated by centrally mediated leptin action [9,10], may also affect peripheral lipid metabolism as well as energy expenditure, as regulated by uncoupling proteins (UCP) [11,12].

On the other hand, Zucker +/fa rats, lean heterozygotes with partial leptin receptor defects, are non-obese and non-hyperphagic, indicating partial leptin receptor insufficiency is not sufficient to cause the fa/fa phenotype. There have been very few studies that address whether this partial leptin signaling insufficiency affects other metabolic parameters. It has been reported that adult mice heterozygous for the ob or db allele display increased body fat compared with their lean +/+ littermates [13]. Thus, these previous findings indicate that even partial insufficiency of leptin signaling can affect peripheral lipid metabolism. Based on this background, we used Zucker +/fa rats to clarify whether or not hepatic lipid metabolism is affected in these animals, and whether this was due to either disruption of peripheral leptin signaling, hyperphagia, or a metabolic disorder.

Materials and methods

Animals and diet

The animals used in this study were male Zucker lean (+/+ and +/fa) and obese (fa/fa) rats. Homozygous lean rats (+/+) were obtained from mating of homozygous (+/+) parents. Lean heterozygotes (+/fa) and obese (fa/fa) rats were produced from crosses of lean heterozygous (+/fa) females and obese (fa/fa) males that received adrenalectomy at 6 weeks of age. After weaning at age 4 weeks, they were allowed free access to standard solid rodent food (CE-2, CLEA Japan) and tap water. Animals were housed in a room-illuminated daily from 0700 to 1900 (a 12:12-h light–dark cycle) and maintained at 21 ± 1 °C with humidity at 55 ± 5%. All studies were conducted in accordance with Oita Medical University Guidelines based on the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Measurement and sampling

Body weight and total calorie intake, calculated by measuring consumption of the rodent food, were recorded weekly. At 18 weeks, they were sacrificed by decapitation. For each rat, after a blood sample was collected, the liver and left epididymal fat were surgically removed and weighed, and a liver tissue sample was taken. Expressions of SREBP-1 and ACC in the liver were investigated by real-time PCR methods. The detailed methods of RNA extraction and PCR were described in previous our studies [14,15].

Assay of serum parameters

All blood samples were immediately centrifuged at 1500 rpm for 15 min at 5 °C and the plasma was stored at −20 °C until each assay. Serum concentrations of glucose, triglyceride and FFA were determined by automatic analyzer using an enzymatic method (SRL, Tokyo, Japan). Serum concentrations of insulin, leptin and adiponectin were assayed with an insulin radioimmunoassay kit (Amersham Pharmatica Bioteck, Little Chalfont, UK), a murine leptin enzyme immunoassay kit (Immune Biological Laboratory, Gunma, Japan) and a murine adiponectin enzyme immunoassay kit (Otsuka pharm, Tokushima, Japan).

Table 1 Calorie intake, body weight, and liver weight in Zucker rats.

	+/+	+/fa	fa/fa
Calorie intake (kcal/day)	75.7 ± 1.8	77.4 ± 2.1	110.1 ± 3.1**
Body weight (g)	450.0 ± 10.8	465.0 ± 10.9	663.7 ± 11.8**
Epididymal fat weight (g)	1.2 ± 0.3	1.4 ± 0.4	10.5 ± 0.9**
Liver weight (g)	13.8 ± 0.3	15.4 ± 0.6*	24.3 ± 0.7**

Values are means ± S.E. for six animals in each group.
* Significant difference from +/+ with $p < 0.05$.
** Significant difference from +/+ with $p < 0.001$.

Assay for triglyceride content and fatty acid composition of liver

All the liver tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until assayed for triglyceride content and fatty acid composition. About 50 mg of each frozen tissue specimen was homogenized in 4.5 ml of phosphate-buffered saline (PBS). Chloroform methanol mixture (2:1 vol/vol) and H_2O were added to the homogenate solutions, which were then centrifuged at 3000 rpm for 5 min. Following centrifugation, the lower chloroform layers were collected, and triglyceride concentrations were assayed by the GPO *p*-chlorophenol method (triglyceride *G*-test, WAKO, Osaka, Japan). From this data, the triglyceride contents of the liver samples were calculated.

In order to assay the fatty acid composition of the samples, the lipid extracts were subjected to methanolysis in 1.37 M HCl in methanol at 100 °C for 2 h, and were evaporated under a stream of nitrogen. Following evaporation, fatty acid methyl esters were extracted with petroleum ether and analyzed by gas chromatography (Shimadzu GC-17A, Shimadzu, Kyoto, Japan) with a 70% sianoplopyl polisilphenirene-ciroxan capillary column (0.25 mm × 25 m; BPX-70, SGE, Ringwood, Austria). Helium was used as the carrier gas and the oven temperature was programmed to hold at 100 °C for the first 2 min, and then to increase from 100 °C to 240 °C at a rate of 5 °C/min and to hold for a final 5 min. The identification and quantification of each fatty acid were made with authentic standard mixture (Funakoshi, Tokyo, Japan) using a Class 5000 (Shimadzu, Kyoto, Japan).

Statistical methods. Results are expressed as mean ± S.E.M. Two-way and one-way ANOVA followed by *post hoc* test were used to determine group difference, with $p < 0.05$ considered to be significant.

Results

Changes in calorie intake, body weight and liver weight. The calorie intake, body and liver weight were significantly increased in Zucker fa/fa rats compared with +/+ rats ($p < 0.001$) (Table 1). There was no difference observed between +/+ and +/fa rats in calorie intake, epididymal fat weight and body weight, but +/fa rats displayed considerably increased liver weights compared with +/+ rats (Table 1).

Changes in serum and hepatic parameters In Zucker fa/fa rats, serum insulin, triglyceride, FFA, and leptin levels were markedly increased in comparison with +/+ rats ($p < 0.001$ for each except FFA, $p < 0.01$ for FFA). With the exception of FFA, the levels of all of these parameters were slightly but significantly elevated in +/fa rats in comparison with +/+ rats ($p < 0.01$ for triglyceride and

Table 2 Serum parameters in Zucker rats.

	+/+	+/fa	fa/fa
Glucose (mg/dl)	107.8 ± 2.1	115.3 ± 2.9	107.3 ± 4.2
Insulin (ng/ml)	1.2 ± 0.1	2.1 ± 0.3*	29.2 ± 3.5***
Triglyceride (mg/dl)	57.0 ± 5.9	87.5 ± 4.5**	388.0 ± 26.5***
FFA (mequiv./l)	0.36 ± 0.03	0.37 ± 0.07	0.56 ± 0.04**
Leptin (ng/ml)	30.7 ± 5.3	57.9 ± 5.8**	859.0 ± 88.4***

Values are means ± S.E. for six animals in each group.
* Significant difference from +/+ with $p < 0.05$.
** significant difference from +/+ with $p < 0.01$.
*** significant difference from +/+ with $p < 0.001$.

Table 3 Hepatic triglyceride content in Zucker rats.

	Hepatic triglyceride content (mg/g liver)
+/+	11.0 ± 1.3
+/fa	19.5 ± 0.8*
fa/fa	41.0 ± 1.7**

Values are means ± S.E. for six animals in each group.
* Significant difference from +/+ with $p < 0.001$.
** significant difference from +/+ with $p < 0.0001$.

leptin, $p < 0.05$ for insulin). Serum glucose levels were not significantly different between the three groups (Table 2). The level of adiponectin was not significantly changed in +/fa rats in comparison with +/+ rats (+/fa 11.1+/−0.3 μg/ml vs. +/fa 10.9+/−0.5 μg/ml; $p > 0.1$). Hepatic SREBP-1 and ACC expressions were both tended to increase but not significantly increased in +/fa rats compared with +/+ rats (SREBP-1: +/+ 100+/−18%; +/fa 125+/−27%, $p > 0.1$; ACC: +/+ 100+/−12%; +/fa 112+/−19%, $p > 0.1$).

Changes in hepatic triglyceride content. Hepatic triglyceride content was 3.7 times higher in Zucker fa/fa rats than in +/+ rats. Interestingly, Zucker +/fa rats displayed hepatic triglyceride levels, which were 1.8 times higher than in +/+ rats (Table 3). In addition, the positive correlation was observed between the levels of circulating and hepatic triglyceride ($r = 0.78$: $p < 0.01$).

Changes in fatty acid composition in the liver. Fig. 1 shows the individual fatty acid composition of fa/fa, +/fa and +/+ liver samples. The major constituents of liver fatty acid were found to be the saturated fatty acids (SFA) palmitate (16:0) and stealinic acid (18:0), the mono-unsaturated fatty acids (MUFA) palmitoleic acid (16:1) and oleic acid (18:1), and the poly unsaturated fatty acids (PUFA) linoleic acid (18:2) and arachidonic acid (20:4). Of the SFAs, the 16:0 fatty acid increased and the 18:0 fatty acid was found to be decreased in fa/fa rats compared with +/+ rats ($p < 0.001$). Of the MUFAs, both the 16:1 and 18:1 fatty acids were present at higher levels in fa/fa rat livers compared with livers from +/+ rats ($p < 0.001$). Of the major PUFAs, the 18:2 and 20:4 fatty acids were found at lower levels in the livers of fa/fa rats compared with those of +/+ rats ($p < 0.001$). In contrast, no remarkable differences in fatty acid composition were detected between +/fa and +/+ rats, except for a slight decrease in 20:4 fatty acids.

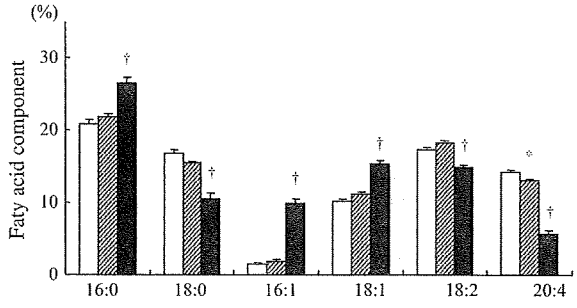

Figure 1 Major fatty acid representation in total liver lipid from 18-week-old +/+ (open bars), +/fa (hatched bars), and fa/fa (filled bars) Zucker rats. Values are means ± S.E. for six animals in each group. *Significant difference from +/+ with $p < 0.05$; †significant difference from +/+ with $p < 0.001$.

Discussion

In this study, we demonstrated that liver triglyceride content was increased, but to different degrees, in Zucker fa/fa rats and +/fa rats, models of complete and partial leptin receptor status, respectively. The most striking difference between these animals was in regard to food intake and body weight, as the fa/fa rats were hyperphagic and obese, while the +/fa rats were normophagic and non-obese. This indicates that the pathogenesis of the increased hepatic triglyceride content in +/fa rats may be different from that of fa/fa rats. At the very least, hyperphagia or obesity per se does not contribute to the development of fatty liver in +/fa rats [16].

Among the serum parameters commonly observed to be elevated in these animal models, hyperleptinemia is not likely to be an inducer of fatty liver, because leptin has been shown to inhibit synthesis of triglyceride in the liver [7]. The positive correlation was observed between the levels of circulating and hepatic triglyceride in the present study. It is reasonable to consider that hypertriglyceridemia is not a cause of fatty liver, but instead results from increased synthesis of hepatic triglyceride and its following secretion from the liver into the circulation. On the other hand, it is highly probable that hyperinsulinemia increases hepatic triglyceride content, since insulin has been shown to accelerate the synthesis of fatty acids and triglycerides through upregulation of sterol regulatory element-binding protein 1c (SREBP1c), a transcriptional factor for fatty acid and triglyceride synthetic enzymes [17,18]. In fact, marked hyperinsulinemia in fa/fa and mild hyperinsulinemia in +/fa rats were observed in present study. Taken together, it is suggested that insufficiency of leptin action due to partial leptin receptor abnormality in Zucker +/fa rats may enhance the development of insulin resistance through abnormal glucose and/or lipid metabolism

in the liver. This would lead to the eventual mild hyperinsulinemia observed in this animal.

Next, we analyzed liver fatty acid composition profiles of the rats since metabolism of hepatic fatty acid was easily affected by dietary factors or insulin action [17–19]. The results revealed changes in lipid profile specific to fa/fa rats, i.e. an increase in monounsaturated fatty acids such as palmitoleic acid and oleic acid, and a concomitant decrease in polyunsaturated fatty acids such as linoleic acid and arachidonic acid. In contrast to fa/fa rats, hepatic fatty acid composition in +/fa rats was not significantly different than that of +/+ rats. These results indicate that neither dietary factors nor mild hyperinsulinemia affect fatty acid metabolism in the liver of +/fa rats. Taken together, partial leptin receptor insufficiency per se rather than changes in several serum parameters including hyperinsulinemia is most likely to induce the increase in hepatic triglyceride content observed in +/fa rats. Of course, complete leptin receptor insufficiency in fa/fa rats must promote the development of fatty liver in conjunction with the influence of hyperphagia, obesity and hyperinsulinemia. In fact, more sever fatty liver was observed in fa/fa rats compared with +/fa rats in this study.

Finally, we must discuss how leptin receptor insufficiency affects lipid metabolism in the liver. Leptin has been shown to upregulate acyl CoA oxidase and carnitine phosphotransferase 1, the enzymes for peroxisomal and mitochondrial β-oxidation, respectively, and to downregulate acyl CoA carboxylase (ACC) and glycerol phosphate acyltransferase, lipogenetic enzymes, in the pancreatic islets [20]. In the liver, leptin has been shown to decrease the expression of SREBP1c mRNA, leading to a concomitant decrease in the levels of its downstream enzymes, such as fatty acid synthase and ACC [21]. These findings indicate that leptin and its peripheral receptors play a role in the prevention of triglyceride accumulation by activation of fatty acid β-oxidation and reduction of triglyceride formation. From this viewpoint, it may be concluded that disruption of leptin action due to leptin receptor insufficiency, even if partial, may promote triglyceride accumulation in the liver. However, in the present study, hepatic SREBP-1 and ACC expression were not significantly increased in +/fa rats compared with +/+ rats. Various kinds of TG synthetic enzymes than SREBP-1 and ACC might play roles in regulating hepatic fat metabolism in +/fa rats. Further studies are needed to be clarified that point.

In summary, this study demonstrates that even partial leptin receptor insufficiency promotes the development of fatty liver and mild insulin resistance independently of hyperphagia and its resultant metabolic disorder. This indicates that low-level insufficiency of leptin action induced by genetic defect or acquired leptin resistance at central as well as peripheral receptors may induce a distortion of peripheral energy metabolism without an overt influence on food intake.

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Knowledge of *Vibrio vulnificus* infection among Japanese patients with liver diseases: A prospective multicenter study

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Knowledge of *Vibrio vulnificus* infection among Japanese patients with liver diseases: A prospective multicenter study

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Summary

Background: *Vibrio vulnificus* (*V. vulnificus*) is a seafood-borne infectious pathogen that can be lethal to humans. The infection has been correlated with pre-existing liver disease, particularly liver cirrhosis. Awareness of *V. vulnificus* infection among Japanese citizens is low, despite the increasing number of patients with hepatocellular carcinoma (HCC). The present study was conducted to assess the level of knowledge of patients with liver disease regarding *V. vulnificus* infection.

Material/Methods: Questionnaires were sent to patients with chronic liver disease who had been treated by liver specialists at 14 medical institutes.

Results: Of 1,336 patients, 304 (22.8%) had liver cirrhosis, and 732 (54.8%) had comorbidities of this disease. Only 14.5% (194/1,336) of patients had knowledge of *V. vulnificus* infection. Of 304 patients with liver cirrhosis, 17.4% (53/304) of the patients had knowledge of *V. vulnificus* infection. Of 60 patients with liver cirrhosis and diabetes mellitus, 11 (18.3%) patients had knowledge of *V. vulnificus* infections. Even when the patients with high risk factors such as liver cirrhosis and diabetes mellitus had knowledge of *V. vulnificus* infections, most ate raw seafood without regard to season.

Conclusions: Patients with chronic liver diseases and their physicians need to be better educated about *V. vulnificus* infection and its prevention.

key words: ***Vibrio vulnificus*** • **liver diseases** • **hepatitis C virus (HCV)** • **hepatocellular carcinoma (HCC)**

Abbreviations: ***V. vulnificus*** – *Vibrio vulnificus*; **HCV** – Hepatitis C virus; **HBV** – Hepatitis B virus; **HCC** – Hepatocellular carcinoma; **PBC** – primary biliary cirrhosis; **AIH** – autoimmune hepatitis; **ICD** – International Classification of Diseases

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