

Table 1
Profile and laboratory data of participating patients infected with HCV genotype 1b at commencement of 48-week peginterferon-ribavirin combination therapy

Demographic data	
Number	114
Gender (M/F)	75 / 39
Age (years)*	54 (30–70)
History of blood transfusion	38 (33.3%)
Family history of liver disease	35 (30.7%)
Body mass index (kg/m ²)*	23.2 (17.6–30.3)
Laboratory data*	
Serum aspartate aminotransferase (IU/l)	60 (17–266)
Serum alanine aminotransferase (IU/l)	81 (25–504)
Serum albumin (g/dl)	3.7 (3.0–4.5)
γ -Glutamyl transpeptidase (IU/l)	67 (15–393)
Leukocytes (/mm ³)	4800 (2300–8800)
Hemoglobin (g/dl)	14.6 (10.6–17.6)
Platelets ($\times 10^4$ /mm ³)	17.6 (6.6–30.9)
ICG R15 (%)	15 (4–49)
Serum iron (μ g/dl)	147 (18–308)
Serum ferritin (μ g/l)	150 (<10–927)
Creatinine clearance (ml/min)	100 (53–146)
Viremia level (KIU/ml)	2000 (67–>5000)
Total cholesterol (mg/dl)	169 (100–236)
High-density lipoprotein cholesterol (mg/dl)	45 (10–83)
Low-density lipoprotein cholesterol (mg/dl)	100 (53–162)
Triglycerides (mg/dl)	100 (33–362)
Uric acid (mg/dl)	5.6 (2.3–8.8)
Fasting blood sugar (mg/dl)	96 (75–257)
Histological findings	
Stage (F1/F2/F3/F4/ND)	51/28/16/1/18
Hepatocyte steatosis (none to mild/moderate to severe/ND)	86/8/20
Treatment	
PEG-IFN α -2b dose (μ g/kg)	1.5 (0.8–1.8)
Ribavirin dose (mg/kg)	10.9 (3.4–14.2)
Amino acid substitutions in the core region**	
aa 70 (wild/non-wild/ND)	54/38/8
aa 91 (wild/non-wild/ND)	58/40/2
aa 70 and aa 91 (double-wild/non-double-wild/ND)	35/61/4

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.

** Amino acid substitutions were evaluated in pretreatment serum samples of 100 patients by PCR with mutation-specific primers.

Two patterns of mutant and competitive were labeled as non-wild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, while the other patterns were considered non-double-wild-type. ND, not determined.

(aa 70: glutamine/histidine, aa 91: methionine) in agarose gel electrophoresis. If the intensities of the bands were similar, the case was regarded as competitive. The detection rate was 94.4%, the sensitivity was 10 KIU/ml, the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases [13]. In this study, the pattern of arginine (wild) at aa 70 and leucine (wild) at aa 91 was evaluated as double-wild-type, while the other patterns were non-double-wild-type. The mutation in this study refers to substitution from consensus sequence. In previous studies, HCV-J was considered as a prototype and the aa substitution was evaluated by comparison with the consensus sequence prepared from 50 clinical trial samples [4,14].

In the present study, the PCR genotyping could be performed in 100 patients; the remaining 14 patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

HCV-RNA was extracted from the serum samples and cDNA was prepared by reverse transcription using MMLV Superscript II reverse transcriptase. The obtained cDNA was amplified by PCR using the following primers: the first PCR was performed using cc11 (sense, 5'-GCC ATG GTG GTC TGC GGA AC-3': 125–144) and e14 (antisense, 5'-GGA GCA GTC CTT CGT GAC ATG-3': 933–953) primers. In the second PCR, for aa 70 the wild-type-specific reaction was performed using 70W2 (sense, 5'-TAT CCC CAA GGC TCG CCG-3': 521–538) and e14, and the mutant-specific reaction was performed using 70M2 (sense, 5'-TAT CCC CAA GGC TCG CCA-3': 521–538) and e14. For aa 91, the wild-type-specific reaction was performed using cc9 (sense, 5'-GCT AGC CGA GTA GTG TT-3': 237–253) and 91W (antisense, 5'-CAT CCT GCC CAC CCC AR-3', R = A or G: 600–616), and the mutant-specific reaction was performed using cc9 and 91M (5'-CAT CCT GCC CAC CCC AT-3': 600–616) [13].

The cycle conditions were 94 °C for 4 min + (94 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min) \times 20 cycles + 72 °C for 7 min in the first PCR; and 94 °C for 1 min + (94 °C for 30 s and 72 °C for 1.5 min) \times 23 cycles + 72 °C for 7 min for aa 70, and 94 °C for 1 min + (94 °C for 30 s and 68 °C for 1.5 min) \times 21 cycles + 72 °C for 7 min for aa 91 in the second PCR. Two microliters of cDNA was used in the first PCR and 1 μ l of the first PCR product was used in the second PCR. For detection, 5 μ l of the second PCR product was electrophoresed for 30 min on 3.0% agarose gel. The final concentration of all primers was 0.2 pmol/ μ l [13].

To avoid false-positive results, the procedures recommended by Kwok and Higuchi [15] to prevent contamination were strictly applied to these PCR assays. No false positive results were observed in this study.

2.5. Statistical analysis

SVR was analyzed on an intention to treat basis. Non-parametric tests were used to compare variables between groups (Mann–Whitney *U* test, χ^2 test and Fisher's exact probability test). Univariate and multivariate logistic regression analyses were used to determine the predictors of SVR and EVR. We also calculated the odds ratios and 95% confidence intervals (95%CI). All *P* values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with SVR and EVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, BMI, aspartate aminotransferase (AST), ALT, albumin, γ -glutamyl transpeptidase (GGT), leukocyte count, Hb, platelets, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, TC, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), TG, uric acid (UA), FBS, hepatocyte steatosis, pathological staging, PEG-IFN dose/body weight, RBV dose/body weight, and aa substitutions in HCV-CR. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL). Sensitivity, specificity, PPV, and NPV were also calculated to determine the reliability of predictors of the response to therapy.

3. Results

3.1. Response to therapy

EVR and SVR were evaluated in all 114 patients. EVR was achieved by 80 of 114 (70.2%) patients, and SVR by 51 of 114 (44.7%) patients. 44.7% (51/114 patients) achieved both EVR and SVR, 29.8% (34/114) were considered non-EVR and non-SVR, 25.4% (29/

Table 2

Factors associated with early virologic response to 48-week peginterferon-ribavirin combination therapy in patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	P
LDL cholesterol (mg/dl)	1: <86	1	<0.001
	2: ≥86	30.29 (4.855–189.0)	
Amino acid substitution in core region	1: double-wild-type ^a	1	0.003
	2: non-double wild-type	0.046 (0.006–0.346)	
γ-Glutamyl transpeptidase (IU/l)	1: <109	1	0.023
	2: ≥109	0.166 (0.035–0.782)	
Ribavirin dose (mg/kg)	1: <11.0	1	0.039
	2: ≥11.0	4.341 (1.075–17.53)	
Leukocyte count (/mm ³)	1: <4500	1	0.041
	2: ≥4500	4.209 (1.061–16.70)	

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.

Normal range for LDL cholesterol: 86–135 mg/dl.

^a Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, and the other patterns were considered non-double-wild-type.

114) were EVR and non-SVR, and 0% (0/114) as non-EVR and SVR. Thus, 63.8% (51/80) of those who achieved EVR also achieved SVR, and none of non-EVR could achieve SVR.

3.2. Predictors of EVR as determined by univariate and multivariate analyses

Univariate analysis identified nine parameters that influenced the EVR: LDL-C (≥86 mg/dl; $P < 0.001$), aa substitutions of HCV-CR (double-wild-type; $P = 0.001$), leukocyte count (≥4500/mm³; $P = 0.003$), GGT (<109 IU/l; $P = 0.008$), TC (≥170 mg/dl; $P = 0.038$), RBV dose/body weight (≥11.0 mg/kg; $P = 0.042$), PEG-IFN dose/body weight (≥1.25 μg/kg; $P = 0.055$), TG (<100 mg/dl; $P = 0.059$), and AST (<60 IU/l; $P = 0.065$). Multivariate analysis that included the above variables identified five parameters that independently influenced the EVR: LDL-C (≥86 mg/dl; $P < 0.001$), aa substitutions of HCV-CR (double-wild-type; $P = 0.003$), GGT (<109 IU/l; $P = 0.023$), RBV dose/body weight (≥11.0 mg/kg; $P = 0.039$), and leukocyte count (≥4500/mm³; $P = 0.041$). Especially, LDL-C (≥86 mg/dl) and aa substitutions of HCV-CR

(double-wild-type) of five parameters increased chances for EVR 20-fold or more (Table 2).

3.3. Assessment of amino acid substitutions and LDL-cholesterol as predictors of EVR

EVR rates of patients with double-wild-type of HCV-CR or high-serum LDL-C levels (≥86 mg/dl) were defined as PPV (prediction of EVR). Non-EVR rates of patients with non-double-wild-type of HCV-CR or low-serum LDL-C levels (<86 mg/dl) were defined as NPV (prediction of non-EVR).

In patients with double-wild-type of HCV-CR, the sensitivity, specificity, PPV, and NPV for prediction of EVR were 46.4%, 88.9%, 91.4%, and 39.6%, respectively. In patients with high serum LDL-C levels, the sensitivity, specificity, PPV, and NPV were 81.0%, 56.3%, 82.1%, and 54.5%, respectively. Thus, evaluation of aa substitutions in HCV-CR indicated high specificity and PPV, while that of serum LDL-C level indicated high sensitivity and PPV for prediction of EVR. Furthermore, when both predictors were used, the sensitivity, specificity, PPV, and NPV were 32.4%, 100%, 100%, and 37.0%, respectively. When one or two predictors

Table 3

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for prediction of early virologic response (EVR) to dual therapy, based on the combination of amino acid substitutions in the core region and low-density lipoprotein cholesterol (LDL-C) level

	Sensitivity	Specificity	PPV ^b	NPV ^c
(A) Double-wild-type of core region ^a	46.4 (32/69)	88.9 (24/27)	91.4 (32/35)	39.3 (24/61)
(B) High level of LDL-C	81.0 (64/79)	56.3 (18/32)	82.1 (64/78)	54.5 (18/33)
(A) and (B)	32.4 (22/68)	100 (27/27)	100 (22/22)	37.0 (27/73)
(A) and/or (B)	97.4 (74/76)	39.3 (11/28)	81.3 (74/91)	84.6 (11/13)

Data in parentheses represent the numbers used for determining the sensitivity, specificity, PPV and NPV.

^a Wild at aa 70 and wild at aa 91 were evaluated as double-wild type, and the other patterns were considered non-double-wild-type.

^b PPV; EVR rates for patients with a combination of double-wild-type of the core region, or high levels (≥86 mg/dl) of LDL-C (prediction of EVR).

^c NPV; non-EVR rates for patients with non-double-wild-type of the core region, or low levels (<86 mg/dl) of LDL-C (prediction of non-EVR).

Table 4

Factors associated with sustained virological response to 48-week peginterferon-ribavirin combination therapy in patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	P
Amino acid substitution in core region	1: double-wild-type ^a	1	0.004
	2: non-double wild-type	0.102 (0.022–0.474)	
LDL cholesterol (mg/dl)	1: <86	1	0.005
	2: ≥86	12.87 (2.177–76.09)	
Gender	1: male	1	0.005
	2: female	0.091 (0.017–0.486)	
ICG R15 (%)	1: <10	1	0.018
	2: ≥10	0.107 (0.017–0.678)	
γ-Glutamyl transpeptidase (IU/l)	1: <109	1	0.032
	2: ≥109	0.096 (0.011–0.819)	
Ribavirin dose (mg/kg)	1: <11.0	1	0.032
	2: ≥11.0	5.173 (1.152–23.22)	

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.

Normal range for LDL cholesterol: 86–135 mg/dl.

^a Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, and the other patterns were considered non-double-wild-type.

were used, the sensitivity, specificity, PPV, and NPV were 97.4%, 39.3%, 81.3%, and 84.6%, respectively. Thus, prediction of EVR by the combination of aa substitutions in HCV-CR and serum LDL-C level had high sensitivity, specificity, PPV, and NPV (Table 3).

3.4. Predictors of SVR as determined by univariate and multivariate analyses

Univariate analysis identified 12 parameters that influenced SVR: histopathological staging of liver fibrosis (F1; $P = 0.002$), leukocyte count ($\geq 4500/\text{mm}^3$; $P = 0.004$), aa substitutions of HCV-CR (double-wild-type; $P = 0.005$), PEG-IFN dose/body weight ($\geq 1.25 \mu\text{g}/\text{kg}$; $P = 0.006$), gender (male; $P = 0.007$), age (< 55 years; $P = 0.009$), RBV dose/body weight ($\geq 11.0 \text{ mg}/\text{kg}$; $P = 0.009$), GGT ($< 109 \text{ IU}/\text{l}$; $P = 0.019$), ICG R15 ($< 10\%$; $P = 0.029$), LDL-C ($\geq 86 \text{ mg}/\text{dl}$; $P = 0.063$), Hb ($\geq 14.0 \text{ g}/\text{dl}$; $P = 0.064$), and AST ($< 60 \text{ IU}/\text{l}$; $P = 0.064$). Multivariate analysis identified six parameters that independently influenced the SVR: aa substitutions of HCV-CR (double-wild-type; $P = 0.004$), LDL-C ($\geq 86 \text{ mg}/\text{dl}$; $P = 0.005$), gender

(male; $P = 0.005$), ICG R15 ($< 10\%$; $P = 0.018$), GGT ($< 109 \text{ IU}/\text{l}$; $P = 0.032$), and RBV dose/body weight ($\geq 11.0 \text{ mg}/\text{kg}$; $P = 0.032$) (Table 4). These results indicate that aa substitutions of HCV-CR and LDL-C levels are significant and independent predictors of both EVR and SVR, especially.

3.5. Assessment of amino acid substitutions and LDL cholesterol as predictors of SVR

Finally, we evaluated the ability to predict SVR by aa substitutions of HCV-CR and serum LDL-C level (each, $P < 0.01$). The SVR rates of patients with double-wild-type of HCV-CR or high serum levels of LDL-C were defined as PPV (prediction of SVR). The non-SVR rates of patients with non-double-wild-type of HCV-CR or low serum levels of LDL-C were defined as NPV (prediction of non-SVR).

In patients with double-wild-type of HCV-CR, the sensitivity, specificity, PPV, and NPV for SVR were 52.4%, 75.9%, 62.9%, and 67.2%, respectively. Thus, aa substitutions in HCV-CR have a high specificity for prediction of SVR. In patients with high-serum levels

Table 5

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for prediction of sustained virological response (SVR), based on a combination of amino acid substitutions in the core region and low-density lipoprotein cholesterol (LDL-C) levels

	Sensitivity	Specificity	PPV ^b	NPV ^c
(A) Double-wild-type of core region ^a	52.4 (22/42)	75.9 (41/54)	62.9 (22/35)	67.2 (41/61)
(B) High level of LDL-C	80.0 (40/50)	37.7 (23/61)	51.3 (40/78)	69.7 (23/33)
(A) and (B)	33.3 (15/45)	87.9 (51/58)	68.2 (15/22)	63.0 (51/81)
(A) and/or (B)	100 (47/47)	22.8 (13/57)	51.6 (47/91)	100 (13/13)

Data in parentheses represent the numbers used for determining the sensitivity, specificity, PPV, and NPV.

^a Wild at aa 70 and wild at aa 91 were evaluated as double-wild type, and the other patterns were considered non-double-wild-type.

^b PPV, EVR rates for patients with a combination of double-wild-type of the core region or high levels ($\geq 86 \text{ mg}/\text{dl}$) of LDL-C (prediction of EVR).

^c NPV, Non-EVR rates for patients with non-double-wild-type of the core region or low levels ($< 86 \text{ mg}/\text{dl}$) of LDL-C (prediction of non-EVR).

of LDL-C, the sensitivity, specificity, PPV, and NPV were 80.0%, 37.7%, 51.3%, and 69.7%, respectively. Thus, serum LDL-C level has high sensitivity in predicting SVR. Furthermore, when both predictors were used, the sensitivity, specificity, PPV, and NPV were 33.3%, 87.9%, 68.2%, and 63.0%, respectively. When one or more of the two predictors were used, the sensitivity, specificity, PPV, and NPV were 100%, 22.8%, 51.6%, and 100%, respectively. These results indicate that the use of the combination of the above two predictors has high sensitivity, specificity, and NPV for prediction of SVR (Table 5).

4. Discussion

We reported previously that substitutions of aa 70 and/or 91 in the HCV core region are an independent and significant predictor of NVR [4,5]. Based on a larger number of patients, the present study also identified aa substitutions in HCV-CR as a predictor of EVR and SVR in patients on 48-week PEG-IFN-RBV dual therapy. Previous studies reported that the HCV core region might be associated with resistance to IFN monotherapy involving the Jak-STAT signaling cascade [16–19]. Our result could be also interpreted to mean that aa substitutions in HCV-CR are associated with those proteins involved in resistance to IFN monotherapy, such as SOCS proteins known to inhibit IFN- α -induced activation of the Jak-STAT pathway and expression of the antiviral proteins 2',5'-OAS and MxA [20]. Furthermore, our result also indicates that aa substitutions in HCV-CR might serve as a surrogate marker for other proteins associated with resistance to the antiviral actions of IFN. Further studies that examine the structural and functional impact of aa substitutions during combination therapy should be conducted to confirm the above finding.

Importantly, our study also identified serum LDL-C levels as a predictor of the response to PEG-IFN-RBV therapy, and we agree with the recent findings of Gopal et al. [21]. Previous studies reported that endocytosis of HCV via the LDL receptor(s) is mediated by the formation of a complex between HCV and VLDL or LDL [22,23]. Furthermore, there is evidence that intracellular cholesterol level modulates LDLr expression, and thus a high LDL-C could downregulate LDLr and diminish the spread of hepatocyte HCV infection. Thus, the correlation between treatment efficacy and LDL-C may be explained by the role of LDL-C in transporting the HCV-LDL complex into the hepatocyte. It should be noted, however, that other *in vitro* studies also showed that statins, which upregulate LDLr, might decrease HCV replication [24–26]. Other mechanisms could also explain the role of LDL-C and the response to PEG-IFN-RBV therapy. For example, high-LDL-C levels

could act by modulating cytokine release [27] and antiviral cellular immune response [28,29]. On the other hand, it is also reported that apolipoprotein E4 allele is associated with high LDL-C levels [30], and with poor response to treatment in patients with genotype 1 HCV [31]. The discrepancy between our results and such findings may be explained by the small number of patients in our study, differences in host factors including race [32–34], and/or differences in viral factors, such as the distribution of genotype 1a or 1b, and geographic diversities of genotype 1b [35]. Further studies of large number of patients matched for race and HCV genotype are required to explore the relationship between serum LDL-C level and the response to PEG-IFN-RBV therapy.

Our results also showed that a high ICG R15 value was a negative predictor of SVR to PEG-IFN-RBV therapy. Previous data indicated that absence of advanced liver fibrosis is a predictor of SVR to IFN monotherapy and IFN-RBV dual therapy [36–38], and that advanced liver fibrosis is usually associated with high rates of ICG R15 [39]. However, our study showed that a milder form of liver fibrosis was not a predictor of response to dual treatment, whereas a low level of ICG R15 was. This discrepant finding may be due to the fact that estimates of liver dysfunction assessed by the degree of liver fibrosis (which is evaluated using only four stages (F1, F2, F3, F4), in contrast to ICG R15), are less sensitive to those by ICG R15. It is also possible that the above discrepancy is related to our exclusion of patients with cirrhosis (F4) (the exclusion was because the Japanese Government Health Insurance system does not provide cover for combination therapy for patients with cirrhosis). Further studies are required to explore the relationship between the severity of histopathological changes in the liver and response to dual therapy especially in patients with cirrhosis.

Our results should be interpreted with caution since we did not include patients of other races or other HCV genotypes. Any generalization of the results should await confirmation by studies of patients of other races infected with other HCV genotypes.

Pretreatment prediction of the response to PEG-IFN-RBV therapy is still incomplete. So far, viral factors (e.g., aa substitutions in HCV-CR), host factors (e.g., LDL-C [21], gender [40], ICG R15, and GGT [41]), and treatment-related factors (e.g., RBV dose [5,42]) have been confirmed to influence the response to such treatment in Japanese patients infected with HCV genotype 1b. Furthermore, evaluation using a combination of predictors indicates the high-sensitivity, specificity, PPV, and NPV of such prediction. We conclude that the response to PEG-IFN-RBV therapy seems to be based on a dynamic tripartite interaction of virus, host, and treatment regimen. Further understanding of the

complex interaction between these factors should facilitate the development of more effective therapeutic regimens.

Acknowledgement

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

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HEPATOLOGY

417-2 α -Tocopherol and ascorbic acid attenuates the ribavirin-induced decrease of eicosapentaenoic acid in erythrocyte membrane in chronic hepatitis C patients

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Key words

fatty acid, hemolytic anemia, interferon, lipid peroxidation, oxidative stress.

Accepted for publication 24 August 2005.

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Abstract

Background: Oxidative damage of the erythrocyte membrane plays an important role in ribavirin-induced anemia. The purpose of the present paper was to assess whether supplementation of α -tocopherol and ascorbic acid (vitamins) causes changes in the erythrocyte membrane fatty acid composition during interferon and ribavirin combination therapy for chronic hepatitis C patients.

Methods: Fatty acid compositions in erythrocyte membrane phospholipids were determined by gas chromatography at 0, 2, 4, 8 weeks, and at the end of combination therapy (26 weeks) for interferon with ribavirin in 32 patients with chronic hepatitis C who were randomized to receive vitamins or not (controls).

Results: Good compliance with orally administered vitamins and ribavirin were confirmed by their concentrations in erythrocytes or plasma. The hemoglobin level was negatively correlated with the ribavirin concentration at 8 weeks ($r = 0.59$, $P = 0.01$) after initiation of therapy in controls, but not in the vitamin group. Among the 26 kinds of fatty acids analyzed, only eicosapentaenoic acid (EPA) significantly decreased at 8 weeks after initiation of therapy ($P = 0.03$) and at the end of therapy ($P = 0.004$) in controls. Vitamins did not inhibit ribavirin-induced anemia, but attenuated the decrease of EPA in erythrocytes. The EPA level was negatively correlated with the drop in hemoglobin levels at 8 weeks after initiation of therapy in controls ($r = 0.58$, $P = 0.015$), but not in the vitamin group.

Conclusions: Supplementation of α -tocopherol and ascorbic acid attenuates the ribavirin-induced decrease of EPA in erythrocyte membrane phospholipids in chronic hepatitis C patients.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, leading to cirrhosis and hepatocellular carcinoma worldwide.¹ A major therapeutic goal in HCV-infected patients is to achieve eradication of the virus, which considerably reduces the risk of development of end-stage liver disease or hepatocellular carcinoma. Combination therapy using interferon (IFN) with or without pegylation and ribavirin has been shown to induce a higher sustained virological response than IFN monotherapy.²⁻⁶ A number of adverse events, however, are increased in combination therapy. Hemolytic anemia is a universal event associated with

ribavirin combination therapy, although the extent of anemia can vary greatly between individuals.⁷ Significant anemia associated with ribavirin therapy can increase fatigue, has a demonstrable effect on quality of life, and is a frequent indication for dose reduction or discontinuation of ribavirin.^{5,6} Adherence to combination therapy has been shown to enhance the sustained virological response in genotype-1-infected patients with chronic hepatitis C.⁸ Therefore, the development of strategies to maximize adherence is an important issue in combination therapy for chronic hepatitis C.

In the present study we focused on oxidative damage to the erythrocyte membrane. α -Tocopherol and ascorbic acid are potent reducing agents that act as antioxidants *in vitro* and *in vivo*.^{9,10}

Experimental data suggest that there are interactions between these antioxidants. α -Tocopherol is an effective chain-breaking antioxidant, thus inhibiting lipid peroxidation.¹⁰ Ascorbic acid can regenerate α -tocopherol from the α -tocopherol radical during lipid peroxidation.¹⁰ Therefore, using a combination of these antioxidants could be more advantageous for antioxidant protection than using a single antioxidant alone. The aim of the present study was to prospectively assess whether supplementation of α -tocopherol and ascorbic acid causes any changes in the erythrocyte membrane fatty acid composition, during IFN and ribavirin combination therapy for chronic hepatitis C patients.

Methods

Patient population

From April 2002 to February 2003, 32 Japanese patients with chronic hepatitis C who met the following criteria were enrolled in this prospective trial by Yamaguchi University Hospital and affiliated institutions. The criteria for enrollment were: a persistently elevated serum alanine aminotransferase (ALT) level for more than 6 months prior to enrollment; HCV-RNA seropositivity; absence of detectable hepatitis B virus surface antigen; exclusion of all other potential causes of chronic liver disease such as autoimmune hepatitis, primary biliary cirrhosis, drug-induced hepatitis, or metabolic liver disease; no history of alcohol abuse, defined as alcohol intake of ≥ 80 g/day for >3 years; no history of depressive illness or thyroid disease; no pregnancy; and a platelet count $\geq 70\,000/\text{mm}^3$, leukocyte count $\geq 3000/\text{mm}^3$ and hemoglobin level ≥ 12 g/dL.

Study design

After all patients had given informed consent, they were randomized to receive daily oral vitamins (500 mg α -tocopherol and 750 mg ascorbic acid; vitamin group) or none (control) by a central randomization schedule in Yamaguchi University, in addition to receiving 6 million units of IFN- α -2b six times weekly for 2 weeks, followed by thrice weekly for 24 weeks with daily oral ribavirin in two divided doses of 600 mg/day for those weighing <60 kg and 800 mg/day for those weighing >60 kg for 26 weeks. During treatment, the dose of ribavirin was adjusted based on the hemoglobin level; ribavirin was decreased by 200 mg/day if the hemoglobin level fell below 10 g/dL, and discontinued when it diminished below 8.5 g/dL. Blood chemistry and blood cell counts were determined every 4 weeks during therapy in addition to 2 weeks after initiation of therapy. The HCV genotypes and HCV-RNA concentrations were determined at baseline. Patients were categorized according to their response to IFN and ribavirin combination therapy: sustained virological responders were defined by the normalization of serum ALT during the 6-month period after completion of treatment, and the absence of detectable serum HCV-RNA tested 6 months after the completion of therapy; those categorized as non-sustained responders did not meet these criteria. Blood samples were obtained at 0, 2, 4, 8, and 26 weeks after initiation of therapy for assessment of the α -tocopherol concentration in erythrocytes, ascorbic acid concentration in plasma, ribavirin concentration in plasma, and fatty acid composition in erythrocyte membrane phospholipids. The

study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics committee.

Hepatitis C virus genotyping and quantification of HCV-RNA

The HCV was genotyped as described previously,¹¹ and HCV-RNA levels were measured using a Cobas Amplicore HCV monitor (Version 2.0; Roche Diagnostics, Tokyo, Japan).

Fatty acid analysis

Blood was drawn into tubes containing disodium ethylenediamine-tetraacetic acid. Plasma and erythrocytes were separated after centrifugation of blood samples at 1600 *g* for 15 min at 4°C. The erythrocyte layer was drawn off into another tube and washed three times with saline solution (4°C) for α -tocopherol determination, and three times with 5 mmol/L phosphate buffer (4°C, pH 6.8) for determination of fatty acid compositions in erythrocyte membrane phospholipids. All samples were stored at -80°C until assayed. Erythrocyte ghosts were obtained according to the method of Hanahan and Ekholm.¹² Total lipid was extracted from the erythrocyte ghosts as described previously.¹³ Phospholipid was separated by 1-D thin-layer chromatography (TLC) using silica gel plates (Silica Gel 60, Merck, Darmstadt, Germany) and a solvent system of petroleum ether:ethyl ether:acetic acid (80:20:1, v/v). The spots corresponding to phospholipids were scraped from the TLC plates and transmethylated using acetyl chloride/methanol (5:50). A known amount of heptadecanoic acid was used as an internal standard. Following transmethylation, fatty acid methyl esters were extracted with petroleum ether and quantified, using a model GC14 A gas chromatograph (Shimadzu, Kyoto, Japan), as described previously.¹⁴ The fatty acid methyl esters were identified according to their retention times by comparison with known standards.

α -Tocopherol concentration in erythrocytes

The α -tocopherol concentration in erythrocytes was determined according to a modified version of the Milne and Borten method.¹⁵ Tocopherols were extracted from saponified erythrocyte samples with ethyl acetate and *n*-hexane (1:9, v/v). The extracts were evaporated under nitrogen and redissolved in ethanol. The α -tocopherol level in erythrocytes was quantified by high-performance liquid chromatographic analysis in TSK-gel ODS-80Ts columns (25.0 \times 4.6 mm; Tosoh, Tokyo, Japan). The mobile phase was methanol:1-butanol (80:20, v/v), including 10 mmol/L of sodium acetate buffer (pH 3.6; 0.1%, v/v) at a flow rate of 1.0 mL/min (model CCPM, Tosoh). The α -tocopherol was monitored at an excitation wavelength of 295 nm and emission wavelength of 325 nm (model 821-FR, Jasco, Tokyo, Japan). The α -tocopherol peak was identified and quantified against authentic D- α -tocopherol (vitamin E reference standards, Eisai, Tokyo, Japan) used as an external standard. The hemoglobin concentration of each erythrocyte sample was detected with the method of Oshiro *et al.*¹⁶ The α -tocopherol concentration was expressed as micrograms per gram of hemoglobin (gHb).

Ascorbic acid concentration in plasma

The plasma ascorbic acid concentration was determined based on the method of Tokumaru *et al.*,¹⁷ which involves chemical derivatization and high-performance liquid chromatographic analysis. The extracted sample was analyzed on μ -Bondasphere 5- μ m C₁₈-100A column (3.9 mm \times 150 mm, Waters, Milford, MA, USA), eluted with 50% acetonitrile containing 0.1% triethylamine at a flow rate of 1 mL/min (Waters 600E Multisolute Delivery System, Waters). The absorption at 505 nm was recorded with a spectrophotometer (Waters 486 Tunable Absorbance Detector, Waters).

Ribavirin concentration in plasma

The serum ribavirin concentration was determined by a validated high-performance liquid chromatography/tandem mass spectrometric assay using ¹³C-ribavirin as an internal standard.^{18,19} The assay was validated with respect to linearity within a range of 50.1–5005 ng/mL, specificity, accuracy (within 15% for all runs), and precision (within 15% for all runs). The assay limit of quantification (LOQ) was 50 ng/mL.

Statistical analysis

Results are expressed as mean \pm SD. Differences in proportion were tested by the χ^2 test with a Yates' correction. Mean quantitative values were compared by Student's *t*-test. Non-parametric data were compared using the Wilcoxon signed rank test. The statistical significance of correlation was determined by the use of simple regression analysis. All reported *P* were two-tailed and *P* < 0.05 was considered to be significant.

Results

Assignment of therapy and completion of the assigned therapy

Among the 32 patients, 14 patients were assigned to take α -tocopherol and ascorbic acid and the remaining 18 were not. One patient in the control group discontinued taking ribavirin at his discretion 2 weeks after initiation of therapy. Because of a fall in the hemoglobin concentration to <10 g/dL, the dose of ribavirin was reduced by 200 mg/day (>4 weeks, <20 weeks) in two of the 14 patients (14.3%) in the vitamin group and in eight of the 17 patients (47.1%) in the control group, respectively. The hemoglobin level did not fall below 8.5 g/dL afterwards in these 10 patients. The rate of dose reduction of ribavirin tended to be lower in the vitamin group than in the control group (*P* = 0.052). One patient in the vitamin group (7.1%) and six patients in the control group (35.3%, *P* = 0.06 vs vitamin group) developed treatment-related adverse events (general fatigue, anorexia and/or depression) that led to discontinuation of therapy between 8 weeks and 26 weeks after its initiation. Blood samples for fatty acid analysis were not obtained from two patients in the vitamin group at the end of therapy. Consequently, 31 patients (14 in the vitamin group and 17 in the control group) were analyzed until 8 weeks after initiation of therapy, and 22 patients (11 patients in each group) were analyzed at the end of therapy. All patients included in the

present study confirmed that they did not self-administer other vitamins or supplements in the course of the study. We also confirmed that all patients did not have special lifestyle or dietary habits, such as vegetarianism.

Clinical and viral characteristics

Table 1 presents the clinical and viral characteristics of patients at baseline who were treated without discontinuance for at least until 8 weeks after initiation of therapy. There were no differences in age, gender, serum ALT level, hemoglobin level, HCV genotype, viral load, or histological diagnosis for grading inflammation, for staging fibrosis, and ribavirin dose/kg bodyweight.

Concentrations of α -tocopherol and ascorbic acid

The α -tocopherol concentration in erythrocytes was significantly higher in the vitamin group than in the control group at 2 weeks after initiation of therapy and thereafter (2.9 \pm 0.7 μ g/gHb vs 3.4 \pm 1.2 μ g/gHb at 0 weeks, 5.7 \pm 2.1 μ g/gHb vs 3.1 \pm 1.5 μ g/gHb at 2 weeks, *P* = 0.0001; 6.3 \pm 1.9 μ g/gHb vs 3.4 \pm 1.1 μ g/gHb at 4 weeks, *P* < 0.00001; 6.3 \pm 1.7 μ g/gHb vs 3.4 \pm 1.1 μ g/gHb at 8 weeks, *P* < 0.00001; 5.7 \pm 1.4 μ g/gHb vs 3.3 \pm 1.0 μ g/gHb at 26 weeks, *P* = 0.0001; Fig. 1). Similarly, the ascorbic acid concentration in plasma was significantly higher in the vitamin group than in the control group at 2 weeks after initiation of therapy and thereafter (51.8 \pm 18.9 nmol/mL vs 50.9 \pm 16.3 nmol/mL at 0 weeks, 68.8 \pm 14.1 nmol/mL vs 45.7 \pm 15.0 nmol/mL at 2 weeks, *P* = 0.0001; 72.3 \pm 14.7 nmol/mL vs 50.9 \pm 12.6 nmol/mL at 4 weeks, *P* = 0.0001; 72.4 \pm 14.6 nmol/mL vs 49.7 \pm 14.3 nmol/mL at 8 weeks, *P* = 0.0001; 80.6 \pm 25.1 nmol/mL vs 55.8 \pm 12.1 nmol/mL at 26 weeks, *P* = 0.008). In addition, the α -tocopherol concentration in erythrocytes and ascorbic acid concentration in plasma significantly increased at 2 weeks after initiation of therapy and thereafter as compared to baseline levels in the vitamin group (Wilcoxon signed rank test; Fig. 1).

Concentrations of ribavirin in plasma

Ribavirin dose/kg bodyweight was significantly correlated with its concentration at 8 weeks after initiation of therapy (*r* = 0.43,

Table 1 Baseline characteristics of patients who were treated without discontinuance until at least 8 weeks after initiation of therapy

	Control group (<i>n</i> = 17)	Vitamin group (<i>n</i> = 14)	<i>P</i>
Age	52.0 \pm 11.0	56.0 \pm 7.9	0.28
Gender (M/F)	7/10	9/5	0.20
ALT (IU/L)	122 \pm 75	87 \pm 50	0.14
Hemoglobin (g/dL)	13.7 \pm 1.3	14.0 \pm 1.1	0.50
Genotype (1a/1b/2a/2b)	0/11/5/1	1/9/4/0	0.56
Viral load (KIU/mL)	603 \pm 238	473 \pm 290	0.18
Histology			
Staging (1/2/3/ND)	2/12/2/1	3/4/2/5	0.86
Grading (1/2/3/ND)	4/7/5/1	4/4/1/5	0.07
Ribavirin dose/kg	11.4 \pm 1.4	11.8 \pm 1.1	0.39

ALT, alanine aminotransferase; ND, not done.

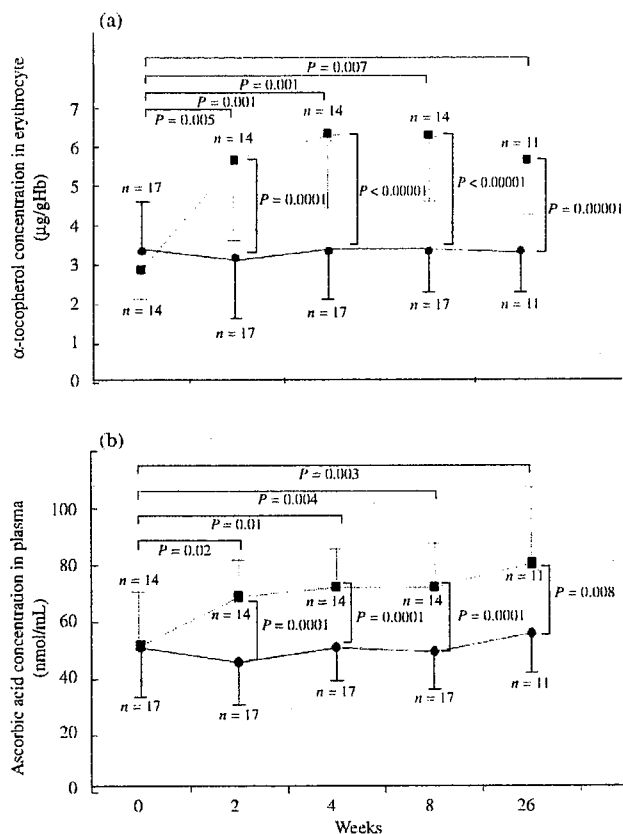


Figure 1 (a) Serial changes of α -tocopherol concentrations in erythrocytes and (b) ascorbic acid concentrations in plasma during combination therapy of interferon with ribavirin. Patients in the vitamin group received daily oral vitamins (500 mg of α -tocopherol and 750 mg of ascorbic acid) throughout the treatment period. Concentrations at each time point were compared by the Student's *t*-test between the (●) control group and (■) vitamin group. Concentrations at each time point also were compared with those at baseline in each group using the Wilcoxon signed rank test.

$P = 0.017$; Fig. 2a). Ribavirin concentrations in plasma were not different between both groups at 2, 4, 8, and 26 weeks after initiation of therapy (1515 ± 421 ng/mL in control group vs 1671 ± 474 ng/mL in vitamin group at 2 weeks, 2056 ± 7981 ng/mL vs 2379 ± 666 ng/mL at 4 weeks, 2218 ± 841 ng/mL vs 2767 ± 699 ng/mL at 8 weeks, 1869 ± 1027 ng/mL vs 2444 ± 862 ng/mL at 26 weeks).

Changes in hemoglobin level

The hemoglobin level, except for that at 2 weeks after initiation of therapy in the vitamin group, decreased significantly at 2 weeks after initiation of therapy and thereafter in both groups as compared to the baseline. However, there was no difference in the hemoglobin level or the degree of decrease from the baseline level throughout the treatment period between the groups.

The hemoglobin level was negatively correlated with the ribavirin concentration at 8 weeks after initiation of therapy in controls

($r = 0.59$, $P = 0.01$; Fig. 2b), but not in the vitamin group ($r = 0.35$, $P = 0.22$). There were also a tendency of negative correlation, but not significant, between two parameters at the end of therapy in controls ($r = 0.54$, $P = 0.08$; Fig. 2d), but not in the vitamin group ($r = 0.07$, $P = 0.83$; Fig. 2e).

Changes in serum alanine aminotransferase levels

The serum ALT level gradually decreased as compared to the baseline level in both groups, but there was no difference in the ALT level throughout the treatment period between the groups (54 ± 32 IU in controls vs 43 ± 31 in the vitamin group at 2 weeks, 33 ± 16 IU vs 32 ± 29 IU at 4 weeks, 27 ± 10 IU vs 29 ± 32 IU at 8 weeks, 17 ± 6 IU vs 25 ± 17 IU at 12 weeks, 17 ± 6 IU vs 52 ± 111 IU at 16 weeks, 18 ± 7 IU vs 21 ± 11 IU at 20 weeks, and 17 ± 8 IU vs 22 ± 16 IU at the end of therapy).

Response to interferon and ribavirin combination therapy

Eleven patients in the control group and 13 patients in the vitamin group completed the scheduled combination therapy, even though the dose of ribavirin was reduced by 200 mg/day at some period in four in the control group and in two in the vitamin group, respectively. Five patients in the control group (45.5%) and six patients in the vitamin group (46.2%) were sustained virological responders.

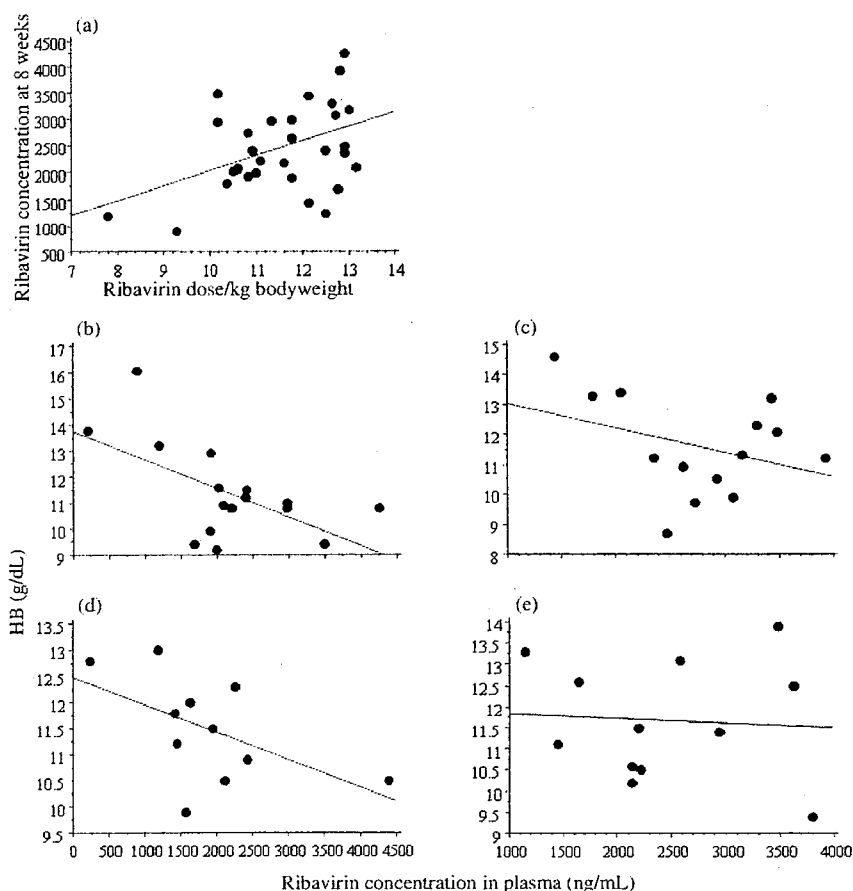
Fatty acid composition in erythrocyte membrane

Fatty acid analysis was performed for 32 patients (14 in the vitamin group and 18 in the control group) at the beginning of therapy; for 31 patients (14 in the vitamin group and 17 in the control group) at 2, 4 and 8 weeks after initiation of therapy; and for 22 patients (11 each in both groups) at the end of therapy, respectively. Among the 26 kinds of fatty acids, only the eicosapentaenoic acid (EPA; 20:5n-3 polyunsaturated fatty acid) level significantly decreased at 8 weeks after initiation of therapy (0.92 ± 0.48 mol% vs 1.18 ± 0.69 mol%, $P = 0.03$, $n = 17$, Wilcoxon signed rank test) and at the end of therapy (0.99 ± 0.57 mol% vs 1.39 ± 0.76 mol%, $P = 0.004$, $n = 11$, Wilcoxon signed rank test) as compared to the baseline level in controls, but not in the vitamin group (Fig. 3). The EPA level was negatively correlated with the drop in the hemoglobin level at 8 weeks after initiation of therapy in controls ($r = 0.58$, $P = 0.015$), but not in the vitamin group ($r = 0.30$, $P = 0.29$; Fig. 4).

Discussion

Combination therapy for pegylated IFN with ribavirin for 48 weeks is accepted as an optimal therapy for chronic hepatitis patients with HCV 1b infection. At the initiation of the present study, however, pegylated IFN was not available and the duration of combination therapy of IFN with ribavirin was limited to 6 months in the Japan health system. Also, ribavirin is dosed at 600 mg/day for those weighing <60 kg and 800 mg/day for those weighing >60 kg in Japan. Therefore, the patients were treated

Figure 2 (a) Correlation of ribavirin dose/kg bodyweight and its concentration at 8 weeks after initiation of therapy, and (b–e) negative correlation between hemoglobin level and ribavirin concentration in patients without supplementation of α -tocopherol and ascorbic acid. (a) Ribavirin dose/kg bodyweight was significantly correlated with its concentrations at 8 weeks after initiation of therapy ($r = 0.43$, $P = 0.017$). (b) The hemoglobin level was negatively correlated with the ribavirin concentration at 8 weeks after initiation of therapy in 16 controls ($r = 0.59$, $P = 0.01$), but not in (c) 14 patients with vitamin supplementation ($r = 0.35$, $P = 0.22$). Because the ribavirin concentration at 8 weeks after initiation of therapy was not determined in one control, the statistical significance of the correlation at 8 weeks after initiation of therapy was analyzed in 16 controls. There were also a tendency of negative correlation, but not significant, between two parameters at the end of therapy in (d) controls ($r = 0.54$, $P = 0.08$), but not in (e) the vitamin group ($r = 0.07$, $P = 0.83$).



with the present regimen, but there was no difference in ribavirin dose/kg bodyweight between both groups.

It is very important to assess the compliance with orally administered drugs when we evaluate their effectiveness. As shown in Fig. 1, we found a significant increase in α -tocopherol concentration in erythrocytes and ascorbic acid concentration in plasma at 2 weeks after initiation of therapy and thereafter in patients who received daily oral vitamins. In contrast, the ribavirin concentration in plasma did not differ between the groups throughout the treatment period. The ribavirin concentration in plasma, which has been shown to be one of the factors influencing ribavirin-induced anemia,^{20,21} was negatively correlated with the hemoglobin level at 8 weeks after initiation of therapy in the control group (Fig. 2), even though we did not find a significant correlation at the end of therapy due to a small number of patients. These results indicated good compliance with orally administered vitamins and/or ribavirin in both groups.

Membrane phospholipids of erythrocytes consist of mainly phosphatidylcholine and phosphatidylethanolamine, both of which are rich in polyunsaturated acids and thus seem to be highly susceptible to peroxidation,^{22,23} and this peroxidation may directly relate to oxidative damage of the erythrocyte membrane. When we consider the report of Franceschi *et al.* that ribavirin treatment *in vitro* induced increases in erythrocyte malondialdehyde and methemoglobin levels,²⁴ the decrease of EPA content in

erythrocyte membrane phospholipids was probably due to peroxidation. We confirmed that increased hepatic expression of 4-hydroxy-2-hexenal, a potent cytotoxic aldehyde originating from the peroxidation of n-3 polyunsaturated fatty acids,²² including EPA, was correlated with a decrease of EPA content in liver tissues of chronic hepatitis C patients.²³ This also supports the decrease of EPA in erythrocytes due to its peroxidation. Thus, the present study provides evidence for membrane oxidative damage induced by ribavirin, apart from alternations of the erythrocyte membrane such as aggregation of band 3 with binding of specific IgG in relation to oxidative damage.²⁴ Supplementation of α -tocopherol and ascorbic acid did not inhibit ribavirin-induced anemia, but tended to lower the frequency of dose reduction of ribavirin during combination therapy (14.3% in the vitamin group and 47.1% in the control group; $P = 0.052$) and the rate of therapy discontinuance (7.1% in the vitamin group vs 35.3% in the control group; $P = 0.06$). The lower frequency of dose reduction of ribavirin or therapy discontinuance, even though it did not necessarily result from the inhibition of ribavirin-induced anemia, appeared to be of clinical importance because adherence to combination therapy has been shown to enhance the sustained virological response in genotype-1-infected patients with chronic hepatitis C.⁸ In addition, supplementation of α -tocopherol and ascorbic acid attenuated the decrease of EPA in erythrocytes during combination therapy (Fig. 3). Because it has been demonstrated that administration of

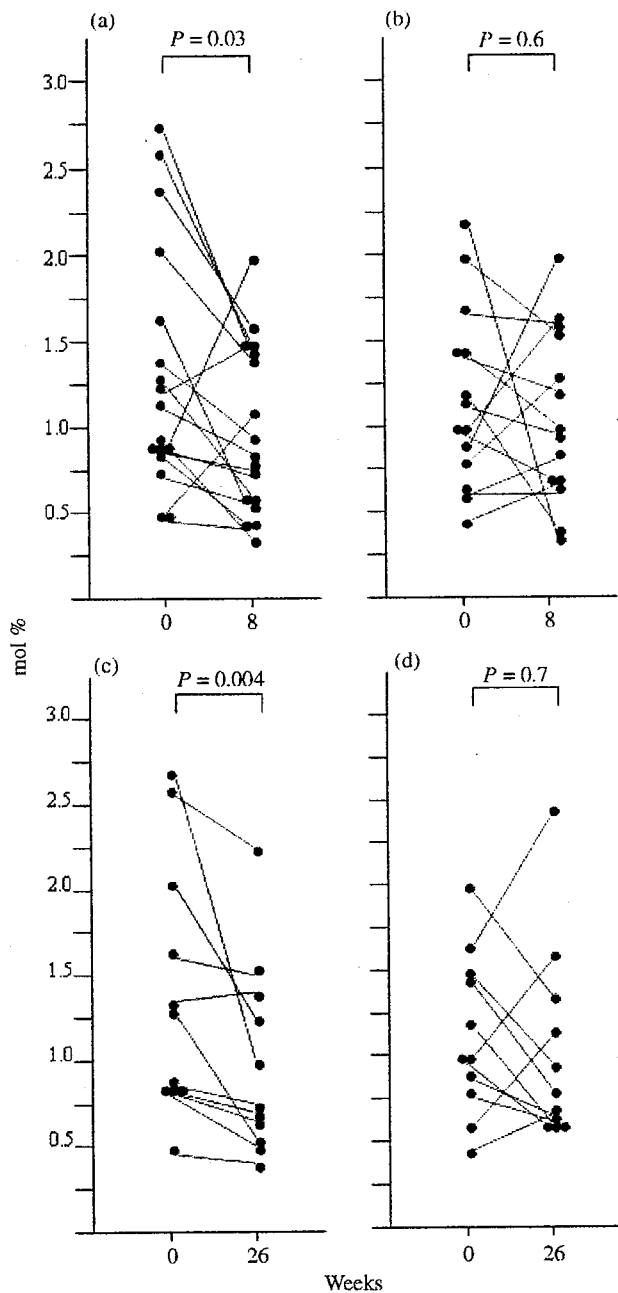


Figure 3 Changes of eicosapentaenoic acid (EPA) content in erythrocyte membrane phospholipids during and at the end of combination therapy of interferon with ribavirin. (a,c) Control group; (b,d) vitamin group. The EPA content significantly decreased at 8 weeks after initiation of therapy and at the end of therapy in (a,c) patients without vitamin supplementation, but (b,d) not in those with it (Wilcoxon signed rank test).

EPA increases erythrocyte fluidity in humans,²⁵ a negative correlation between the EPA level in the erythrocyte membrane and the drop in the hemoglobin level in controls, as shown in Fig. 4, suggested the potential usefulness of EPA supplementation as a

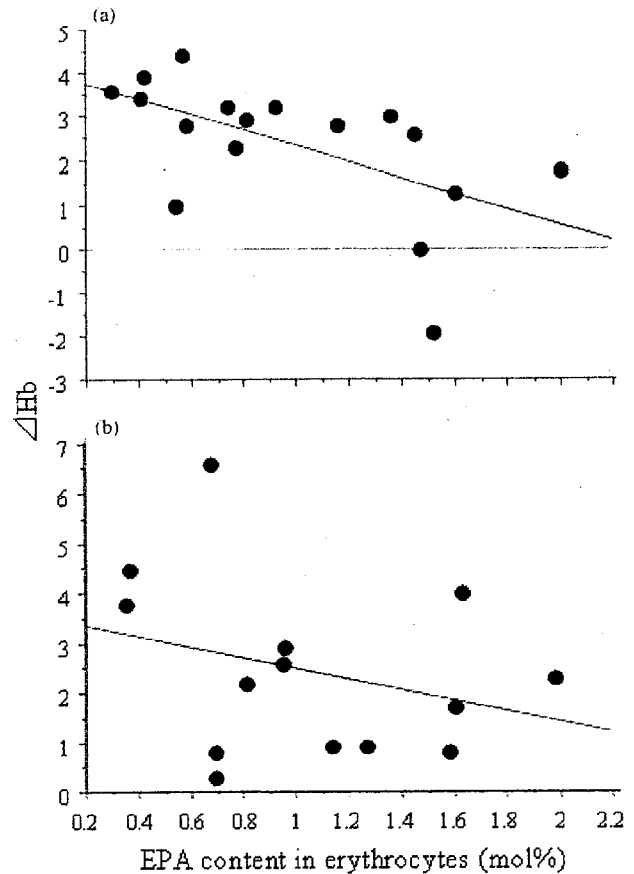


Figure 4 Negative correlation between eicosapentaenoic acid (EPA) content in erythrocytes and the drop in hemoglobin level at 8 weeks after initiation of combination therapy of interferon with ribavirin. The EPA level was negatively correlated with the drop in the hemoglobin level at 8 weeks after initiation of therapy in (a) controls ($r = 0.58$, $P = 0.015$), but not in (b) the vitamin group ($r = 0.30$, $P = 0.29$). Δ Hb: Hemoglobin (Hb) level at baseline minus Hb level at 8 weeks after initiation of therapy.

therapeutic tool for preventing ribavirin-induced anemia. Mabile *et al.* reported that n-3 polyunsaturated fatty acid incorporation into erythrocyte membranes by fish oil consumption increased the resistance to *ex vivo* oxidative stress-induced hemolysis.²⁶ And a pilot study demonstrated that oral administration of ethyl ester EPA (1800 mg/day) for 2 months increased the hemoglobin level in patients with chronic hepatitis C, who had developed anemia while receiving combination therapy consisting of IFN with ribavirin.²⁷ However, we need to determine the adequate dose and duration of EPA administration in further studies, because excess intake of polyunsaturated fatty acids (i.e. EPA and docosahexaenoic acid [22:6n-3] in fish oil) has been shown to lead to acceleration of membrane lipid peroxidation of erythrocytes in mice.²⁸

In conclusion we have shown a decrease of EPA content in erythrocyte membrane phospholipids in patients with chronic hepatitis C who developed anemia while receiving combination therapy consisting of IFN with ribavirin, and that supplementation of

α -tocopherol and ascorbic acid attenuated a decrease of EPA in erythrocyte membrane phospholipids. These results indicate the potential usefulness of EPA supplementation combined with α -tocopherol and ascorbic acid as a therapeutic tool for preventing ribavirin-induced anemia.

Acknowledgments

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (No. 15590653), and in part by grants from the Ministry of Health, Labor and Welfare, Japan.

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Timing of interferon therapy and sources of infection in patients with acute hepatitis C

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Received 13 June 2005; received in revised form 22 August 2005; accepted 31 August 2005

Available online 15 December 2005

Abstract

Background/Aims: Controversy over the selection of patients and optimum therapeutic method for acute hepatitis C has continued. The aims of this study were to investigate the source of infection, and to evaluate the timing of interferon (IFN) therapy in patients with acute hepatitis C in Japan.

Methods: The records of 102 patients from 12 facilities in Japan who developed acute hepatitis C after 1990 were investigated. In the patients treated with IFN, we performed multivariate analysis to investigate factors related to sustained virological response (SVR).

Results: Medical procedure was the most common source of infection, accounting for 32.4% in the 102 patients (33/102). Of 81 patients treated with IFN, 71 patients were followed after IFN therapy, and 57/71 (80.3%) had SVR. The SVR rate was significantly higher in patients treated with IFN within 24 weeks from onset of symptoms than the SVR rate in those treated after 25 weeks ($P=0.0016$). Multivariate analysis revealed that only the duration between onset of symptoms and initiation of IFN therapy (within 24 weeks) was related to SVR.

DOI of related article: 10.1016/j.hepres.2005.10.002.

Abbreviations: HCV, hepatitis C virus; IFN, interferon; ALT, alanine aminotransferase; SVR, sustained virological response; Peg-IFN, pegylated interferon

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Conclusions: Our multicenter cooperative survey revealed that medical procedure was the most frequent source of infection in acute hepatitis C. As concerns the therapy, interferon treatment should be initiated within 24 weeks after onset of symptoms.
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Keywords: Hepatitis C virus (HCV); Acute hepatitis; Medical procedure; Interferon

1. Introduction

There are about 170 million people infected with the hepatitis C virus (HCV) worldwide [1], and the infection progresses to hepatic cirrhosis in 10–30% [1,2]. Since patients often lack subjective symptoms even in acute hepatitis C [3], infection is often realized by patients when the pathology progresses to hepatic cirrhosis and hepatocellular carcinoma. There are a variety of sources of infection, such as medical procedure, intravenous drug use, and sexual behavior [4,5]. In addition, vertical transmission of HCV has been reported, and it seems that maternal viral load is significant for infection to fetus [6]. On the other hand, as a therapy for acute hepatitis C, interferon (IFN) administration has been established to be effective [4,5,7–13].

Although the initial prevention of hepatitis C virus (HCV) infection is ideal, the most effective method of preventing progression to the chronic hepatitis C is still controversial in the acute phase. In Japan, the development of acute hepatitis C due to blood transfusion has markedly decreased after introduction of the HCV antibody test for screening of blood donors [14]. However, infection from intravenous (i.v.) drug use and incidences due to accidental contamination of medical staff are still important problems [15,16]. Investigation for the sources of infection in acute hepatitis C is very important for the prevention. In this study, we investigated a national survey on the route of infection of acute hepatitis C and the therapeutic effectiveness according to the timing of IFN therapy. This survey consists of the largest number of case reports and may reflect the current situation of acute hepatitis C in Japan.

2. Patients and methods

2.1. Patients

A retrospective study was performed in patients of 12 facilities nationwide who developed acute hepatitis C after 1990. The total number of patients at the facilities was 102. Informed written consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Age, gender, source of infection, HCV serotype or genotype, HCV-RNA level, histology of liver biopsy, fluctuation in alanine aminotransferase (ALT) level, presence or absence of IFN therapy, course when not treated with IFN, duration between onset of symptoms and IFN therapy, type of IFN, total dose of IFN, administra-

tion method, total duration of administration, and therapeutic results were investigated in each patient.

2.2. Diagnosis of acute hepatitis C

The diagnostic criteria of acute hepatitis C were HCV-RNA detectable at the time of an elevated ALT level, followed by development conversion of HCV antibody. Patients in whom HCV antibody was already positive at the onset were excluded.

2.3. Natural course

In patients who followed the natural course without any treatments, the chronic hepatitis was defined as persistence of HCV-RNA positivity for 6 months or longer, and resolution was defined as a disappearance of serum HCV-RNA within 6 months followed by persistent negativity for 6 months or longer.

2.4. Definition of fluctuation of ALT

In patients diagnosed with acute hepatitis C, when one peak of the serum ALT level was observed, the fluctuation was designated as monophasic, and when two or more peaks were observed, the fluctuation was designated as bi- or multiphasic.

2.5. Serologic tests

Anti-HCV antibody was determined using a second-generation or third-generation enzyme-linked immunosorbent assay (Ortho Diagnostics Systems, Tokyo, Japan). Hepatitis C virus RNA was quantified by using the bDNA signal amplification assay (Chiron Corp.) or the Cobas Amplicor HCV Monitor test ver1.0 or 2.0 (Roche Diagnostic Systems, Tokyo, Japan). The data were represented as Meq/ml, K copies/ml, and KIU/ml, respectively. Detection of HCV-RNA to determine the response of IFN treatment was used by Amplicor HCV (Roche Diagnostics K.K., Japan). Hepatitis C virus serotype was determined using the genotyping enzyme-linked immunosorbent assay (International Reagents Corporation, Tokyo, Japan) to be type 1 or 2 [17].

2.6. IFN therapy

For IFN, IFN- α (natural form, gene recombinant, or consensus IFN), or IFN- β was used (Table 4). No concurrent treatment with IFN and ribavirin was administered to any patient. Among patients treated with IFN, the sustained

virological response (SVR) was defined undetectable HCV-RNA in serum at least 6 months after cessation of therapy. Non-response was defined as detectable HCV-RNA for 6 months after cessation of therapy.

2.7. Statistical analysis

Data were expressed as the mean \pm standard deviation for continuous variables and as counts for categorical variables. The results were compared using the Chi-square test, Fisher's exact probability test, or Mann-Whitney *U*-test, depending upon the type of data analysed. Logistic regression was used to analyse the factors contributing to SVR with IFN therapy. *P* values <0.05 were considered significant. Statistical analyses were performed by using Stat View software (version 5.0; SAS Institute Inc., Cary, NC).

3. Results

3.1. Patient characteristics

The baseline characteristics of the 102 patients in this study are shown in Table 1. The distribution of patients by gender and age is shown in Table 2.

3.2. Natural course

The natural course of the disease was followed in 21 patients, and the course could be followed to the outcome

Table 1

Base-line characteristics of 102 patients

Age	38.6 \pm 16.2 (16–84)
Male/female (mean age)	46 (39.2 \pm 16.0)/56 (38.2 \pm 16.5)
Source of infection (%)	
Medical procedure	33 (32.4)
Accidental needle stick	21 (20.6)
Sexual behavior	8 (7.8)
Drug abuse	6 (5.9)
Tattoo	3 (2.9)
Unknown	31 (30.4)
Viral load (high ^a /low/N.D.)	46/45/11
HCVserotype(1/2/N.D.)	54/23/25
IFN/without IFN	81/21

N.D., not determined; IFN, interferon. Details of the routes in medical procedure: surgery 14, blood transfusion 5, endoscopy 3, intravenous injection 4, invasive procedure 3, dental therapy 3, dialysis 1.

^a Viral load (high): more than 100 KIU/ml or 1 Meq/ml.

in 18 patients (the prognosis was unknown in three patients) (Table 3). The disease progressed to chronic hepatitis C in 61.1% of the patients and resolved spontaneously in 38.9% of the patients. The age and the fluctuation pattern of the ALT level were significantly different between the two groups. As for gender, serum HCV-RNA level, and serogroup, no correlation with spontaneous resolution or chronic hepatitis C was observed.

3.3. IFN therapy

Table 4 shows the backgrounds of the 81 patients treated with IFN. Of 71 patients in whom the effect was clarified,

Table 2

Distribution of patients according to gender and age

Age (years)	Number of patients					
	Medical procedure (M/F)	Accidental needlestick (M/F)	Sexual behavior (M/F)	Drug abuse (M/F)	Tattoo (M/F)	Unknown (M/F)
<19	0/1	0/0	0/0	0/1	0/0	0/1
20–29	5/1	3/8	1/3	2/1	3/0	2/6
30–39	4/3	3/3	2/1	0/1	0/0	3/3
40–49	2/4	0/4	1/0	0/1	0/0	2/3
50–59	4/3	0/0	0/0	0/0	0/0	2/3
60–69	4/1	0/0	0/0	0/0	0/0	2/0
70–79	0/0	0/0	0/0	0/0	0/0	1/1
>80	0/1	0/0	0/0	0/0	0/0	0/2
Total	19/14	6/15	4/4	2/4	3/0	12/19

M, male, F, female.

Table 3

Base-line characteristics of 18 untreated patients

	Resolved group (seven cases)	Chronic group (11 cases)	<i>P</i> value
Age	64.4 \pm 15.2	45.6 \pm 14.3	0.0331 ^a
Gender (male/female)	2/5	4/7	>0.9999
HCV RNA level (high ^b /low/N.D.)	2/4/1	6/4/1	0.6084
Serogroup (1/2/N.D.)	4/0/3	4/2/5	0.4667
Fluctuation of ALT level (monophasic/bi- or multiphasic/N.D.)	5/0/2	0/8/3	0.0008 ^a

N.D., not determined; ALT, alanine aminotransferase. Fluctuation of ALT level: monophasic; one peak of the serum ALT was observed, bi- or multiphasic; two or more peaks of the serum ALT were observed (N.D. was excluded from statistical comparisons).

^a Statistically significant.

^b Viral load (high): more than 100 KIU/ml or 1 Meq/ml.

Table 4
Base-line characteristics of 81 patients treated with interferon

Age	38.6 ± 16.2
Gender (male/female)	43/38
HCV RNA level (high ^a /low/N.D.)	38/36/7
HCV serogroup (1/2/N.D.)	46/21/14
Fluctuation of ALT level (monophasic/bi- or multiphasic/N.D.)	21/53/7
Type of IFN (α/β)	63/18
Total IFN dose (MU)	470 ± 228.1 (52–972)
Duration of IFN administration (w)	17.6 ± 8.9 (4.0–42.0)
Outcome (SVR ^b /NR/N.D.)	57/14/10

N.D., not determined; ALT, alanine aminotransferase; IFN, interferon; MU, million units; SVR, sustained virological response; NR, non-response: detectable HCV RNA in serum for 6 months after cessation of therapy.

^a HCV RNA level (high): more than 100 KIU/ml or 1 Meq/ml.

^b Sustained virological response: undetectable HCV RNA in serum at least 6 months after cessation of therapy.

57 patients (80.3%) had SVR. Table 5 shows the results of the logistic regression analysis of SVR-related factors. Age, gender, serogroup, HCV-RNA level, fluctuation of ALT, duration between onset and initiation of IFN, type of IFN, total IFN dose, and duration of IFN administration were evaluated statistically by univariate and multivariate analysis. On multivariate analysis as well as univariate analysis, the duration between onset of symptoms and initiation of IFN therapy was the only factor related to SVR.

The SVR rate according to the duration before initiation of IFN therapy was investigated (Fig. 1), and the SVR rate was found to be significantly higher in patients treated before 24 weeks than in patients treated after 25 weeks. However, immediate administration has not been associated with higher SVR rate (0–8 weeks versus 9–24 weeks).

On comparison of the SVR rate by the source of infection, the SVR rate was 100% in the patients infected by accidental needlestick (19/19) (the prognosis was unknown in two of 21 patients infected by needlestick). This was significantly higher than that in patients infected via other routes (19/19

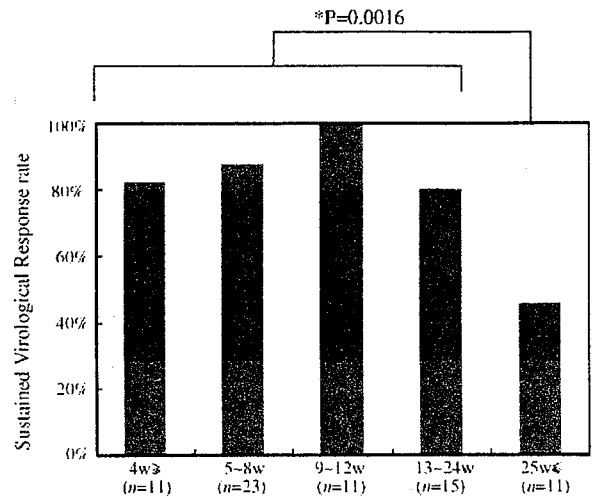


Fig. 1. Sustained virological response rate according to duration between onset of symptoms and initiation of IFN therapy. The groups treated with IFN 0–24 weeks after onset of symptoms and treated after 25 weeks were compared. Comparison by the Chi-square test. (*) Statistically significant; w, week.

versus 38/52, $P < 0.05$). The duration between onset of symptoms and initiation of IFN therapy was investigated according to the source of infection, and the duration was shortest in the needlestick group (9.7 ± 5.3 weeks).

4. Discussion

We examined the source of infection and optimal timing of therapy in patients with acute hepatitis C at 12 facilities in Japan. Since there has been no study performed in more than 100 patients with acute hepatitis C in Japan, this study may reflect the current situation in Japan. HCV serogroup of 25 patients were not determined (Table 1). Several reasons are considered. Firstly, the study is retrospective. Secondly,

Table 5
Logistic regression analysis of odds ratio for sustained virological response

Variable	Odds ratio	95% CI	P value
Univariate			
Age(40>/40≤)	2.48	0.73–8.46	0.147
Gender (female/male)	2.48	0.74–8.33	0.143
Serogroup (1/2)	1.03	0.23–4.54	0.969
HCV RNA level (high ^a /low)	1.75	0.46–6.68	0.413
Fluctuation of ALT (monophasic/bi- or multiphasic)	1.57	0.38–6.45	0.531
Duration between onset and initiation of IFN (≤24w/≥25w)	7.50	1.85–30.48	0.005 ^b
Type of IFN (alpha/beta)	4.33	0.52–36.18	0.176
Total IFN dose (>300MU/≤300MU)	2.27	0.63–8.15	0.208
Duration of IFN administration (≥24w/<24w)	1.43	0.44–4.67	0.551
Multivariate			
Duration between onset and initiation of IFN (≤24w/≥25w)	15.78	1.37–181.61	0.027 ^b

ALT, alanine aminotransferase; IFN, interferon; MU, million units; 95% CI, 95% confidence interval.

^a HCV RNA level high: More than 100 KIU/ml or 1 Meq/ml.

^b Statistically significant.

titer of anti-HCV is often low in early phase of acute hepatitis C. Many patients were considered to be infected during a medical procedure. Studies on risk of surgery for the development of acute hepatitis C have been reported previously [18]. Alfonso et al. performed a large-scale surveillance in Italy and found that 25.5% of patients (261/1023) with acute hepatitis C had undergone an invasive procedure. Therefore, medical care should be recognized as an important source of infection in the sporadic incidence of acute hepatitis C. On the other hand, in blood donors of Western Mexico, the most frequent risk factors for HCV transmission were transfusion (42%) and household exposure (14.8%) [19]. Therefore, the main risk factors for infection may differ with countries.

Since IFN therapy for acute hepatitis C is not covered by the health care insurance, the therapy could not be administered to all patients. The progression to the chronic hepatitis C in the 18 patients with natural courses without IFN therapy was almost consistent with previous reports [20,21]. As shown in Table 3, a significant difference was observed in age, but this may have been due to the two patients in their 80s in the spontaneous resolution group (data not shown). The important point is that the ALT fluctuation was monophasic in all patients in the spontaneous resolution group. In contrast, the fluctuation was bi- or multiphasic in patients who progressed to chronic hepatitis C. As a characteristic of acute hepatitis C in which spontaneous elimination of the virus is likely to occur, it has been reported that many cases are accompanied by subjective symptoms, such as jaundice and influenza-like symptoms [22,23]. Subjective symptoms are sometimes influenced by the patient's subjective sense. In contrast, the fluctuation of the ALT level may be a more objective index. Hofer et al. observed the natural course for at least 30 days after onset, and when serum HCV-RNA became negative during this period, the disease was resolved at a high rate, suggesting that IFN therapy should be administered to patients in whom negative conversion of HCV-RNA did not occur within 30 days [22]. Combined with our results, it might be likely that the disease resolves spontaneously in patients in whom the ALT level followed the monophasic course, as well as in those in whom the disease is symptomatic and negative conversion of HCV-RNA occurs in the early stage.

As the results of IFN therapy, the SVR rate was 80.3% (57/71) as shown in Table 4. Our present study, albeit retrospective analysis, revealed that therapy initiated within 24 weeks was the only factor related to the SVR in both univariate and multivariate analysis (Table 5). In the randomized controlled study by Hwang et al., the factor related to SVR was the HCV-RNA level before initiation of therapy [9]. However, there were only 33 patients, which may have led to a result different from our results. On the other hand, Nomura et al. recently performed a randomized controlled trial in patients with acute hepatitis C, and their results demonstrate that the SVR rate was significantly higher in the early-intervention group (IFN therapy was initiated 8 weeks

after the onset) than in the late-intervention group (IFN therapy was initiated after 1 year observation from the onset) (87% versus 40%) [24]. Otherwise, Gruner et al. prospectively investigated the T-cell dynamics in patients with acute hepatitis C, and found that activity of HCV-specific IFN- γ -producing T cells started to decrease 24 weeks after onset [25]. In addition, T cell actions have been reported to be important for elimination of HCV in the early stage of infection [26–30], and the defective functions of HCV-specific T cells might contribute to viral persistence in chronically infected patients [31]. It is interesting that our results support their reports.

Next, we evaluated the optimal timing of initiation of therapy within 24 weeks. In our previous study, we administered therapy after observation of the course for about 4 weeks when signs of the chronic hepatitis began to appear, not immediately after the onset, and obtained good results [32,33]. Licata et al. investigated the optimum timing of IFN therapy by meta-analysis [34]. Their analysis shows that delaying therapy 2 months after the onset of the disease does not affect the efficacy of treatment, therefore, they suggest that patients should be treated within 60 days from the onset to avoid the unnecessary treatment of affected patients who would spontaneously recover. In our study, the highest SVR rate was obtained in the group treated 9–12 weeks after onset of symptoms as shown in Fig. 1, which was consistent with their analytical results.

The SVR rate obtained by combination therapy with Pegylated-IFN (Peg-IFN) and ribavirin for chronic hepatitis C was 30–54% [35–37], but for acute hepatitis C, the therapeutic result was good even when IFN was administered alone. To elucidate this difference, it may be important to investigate not only the T-cell dynamics but also viral genome in various aspects [7]. In our present study, no patients were treated with Peg-IFN. Recently, the efficacy of Peg-IFN monotherapy with acute hepatitis C has been reported. Santantonio et al. evaluated the delaying Peg-IFN therapy, targeting sixteen patients who failed to spontaneously clear the virus within 12 weeks from the onset. They reported that 15/16 patients (94%) showed SVR [38]. Since the highest SVR was obtained in the group treated 9–12 weeks after onset in our study, it is important to start the IFN therapy in optimal timing regardless of the kind of IFN. The high SVR has been obtained by IFN monotherapy, so that, it is necessary to investigate whether ribavirin should be administered concurrently with IFN.

In conclusion, the major sources of infection of acute hepatitis C in Japan were the medical procedure and accidental needlestick. The disease may be likely to resolve spontaneously in patients in whom fluctuation of the ALT level follows the monophasic course. The SVR rate was significantly higher in the group treated with IFN within 24 weeks after the onset of symptoms than in the group treated after 25 weeks. In cases of acute hepatitis C, it is desirable to administer IFN at least within 24 weeks when the ALT level starts to follow a multiphasic course.

Acknowledgments

This study was supported, in part, by a grant-in-aid from the Ministry of Health, Welfare, and Labour of Japan. The authors thank Kendo Kiyosawa, M.D., Keisuke Hino, M.D., Kyosuke Kaji, M.D., Akihiro Iwamitsu, M.D., Tomoo Naito, M.D., Yasuhito Tanaka, M.D., for assistance with data collection.

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Applied nutritional investigation

Vitamin E and C supplementation prevents decrease of eicosapentaenoic acid in mononuclear cells in chronic hepatitis C patients during combination therapy of interferon α -2b and ribavirin

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Manuscript received January 20, 2005; accepted May 9, 2005.

Abstract

Objective: We investigated the effects of vitamin E and C supplementation on the fatty acid composition of mononuclear cells and on the clinical observations in patients who had chronic hepatitis C and received interferon- α -2b (IFN- α -2b) and ribavirin combination therapy.

Methods: Patients were randomly allocated to receive daily 500 mg of vitamin E and 750 mg of vitamin C (vitamin group, $n = 14$) or no supplement (non-vitamin group, $n = 16$) in addition to IFN- α -2b and ribavirin therapy. The fatty acid composition of mononuclear cell phospholipids was analyzed before and at 2, 4, and 8 wk after treatment.

Results: After vitamin supplementation, plasma and red blood cell α -tocopherol and plasma ascorbic acid levels increased in the vitamin group. Serum levels of alanine aminotransferase decreased significantly after 2 wk of treatment in both groups. At the start of treatment, a lower level of eicosapentaenoic acid (EPA) and a higher level of the molar ratio of arachidonic acid to EPA in mononuclear cells were observed in the present patients compared with healthy volunteers, and a significant correlation between the molar ratio and serum alanine aminotransferase level was found. The EPA level of mononuclear cells was maintained in the vitamin group during treatment, whereas a significant decrease was observed in the non-vitamin group at 4 and 8 wk after treatment.

Conclusions: Antioxidant vitamin supplementation during IFN- α -2b and ribavirin therapy prevented a decrease in EPA of mononuclear cell phospholipids. If a further decrease in the ratio of arachidonic acid to EPA can be achieved by using oral EPA supplementation, the efficacy of IFN- α -2b and ribavirin therapy may be improved. © 2006 Elsevier Inc. All rights reserved.

Keywords:

Vitamin E; Vitamin C; Eicosapentaenoic acid; Arachidonic acid; Interferon; Ribavirin; Hepatitis C patients; Mononuclear cell

Introduction

Antiviral therapy using interferon- α -2b (IFN- α -2b) is an important component in the treatment of patients who have hepatitis C virus (HCV). IFN- α -2b therapy combined with

ribavirin, a purine nucleoside analog, is reported to induce a higher sustained viral response compared with IFN- α -2b monotherapy [1]. The principal mechanism of action of ribavirin in the combination therapy has been postulated to be that ribavirin serves as an immunomodulator to enhance the production of type I cytokines, including interleukin-2, IFN- γ , and tumor necrosis factor- α (TNF- α) [2]. However, hemolytic anemia is a known side effect of ribavirin therapy [3]. The mechanism by which ribavirin causes hemolysis remains uncertain but may be related to its accumulation in erythrocytes,

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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