switched to the combination of PEG-IFN and RBV in recent years, it is important to know if a larger dose of IFN is beneficial to patients with chronic hepatitis C.

Many molecular mechanisms through which HCV evades host innate immunity have been reported to date. HCV core, E2 and NS5A proteins have been reported to inhibit the IFN signaling system [Gale et al., 1997; Taylor et al., 1999; Blindenbacher et al., 2003; Bode et al., 2003; Foy et al., 2003; Lin et al., 2006; Ciccaglione et al., 2007]. Variations of amino acid (aa) sequences in the E2 and the NS5A region have been reported to correlate with the effect of IFN therapy [Enomoto et al., 1996; Chayama et al., 1997, 2000; Polyak et al., 1998, 2000; Hashimoto et al., 1999; Puig-Basagoiti et al., 2001; Pascu et al., 2004; Gaudy et al., 2005; Brillet et al., 2007; Torres-Puente et al., 2008]. Recently, Akuta et al. [2005, 2006, 2007a, b] reported that substitution of aa 70 and/or 91 in the core region is an independent and significant predictor of non-virological response.

The aim of the present study was to evaluate the therapeutic efficacy and safety of a large dose of IFN-α-2b combined with RBV. For this purpose, a randomized trial was conducted to compare the therapeutic effects of high-dose (10 MU) versus standard dose (6 MU) of IFN-α-2b combined with RBV in patients with high HCV viral titers. The second endpoint of this study was to analyze the predictive factors associated with virological response including aa substitutions in the core region and the NS5A region.

PATIENTS AND METHODS

Patient Selection

Two hundred adult patients enrolled into the study. The inclusion criteria were positivity for antibody to HCV, HCV RNA levels higher than 100 KIU/ml, and the diagnosis of chronic hepatitis C was confirmed by liver biopsy. The liver biopsy specimens were evaluated as described by Desmet et al. [1994], and classified into F0 to F3. None of the patients included in this study had liver cirrhosis (F4). Other exclusion criteria included leukocytopenia (leukocyte $<4{,}000/mm^3$) and anemia (hemoglobin concentration <10 g/dl). Patients with human immunodeficiency or hepatitis B super infection, previous organ transplantation, other causes of liver disease, poorly controlled diabetes, de-compensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, hemophilia or auto-immune type diseases were also excluded.

Study Design

The double-blind, multi-center randomized clinical trial was conducted in 23 centers in Hiroshima city (The Hiroshima Liver Study Group). The study was approved by the Ethics Committee of Hiroshima University. Written informed consent was obtained form all participants. Eligible patients were assigned randomly into either of the two groups without further stratification using sequentially numbered cards in sealed envelopes. Patients were randomized to treatment with combination of IFN-α-2b (Intron A, Shering Plough, Kenilworth, NJ) at a dose of 6 MU (Group A) or 10 MU (Group B) plus RBV (Rebetol, Shering Plough). IFN-α-2b was administered intramuscularly daily over the initial 2 weeks and three times weekly in the remaining 22 weeks. The dose of RBV was adjusted according to body weight (600 mg/day for ≤60 kg, 800 mg/day for >60 kg). Adverse events were monitored clinically by careful interview and hematological examination throughout the study. The dosage of RBV was reduced in patients who experienced a decrease in hemoglobin concentration to <10 g/dl.

Blood samples were taken 2 and 4 weeks after the beginning of therapy and every 4 weeks thereafter. Biochemical and hematological tests were performed in each center, including alanine amino transferase (ALT). Part of the serum samples were kept frozen at −80°C until further analysis. Viral genotypes were determined by phylogenetic analysis after reverse transcription (RT)-polymerase chain reaction (PCR) and direct sequencing.

Assessment of Efficacy

Serum HCV RNA was detected by nested PCR assay (Cobas Amplicor HCV test v 2.0, Roche Diagnostics, Tokyo, Japan; limit of detection, 50 IU/ml) at weeks 2, 4 and every 4 weeks during treatment and 24 weeks after the cessation of therapy. Positive samples were analyzed further by quantitative assay (Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics; limit of detection, 500 IU/ml).

The primary endpoint of this study was sustained virological response, defined as undetectable serum HCV RNA by qualitative PCR test and normalization of ALT 24 weeks after the treatment. Non-virological response was applied to those patients with positive qualitative HCV RNA PCR tests in all examinations. Virological response was used to define the remaining patients who became PCR negative at least once during the treatment.

Nucleotide Sequencing of the Core and NS5A Gene

The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR] [Enomoto et al., 1996]) sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: The first-round PCR was performed with primers CC11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and the second-round PCR with primers CC9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse) as described by Akuta et al. [2005, 2006, 2007a, b]. After denaturation at 95°C for 5 min, 35

cycles of amplification were set as follows; denaturation for 30 sec at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. (b) Nucleotide sequences of ISDR in NS5A: PCR was performed with IM11 (forward, 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5OA2KI (reverse, 5′-CCC GTC CAT GTG TAG GAC AT-3′). After denaturation at 98°C for 30 sec, 35 cycles of amplification were set as follows; denaturation for 10 sec at 98°C, annealing of primers for 30 sec at 66°C, and extension for 15 sec at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated in a 2% agarose gel and purified by GENECLEAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [Kato et al., 1990].

Quantitation of HCV Core Antigen

HCV core antigen levels were measured using stored serum samples just before and 4 weeks after the start of the therapy as described previously [Aoyagi et al., 1999].

Statistical Analysis

The baseline characteristics of the patients in the two groups were compared and the differences were assessed by Chi-square test with Yate's correction and Mann–Whitney *U*-test. To assess the sustained virological response rates, an intention-to-treat (ITT) analysis and a per-protocol (PP) analysis were conducted. The response rates and substitutions in the core region and the ISDR were compared by Fisher's exact test. All *P* values reported are two-sided and those less than 0.05 were considered significant. To determine the predictors of sustained virological and non-virological responses, univariate and multivariate logistic regression analyses were carried out. Potential predictive factors included the following variables: age, sex, alcohol consumption, past history of IFN monotherapy, body mass index, ALT, hemoglobin, platelets, HCV RNA level, genotype, liver histology, total RBV dose (adjusted for body weight [mg/kg]) and total dose of IFN-α-2b. The odds ratio and 95% confidence intervals (95% CI) were also calculated. Variables with 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. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

Patient Demographics

Patient enrollment started in January 2002, and the trial ended in March 2005. The disposition of patients throughout the trial is shown in Figure 1. A total of 200 patients were randomized to treatment, and 198 patients met the eligibility criteria and underwent

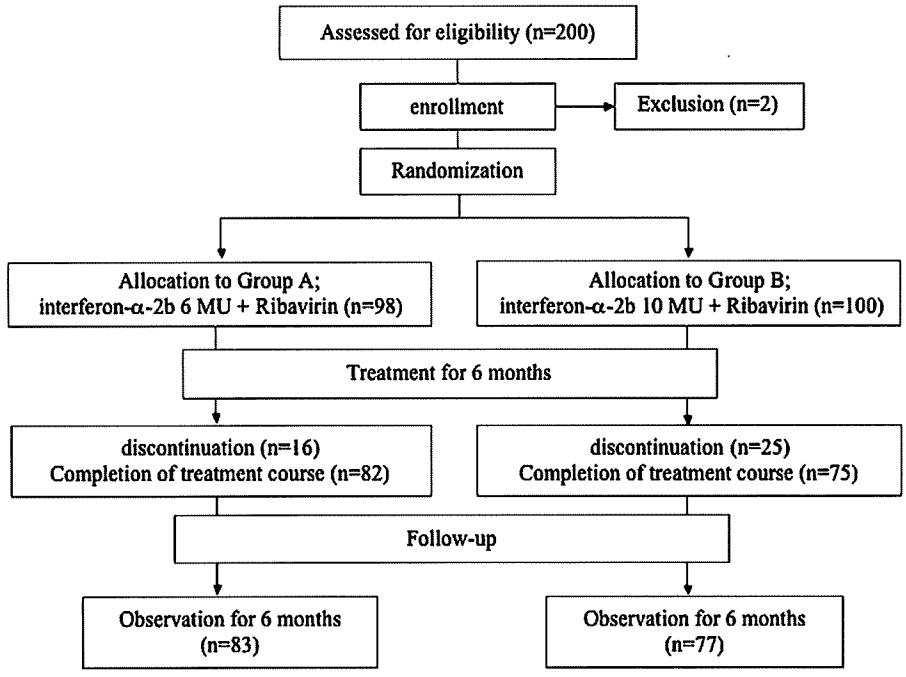

Fig. 1. Flow chart of number of patients throughout the trial. A total of 200 patients were included in this study. One hundred ninety-eight patients met the eligibility criteria and they underwent randomization, 98 patients in Group A and 100 patients in Group B.

TABLE I. Baseline Characteristics of the Patients

Characteristic	Group A (n = 98)	Group B (n = 100)	P
Age (years)[a]	55 ± 10.3	55 ± 11.0	0.43
Male sex (%)	63	75	0.07
Alcohol consumption (%)[b]	23	20	0.61
Past history of IFN monotherapy (%)	33	35	0.72
Body-mass index (kg/m^2)[a]	23.3 ± 2.9	24.2 ± 3.6	0.05
ALT (IU/L)[a]	79.2 ± 45.3	109.4 ± 111.2	0.31
Hemoglobin (g/dl)[a]	14.2 ± 1.4	14.5 ± 1.2	0.02
Platelets ($\times 10^4/mm^3$)[a]	14.8 ± 4.8	16.5 ± 5.0	<0.05
HCV RNA (KIU/ml) (%)			
100–850	49	47	
≥850	51	53	0.80
Genotype (%)			
1b	82	72	
2a/2b	17	28	0.32
3a/3b	1	0	
Liver histology[a,c]	2.0 ± 0.84	1.8 ± 0.82	0.05

ALT, alanine aminotransferase.
[a]Values are mean ± SD.
[b]Percentage of patients who consumed alcohol at >30 g/day.
[c]Liver fibrosis was scored 0 (F0), no fibrosis; 1 (F1), periportal expansion; 2 (F2), portoportal septa; 3 (F3), portocentral linkage or bridging fibrosis.

randomization. Ninety-eight patients were assigned to Group A and 100 patients to Group B. Patients were observed for 24 weeks after the treatment. Sixteen patients of Group A and 25 patients of Group B discontinued the treatment because of adverse events. Table I lists the baseline characteristics of the patients. Hemoglobin concentrations and platelet counts were higher in group B patients. The other parameters were similar between the two groups.

Overall Sustained Virological Response

The effect of therapy in the two groups is summarized in Table II. The sustained virological response rate was lower significantly in patients of group B with genotype 2a/b relative to those of group A (ITT analysis). This reflects the fact that a larger number of patients dropped out form the protocol because of the adverse effects (1 [6%] of 16 in group A and 10 [43%] of 23 in group B, $P = 0.02$). All patients who stopped treatment did not achieve sustained virological response. Patients with genotype 1b had a lower sustained virological response rate than those with genotype 2a/b (33/124 [27%] vs. 26/39 [67%], $P < 0.01$).

TABLE II. Rates of Sustained Virological Response According to Adherence

Genotype	Group A	Group B	P
1b	n = 68	n = 56	
ITT	16/68 (24%)	17/56 (30%)	0.39
PP	16/53 (30%)	17/41 (41%)	0.25
2a/b	n = 16	n = 23	
ITT	15/16 (94%)	11/23 (48%)	0.005
PP	15/15 (100%)	11/13 (85%)	0.21

ITT, intention to treatment analysis; PP, per protocol analysis; IFN, interferon; RBV, ribavirin.

Dose Reduction or Discontinuation and Adverse Events

Table III summarizes the laboratory abnormalities and the dose reduction and discontinuation of IFN-α-2b and RBV due to adverse events. The overall discontinuation rate was 16% for group A and 25% for group B (not significant). The most frequent adverse event associated with dose reduction was anemia. A larger number of patients of group B developed depression ($P = 0.02$).

Predictive Factors Associated With Sustained Virological Response

Univariate analysis identified three parameters that correlated with sustained virological response: age (<60 years, $P = 0.007$); genotype (2a/b, $P < 0.001$); and platelet count ($>15 \times 10^4/mm^3$, $P = 0.01$). Multivariate analysis including the above variables identified two parameters that independently predicted sustained virological response: age ($P = 0.02$) and genotype ($P < 0.001$) (Table IV).

TABLE III. Dose Reduction or Discontinuation and Adverse Events

	Group A (n = 98) % (n)	Group B (n = 100) % (n)	P
Discontinuation	16 (16)	25 (25)	0.13
Dose reduction or discontinuation of			
IFN	20 (20)	41 (41)	0.002
RBV	36 (35)	50 (50)	0.04
IFN and/or RBV	37 (36)	55 (55)	0.01
Depression	0 (0)	7 (7)	0.02

IFN, interferon; RBV, ribavirin.

TABLE IV. Factors Associated With Sustained Virological Response to Combination Therapy of Interferon Plus Ribavirin by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	*P*
Age (years)	0: ≥60	1	
	1: <60	2.420 (1.173–5.002)	0.020
Genotype	0: 1b	1	
	1: 2a/b	5.301 (2.401–11.702)	<0.001

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

Analysis of aa Sequences in the Core Gene in Genotype 1b Patients

The relationship between aa substitutions in the core region and the viral response to therapy was investigated in patients with genotype 1b using 93 available serum samples. Figure 2 shows the sequences of aa 61–110 of the HCV core region in 93 patients just before commencement of treatment. Table V summarizes the relationship between the response to IFN therapy and

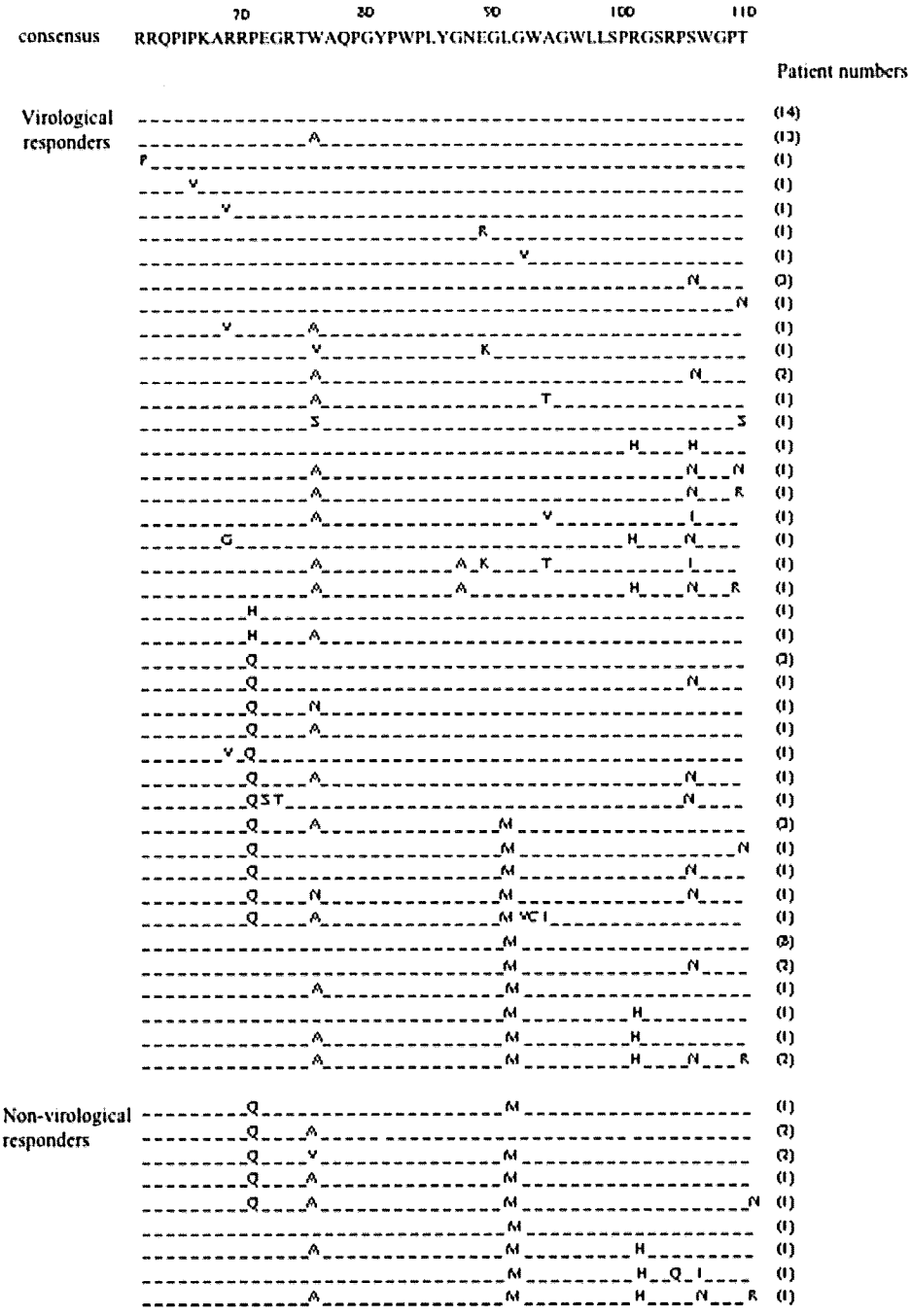

Fig. 2. Sequences of amino acids 61–110 in the core region at commencement of combination therapy in 93 patients infected with hepatitis C virus genotype 1b. Dashes indicate amino acids identical to the consensus sequence of genotype 1b, and substituted amino acids are shown by standard single-letter codes.

TABLE V. Amino Acid Substitutions in the Core Region in Non-Virologic Responders and Virological Responders in 93 Patients With HCV Genotype 1b

Presence of substitution site	Non-virological response (n = 11) % (n)	Virological response (n = 82) % (n)	P
aa 70	64 (7)	23 (19)	0.01
aa 75	73 (8)	45 (37)	0.11
aa 91	82 (9)	30 (25)	0.001
aa 106	27 (3)	31 (26)	1.0
aa 110	18 (2)	12 (10)	62
aa 70 and 91	45 (5)	10 (8)	0.006
aa 70 and/or 91	100 (11)	44 (36)	<0.001

aa, amino acid.

substitutions of aa. Among aa substitutions, only substitutions of aa 70 and 91 were associated with non-virological response. All non-virological responders had aa substitutions at 70 or 91, or both substitutions. In contrast, only 36 of 82 (44%) virological responders had these substitutions ($P < 0.001$, Table V). In contrast to non-virological response, these substitutions were not predictive for sustained virological response ($P = 0.11$–0.82).

Next, the effect of substitutions of aa 70 and 91 in the core region on early viral kinetics was analyzed by dividing patients into four groups according to the pattern of aa substitutions. As shown in Figure 3, the most rapid decrease in core antigen was noted in patients where both aa 70 and 91 were wild-type (double-wild). In contrast, the poorest reduction was noted in patients with both of aa 70 and 90 substitutions (double-mutant). Patients with either of the two aa substitutions (mutant/wild or wild/mutant) showed decrease in between the above two groups. HCV core antigen decreased below the detectable limit (20 fmol/L) at week 4 in 37 of 40 (93%) patients who had neither aa 70 nor aa 90 substitutions. In contrast, it decreased below the detectable limit in only 5 of 12 patients (42%) who had both aa 70 and 91 substitutions ($P = 0.031$).

Analysis of Nucleotide Sequence of the NS5A Gene

The aa sequences of ISDR in the NS5A gene were determined in 40 patients where PCR for this region was positive. Seventeen of 40 patients had no aa

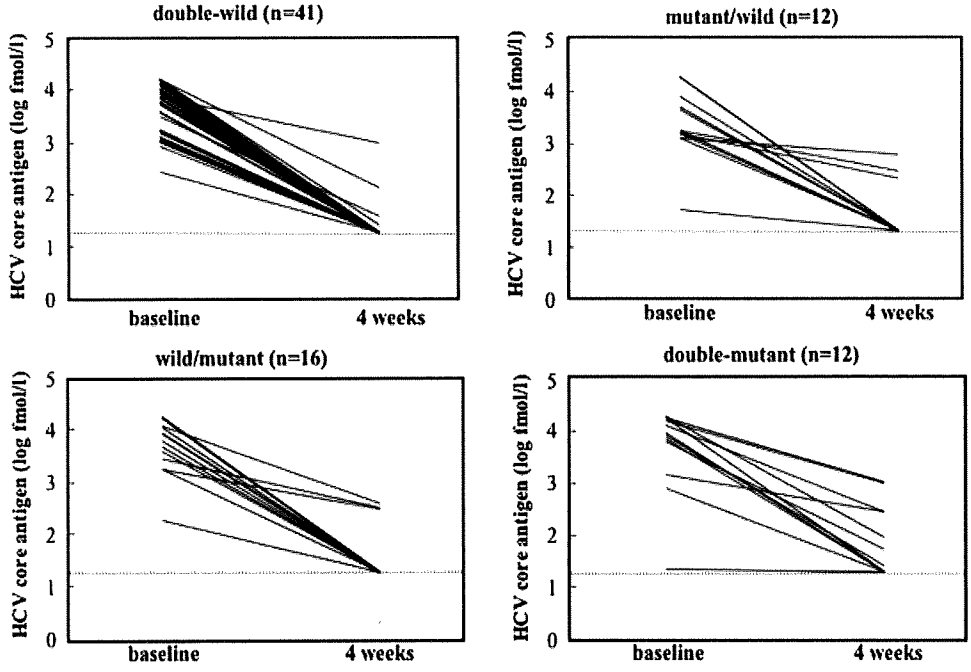

Fig. 3. Reduction of amount of HCV core antigen based on the presence of substitutions at amino acid 70 or 91. Eighty-one patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. The response was divided into four patterns based on the presence of substitution(s) at aa 70 and/or 91. Double-wild; no substitution, neither at aa 70 nor aa 91, mutant/wild; substitution only at aa 70, wild/mutant; substitution only at aa 91, double-mutant; substitutions at both aa 70 and 91. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.

TABLE VI. Amino Acid Substitutions in the IFN-Sensitive Determining Region (ISDR) in Non-Virologic Responders and Virological Responders in 40 Patients With HCV Genotype 1b

ISDR[a]	Non-virological response (n = 8) % (n)	Virological response (n = 32) % (n)	P
Wild-type (n = 17)	36 (6)	64 (11)	
Mutant-type (n = 23)	9 (2)	91 (21)	0.012

aa, amino acid.
[a]Absence of amino acid substitutions was evaluated as wild-type, and presence of one or more amino acid substitutions as mutant-type.

substitutions in ISDR (wild-type), while the remaining 23 patients had one or more substitutions (mutant-type). The relationship between aa substitutions of ISDR and effects of treatment was analyzed. The existence of aa substitution in the ISDR was not predictive for sustained virological response ($P = 0.137$), however, such substitution was observed frequently in virological responders compared to non-virological responders (66% vs. 25%, $P = 0.012$) (Table VI). The use of a different categorization based on the number of substitutions in the ISDR (0/1 vs. ≥ 2) yielded similar results, that is, not predictive for sustained viral response but predictive for virological responders (data not shown).

HCV core antigen decreased more rapidly in patients with ISDR mutant-type compared to those with wild-type (Fig. 4). HCV core antigen decreased below the detectable limit at week 4 in only 6 of 17 (35%) patients with wild-type. In contrast, it decreased below the detectable limit in 19 of 23 (83%) in patients with ISDR mutant-type ($P = 0.006$).

Predictive Factors Associated With Sustained Virological Response and Non-Virological Response in Patients With Genotype 1b

Finally, the predictive factors associated with sustained virological response and non-virological response were analyzed in patients with genotype 1b, including aa substitutions in the core region and ISDR. Univariate analysis showed two parameters correlated with sustained virological response: age (<60 years, $P = 0.004$) and presence of aa substitutions in the core (aa 70 and/or 91, $P = 0.04$). However, multivariate analysis, including the above variables, identified no parameters that influenced sustained virological response independently (age, $P = 0.89$; core, $P = 0.07$). Univariate analysis showed two parameters correlated with non-virological response: age (<65 years, $P = 0.02$) and aa substitutions in the core (double-mutant, $P = 0.01$). Multivariate analysis including the above variables identified aa substitutions in the core as an independent factor that influenced non-virological response (age, $P = 0.40$; core, $P = 0.03$) (Table VII).

DISCUSSION

Treatment of patients with chronic HCV infection had improved by the advent of PEG-IFN and RBV combination therapy. However, a substantial number of patients do not respond to the combination therapy [Taliani et al., 2006]. Several studies described attempts to improve the sustained virological response rate in such patients. Recent trials showed that a longer treatment period results in a higher sustained virological response rate [Berg et al., 2006; Sánchez-Tapias et al., 2006]. However, there are no conclusive studies that compared a larger dose of IFN with standard dose. Although the treatment had shifted in recent years to PEG-IFN and

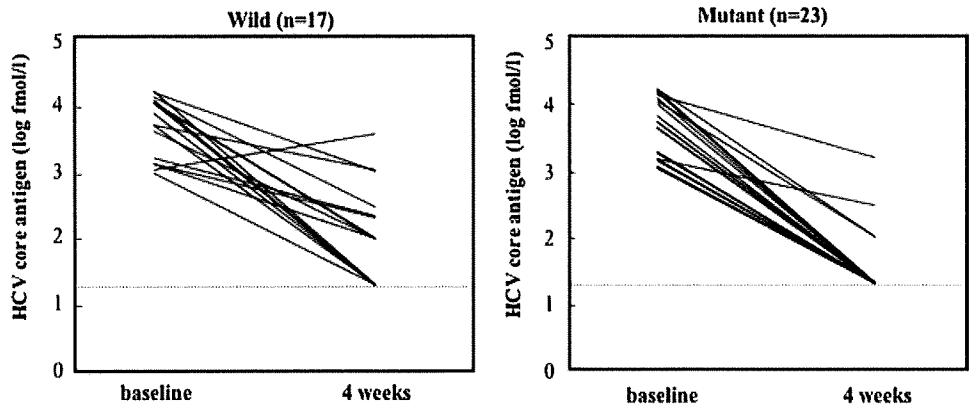

Fig. 4. Reduction of amount of HCV core antigen based on the presence of substitutions in the ISDR. Sixty-five patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. Patients were divided into two groups based on the presence of amino acid substitution(s) in the ISDR. Wild-type; absence of substitutions, mutant-type; presence of one or more substitutions. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.

TABLE VII. Factors Associated With Non-Virological Response to Combination Therapy of Interferon Plus Ribavirin Identified by Multivariate Analysis in Patients With Genotype 1b

Factor	Category	Odds ratio (95% CI)	*P*
Amino acid substitutions in the core region[a]	0: No double-mutant	1	0.028
	1: Double-mutant	7.000 (1.238–39.566)	

Only the variable that achieved statistical significance ($P < 0.05$) on multivariate logistic regression is shown.
[a]The mutant aa 70 and 91 pattern was evaluated as double-mutant, and other patterns as non-double-mutant.

RBV combination therapy, a different dose of IFN was used in the present study to test whether a larger dosage of IFN improves the outcome of IFN therapy.

In this study, the larger dose did not increase sustained virological response nor decrease non-virological response. Instead, the dose reduction of IFN and/or RBV was significantly higher in the higher dose group (Table III). Furthermore, the incidence of depression was significantly higher in the high-dose group (Table III). These results suggest that a high dose of IFN is not beneficial to patients who receive IFN and RBV combination therapy, and probably who will receive the PEG-IFN and RBV combination therapy.

The predictive factors for sustained virological response and non-virological response to the combination therapy for patients with genotype 1b were analyzed. Logistic regression analyses identified pretreatment substitutions at both aa 70 and 91 in the core region (double-mutant) as a singular predictive factor for non-virological response (Table VII). Furthermore, the existence of aa substitution in the ISDR was significantly more frequent in virological responders compared to non-virological responders (Table VI), in agreement with previous reports [Puig-Basagoiti et al., 2001; Pascu et al., 2004]. It has been reported that the numbers of aa substitutions in the ISDR correlate with serum HCV RNA levels [Enomoto et al., 1996]. However, no apparent correlation was observed in this study. As shown in Figures 3 and 4, patients who had substitutions of aa 70 and/or 91 in the core region or no aa substitutions in ISDR had poor initial reduction in the HCV core antigen. These results are consistent with recent studies that have shown the importance of a rapid initial decline of the viral load in obtaining a better response rate [Fried et al., 2002; Davis et al., 2003]. These results suggest that aa substitution analysis should provide important information on treatment of patients with genotype 1b.

The core protein of the HCV has been reported to disturb the IFN signaling by interacting with STAT1 SH2 domain [Lin et al., 2006] or repressing IRF1 [Ciccaglione et al., 2007]. These studies did not analyze the effect of aa substitutions in the core region. Further study is necessary to clarify the effect of aa substitutions in the core region and to identify a molecular target to improve the therapy.

Although aa substitution in the core region was identified as an important predictor in patients with genotype 1b in this study, aa substitutions of the core region and ISDR in patients with genotype 2a/b infection were not analyzed. Although the sustained virological response rate in patients who completed the therapy was high (26/28 [93%], per protocol analysis), few patients were unable to achieve sustained virological response. Furthermore, a significant number of patients could not complete the treatment course because of adverse effects. A more effective and easy to complete therapy should be developed to treat such patients. The predictive factors in such patients should also be clarified.

The recent development of a new type of drug targeting NS3/4 protease may improve the outcome of treatment in patients with chronic hepatitis C [Reesink et al., 2006; Forestier et al., 2007; Kieffer et al., 2007; Sarrazin et al., 2007a,b]. However, drug resistant mutants might emerge against such a small molecule therapy targeting viral enzyme(s). The functions of virus proteins that resist IFN including core, ISDR and PePHD should be clarified further to develop a better therapy that can achieve a higher sustained virological response rate with fewer and milder side-effects.

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Enhancement of tumor-specific T-cell responses by transcatheter arterial embolization with dendritic cell infusion for hepatocellular carcinoma

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Transcatheter arterial embolization (TAE) destroys a tumor by the induction of necrosis and/or apoptosis and causes inflammation with cytokine production, which may favor immune activation and presentation of tumor-specific antigens. In the current study, we attempted to identify the effect of TAE on tumor-specific T-cell responses and the additional effect of dendritic cell (DC) infusion performed during TAE. The prevalence of tumor antigen-specific T cells was determined by interferon-γ enzyme-linked immunospot analysis using alpha-fetoprotein (AFP) and tumor antigen-derived peptides in 20 and 13 patients with hepatocellular carcinoma (HCC) who received TAE and TAE with DC infusion, respectively. The increased frequency of AFP-specific T cells was observed in 6 of 20 patients after TAE. It was observed more frequently in patients with DC infusion than in those with TAE alone. However, tumor recurrence was not completely prevented in patients albeit displayed enhanced immune responses. The evidence that the enhanced immune responses were transient and attenuated within 3 months was provided in time-course analysis. In conclusion, TAE with DC infusion enhances the tumor-specific immune responses more effectively than TAE alone. Although the effect is not sufficient to prevent HCC recurrence, these results may contribute to the development of novel immunotherapeutic approach for HCC.

Hepatocellular carcinoma (HCC) is one of the most common malignancies and has gained major clinical interest because of its increasing incidence. Although current advances in therapeutic modalities have improved the prognosis of patients with HCC, the survival rate is still unsatisfactory.[1–4] One of the reasons for the poor prognosis is the high rate of recurrence after treatment.[5] Therefore, the development of new antitumor therapies to protect against recurrence is important to improve the prognosis for HCC.

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Several recent studies of cancer treatment causing tumor necrosis or apoptosis have shown that they induce the activation of tumor-specific immune responses.[6–10] The mechanism to activate host immune responses against tumors is still unknown; however, several studies *in vitro* or *in vivo* suggest that cytokine production, attracting leukocyte infiltration, increase of tumor antigen uptake by macrophages or dendritic cells (DCs) and release of heat shock protein caused by inflammation at the tumor site are associated with the phenomenon.[11–17]

Transcatheter arterial embolization (TAE) has been used extensively in the Western world and Asia to treat unresectable HCCs.[18–20] Although several previous randomized controlled trials have failed to show a survival benefit in patients treated with TAE compared to untreated patients,[21,22] recent studies demonstrated a survival benefit for TAE *versus* conservative treatment in carefully selected patients.[23–25]

Histological assessment of resected HCC after TAE shows that the treatment induces necrotic and apoptotic changes in the tumor.[26–29] Moreover, it is reported that the serum levels of macrophage-colony stimulating factor and the lipopolysaccharide-stimulated production of interleukin-1 beta, IL-6 and tumor necrosis factor-alpha in peripheral whole blood were increased after TAE.[30–32] Taken together with the previously described knowledge of immune responses after treatment to induce tumor necrosis or apoptosis, these observations support the hypothesis that the induction of apoptotic or necrotic cell death and inflammatory cytokines by TAE favors immune activation and induction of tumor-specific T-cell

Key words: immune response, AFP, CTL, immunotherapy, epitope
Abbreviations: HLA: human leukocyte antigens; IFN: interferon; HCV: hepatitis C virus; ELISPOT: enzyme-linked immunospot; TAE: transcatheter arterial embolization; MRP: multidrug resistance-associated protein; hTERT: human telomerase reverse transcriptase
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responses. In a previous study, we also made a preliminary report that immune responses specific for tumor antigens were enhanced after HCC treatments.[7,10] In addition, we have recently developed a new immunotherapeutic approach for HCC using DC infusion performed during TAE, showing the potential to enhance tumor-specific immune responses.[7]

In the current study, we first attempted to identify the effect of TAE for tumor-specific T-cell responses in patients with HCC. Next, we examined the additional effects of DC infusion to the tumor site after TAE. Finally, we analyzed the relationship between clinical characteristics of patients and T-cell responses after TAE and evaluated whether the activation of tumor-specific T-cell responses can prevent HCC recurrence.

Material and Methods

Patient population

The study examined 33 patients with HCC, consisting of 25 men and 8 women ranging from 48 to 83 years old with a mean age of 66 ± 9 years. Twenty patients were treated by TAE. Thirteen patients were treated by TAE with DC infusion as a part of clinical study, which was approved by ethical committee of Kanazawa University Graduate School of Medical Science and registered in September 2003. The patients who received TAE with DC infusion were selected according to the criteria we previously reported.[7] All subjects were negative for Abs to human immunodeficiency virus (HIV) and gave written informed consent to participate in this study in accordance with the Helsinki declaration.

Treatment of hepatocellular carcinoma

HCCs were detected by imaging modalities such as dynamic CT scan, MR imaging and abdominal arteriography. The diagnosis of HCC was histologically confirmed by taking US-guided needle biopsy specimens, surgical resection or autopsy in 18 cases. For the remaining 15 patients, the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT.[33] The tumor size was categorized as "small" (≤2 cm) or "large" (>2 cm), and tumor multiplicity was categorized as "multiple" (≥2 nodules) or "solitary" (single nodule). The TNM stage was classified according to the Union Internationale Contre Le Cancer (UICC) classification system (6th version).[34]

Twenty patients were treated by TAE as previously described.[19,35] In brief, after evaluation of the feeding arteries and surrounding vascular anatomy, a microcatheter (Microferret, Cook, Bloomington, IN) was inserted into the segmental or subsegmental artery with a coaxial method using a 0.016-inch guidewire (Radifocus GT wire, Terumo, Tokyo, Japan). A mixture of the anticancer drug and iodized oil was administered, and the feeding artery was embolized with gelatin sponge particles (Gelfoam; Pharmacia Upjohn, Kalamazoo, MI). The mixture of anticancer drug and iodized oil contained 10–30 mg of Epirubicin (Farmorubicin; Kyowa Hakko Kogyo, Tokyo, Japan), 1–3 ml of iodized oil (Lipiodol Ultra Fluide) and 0.5–1.0 ml of iohexol (Omnipaque 300).

Preparation and injection of autologous DCs

DCs were generated as previously described.[7] In 6 patients, DCs were pulsed with 0.1 KE/ml OK-432 (Chugai Pharmaceutical, Tokyo, Japan), which is a biological response modifier derived from the weakly virulent Su strain of Streptococcus pyogenes,[36,37] for 3 days before injection. The cells were harvested for injection; 5×10^6 cells were reconstituted in 5-ml normal saline containing 1% autologous plasma, mixed with gelatin sponge particles and infused through an arterial catheter following iodized oil injection during TAE.

After TAE or TAE with DC infusion, 26 patients received percutaneous tumor ablation by ethanol injection (PEIT), microwave coagulation (MCT) or radiofrequency (RF). Twenty-one patients were diagnosed with complete necrosis of the tumor lesion using dynamic CT after the completion of treatment. Follow-ups were conducted at outpatient clinics using blood tests and dynamic CT every 3 months for 1 year.

Laboratory and virologic testing

Blood samples were tested for HBsAg and HCVAb by commercial immunoassays (Fuji Rebio, Tokyo, Japan). HLA-based typing of PBMC from patients was performed using complement-dependent microcytotoxicity with HLA typing trays purchased from One Lambda. The serum alpha-fetoprotein (AFP) level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan), and the pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathologic study of primary liver cancer.[38] The severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet *et al.*[39]

Interferon-γ enzyme-linked immunospot assay

The prevalence of tumor antigen-specific T cells was determined by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis (Mabtech, Nacka, Sweden) as previously described.[10,40] HLA-A24-restricted AFP-derived peptides (10 μg/ml), which were AFP_{357} (EYSRRHPQL), AFP_{403} (KYIQESQAL) and AFP_{434} (AYTKKAPQL),[10] and 20 μg/ml AFP derived from human placenta (Morinaga Institute of Biological Science, Yokohama, Japan, purity >98%) were added directly to the wells. These 3 AFP-derived peptides could induce CTLs showing cytotoxicity against hepatoma cells and were frequently recognized by PBMCs of patients with HCC as we previously reported,[10] and therefore, we selected them as an immunogenic peptide. The HLA-A24-restricted AFP and CMV-derived peptides were used only for HLA-A24 or A23 positive patients. Other tumor antigen-derived peptides consisted of $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), $MRP3_{765}$ (VYSDADIFL), $hTERT_{167}$ (AYQVCGPPL), $hTERT_{324}$

(VYAETKHFL) and $hTERT_{461}$ (VYGFVRACL), which we previously reported that they were useful for analyzing host immune responses to HCC.[40,41]

PBMCs were added to the wells at 3×10^5 cells/well. In the assay using PBMC depleted $CD4^+$ or $CD8^+$ cells, the number of cells was adjusted to 3×10^5 cells/well after the depletion. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system using CD4 or CD8 MicroBeads (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions. After the depletion, 1×10^6 cells were stained with CD4 and CD8 antibodies (Becton Dickinson, Tokyo, Japan) and analyzed by FACSCalibur (Becton Dickinson, Tokyo, Japan) to confirm the ratio of $CD4^+$ and $CD8^+$ cells. Data analysis was undertaken with CELLQuest™ software (Becton Dickinson, San Jose, CA).

Plates were analyzed with a KS ELISpot Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of antigen. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least 2-fold greater than the number of spots in the absence of antigen. Negative controls consisted of incubation of PBMCs with a peptide representing an HLA-A24-restricted epitope derived from HIV envelope protein ($HIVenv_{584}$) and were always <5 spots per 3×10^5 cells.[42] The positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$).[43] All peptides used in this study were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). ELISPOT analysis was performed before and 2–4 weeks after TAE. In patients receiving additional treatment for complete ablation of tumor, analysis was performed just before the additional treatment. An increase of antigen-specific T cells was defined as significant when T-cell responses changed to positive or if the number of spots detected after TAE was at least 2-fold greater than the number of spots detected before treatment.

Statistical analysis

Unpaired Student's *t*-test was used to analyze the effect of variables on immune responses in patients with HCC. Fisher's exact test (2-sided *p*-value) was used to analyze the frequency of positive immune responses in patients between with TAE and TAE with DC infusion.

Results

T-cell responses to AFP in the patients who received TAE

The frequency of AFP-specific T cells before and after TAE was tested *ex vivo* in an IFN-γ ELISPOT assay. The serum AFP level and number of peripheral lymphocytes and antigen-specific T cells are shown in Table 1. Before treatment, 2 patients showed a specific T-cell response to AFP-derived peptides and 3 patients to protein in 20 patients (Patients 1–20). After treatment, a T-cell response to AFP-derived peptides and protein was detected in 4 and 3 patients, respectively.

When an increase of antigen-specific T cells was defined as significant if T-cell responses changed to positive or the number of spots detected after TAE was at least 2-fold greater than the number of spots detected before treatment, 6 of 20 (30%) patients (Patients 4, 6, 7, 11, 18 and 20) showed a significant increasing of AFP-specific T-cell frequency after treatment. It was observed even in the patient (Patients 6, 7 and 18) who had no T cells specific to corresponding AFP-derived peptides before treatment. When a decrease of antigen-specific T cells was defined as significant if T-cell responses changed from positive to negative or the number of spots detected after TAE was less than half of the number of spots detected before treatment, 4 of 20 (20%) patients (Patients 5, 14, 15 and 16) showed a significant decreasing of AFP-specific T-cell frequency after treatment.

AFP-specific IFN-γ-producing T cells were also analyzed by ELISPOT assay using PBMC depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cells is responsive to whole AFP. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system, and the results were confirmed by flow cytometric analysis (Fig. 1*a*). After depletion of $CD4^+$ or $CD8^+$ cells, the ratio of each cell population was decreased to less than 0.1% of PBMCs. The IFN-γ ELISPOT assay showed that IFN-γ-producing T cells against AFP consisted of both $CD8^+$ and $CD4^+$ cells (Fig. 1*b*).

To confirm the effect of TAE for host immune responses to HCC, we also examined the frequency of tumor antigen-specific T cells in 4 patients (Patients 5, 8, 10 and 14) using MRP3- or hTERT-derived peptides that we previously identified as useful for analyzing host immune responses to HCC.[40,41] A significant increasing of MRP3- or hTERT-specific T-cell frequency was observed in all patients after TAE (Table 2).

T-cell responses to AFP in the patients who received TAE with DC infusion

In 13 patients receiving TAE with DC infusion (Patients 21–33), 2 patients showed a specific T-cell response with AFP-derived peptides and 2 patients with protein before treatment (Table 3). After treatment, 8 patients showed a specific T-cell response to AFP-derived peptides and 3 patients to protein.

Next, we compared TAE with DC infusion with TAE alone regarding the effect to AFP-specific immune response. Table 4 shows the clinical features of patients with HCC who received TAE and TAE with DC infusion and they were not statistically different except liver function.

The frequency of patients who showed both positive and increasing T-cell response with AFP-derived peptides or protein after treatment was significantly higher in patients receiving TAE with DC infusion than in those receiving TAE alone ($p = 0.04$) (Fig. 2*a*). On the other hand, the frequency of patients who showed both positive and increasing T-cell

Table 1. T cell response to AFP and AFP-derived peptides by ELISPOT assay before and after TAE

				Before treatment								After treatment							
Patient	HLA	Additional treatment	Complete ablation	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT
1	A2	RF	C	<10	1,600	ND	ND	ND	1	ND	0	<10	1,400	ND	ND	ND	0	ND	1
2	A26,A31	RF	C	61	1,700	ND	ND	ND	0	ND	**13**	23	900	ND	ND	ND	0	ND	0
3	A11,A26	No	–	100	1,700	ND	ND	ND	5	ND	1	50	1,500	ND	ND	ND	0	ND	0
4	A24	RF	C	18	700	0	7	0	6	0	**25**	16	500	1	**10**	1	1	2	**16**
5	A24,A33	RF	C	2,357	1,200	**13**	2	6	0	**13**	0	700	1,100	2	1	1	0	9	0
6	A24	RF	C	14	1,800	0	0	0	0	0	**42**	<10	1,400	**53**	**27**	**38**	**14**	**36**	**108**
7	A23,A33	No	–	96	500	0	0	0	5	**291**	0	138	800	**46**	0	0	3	**484**	0
8	A24,A26	No	–	142	600	1	0	0	0	0	0	126	500	2	0	0	0	**166**	1
9	A2,A24	RF	C	<10	700	6	1	0	0	9	0	<10	700	0	0	0	0	**32**	**15**
10	A24	PEIT	C	<10	1,300	8	4	8	8	**146**	5	<10	1,300	0	1	1	0	1	1
11	A24,A26	PEIT	N	18	1,100	0	0	0	1	ND	0	13	400	0	0	0	**15**	**10**	**55**
12	A24,A33	RF	N	11	800	3	2	0	4	**94**	**10**	11	700	0	0	0	0	**24**	0
13	A11,A24	PEIT	C	52	1,300	0	2	5	1	2	0	24	1,200	0	0	0	0	0	3
14	A24	RF	C	54	2,400	**25**	5	4	8	**12**	0	67	1,700	0	0	0	0	0	0
15	A2,A24	RF	N	62	1,200	0	3	0	**25**	2	3	14	800	0	0	0	8	0	0
16	A3,A24	RF	C	2,876	900	0	1	0	**13**	0	5	3,285	700	0	0	0	0	0	0
17	A24,A33	No	–	205	400	4	2	3	2	**26**	9	220	100	2	1	0	1	**39**	1
18	A24,A30	RF	C	18	1,100	4	0	3	8	**14**	7	13	900	1	**16**	1	5	**12**	0
19	A2,A24	RF	C	330	1,500	2	0	0	0	**18**	1	36	1,100	0	4	0	3	8	1
20	A2,A33	RF	C	10	1,400	ND	ND	ND	**10**	ND	**68**	<10	800	ND	ND	ND	**31**	ND	**101**

Abbreviations: Lymph., number of lymphocytes; RF, radiofrequency ablation; PEIT, percutaneous ethanol injection therapy; No, no treatment; C, completed; N, not completed; –, not determined; ND, not done. The bold letters show the positive responses in ELISPOT assays.

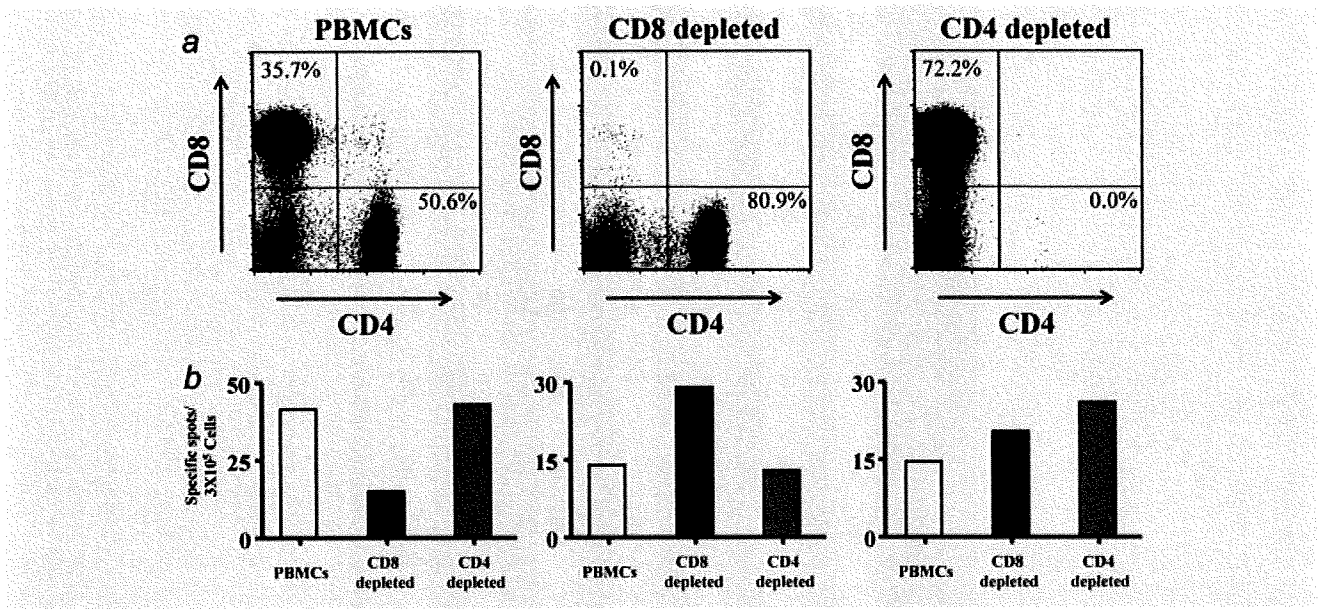

Figure 1. IFN-γ production of CD4- or CD8-depleted T cells against whole AFP. AFP-specific IFN-γ-producing T cells were analyzed by ELISPOT assay using PBMC depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cells is responsive to whole AFP. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system and the results were confirmed by flow cytometric analysis (*a*). IFN-γ ELISPOT assay using nontreated PBMCs and PBMC depleted $CD4^+$ or $CD8^+$ cells showed that T cells producing IFN-γ against whole AFP consisted of both $CD8^+$ and $CD4^+$ cells (*b*). Assays were performed in 5 patients and the representative result is shown.

Table 2. T cell response to other tumor antigen-derived peptides by ELISPOT assay before and after TAE

	Before treatment						After treatment					
Patient	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$
5	2	7	8	0	3.5	7.5	0	0	0	7	3	**35**
8	6	6	1	3	ND	ND	**17**	**18**	**22**	**18**	**14**	9
10	0	1	3	0	5	7	0	4	7	6	11	4
14	6	5	0	9	5	**13**	6	14	22	8	**10**	7

Abbreviation: ND, not done. The bold letters show the positive responses in ELISPOT assays.

response with CMV-derived peptide or tetanus toxoid was not different between the 2 groups (Figs. 2*b* and 2*c*).

In the comparison of the mean values of spots generated with AFP-derived peptides, protein, CMV-derived peptides or tetanus toxoid, no significant difference was observed between patients with TAE alone before and after treatment (Figs. 3*a*–3*d*). In contrast, the mean values of spots generated with AFP-derived peptides were significantly higher in patients after TAE with DC infusion than in those before treatment (Fig. 3*e*). The mean values of spots generated with protein, CMV-derived peptides or tetanus toxoid were not significantly different between patients before and after TAE with DC infusion (Figs. 3*f*–3*h*). Based on the above results, we considered that the main difference between TAE alone and TAE with DC infusion was the response to HLA-A24-restricted AFP-derived epitopes. Therefore, to analyze the difference between TAE alone and TAE with DC infusion more precisely, we selected the patients with HLA-A24 or A23 and compared the clinical parameters of both groups. However, there were no statistical differences except liver function in the 2 groups (Table 5).

Enhancement of AFP-specific T-cell responses and treatment outcome

To evaluate the effect of immune enhancement by TAE or TAE with DC infusion for the treatment outcome, we analyzed the clinical course of 17 patients who received complete ablation by additional RFA, PEIT or MCT after these treatments and could be followed up using dynamic CT every 3 months (Table 6). Seven patients showed increasing specific spots for AFP or AFP-derived peptides in ELISPOT assay after TAE. HCC recurrence within 3 months after complete ablation was observed in 3 patients who showed increasing AFP-specific T-cell responses after TAE. Furthermore, recurrence within 6 months after complete ablation was observed

Table 3. T cell response to AFP and AFP-derived peptides by ELISPOT assay before and after TAE with DC infusion

				Before treatment								After treatment							
Patient	HLA	Additional treatment	Complete ablation	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT
21	A24	No	–	332	1,100	7	1	4	ND	**10**	ND	819	800	**11**	0	**10**	ND	**188**	ND
22	A24,A26	RF	N	341	700	0	**26**	5	ND	**68**	ND	237	500	ND	**59**	ND	ND	**81**	ND
23	A11,A24	No	–	41	600	0	2	5	1	2	0	43	400	0	0	0	0	0	3
24	A2,A24	MCT	C	1,260	800	3	8	7	ND	**19**	ND	614	1,300	**26**	4	7	ND	**12**	ND
25	A24,A33	RF	C	11	1,500	0	1	0	**31**	5	**15**	19	900	1	4	**15**	**26**	3	4
26	A24,A33	RF	C	<10	2,000	0	0	0	0	0	0	<10	1,700	0	**16**	0	0	0	0
27	A24,A26	RF	C	16	700	0	0	0	1	1	0	16	700	2	1	**15**	9	0	1
28	A11,A31	RF	N	31	800	ND	ND	ND	3	ND	0	33	700	ND	ND	ND	0	ND	0
29	A11,A33	No	–	<10	1,100	ND	ND	ND	0	ND	0	<10	700	ND	ND	ND	0	ND	1
30	A2,A11	RF	C	13	1,300	ND	ND	ND	8	ND	1	14	1,500	ND	ND	ND	**12**	ND	7
31	A24,A33	RF	C	1,014	800	0	0	0	0	1	0	15	300	0	0	**20**	0	0	0
32	A11,A24	RF	C	<10	1,000	3	3	**11**	**48**	**97**	0	10	1,200	**23**	**20**	**20**	**45**	**91**	**23**
33	A2,A26	RF	C	29	1,300	ND	ND	ND	0	ND	0	27	1,300	ND	ND	ND	0	ND	0

Abbreviations: Lymph., number of lymphocytes; RF, radiofrequency ablation; PEIT, percutaneous ethanol injection therapy; MCT, microwave coagulation therapy; C, completed; N, not completed; –, not determined; ND, not done. The bold letters show the positive responses in ELISPOT assays.

Table 4. Patient characteristics

	Patients treated by TAE (n = 20)	Patients treated by TAE with DC (n = 13)	p-value[1]
Age (years)[2]	66.6 ± 7.8	65.7 ± 10.0	NS
Sex (M/F)	14/6	11/2	NS
HLA (A23 or 24/others)	16/4	9/4	NS
ALT (IU/l)	51.0 ± 47.4	86.9 ± 62.8	NS
Total bilirubin (g/dl)	1.3 ± 0.9	1.5 ± 0.9	NS
Albumin (g/dl)	3.7 ± 0.7	3.2 ± 0.6	NS
AFP level (ng/ml)	322.7 ± 793.0	239.8 ± 418.2	NS
Diff. degrees of HCC (well/moderate or poor/ND[1])	2/6/12	4/4/5	NS
Tumor size (small/large[3])	4/16	1/12	NS
Tumor multiplicity (multiple/solitary)	18/2	12/1	NS
TNM stage (I, II/III, IV)	19/1	11/2	NS
Histology of nontumor liver (LC/chronic hepatitis)	15/5	10/3	NS
Liver function (Child A/B or C)	14/6	3/10	0.02
Etiology (HCV/HBV/others)	12/2/6	13/0/0	NS

[1]Abbreviations: NS, no statistical significance; ND, not determined. [2]Data are expressed as the mean ± SD. [3]Small: ≤2 cm, large: >2 cm.

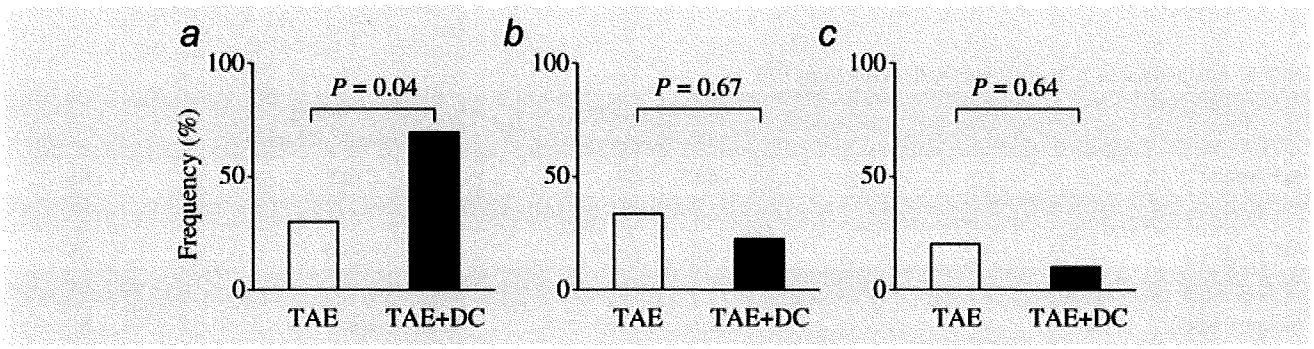

Figure 2. Frequency of the patients who showed enhancement of T-cell responses after treatment. The prevalence of antigen-specific T cells was determined by IFN-γ ELISPOT analysis using alpha-fetoprotein (AFP) and AFP-derived peptides (*a*), CMV pp65-derived peptide (*b*) or tetanus toxoid protein (*c*) in 20 and 13 patients with HCC who received TAE and TAE with DC infusion, respectively.

in 4 and 6 patients who did and did not show increasing AFP-specific T-cell responses, respectively.

Kinetics of AFP-specific T-cell responses before and after TAE

Next, we examined the kinetics of AFP-specific T cells in 8 patients who showed increasing frequency of IFN-γ-producing T cells against AFP or AFP-derived peptides after TAE. The frequency was examined by ELISPOT assay before and 2–4 weeks and 3 months after TAE. Thirteen kinds of AFP-specific T cells showed increasing frequency 2–4 weeks after TAE (Fig. 4); however, the increase was transient and most cell types decreased 3 months after TAE. Three patients showed more than 10 specific spots for AFP or AFP-derived peptides 3 months after TAE (Patients 6, 11 and 30). In analysis of the correlation between the maintenance of AFP-specific T-cell responses and HCC recurrence, 1 patient (Patient 6) had HCC recurrence after 6 months and 1 patient (Patient 30) did not show recurrence. Another patient (Patient 11) did not receive curative ablation and was not analyzed. There was no difference in the kinetics of AFP-specific T cells between patients who received TAE with and without DC infusion.

Discussion

In a previous study, we made a preliminary report that immune responses specific for tumor antigens were enhanced after HCC treatments.[7,10] Similarly, as in our previous or other group's results,[8] we observed enhancement of AFP-specific immune responses in 6 of 20 patients with TAE alone in this study. The enhancement of tumor antigen-specific immune responses was also observed in the cases using MRP3- or hTERT-derived peptides.

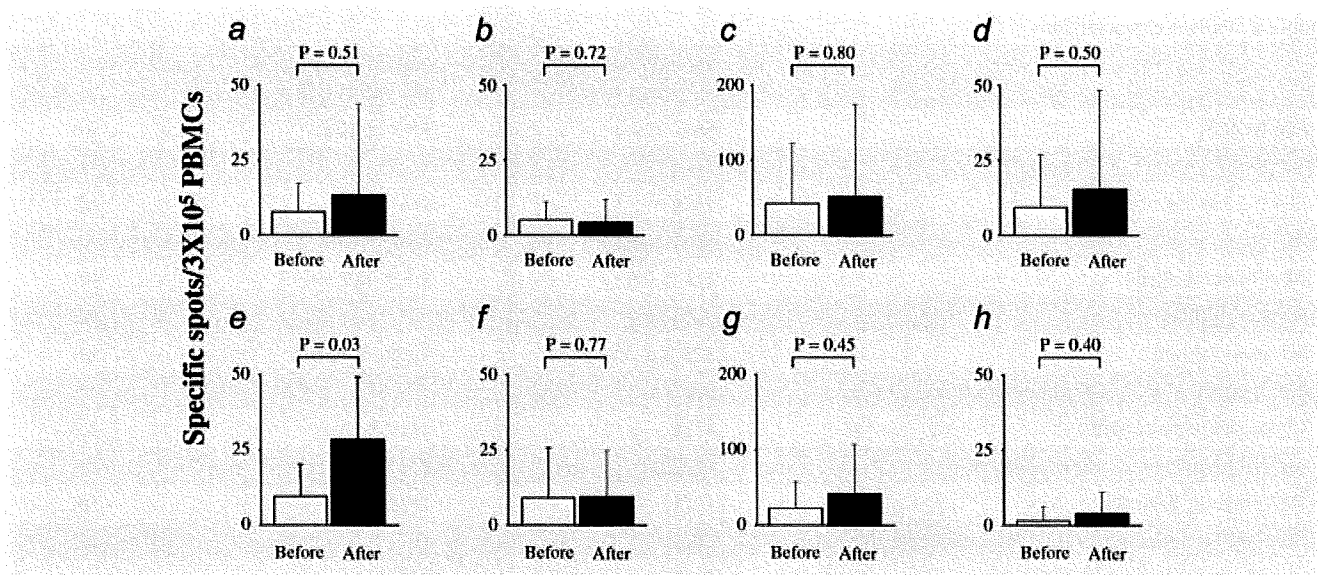

Figure 3. Comparison of direct *ex vivo* analysis (IFN-γ ELISPOT assay) before and after treatment of HCC. The assay was performed using PBMCs of patients who received TAE for AFP-derived peptides (*a*), AFP (*b*), CMV pp65-derived peptide (*c*) or tetanus toxoid protein (*d*). The same assay was performed using PBMCs of patients who received TAE with DC infusion for AFP-derived peptides (*e*), AFP (*f*), CMV pp65-derived peptide (*g*) or tetanus toxoid protein (*h*). AFP and CMV pp65-derived peptides were tested in only HLA-A24 or A23 positive patients. Data are expressed as the mean + SD of specific spots.

Table 5. Characteristics of the patients with HLA-A24 or A23

	Patients treated by TAE (n = 16)	Patients treated by TAE with DC (n = 9)	p-value[1]
Age (years)[2]	65.7 ± 7.8	67.8 ± 10.8	NS
Sex (M/F)	10/6	7/2	NS
ALT (IU/l)	55.9 ± 51.9	75.4 ± 53.0	NS
Total bilirubin (g/dl)	1.4 ± 0.8	1.4 ± 1.1	NS
Albumin (g/dl)	3.6 ± 0.7	3.1 ± 0.6	NS
AFP level (ng/ml)	392.1 ± 877.8	337.2 ± 477.1	NS
Diff. degree of HCC (well/moderate or poor/ND[1])	2/5/9	3/3/3	NS
Tumor size (small/large[3])	3/13	0/9	NS
Tumor multiplicity (multiple/solitary)	15/1	8/1	NS
TNM stage (I, II/III, IV)	15/1	7/2	NS
Histology of nontumor liver (LC/chronic hepatitis)	13/3	8/1	NS
Liver function (Child A/B or C)	10/6	0/9	0.003
Etiology (HCV/HBV/others)	11/1/4	9/0/0	NS

[1]Abbreviations: NS, no statistical significance; ND, not determined. [2]Data are expressed as the mean ± SD. [3]Small: ≤2 cm, large: >2 cm.

The precise mechanism of this phenomenon is still unknown; however, in recent studies, several treatments to destroy tumor cells by necrosis and/or apoptosis have induced antitumor immune responses in animal models[14,44] and even in humans.[6–10] In the study of *in situ* tumor ablation, it is reported that tumor ablation creates a tumor antigen source for the induction of antitumor immunity.[9,44] In another study regarding photodynamic therapy (PDT),[45] it is reported that acute inflammation, expression of heat-shock proteins and providing tumor antigens to DCs caused by PDT induce tumor-specific immune responses.

Based on these results, we hypothesize that DC infusion with TAE can induce antitumor immune responses more effectively than TAE alone. According to DC research in recent years, successful enhancement of the antitumor immune response has been reported by intratumoral

Table 6. Enhancement of AFP-specific T cell response and treatment outcome

	Enhancement of AFP-specific T cell response	Recurrence, 3 months	Recurrence, 6 months
Patient 1	–	N	U
Patient 2	–	N	M
Patient 4	+	M	ND
Patient 5	–	N	M
Patient 6	+	N	U
Patient 9	–	N	M
Patient 10	–	N	N
Patient 13	–	N	N
Patient 14	–	N	N
Patient 16	–	N	M
Patient 19	–	N	U
Patient 24	+	U	ND
Patient 25	+	M	ND
Patient 26	+	N	N
Patient 30	+	N	N
Patient 31	+	N	N
Patient 33	–	N	N

Abbreviations: N, no recurrence; U, uninodular recurrence; M, multinodular recurrence; ND, not determined.

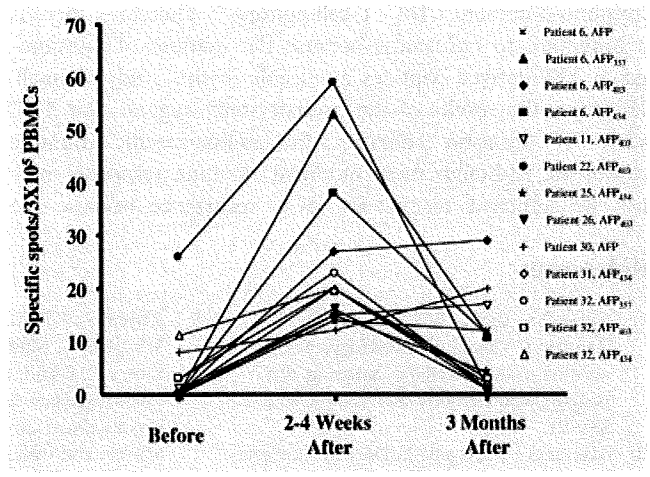

Figure 4. Kinetics of AFP-specific T-cell responses determined by IFN-γ ELISPOT assay before and after TAE. PBMCs were obtained before and 2–4 weeks and 3 months after TAE. Each graph indicates the kinetics of T cells specific for each antigen in each patient. Some patients received additional treatments as indicated in Tables 1 and 3 for a curative treatment after the measurement of T-cell responses at 2–4 weeks after TAE.

administration of DC in combination with tumor ablation.[46,47] Furthermore, immunotherapies using DC have been performed in patients with HCC and their antitumor effects are reported.[48–50] These results support our hypothesis and therefore, in the next step, we examined the immunological effects of DC infusion with TAE.

The comparison of frequency in patients who showed enhancement of AFP-specific immune responses revealed more frequency in patients with DC infusion than in those with TAE alone. On the other hand, there were no differences in the 2 groups in the comparison of frequency for patients who showed enhancement of CMV or TT-specific immune responses. These results suggest that DC infusion with TAE affects tumor-specific immune responses and that the effects are limited to the tumor area.

Some patients with TAE alone showed disappearance of AFP- or control antigen-specific T cells. Although the mechanism of this phenomenon is unknown, anticancer drugs used in TAE might suppress the immune responses, because most of the patients showed decreasing the number of lymphocytes after TAE. These results suggest that TAE alone might give a chance to enhance tumor-specific T-cell responses in only some patients. Further analysis using many more patients with TAE is necessary to make clear the differences in the patients with and without enhancement of T-cell responses. In contrast, disappearance of AFP- or control antigen-specific T cells was not observed in the patients with DC infusion, suggesting strong immunostimulating effect of this treatment.

In analysis of the association between the enhancement of AFP-specific T cells and clinical responses, no correlation could be shown, suggesting that enhancement of T-cell response associated with TAE or TAE with DC infusion may not have protective effect against HCC recurrence. To clarify the mechanism in more detail, we examined the kinetics of AFP-specific T-cell response. Increased frequency of AFP-specific T cells was transient and fell in 4 of 8 patients 3 months after treatment (Fig. 4). Similar to our results, Ayaru *et al.* also reported that the frequency of AFP-specific $CD4^+$ T cells fell in all patients by 1–3 months after TAE.[8] In addition, our results suggest that DC infusion with TAE is not effective to maintain the increased frequency of AFP-specific T cells.

Recent genome profiling studies of HCC show that HCC is a very heterogenous tumor.[51] Furthermore, HCC has multicentric carcinogenesis and develops at different time points. These characters of HCC may also be another reason for no correlation between the enhancement of AFP-specific T cells and clinical responses. The identification of many more tumor antigens and their T-cell epitopes is necessary for more precise analysis of the relationship between antitumor immune response and clinical response, and for immunotherapy.

In the recent study, it is reported that $CD8^+$ T-cell response to AFP is multispecific and AFP-specific IFN-γ-producing $CD8^+$ T cells are directed against different epitopes spreading over the entire AFP sequence with no single

immuno-dominant $CD8^+$ T-cell epitope.[52] Therefore, there is a limitation to our study, because the number of immunogenic AFP-derived peptides applicable in this study is small. However, the results of the present study suggest that TAE with DC infusion enhances the tumor-specific immune responses. Although these modified immune responses may not be sufficient to prevent HCC recurrence because the enhanced immune responses are transient and attenuate within 3 months, these results may contribute to the development of novel immunotherapeutic approach for HCC.

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