Table 4. Determination of HBV and HCV infection status using stored serum samples

	HCC Cases (N=211)	Controls (N=640)
	n (%)	n (%)
Etiology (HBV/HCV status)*		
HBV −/HCV −	45 (21.3)	579 (90.5)
HBV +/HCV −	29 (13.7)	18 (2.8)
HBV −/HCV +	132 (62.6)	41 (6.4)
HBV +/HCV +	5 (2.4)	2 (0.3)

* HBV infection (HBV +) status was defined by HBsAg-positive or anti-HBc-high titer-positive. HCV infection (HCV +) status was defined by HCV RNA-positive.

qualitative PCR assay detected as HCV RNA positive in 100% of serum samples containing 100 IU/ml of HCV RNA, and 50% of the samples containing 60 IU/ml of HCV RNA (Table 3).

Shown in Table 4, among HCC cases, 13.7% were found to be HBV only infections, 62.6% were HCV only infections, and 2.4% were positive for both; while the remaining subjects negative for both. Among the controls, 2.8% were found to be HBV only infections, 6.4% were HCV only infections, and 0.3% were positive for both. These distributions of HBV and HCV infection status were similar to those in previous reports on Japanese populations (29, 30). We plan an extending this ongoing HCC study by using stored sera after taking into account HBV and HCV infection status.

Conclusions

The identification of HBV and HCV infections in individuals of a defined cohort can be accomplished using both frozen and freeze-dried sera, and that the identification can be accurate and reliable depending on the method, condition, and duration of storage and associated optimizations. Although freeze-dried sera cannot provide live viruses that can be grown and studied in culture, the loss of hepatitis viral infectivity has the advantage of safety during testing or shipping. Therefore it is expected that the use of freeze-dried sera, stored for long periods, will be useful in future viral hepatitis studies e.g. evolution of the hepatitis virus as well as the natural history of viral liver diseases. In addition long-term storage of frozen and freeze-dried sera from cases of specific diseases, including intractable diseases that cannot be diagnosed or treated with present-day medicine, may be useful for future generations.

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Involvement of Dendritic Cell Frequency and Function in Virological Relapse in Pegylated Interferon-α and Ribavirin Therapy for Chronic Hepatitis C Patients

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A combination of pegylated interferon α (PEG-IFNα) and ribavirin has been used widely. Enhancement of immune response against hepatitis C virus (HCV) is known to be involved in the efficacy of the combination therapy. The aim of the study was to elucidate whether the frequency or function of immunocompetent blood cells is related to the outcome of the therapy. Twenty-five chronic hepatitis C patients with high viral load of HCV genotype 1 who underwent 48 weeks of PEG-IFNα2b and ribavirin therapy were examined. During the treatment, frequencies of dendritic cell subsets, helper T cell subsets, and NK cells were phenotypically determined. In some patients, the ability of dendritic cells to stimulate allogeneic $CD4^+$T cells was examined at the end and after the therapy. Among the 25 patients, 11 showed a sustained virological response, 11 a transient response, and 3 no response. In comparison with sustained virological responders, non-sustained virological responders showed impaired dendritic cell function at the end and after the treatment. The transient responders showed a decline of plasmacytoid dendritic cell frequency from Weeks 1–12 and impaired dendritic cell function as well. Even in patients who attained negative serum HCV RNA at Week 12, the transient responders showed a significant decrease of plasmacytoid dendritic cell frequency and impaired dendritic cell function. In conclusion, in PEG-IFNα and ribavirin combination therapy for chronic hepatitis C patients, the early-phase plasmacytoid dendritic cell frequency and/or end-of-treatment dendritic cell function are related to the virological outcome of the therapy.
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KEY WORDS: chronic hepatitis C; PEG interferon; ribavirin; dendritic cell

INTRODUCTION

Hepatitis C virus (HCV) infection causes various types of liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [Seeff, 2002]. The most effective way to prevent the progression of disease is to eradicate HCV from the infected hosts [Alter et al., 1989]. At present, combination therapy with pegylated interferon alpha (PEG-IFNα) and ribavirin is considered as the standard treatment for chronic HCV infection. The rate of the sustained virological response achieved by the combination therapy has been up to 50% in patients with HCV genotype 1 and a high HCV RNA titer; however, half of the patients do not attain sustained virological response [Manns et al., 2001; Fried et al., 2002]. In addition to HCV genotype and HCV quantity, several factors have been reported as

Abbreviations: HCV, hepatitis C virus; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells; NK, natural killer; MLR, mixed leukocyte reaction

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therapeutic determinants in PEG-IFNα and ribavirin combination therapy, such as liver fibrosis, age, gender, and ethnicity [Manesis et al., 1997; Poynard et al., 1998; Jacobson et al., 2005]. It is accepted that initial changes of serum HCV RNA titer from the beginning of the therapy, i.e., HCV dynamics, correlates well with the clinical outcomes of the treated patients [Davis et al., 2003; Hayashi and Takehara, 2006]. In PEG-IFNα and ribavirin therapy, an early virological response is defined as a reduction in serum HCV RNA quantity by at least 2 $\log_{10}$ units or to an undetectable level by a sensitive qualitative PCR after the first 12 weeks of the treatment or negative serum HCV RNA at Week 24 of the therapy [Davis et al., 2003]. It has been reported that the patients who fail to attain early virological response at Week 12 or 24 are not likely to gain sustained virological response after 48 weeks of the combination therapy, suggesting that early virological response can serve as a negative predictor of sustained virological response [Ferenci, 2004; Ferenci et al., 2005]. Prolongation of the duration of PEG-IFNα and ribavirin combination therapy from 48–72 weeks is likely to improve sustained virological response rate by decreasing relapsers [Berg et al., 2006]. Therefore, identifying potential relapsers during therapy and providing additional weeks of treatment may be clinically important, since it can offer them a better chance of attaining sustained virological response. However, no reliable marker is currently available for predicting virological relapse in PEG-IFNα and ribavirin therapy.

In chronic hepatitis C, multifaceted immune dysfunction may be implicated in the persistence of HCV including dendritic cells, NK cells, and T cells [Kanto et al., 1999; Auffermann-Gretzinger et al., 2001; Rosen et al., 2002; Nattermann et al., 2006]. It is reported that sustained viral responders maintained vigorous and multispecific HCV-specific $CD4^+$ Th1 responses, suggesting that the restoration of $CD4^+$ T cell responses may be related to successful HCV eradication [Kamal et al., 2002]. However, it is not known whether the frequency or the function of other immune cells during the combination therapy has any relationship to the therapy outcome.

In the present study, in order to determine immunological markers correlated with the efficacy of the treatment, the frequency of peripheral blood cell subsets and their dynamics were studied during and after the combination therapy. The function of dendritic cells from the patients was examined to clarify whether it was correlated with the therapeutic efficacy. This study supports the view that the reactivity of the immune system to the combination therapy is involved critically in the outcome of the treatment.

MATERIALS AND METHODS

Patients

Among chronic hepatitis C patients who had been followed at Osaka University Hospital, Osaka Koseinenkin Hospital, and Osaka National Hospital, 32 patients who received PEG-IFNα2b and ribavirin combination therapy for 48 weeks were enrolled in the present study. The study was approved by the ethical committee of the Osaka University Graduate School of Medicine. Written informed consent was obtained from all patients. At enrollment, the patients were confirmed to be positive for both serum anti-HCV antibody and HCV RNA, but were negative for other viral infections, including hepatitis B virus and human immunodeficiency virus. All the patients were infected with HCV genotype 1b with a serum HCV RNA quantity of more than 100 kilocopies/ml, as determined by methods described elsewhere [Pawlotsky et al., 2000]. All patients had shown persistent or fluctuating serum alanine aminotransferase abnormalities at enrollment. The presence of other causes of liver disease, such as autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, alcohol abuse, and metabolic disorders was excluded by laboratory and imaging analyses. With all patients, a combination of biochemical markers and ultrasonography or computed tomography scan analyses ruled out the presence of cirrhosis and tumors in the liver. Histological analyses of liver disease were performed with liver tissue obtained by ultrasonography-guided biopsy. The activity and stage of the disease were assessed by two independent pathologists according to the classification proposed by Desmet [Desmet et al., 1994].

Study Design

All patients were treated with PEG-IFNα2b subcutaneously at a dose of 75 μg/week (body weight > 40 kg and ≤ 60 kg) or 105 μg/week (body weight > 60 kg and ≤ 80 kg) or 135 μg/week (body weight > 80 kg and ≤ 100 kg) and oral ribavirin at a dose of 600 mg/day (body weight > 40 kg and ≤ 60 kg) or 800 mg/day (body weight > 60 kg and ≤ 80 kg) or 1000 mg/day (body weight > 80 kg and ≤ 100 kg). Ribavirin was administered divided into two doses per day. All patients were treated for 48 weeks and followed for 24 weeks after the cessation of therapy. The early responders were defined as those who showed a reduction in serum HCV RNA quantity to an undetectable level by qualitative PCR at Week 12 of the therapy. Virological response was estimated at 24 weeks after cessation of the treatment. Sustained virological response was defined as the maintenance of negative serum HCV RNA by PCR for more than 6 months after completion of the therapy. Transient response was defined as the reappearance of serum HCV RNA within 6 months after cessation of therapy in patients who had achieved negative serum HCV RNA at the end of the treatment. No response meant that there was persistently positive serum HCV RNA throughout the therapy period. Non-sustained virological response group is comprised of transient responders and no responders.

Analysis of Dendritic Cell Subsets, Helper T Cells, and NK Cells

For the numerical analyses of blood dendritic cells, helper T cells, and NK cells, venous blood was drawn

from patients before treatment and at Weeks 1, 4, 8, 12, 24, and 48 during the therapy. Peripheral blood mononuclear cells (PBMCs) were collected by density-gradient centrifugation on a Ficoll–Hypaque cushion. After viable PBMCs had been counted, the cells were stained with combinations of various antibodies for phenotypic markers.

The following monoclonal antibodies were purchased from BD Biosciences (San Jose, CA): anti-lineagemarker (Lin; CD3 (clone SK7), CD14 (clone MφP9), CD16 (clone 3G8), CD19 (clone SJ25C1), CD20 (clone L27), and CD56 (clone NCAM16.2)), anti-CD4 (clone RPA-T4), anti-CD11c (clone B-ly6), anti-CD123 (clone 7G3), anti-CD3 (clone UCHT1), anti-CD45RO (clone UCHL1), anti-CD56 (clone B159), anti-HLA-DR (clone L243), anti-CCR4 (clone 1G1). Anti-CXCR3 (clone 49801) monoclonal antibody was purchased from R&D Systems (Minneapolis, MN). Staining was performed with FITC, PE, PerCP, and APC conjugated antibodies as described previously. The acquisitions and analyses of data were performed with FACSCalibur (BD Biosciences) and CellQuest software.

Blood dendritic cells were defined as Lin^- and $HLA\text{-}DR^+$ cells. Myeloid dendritic cells are Lin^-, $HLA\text{-}DR^+$, $CD11c^+$, $CD123^{low}$ cells, and plasmacytoid dendritic cells are Lin^-, $HLA\text{-}DR^+$, $CD11c^-$, and $CD123^{high}$ cells, respectively. Helper T cell subpopulations were defined by the pattern of CXCR3 and CCR4; Th1 cells are $CD4^+$, $CD45RO^+$, $CXCR3^+$, and Th2 cells are $CD4^+$, $CD45RO^+$, and $CCR4^+$, respectively. NK cells were defined as $CD3^-$, $CD56^+$ cells. The percentages of dendritic cell subsets and NK cells in PBMCs or Th1 and Th2 cells in $CD4^+$ T cells were determined by FACS. In order to examine the dynamics of dendritic cell subsets after initiation of the treatment, we used the ratio of frequencies at each time point to those before the therapy.

Allogeneic Mixed Leukocyte Reaction With Dendritic Cells

In some patients, we examined whether the allostimulatory ability of dendritic cells was related to the clinical outcomes. At the end of treatment and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from PBMC obtained from the patients according to methods reported previously [Romani et al., 1994]. As controls, monocyte-derived dendritic cells were generated simultaneously from healthy donors. As responder cells in mixed leukocyte reaction (MLR), naive $CD4^+$ T cells were isolated from PBMC of irrelevant healthy donors by using a naive $CD4^+$ T cell enrichment kit (Stemcell Technologies, Vancouver, BC). Allogeneic MLR with monocyte-derived dendritic cells was performed as reported previously [Kanto et al., 1999]. In order to compare the ability of monocyte-derived dendritic cells among patients, we determined the MLR ratio between patients and controls as counts per minute (cpm) of ^3H-thymidine incorporated into $CD4^+$ T cells at the T cell/dendritic cell ratio of 10/1.

Statistical Analyses

For statistical analysis, the non-parametric Mann–Whitney *U*-test was used between the groups. To analyze paired data, we used Wilcoxon's signed rank test. Differences of continuous variables between groups were compared by two-way ANOVA. *P*-values of less than 0.05 were considered to be statistically significant. These statistical analyses were performed with StatView software (Cary, NC).

RESULTS

Outcome of the PEG-IFNα and Ribavirin Therapy

Among the 32 patients who received PEG-IFNα2b and ribavirin combination therapy, 25 completed the therapy while 7 patients dropped out due to various adverse effects. Among the 25 patients who completed the therapy, 11 (44%) achieved sustained virological response, 11 (44%) showed transient response, and 3 (12%) showed no response (Table I). There was no difference in the baseline clinical parameters among these groups (Table I). With regard to HCV RNA at Week 12 in patients who completed the therapy, 11 were negative for HCV RNA (early responders), while the remaining 14 were not. Among 11 patients with early response, 7 were sustained virological responders and 4 were transient responders. Among 14 patients who were positive for serum HCV RNA at Week 12, 4 patients achieved sustained virological response, 7 showed transient response, and 3 showed no response. Details of the therapeutic response in the current study are shown in Figure 1.

TABLE I. Baseline Clinical Characteristics of the Patients

	All patients	SVR	TR	NR
Age[a]	50.0 ± 10.9	46.7 ± 12.4	54.1 ± 8.9	46.7 ± 9.3
Sex (M/F)	20/5	9/2	8/3	3/0
ALT (IU/l)[a]	99.3 ± 47.8	97.5 ± 50.9	103 ± 51.3	94.0 ± 34.6
HCV RNA (kilo copies/ml)[a]	3146 ± 2675	3685 ± 3023	2743 ± 2338	2647 ± 3163
Activity (minimal/mild/moderate)	7/7/11	5/3/3	1/4/6	1/0/2
Fibrosis (mild/moderate/severe)	11/12/2	6/5/0	3/7/1	2/0/1

ALT, alanine aminotransferase.
Histological activity and fibrosis were assessed according to the classification proposed by Desmet.
[a]Mean ± SD.

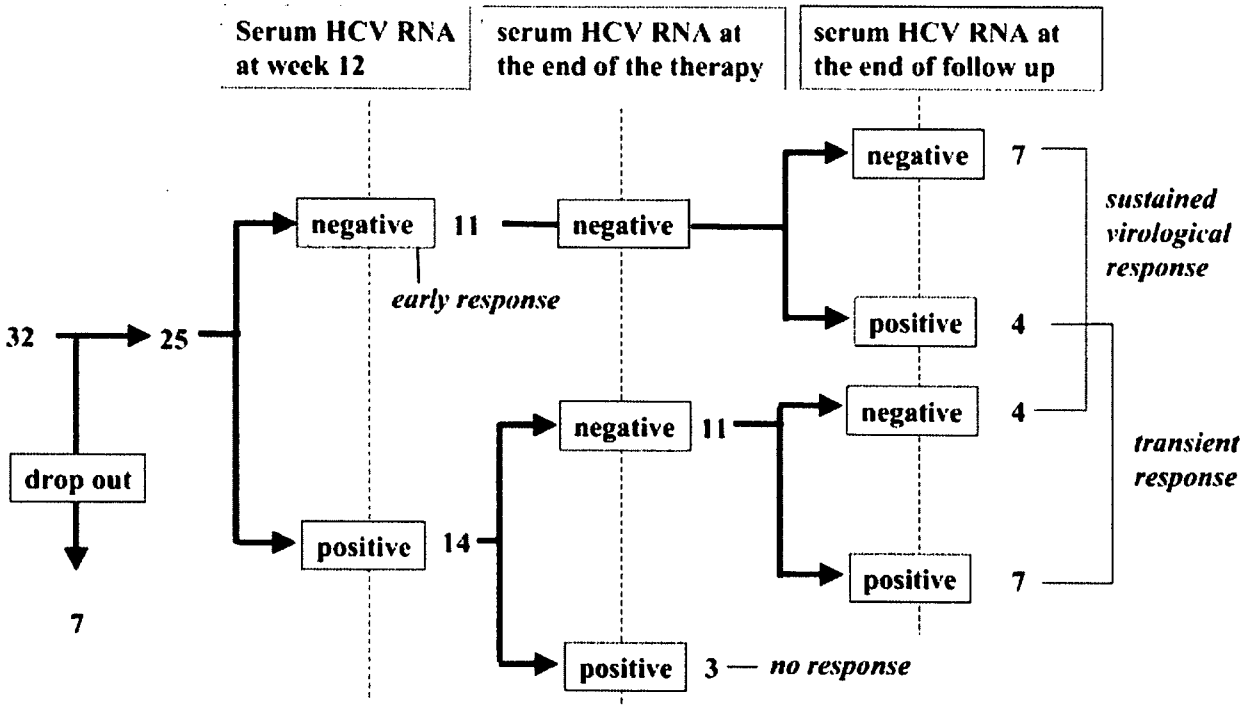

Fig. 1. Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFNα2b and ribavirin combination therapy. Thirty-two patients received the therapy, but seven dropped out due to various adverse effects. Among the 25 who completed the therapy, 11 achieved sustained virological response, 11 were transient responders, and 3 were non-responders. The early responders were defined as those who showed a reduction in HCV RNA quantity to an undetectable level by qualitative PCR at Week 12 of the therapy. According to this criterion, 11 patients were early responders and were further categorized into 7 sustained virological response (sustained virological responders with early response) and 4 transient response (transient responders with early response). Of the other 14 patients who were not early responders, 4 were sustained virological responders, 7 were transient responders, and 3 were non-responders.

Non-Sustained Virological Responders Had a Lower MLR Ratio Than Sustained Virological Responders

In order to clarify whether the frequency and function of immune cells are involved in the outcomes of the combination therapy, these parameters were compared between sustained virological responders and non-sustained virological responders, including transient responders and no responders. The pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1, and Th2 were not different between the sustained virological responders and non-sustained virological responders (Fig. 2A). As for the changes of dendritic cell subsets during the therapy, frequencies of both plasmacytoid dendritic cells and myeloid dendritic cells at each time point did not differ between sustained virological responders and non-sustained virological responders (Fig. 2B,C). The percentages of NK cells in non-sustained virological responders tended to be higher than those in sustained virological responders from Weeks 4–48, which did not reach statistical significance ($P = 0.0533$ ANOVA) (Fig. 2F). The frequencies of Th1 and Th2 did not differ between these two groups (Fig. 2G,H). As for dendritic cell function, dendritic cells from the non-sustained virological responders showed a lower MLR ratio than those from the sustained virological responders at the end ($P < 0.01$) and at 4 weeks after the completion of therapy ($P < 0.005$) (Fig. 3). These results show that lesser ability of dendritic cells at the end of treatment may be related to non-sustained virological response.

Transient Responders Had a Lower MLR Ratio in Dendritic Cell Function Than Sustained Virological Responders in the Course of Combination Therapy

In order to elucidate if the above-mentioned immunological markers are related to virological relapse, a

Fig. 2. Pretreatment frequency of blood cells and its changes during 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (**A**), during the combination therapy (**B**, **C**, **F**–**H**) and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (**D**, **E**) were determined as described in Materials and Methods, which were compared between sustained virological responders and non-sustained virological responders. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict non-sustained virological responders. The results are expressed as the mean ± SEM of 11 sustained virological responders and 14 non-sustained virological responders. PBMC, peripheral blood mononuclear cells; NK, natural killer.

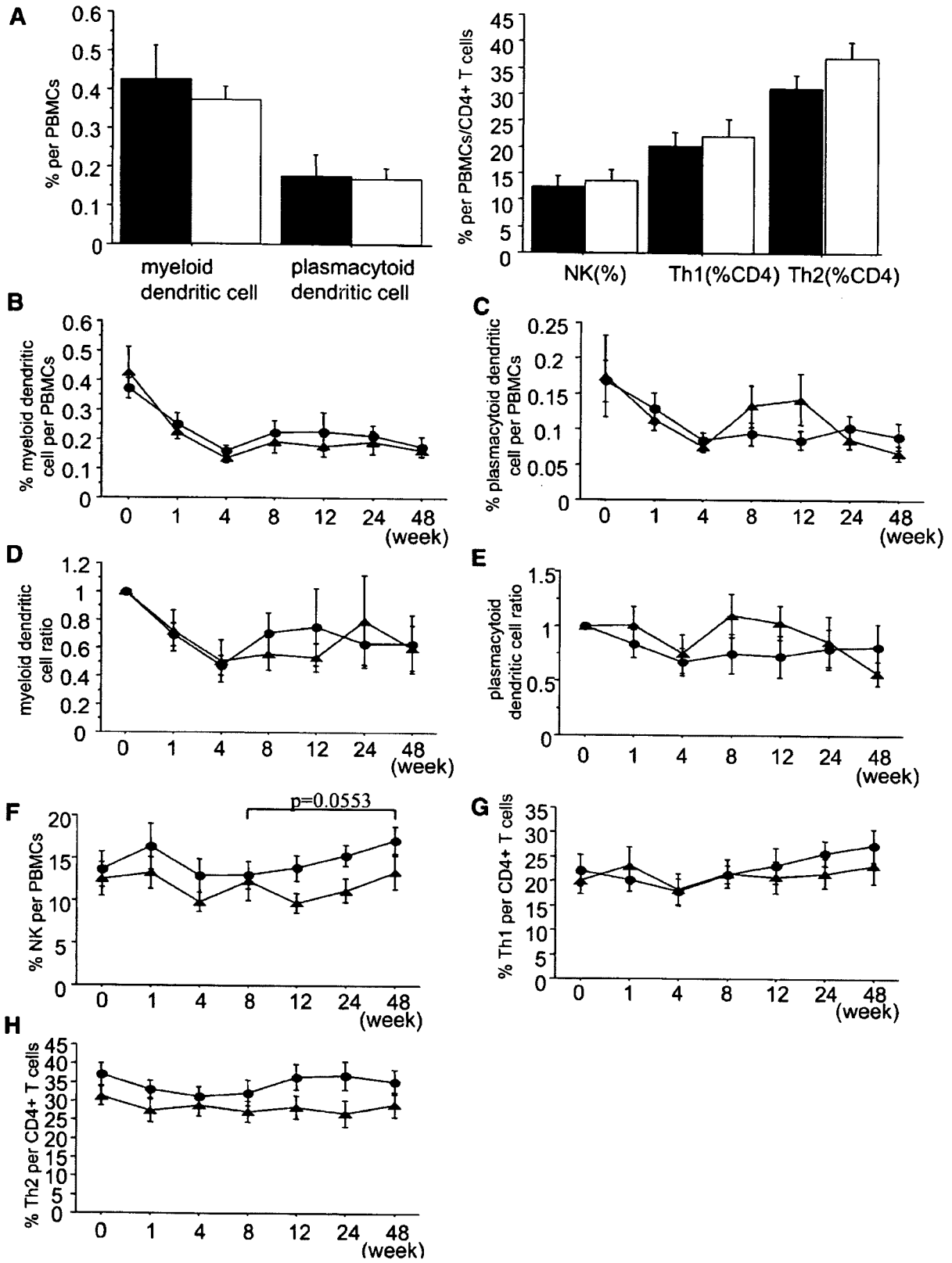
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK(%)
Th1(%CD4)
Th2(%CD4)
B
% myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
plasmacytoid dendritic cell ratio
F
p=0.0553
% NK per PBMCs
G
% Th1 per CD4+ T cells
H
% Th2 per CD4+ T cells
0 1 4 8 12 24 48
(week)

Fig. 2.

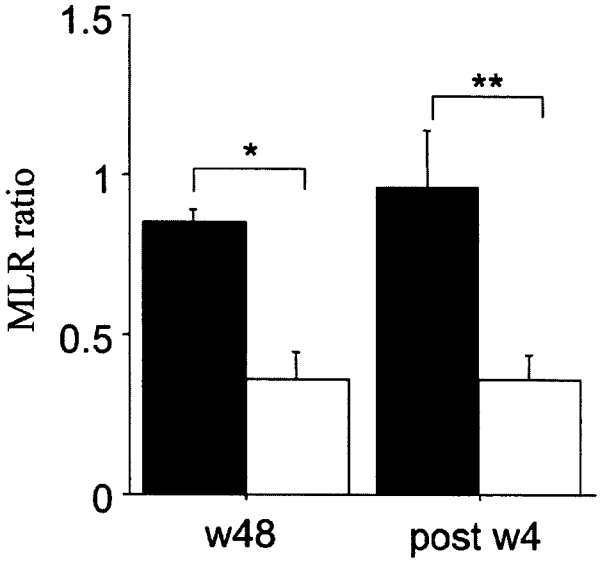

Fig. 3. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined from the counts per minute (cpm) of ^{3}H-thymidine incorporated into CD4$^+$ T cells at T cell/dendritic cell ratio of 10/1. The results are expressed as the mean ± SEM of 11 sustained virological responders and 14 non-sustained virological responders. Black bars indicate sustained virological responders and white bars indicate non-sustained virological responders. $^*P < 0.01$, $^{**}P < 0.005$.

comparison was undertaken between sustained virological responders and transient responders. The pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1, and Th2 were not different between the sustained virological responders and transient responders (Fig. 4A).

The percentages of myeloid dendritic cells and plasmacytoid dendritic cells were not different between the sustained virological responders and transient responders at each time point (Fig. 4B,C). The transient responders tended to show a lower plasmacytoid dendritic cell ratio than sustained virological responders from Weeks 1–12 ($P = 0.0553$, ANOVA) (Fig. 4E), suggesting that plasmacytoid dendritic cell is likely to decrease in the early phase in transient responders whereas those in sustained virological responders tend to be maintained. By contrast, no difference was observed in the myeloid dendritic cell ratio between the groups (Fig. 4D). The percentages of NK cells in transient responders were significantly higher than those in sustained virological responders from Weeks 8–48 ($P < 0.05$) (Fig. 4F). The frequencies of Th1 or Th2 at each point during therapy did not differ between the sustained virological responders and transient responders (Fig. 4G,H).

With regard to the dendritic cell function, the transient responders showed a lower MLR ratio than the sustained virological responders from Weeks 4–48 after the end of the therapy ($P < 0.05$) (Fig. 5). These results suggest that sustained impairment of dendritic cell function at the end and after the treatment may be related to the virological relapse after cessation of the therapy.

Early-Phase Decline of Plasmacytoid Dendritic Cell Frequency and Sustained Impairment of Dendritic Cell Ability Are Related to Transient Response in the Combination Therapy Even in Patients Who Lost Serum HCV RNA at Week 12 of the Treatment

In order to estimate more precisely the involvement of immunological markers in the outcomes of the combination therapy, we examined the above-mentioned parameters in patients who attained negative serum HCV RNA at Week 12 (early response group), as they were considered to be comparable with respect to the virological response to the therapy. Among 11 patients who were clear of serum HCV at Week 12, 7 were categorized into sustained virological response (sustained virological responders with early response) and the remaining 4 into transient response (transient responders with early response) (Fig. 1). Among patients with early response, the pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, Th1, Th2, and NK cells (Fig. 6A) and those at any points during the therapy did not differ between sustained virological responders and transient responders (Fig. 6B,C,F–H). The plasmacytoid dendritic cell ratios in transient responders were lower than those in sustained virological responders from Weeks 1–12 ($P < 0.05$, ANOVA) (Fig. 6E), whereas the myeloid dendritic cell ratio did not differ between the groups (Fig. 6D).

As for MLR, dendritic cells from the transient responders showed a lower MLR ratio than those from the sustained virological responders at the end and at 4 weeks after the completion of therapy (Fig. 7) ($P < 0.001$).

DISCUSSION

In the PEG-IFNα and ribavirin therapy for chronic hepatitis C, viral and host factors are critically involved in the efficacy of treatment. As for viral factors, HCV

Fig. 4. Pretreatment frequency of blood cells and its changes during 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (**A**), during the combination therapy (**B**, **C**, **F–H**), and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (**D**, **E**) were determined as described in Materials and Methods, which were compared between sustained virological responders and transient responders ones. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict transient responders. The results are expressed as the mean ± SEM of 11 sustained virological responders and 11 transient responders. PBMC, NK are shown in Figure 2. $^*P < 0.05$ (sustained virological responders vs. transient responders).

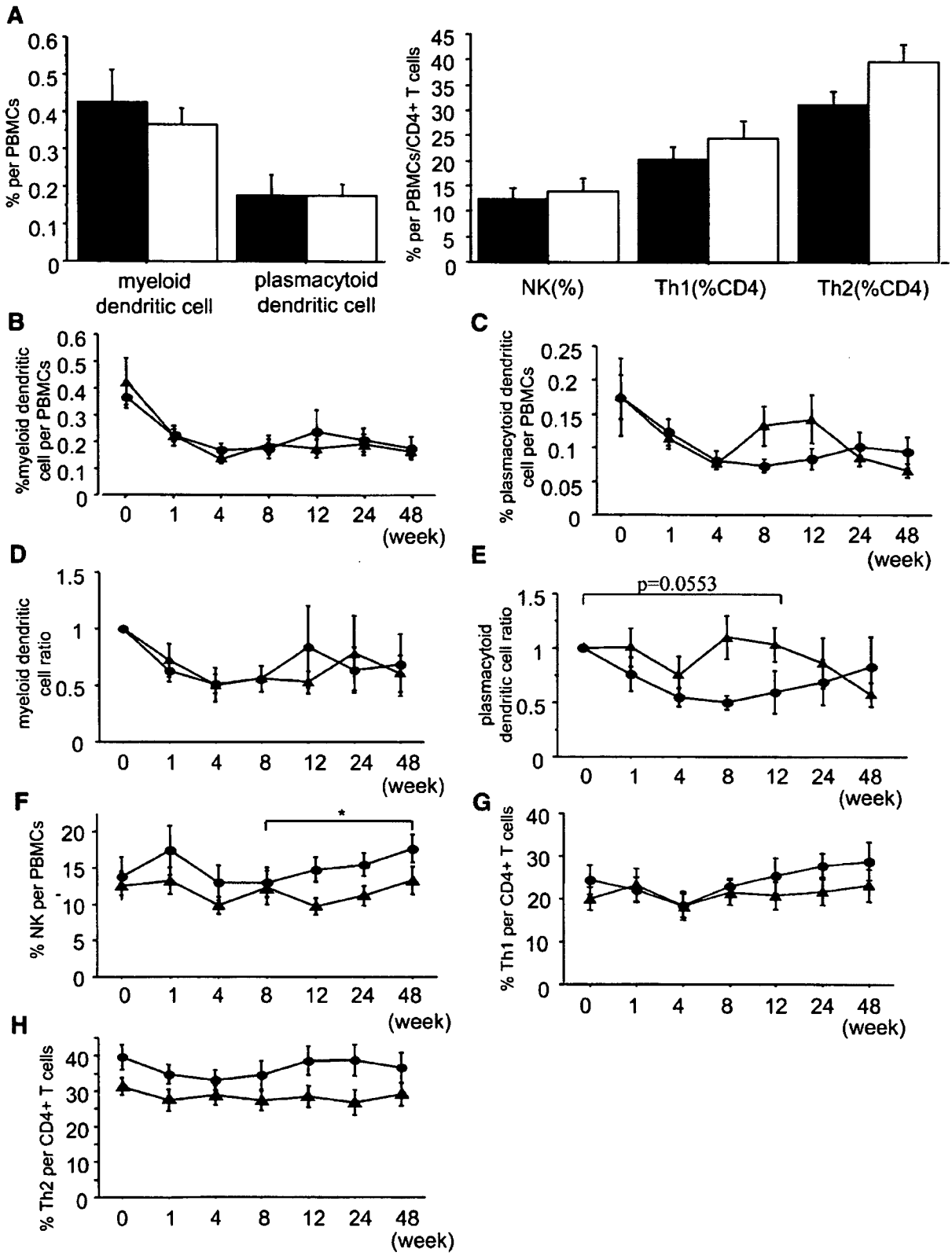
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK(%)
Th1(%CD4)
Th2(%CD4)
B
%myeloid dendritic cell per PBMCs
(week)
C
% plasmacytoid dendritic cell per PBMCs
(week)
D
myeloid dendritic cell ratio
(week)
E
p=0.0553
plasmacytoid dendritic cell ratio
(week)
F
*
% NK per PBMCs
(week)
G
% Th1 per CD4+ T cells
(week)
H
% Th2 per CD4+ T cells
(week)

Fig. 4.

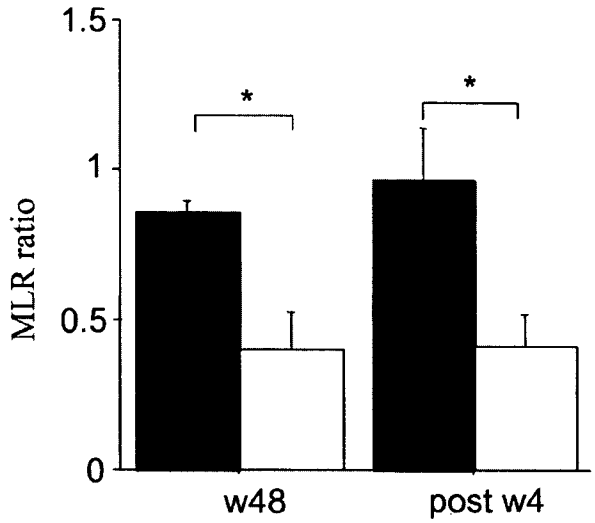

Fig. 5. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined as the same as Figure 3. The results are expressed as the mean ± SEM of 11 sustained virological responders and 11 transient responders. Black bars indicate sustained virological responders and white bars indicate transient responders. $^*P < 0.05$.

genotypes and baseline HCV RNA titers are major determinants dictating therapeutic outcomes. In addition, failure of rapid decline in serum HCV RNA from the beginning of the treatment, i.e., non-early virological response, has been used as a negative predictor for sustained virological response. Alternatively, the enhancement of immunity has been implicated to play a key role in the successful responses in PEG-IFNα and ribavirin therapy. However, it is yet to be determined which parameters are practically feasible for the assessment of treatment-induced immune responses correlating with therapeutic efficacy.

In the present study, it was determined whether the frequencies of dendritic cells, NK cells, Th1 and Th2 cells, as well as dendritic cell function in patients are related to the outcome of the PEG-IFNα and ribavirin therapy. By comparing these markers in the course of the treatment between sustained virological responders and non-sustained virological responders, it was demonstrated that non-sustained virological responders showed impaired dendritic cell function in MLR than sustained virological responders. When the analyses were extended to comparison between sustained virological responders and transient responders, transient responders exhibited (1) lower plasmacytoid dendritic cell ratio, (2) higher NK cell frequency, and (3) impaired dendritic cell function than sustained virological responders. Of particular interest were the findings of a lower plasmacytoid dendritic cell ratio as well as lower MLR even in transient responders with early response compared to sustained virological responders with early response. Since patients with early response are defined as those who showed negative serum HCV RNA at Week 12, they are considered to be similar in virological response to the combination therapy. Thus, such parameters could serve as immunological markers for virological relapse, presumably being independent of the early virological response.

In general, homeostasis of blood cell number is regulated by their life span and their recruitment from the bone marrow to circulating blood. A reduction of blood cell numbers is frequently observed in patients who are treated with PEG-IFNα and ribavirin combination therapy, which may be due to bone marrow suppression, enhancement of cellular apoptosis, or alteration of localization. However, the dynamics of dendritic cell subsets or NK cells under combination therapy is yet to be clarified. Some investigators have reported that the frequency or the absolute number of blood dendritic cell is dynamically changed by various stresses, such as infection [Hotchkiss et al., 2002] or surgery [Ho et al., 2001]. The present study showed that reduction of plasmacytoid dendritic cells after the introduction of combination therapy is much greater in the transient responders than in the sustained virological responders. IFNα is reported to act as a regulatory factor on $CD11c^-$ dendritic cells to sustain their viability and to inhibit gaining the ability to stimulate Th2 development [Ito et al., 2001]. Thus, patients who respond well to IFNα, as demonstrated by better plasmacytoid dendritic cell survival during the treatment, are likely to have better chances to eradicate HCV. Limited information is available about the factors influencing the number of NK cells. In chronic HCV infection, it has been reported that the progression of liver disease is associated with a decrease of peripheral as well as liver-residing NK cells [Kawarabayashi et al., 2000]. It is plausible that the lower frequency of peripheral NK cells in the sustained virological responders compared to the transient responders, as shown in this study, may be related to the accumulation of NK cells in the liver, where they presumably produce IFNγ to suppress HCV replication. Further study is needed to disclose the reasons for the dynamics of these cells being related to the virological response in the combination therapy.

Fig. 6. Pretreatment frequency of blood cells and changes during 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (**A**), during the combination therapy (**B**, **C**, **F**–**H**) and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (**D**, **E**) were determined as described in Materials and Methods, which were compared between sustained virological responders and transient responders ones. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict transient responders. The results are expressed as the mean±SEM of seven sustained virological responders with early response and four transient responders with early response. PBMC, NK are shown in Figure 2. $^*P < 0.05$ (sustained virological responders vs. transient responders).

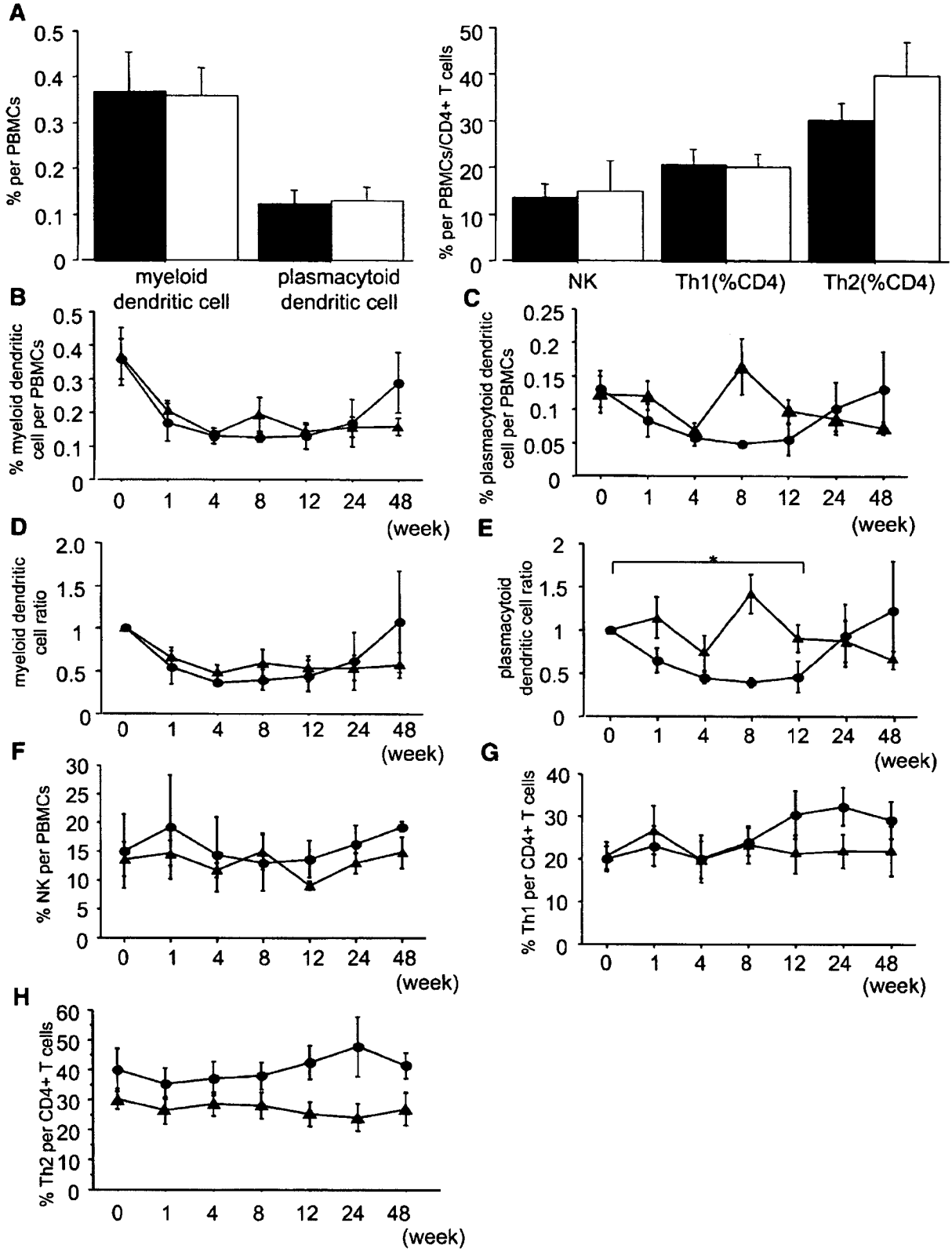
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK
Th1(%CD4)
Th2(%CD4)
B
% myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
plasmacytoid dendritic cell ratio
F
% NK per PBMCs
G
% Th1 per CD4+ T cells
H
% Th2 per CD4+ T cells
(week)

Fig. 6.

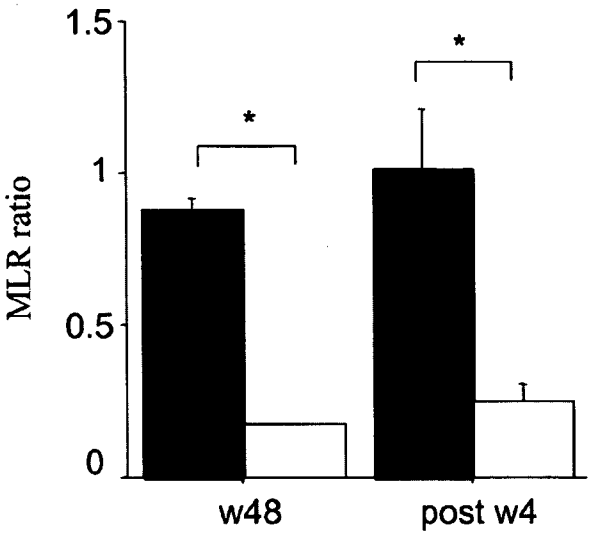

Fig. 7. Allostimulatory activity of dendritic cells in the patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. At the end of treatment (Week 48) and at Week 4 after the completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined as the same as Figure 3. The results are expressed as the mean ± SEM of seven sustained virological responders with early response and four transient responders with early response. Black bars indicate sustained virological responders and white bars indicate transient responders, respectively. *$P < 0.05$.

In the present study, non-sustained virological responders or transient responders showed a lesser capacity for dendritic cell function than sustained virological responders at the end and after cessation of the therapy. Even in the patients who lost serum HCV RNA at Week 12, the dendritic cell function was lower in transient responders than sustained virological responders. One of the mechanisms of impaired dendritic cell function in non-sustained virological responders or transient responders may be residual HCV both in serum and in cells. It is reported that the relapse rate was higher in the patients who were positive for HCV RNA by sensitive transcription-mediated amplification (TMA) at the end of combination therapy than those who were negative for it, even when they were negative for HCV RNA by conventional PCR [Gerotto et al., 2006]. Other investigators have shown that residual HCV is detectable by means of sensitive PCR in blood cells from patients who cleared HCV from the serum by IFNα and ribavirin combination therapy [Pham et al., 2004], supporting the possibility that blood cells are reservoirs of HCV replication. Taking these findings into consideration, it is conceivable that a small quantity of HCV might exist in the blood cells in some transient responders. Since direct HCV infection of monocytes or blood dendritic cells is considered to be one of the mechanisms of the functional impairment of dendritic cell [Navas et al., 2002; Goutagny et al., 2003; Ducoulombier et al., 2004], persistent HCV may delay the restoration of dendritic cell function in non-sustained virological responders or transient responders compared to sustained virological responders.

In summary, it was shown that the frequencies of plasmacytoid dendritic cells or NK cells and dendritic cell function might be related to the outcomes of the combination therapy. Since the present study was performed with a relatively small number of patients, a greater number of patients should be examined in order to validate the feasibility of using these as immunological markers of relapse. The prediction of virological non-response or relapse during therapy can help improve the clinical outcomes of treated patients, as prolongation of combination therapy offers potential relapsers a better chance of sustained virological response by suppressing HCV reappearance.

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Original Article

Efficacy of low dose long-term interferon monotherapy in aged patients with chronic hepatitis C genotype 1 and its relation to alpha-fetoprotein: A pilot study

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Aim: The objective of this study was to examine the efficacy and safety of low dose long-term interferon (IFN) therapy in aged patients with chronic hepatitis C genotype 1.

Methods: The IFN therapy was performed in Shin-Kokura Hospital on 44 patients aged 60 or older with chronic hepatitis C. All patients had high viral loads of genotype 1. Three million units of natural IFN-α was administered intramuscularly or intrasubcutaneously, three times a week for three years. A control group of 44 subjects not treated with IFN, matched for age, gender and hepatic histology, was formed.

Results: Two of the 44 patients showed a sustained virological response. Alanine aminotransferase was below the upper limit of normal in 59% (23/39) of the patients and alpha-fetoprotein was less than 40 ng/mL in 97% (38/39) on the completion of treatment. Sustained biochemical response was observed in 53% (19/36) of the patients. In the liver cirrhosis group, serum albumin values and platelet counts increased in 38% (6/16) and 33% (6/18) of patients, respectively. Hepatocellular carcinoma (HCC) appeared in three patients by 13 months after the start of treatment, but no cases were reported thereafter. The cumulative non-carcinogenesis rate of HCC in the liver cirrhosis group was significantly higher in the IFN treatment group compared to the control group (log–rank test, $P = 0.046$).

Conclusion: Low dose long-term interferon monotherapy to prevent carcinogenesis of HCC was considered useful in aged patients for whom peg-interferon and ribavirin combination therapy is difficult.

Key words: aged patients, alpha-fetoprotein, chronic hepatitis C, interferon, long-term treatment

INTRODUCTION

CURATIVE TREATMENT OF chronic hepatitis C currently depends mainly on peg-interferon (peg-IFN) and ribavirin combination therapy.[1,2] It is the treatment of choice and shows greater therapeutic effects than interferon (IFN) monotherapy, especially in patients with high viral loads. However, many patients, especially aged patients, often discontinue ribavirin combination therapy because of adverse reactions such as anemia.[3] In patients with high viral loads of genotype 1, the rate of sustained virological response (SVR) is about 50% for peg-IFN and ribavirin combination therapy, but the SVR rate drops in patients who are elderly or have obesity and/or steatosis.[4–6] In aged patients, peg-IFN and ribavirin combination therapy is also often difficult because of morbid conditions such as diabetes or hypertension.

In Japan, with the aging of patients with hepatitis C, the number of patients over 60 years is increasing, but in such aged patients, the incidence of hepatocellular carcinoma (HCC) is high[7] and IFN therapy is required to prevent the development of HCC. Few reports are available on the effects of long-term IFN therapy in aged patients. Alpha-fetoprotein (AFP) is a tumor marker for HCC and cases of high blood levels of AFP are often found among patients with chronic hepatitis C or liver cirrhosis.[8,9] It is known that AFP values decrease when effects are obtained from IFN therapy. However, several reports have called attention to AFP values as an effect of IFN therapy, independent of SVR.[10] In the present study, we performed low dose long-term IFN

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monotherapy in aged patients (60 years or older) with hepatitis C to prevent carcinogenesis of HCC, and examined the efficacy and changes in AFP values.

METHODS

Patients

THE SUBJECTS WERE 44 patients (22 males and 22 females), aged 60 years or above with hepatitis C who started low dose IFN monotherapy in Shin-Kokura Hospital between 1994 and 2002 (Table 1). The age at the start of treatment ranged from 61 to 76, with a median age of 67.2. Twenty-six patients had chronic hepatitis (CH) and 18 had liver cirrhosis (LC). The virus was genotype 1 with levels of 100 KIU/mL or 1.0 Meq/mL or higher. The subjects included 11 patients taking a glycyrrhizin preparation for at least one year before IFN administration and all 11 patients had alanine aminotransferase (ALT) levels of 60 IU/L or higher at registration. The glycyrrhizin preparation was discontinued at least one month before registration.

A group of 44 patients receiving IFN therapy and a control group of 44 subjects not treated with IFN, matched for age, gender and hepatic histology were formed. The control group was observed for at least seven years. This study was not randomized.

Chronic hepatitis (CH) patients were F1–F3 based on liver biopsies. Liver cirrhosis was diagnosed by liver biopsy or clinically. All subjects were Child's A. The 88 subjects were divided into the following four groups and the incidence of HCC was examined. CH-T group ($n = 26$): CH patients receiving IFN therapy; LC-T group ($n = 18$): LC patients receiving IFN therapy; CH-c group ($n = 26$): CH controls and LC-c group ($n = 18$): LC controls.

Within one month before the start of treatment, all subjects were subjected to abdominal ultrasonography (US) and dynamic computed tomography (CT) to check for HCC.

Low dose long-term IFN monotherapy

IFN therapy was performed using 3 million units of IFN-α (human lymphoblastoid interferon (HLBI), Sumiferon; Dainippon Sumitomo Pharma, Osaka, Japan) administered intramuscularly or intrasubcutaneously 2–3 times per week for three years. The patients were observed for at least two years thereafter.

Blood testing

A serum hepatitis C virus (HCV) qualitative test was performed using an Amplicor-HCV kit, version 2.0 (Roche Molecular Diagnostics Co, Tokyo, Japan).[11] Serum HCV-RNA levels were quantified using branched DNA (bDNA) probe assay (version 2; Chiron, Dai-ichi Kagaku, Tokyo)[12,13] or combined PCR assay (Amplicor-HCV monitor assay, Roche Molecular Diagnostics Co., Tokyo, Japan).[14] In this study, a high viral load was designated as a serum HCV-RNA level of higher than 10^6 equivalents/mL using bDNA assay, or higher than 10^5 IU/mL serum using Amplicor-HCV monitor assay. The white blood cell count, red blood cell count, hemoglobin concentration, platelet count, serum ALT levels, serum albumin levels and serum AFP levels were measured before treatment and once every four weeks. The upper limit of the normal range (ULN) for ALT was 40 IU/L.

The efficacy of treatment was assessed based on HCV-RNA negativity and sustained control of ALT below the ULN. Patients who tested as HCV-RNA-negative at 24 weeks after completion of the IFN treatment were considered to be in SVR. Patients who tested

Table 1 Baseline characteristics of patients

	IFN therapy group			Control group		
	CH-T† $n = 26$	LC-T‡ $n = 18$	Total $n = 44$	CH-c† $n = 26$	LC-c‡ $n = 18$	Total $n = 44$
Mean age (years) (range)	66.1 (61–74)	68.7 (61–76)	67.2 (61–76)	65.8 (60–71)	67.9 (61–73)	67.3 (60–73)
Sex (M : F)	12 : 14	10 : 8	22 : 22	12 : 14	10 : 8	22 : 22
ALT (IU/L)	80	47	67	82	48	68
Plat (/μL)	165 000	72 000	127 000	179 000	87 000	141 000
AFP (ng/mL)	65	54	61	67	60	64

†No significant difference between CH-T group and CH-c group; ‡No significant difference between LC-T group and LC-c group.
ALT, alanine aminotransferase; AFP, alpha-fetoprotein; CH-c, chronic hepatitis control group; CH-T, chronic hepatitis IFN therapy group; LC-c, liver cirrhosis control group; LC-T, liver cirrhosis IFN therapy group.

as HCV-RNA positive but showed sustained control of ALT below ULN at six months after the completion of the IFN treatment were considered to have a sustained biochemical response (SBR). The remainder of the patients were considered to be non-responders.

Abdominal ultrasonography was performed once every three months and CT once a year. HCV-RNA was measured once every six months. Treatment was discontinued when serious adverse reactions appeared, when the platelet count fell below 25 000/μL, when HCC occurred, or when the patient desired discontinuation.

Informed consent

The study protocol was approved by the institutional ethics committee of Shin-Kokura Hospital and all patients gave their informed consent to participate in this study. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice.

Statistical analysis

Differences between the groups were analyzed using Fisher's exact test. Continuous variables were compared using the Mann–Whitney test. The cumulative non-carcinogenesis rates in the IFN treatment and control groups were compared using the log-rank test. All tests were two-sided, and *P*-values less than 0.05 were considered to be significant.

RESULTS

The negativity of HCV-RNA

HCV-RNA STATUS became negative during IFN treatment in six patients in the CH-T group and two patients in the LC-T group. At six months after completion of IFN treatment, HCV-RNA negativity was maintained and SVR was obtained in only two patients of the CH-T group. In these two patients, HCV-RNA became negative within one year after the start of IFN treatment and negativity was maintained thereafter. No patients with SVR were observed in the LC-T group.

Changes in ALT (Table 2)

The mean values of ALT in the IFN treatment group were 67 IU/L before treatment, 46 IU/L after six months, 44 IU/L on completion and 48 IU/L two years after completion of treatment. The mean ALT values at six months, three years and five years after registration in the control group were 72 IU/L, 78 IU/L and 70 IU/L, respectively. The ALT values in the IFN therapy group were significantly lower at all times compared to the control group ($P < 0.05$). The mean ALT levels in the CH-T and LC-T groups before treatment were 80 IU/L and 47 IU/L, decreasing to 51 IU/L and 39 IU/L, respectively, after six months ($P < 0.05$). Thereafter, no changes occurred during or after treatment. The rate of ALT levels of ULN or lower in the CH-T and LC-T groups were 58% and 50% after six months, 54% and 56% on completion of IFN treatment and 52% and 53% two years after completion of treatment, respectively. In both the CH-T and LC-T groups, a significantly higher

Table 2 Efficacy of the low dose, long-term interferon monotherapy (serum ALT and AFP levels)

	Before	After 6 months	On completion	After 2 years
The rate of decrease to less than 40 IU/L of ALT				
CH-T	0/26	15/26***	14/23***	11/21***
(%)	0	58	61	52
LC-T	2/18	9/18*	9/16**	8/15*
(%)	11	50	56	53
Total	2/44	24/44***	23/39***	19/36***
(%)	5	55	59	53
The rate of decrease to less than 40 ng/mL of AFP				
AFP: >40 ng/mL	0/18	14/18***	14/15***	13/14***
(%)	0	78	93	93
Total	26/44	40/44***	38/39***	35/36***
(%)	59	91	97	97

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
ALT, alanine aminotransferase; AFP, alpha-fetoprotein; CH-T, chronic hepatitis IFN therapy group; LC-T, liver cirrhosis IFN therapy group.

rate of ALT levels of ULN or lower was found at all times, when compared with before treatment ($P < 0.05$). The rate of decrease to ULN or lower of ALT during and after IFN treatment ranged from 50% to 56%. In 11 patients who used a glycyrrhizin preparation before IFN therapy, eight patients showed ALT of less than 60 IU/L and seven patients were at ULN or lower at six months after the start of IFN therapy. SBR was achieved in 19 patients in total (53%), 11 in the CH-T group and eight in the LC-T group.

Changes in AFP (Figs 1,2 and Table 2)

The mean AFP value before treatment was 61 ng/mL. The mean AFP values were 46 ng/mL after three months of treatment, 31 ng/mL after six months of treatment, 21 ng/mL on completion of treatment and 25 ng/mL two years after completion of treatment. The values were all significantly lower than before treatment ($P < 0.001$). The mean AFP values at three months, six months, three years and five years after registration in the control group were 67 ng/mL, 66 ng/mL, 82 ng/mL and 74 ng/mL, respectively. The AFP values in the IFN therapy group were significantly lower at all times compared to the control group ($P < 0.001$). The AFP value before treatment was 40 ng/mL or higher in 18 patients.

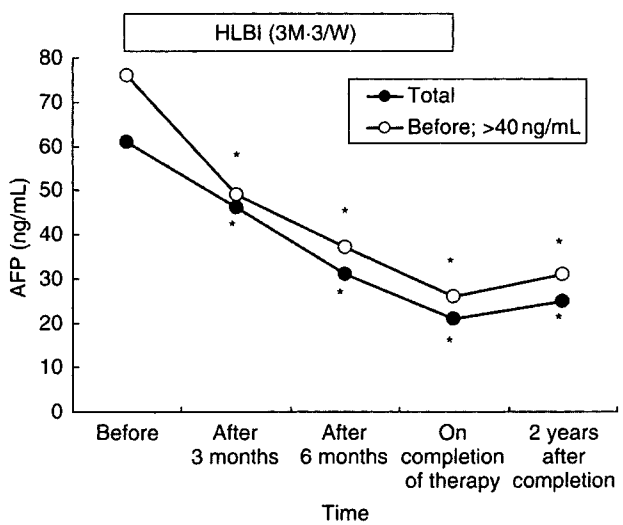

Figure 1 Efficacy of low dose long-term interferon monotherapy (serum AFP levels). Changes in mean alpha-fetoprotein (AFP) levels in the third and sixth months of therapy, on completion and two years after completion of therapy. (●) Total, (○) before start of treatment >40 ng/mL. *$P < 0.001$ (before therapy vs. third month, sixth month, on completion and two years after completion).

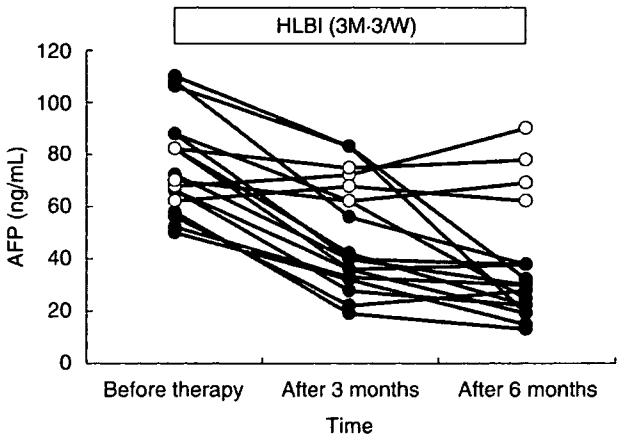

Figure 2 Efficacy of low dose interferon monotherapy (serum AFP levels). Changes in mean alpha-fetoprotein (AFP) values in the third and sixth months in 18 patients with AFP values before treatment of 40 ng/mL or higher. (●) AFP decreased cases, (○) AFP unchanged or increased cases.

Figure 2 shows the courses of these 18 patients at three months and six months after the start of treatment. The mean AFP values were 75.9 ng/mL before treatment, 49.4 ng/mL after three months, 37.1 ng/mL after six months, 26.2 ng/mL on completion and 31.4 ng/mL two years after completion of treatment. The AFP value after three months and thereafter was significantly lower than that before treatment ($P < 0.001$). After six months of treatment, the AFP value had decreased to less than 40 ng/mL in 14 out of 18 patients. AFP values did not change in three of the four remaining patients and increased in one patient (Fig. 2). HCC appeared in three out of four patients with no decreases in AFP. In the 18 patients with AFP values of 40 ng/mL or higher before treatment, the rates of decrease to less than 40 ng/mL were 78% (14/18) after six months, 93% (14/15) on completion and 93% (13/14) two years after completion of treatment. The values were all significantly higher than before treatment ($P < 0.001$) (Table 2).

Changes in serum albumin values and platelet counts

Among the 16 patients showing serum albumin values of less than 3.9 g/dL before treatment, six patients (38%) showed increases of at least 0.3 g/dL on completion of treatment when compared to before treatment. The mean value of 3.8 g/dL for serum albumin after completion of treatment was higher than the 3.5 g/dL value before treatment.

The platelet count in the CH-T group decreased up to three months after the start of IFN therapy, but increased to the value before treatment thereafter. In the LC-T group, the platelet count increased in six patients (33%) on completion of treatment.

Incidence of HCC (Fig. 3)

Figure 3 shows the incidence of HCC in each group. HCC appeared during IFN treatment in three patients in the CH-T and LC-T groups. The time of discovery of HCC in these three patients was at eight months, at nine months and at 13 months after the start of treatment. The three HCC patients were all male and they were 68, 73 and 70 years of age. The maximum diameters of HCC were 11 mm, 18 mm and 14 mm, and HCC was limited to one tumor in each case. HCC occurred in one patient in the CH-T group and two patients in the LC-T group. The liver tissue of the three patients showed F3 chronic hepatitis in one patient and F4 liver cirrhosis in the other two patients. No new cases of HCC appeared until the completion of IFN treatment thereafter. One patient in the LC-T group had HCC at three years and three months after the completion of treatment. The AFP value for this patient was 58 ng/mL before treatment, dropping to 22 ng/mL after six months of treatment. Thereafter, the value remained at less than 30 ng/mL until the completion of treatment. The value at the time of carcinogenesis was 36 ng/mL. HCC was observed at four years after the completion of treatment in the CH-T group. The annual incidences of HCC in the CH-c and LC-c control groups were 3.5% and 8.7%, respectively. The incidence was lowest in the CH-T group and highest in the LC-c group. The incidences were the same in the LC-T group treated with IFN and in the CH-c control group (Fig. 3). The cumulative non-carcinogenesis rate of HCC in the chronic hepatitis group showed no significant difference when compared between the IFN treatment and control groups. However, the cumulative non-carcinogenesis rate of HCC in the liver cirrhosis group was significantly higher in the IFN treatment group compared to the control group (log-rank test, $P = 0.046$).

Safety

IFN treatment was discontinued in two patients, one with stomach cancer and one with lung cancer in the CH-T and LC-T groups. Three other patients developed HCC and so treatment was discontinued in a total of five patients. One patient dropped out because of a cerebral hemorrhage during observation after treatment. This patient was a 68-year-old male who had been treated for diabetes before IFN treatment. The diabetes was not aggravated in observations during or after treatment. Two other patients did not come to the hospital and dropped out. Treatment was not discontinued in any patients due to adverse reactions to IFN.

DISCUSSION

IT HAS BEEN 15 years since IFN treatment of chronic hepatitis C started in 1992. The mortality of HCC has tended to increase every year. Patients with chronic hepatitis C are getting older every year. For conventional IFN monotherapy for six months, the SVR rate (sustained negativity of HCV-RNA) was low, at about 20%.[15,16] The SVR rate was increased to about 50% when peg-IFN and ribavirin combination therapy was used. It is well known that the incidence of HCC drops when

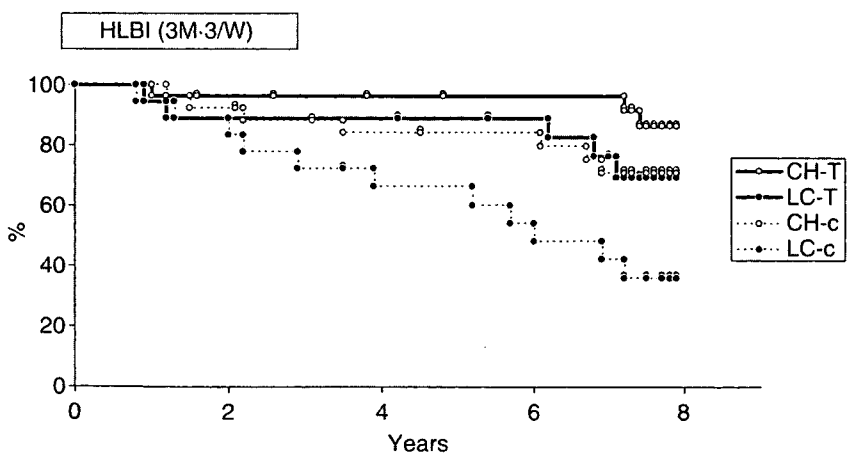

Figure 3 Course of non-carcinogenesis rate using interferon therapy. CH-c, chronic hepatitis control group; CH-T, chronic hepatitis IFN therapy group; LC-c, liver cirrhosis control group; LC-T, liver cirrhosis IFN therapy group. $P = 0.134$ (CH-T vs. CH-c using log-rank test), $P = 0.046$ (LC-T vs. LC-c using log-rank test).

negativity of the HCV is maintained.[17] However, even if HCV-RNA is not negative, the incidence of HCC has been found to decrease when hepatic function becomes normal.[18,19] The primary goal of IFN treatment is to eradicate the HCV, but in aged patients, combination therapy with ribavirin is quite often difficult[3] and it is important to improve hepatic function using low dose IFN monotherapy and prevent onset of HCC.

In the present study, ALT was decreased significantly three months after the start of treatment. After six months, ALT was at the ULN or lower in 55% of patients and no change occurred thereafter. The same results were obtained in both the CH-T and LC-T groups, that is the rate of decrease of ALT to ULN or lower using low dose IFN treatment was about 50–60%. The results of IFN therapy are assessed by HCV-RNA negativity, but the present patients were aged patients with high viral loads of genotype 1 and the SVR rate was low. Therefore, the ALT normalization rate was lower than that in previous reports. However, in this study, the SBR rate was 53%. Even if HCV-RNA does not become negative, prevention of onset of HCC can be expected with normalization of hepatic function, and low dose IFN therapy is considered useful.

Patients who did not show a decrease in ALT after long-term administration of a glycyrrhizin preparation were treated with IFN and ALT decreased in about 60% of cases. This result suggests that treatment with small doses of IFN is useful in improving ALT in patients with chronic hepatitis C or chronic liver cirrhosis showing no decrease in ALT after administration of a glycyrrhizin preparation.

AFP is a tumor marker of HCC and it becomes positive in about 65% of HCC patients. AFP is also useful as a parameter for the early diagnosis of HCC and observation of its course after treatment.[20–22] AFP is not only secreted by HCC, but is also produced in regenerated liver after it is injured.[23] Even if HCC is not confirmed by US and CT, high levels of AFP are seen in some patients. Many reports have cited elevated AFP baselines as an independent HCC risk factor.[8,9] In the present study, AFP was significantly decreased at three months after the start of treatment. In 14 of the 18 patients (78%) with AFP levels of 40 ng/mL or higher before treatment, the value had dropped to less than 40 ng/mL after six months (Fig. 2). HCC appeared during treatment in three of the four patients with no decrease in AFP. The three HCC patients developed the disease between eight and 13 months after the start of treatment and no cases appeared during treatment thereafter. These results indicate that a high AFP value is a useful factor in determining onset of HCC. In these three patients, HCC was not discovered using US or CT before the start of IFN treatment, but they appeared to be in a precancerous condition for HCC.

The remaining patients did not develop HCC during treatment and no HCC was found for three years after the completion of treatment. The onset of HCC seems to be delayed for six years by IFN treatment for three years, and the HCC onset rate was the same as that in the chronic hepatitis control group (CH-c group) in observation of the course for seven or eight years.

Murashima *et al.* reported that IFN therapy universally reduced the AFP baseline.[10] In this study AFP levels were reduced by IFN therapy in patients who did not develop HCC. However, even in patients whose ALT values did not become normal, decreases in AFP were observed. The mechanism of the decrease of AFP by IFN therapy was unclear. AFP is considered to be a good marker for estimating the effects of long-term IFN therapy to prevent carcinogenesis of HCC, in the same way as ALT.

In a study by Arase *et al.*, IFN monotherapy was found to show greater effects by long-term administration for one year and six months.[24] In order to reduce adverse reactions such as pancytopenia, a low dose of IFN was given and thus it was possible to perform the treatment for a longer period. It has been reported that long-term administration for two years or longer is effective.[25] It was also reported that even when HCV-RNA did not become negative after discontinuation of administration for three years, normal hepatic function was maintained for long periods.[26] No discontinuations due to adverse reactions, such as pancytopenia, were mentioned in any of these reports. Therefore, an administration period of two–three years is considered necessary. IFN treatment appears to be relatively safe as long as the dose is low. In our study, a total of five patients discontinued treatment during the three-year long-term treatment, one with stomach cancer, one with lung cancer and three with HCC. Treatment was not discontinued in any patients because of direct adverse reactions to IFN. Low dose long-term IFN therapy is considered safe and useful in the prevention of the onset of HCC.

The rate of change of HCV-RNA to negative is high during IFN and ribavirin combination therapy, but many recurrences occur after the completion of therapy. Sustained negativity of HCV-RNA can be achieved during treatment by continuing IFN monotherapy.[27] In the present study, two out of eight patients in whom HCV-RNA became negative during IFN therapy remained negative thereafter and were classified as SVR.

It is important to continue low dose treatment for a long time to maintain HCV-RNA negativity.

Prevention of onset of HCC during treatment has been confirmed, but HCC occurred again from three years after the completion of therapy. The treatment period for IFN is a topic for future study. The present study was a pilot study with no randomization and a small number of patients. A large-scale randomized clinical trial will be necessary in the future to determine if IFN low dose therapy is effective or not in prevention of the onset of HCC.

CONCLUSION

LOW DOSE LONG-TERM IFN therapy was performed in aged patients. Improvement of hepatic function disorders was seen in about 60% of patients and the AFP values decreased in about 90% of patients. In three years of treatment, no discontinuations took place because of adverse reactions, such as pancytopenia. Onset of HCC occurred in three patients by 13 months after the start of treatment, but no cases appeared thereafter. In aged patients in whom peg-IFN and ribavirin combination therapy is difficult, low dose long-term IFN monotherapy appears to be useful in the prevention of onset of HCC.

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