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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-DR^+ cells. Myeloid dendritic cells are Lin^-, HLA-DR^+, $CD11c^+$, $CD123^{low}$ cells, and plasmacytoid dendritic cells are Lin^-, HLA-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.

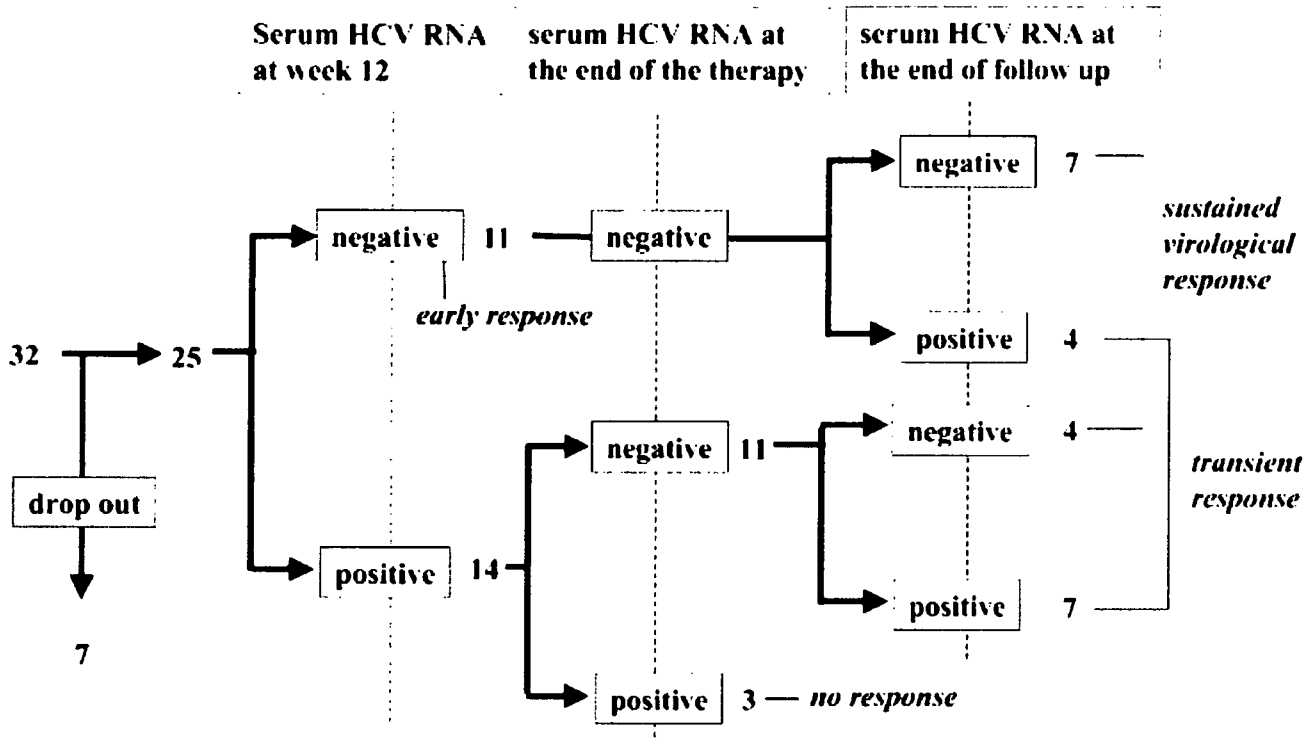

Fig. 1. Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFNα2b and ribavirin combination therapy. Thirty-two patients received the therapy, but seven dropped out due to various adverse effects. Among the 25 who completed the therapy, 11 achieved sustained virological response, 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.

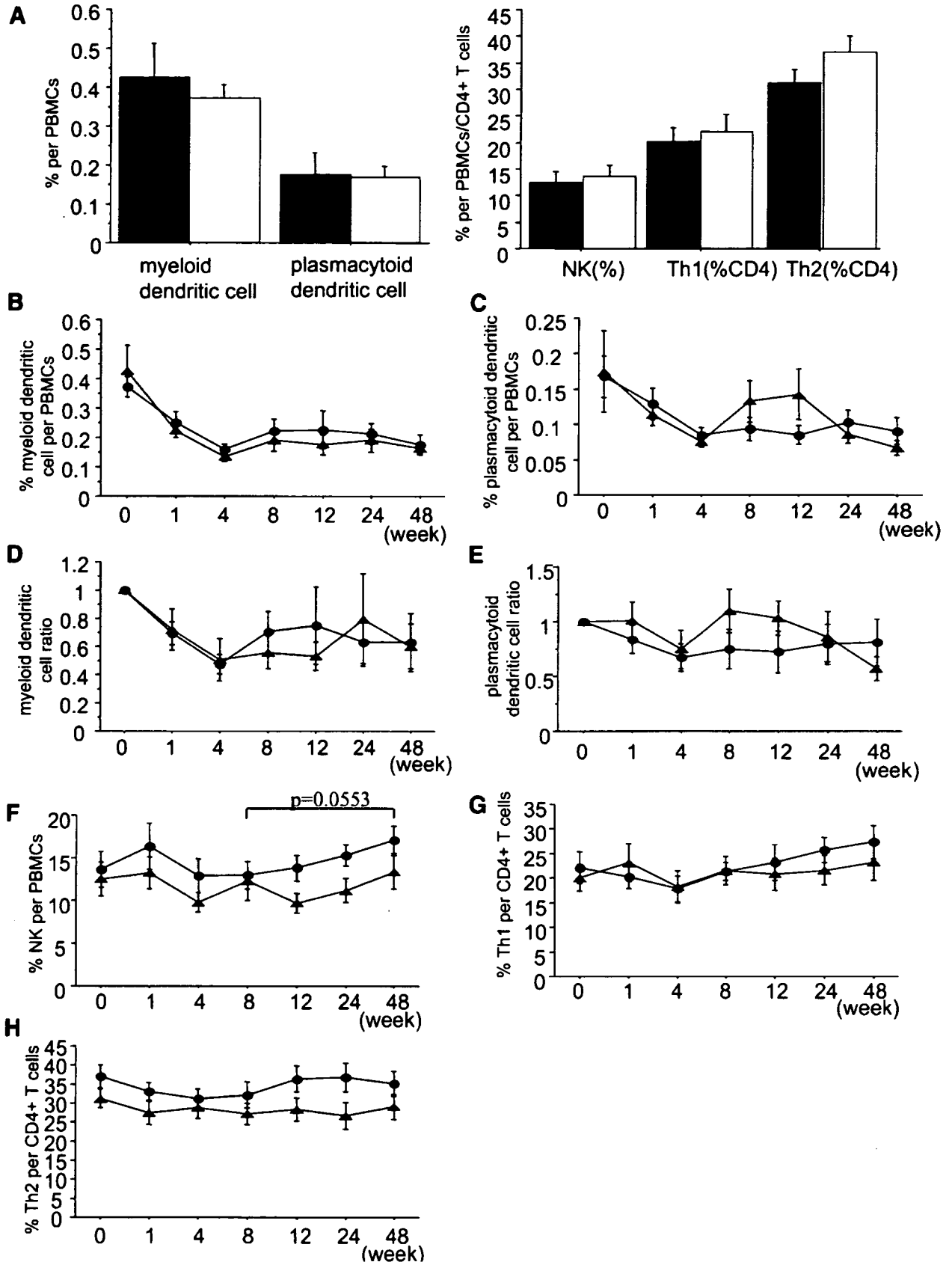
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK(%)
Th1(%CD4)
Th2(%CD4)
B
% myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
plasmacytoid dendritic cell ratio
F
% NK per PBMCs
p=0.0553
G
% Th1 per CD4+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

Fig. 2.

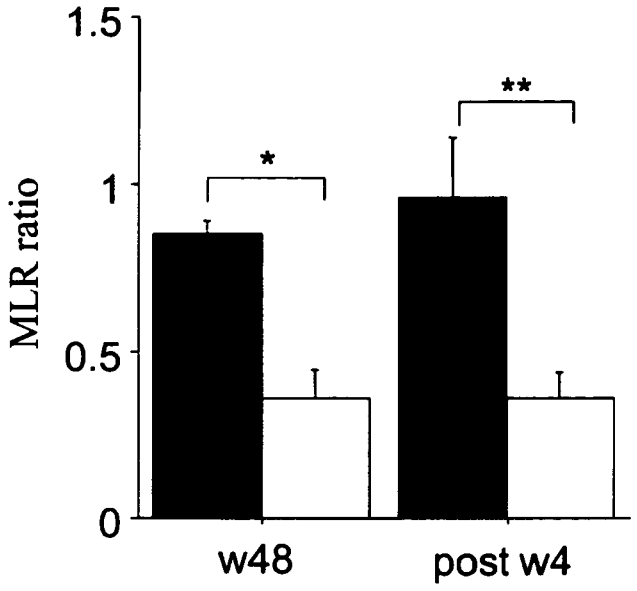

Fig. 3. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic 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).

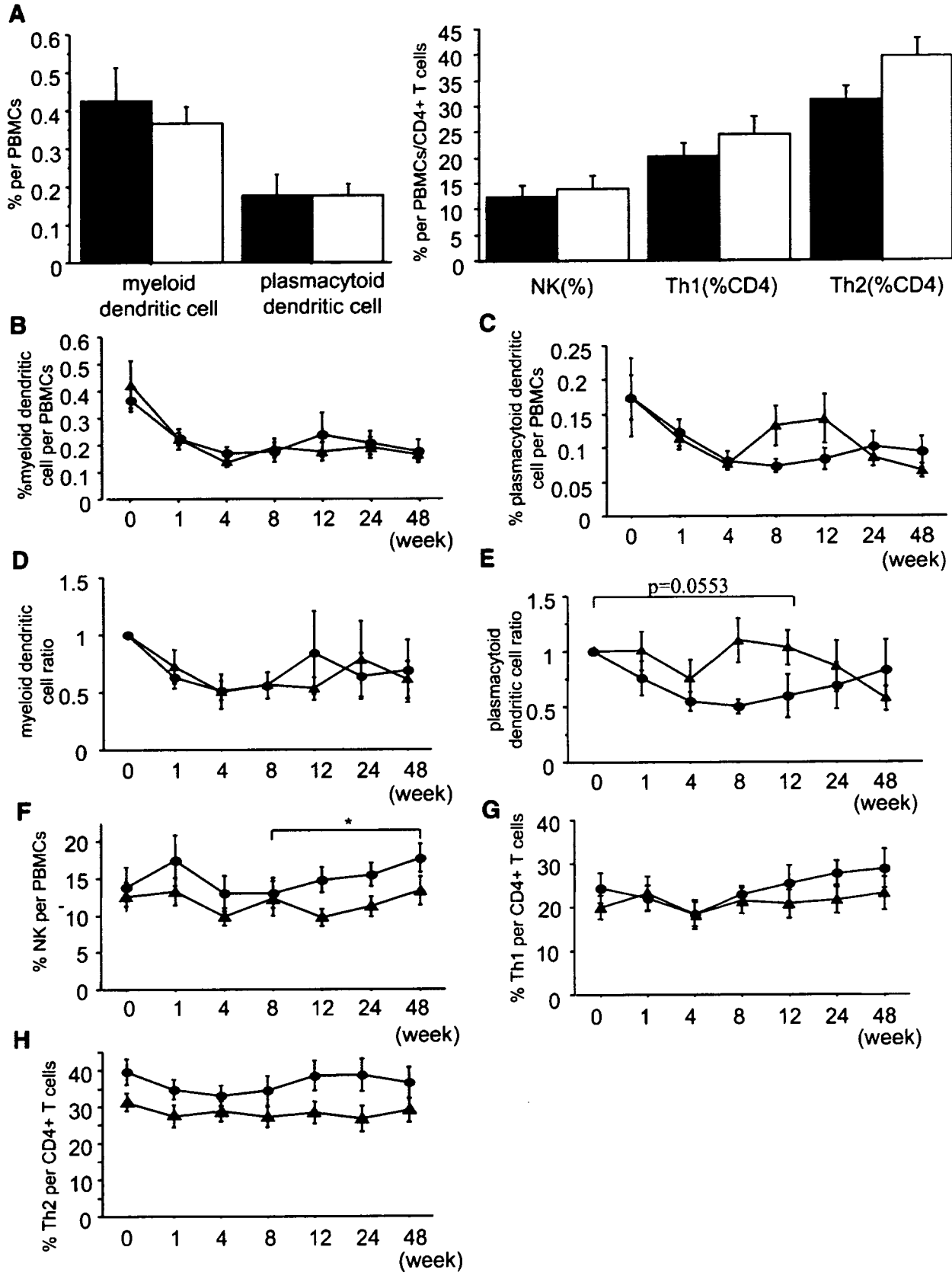
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK(%)
Th1(%CD4)
Th2(%CD4)
B
%myeloid dendritic cell per PBMCs
(week)
C
% plasmacytoid dendritic cell per PBMCs
(week)
D
myeloid dendritic cell ratio
(week)
E
p=0.0553
plasmacytoid dendritic cell ratio
(week)
F
*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

Fig. 4.

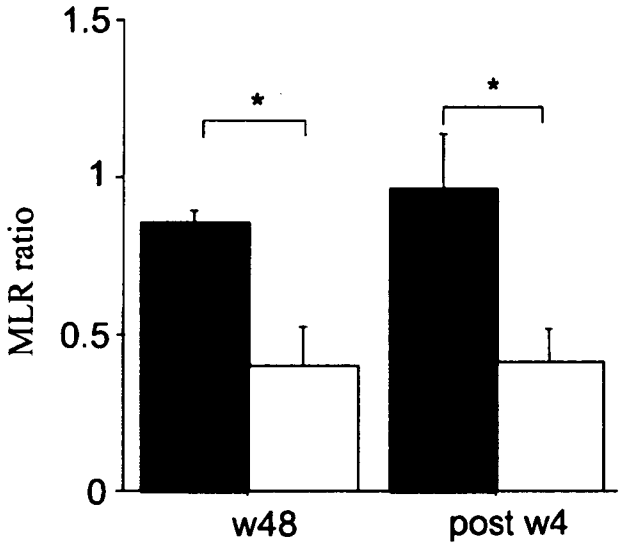

Fig. 5. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were 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).

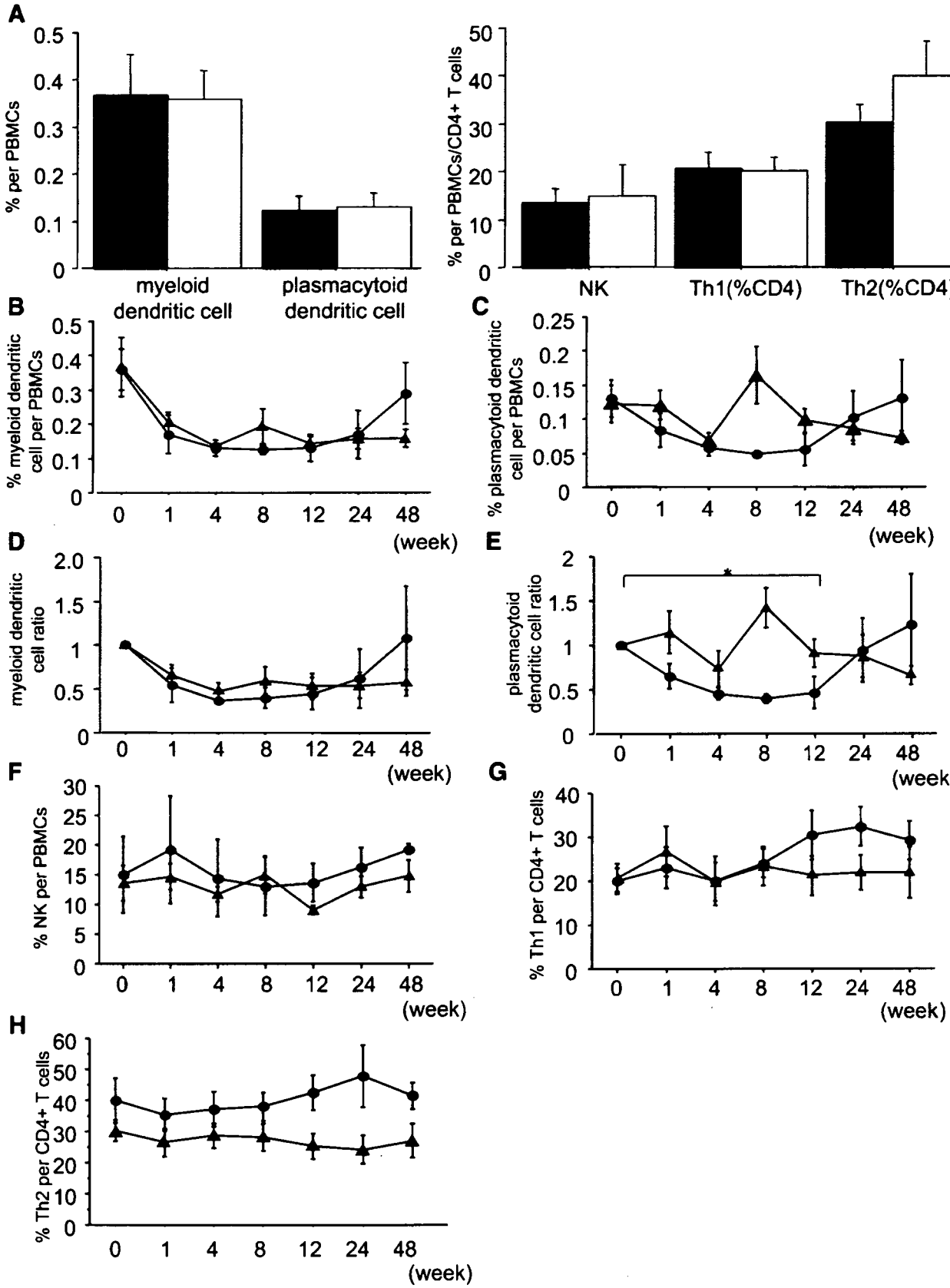
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK
Th1(%CD4)
Th2(%CD4)
B
% myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
plasmacytoid dendritic cell ratio
F
% NK per PBMCs
G
% Th1 per CD4+ T cells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

Fig. 6.

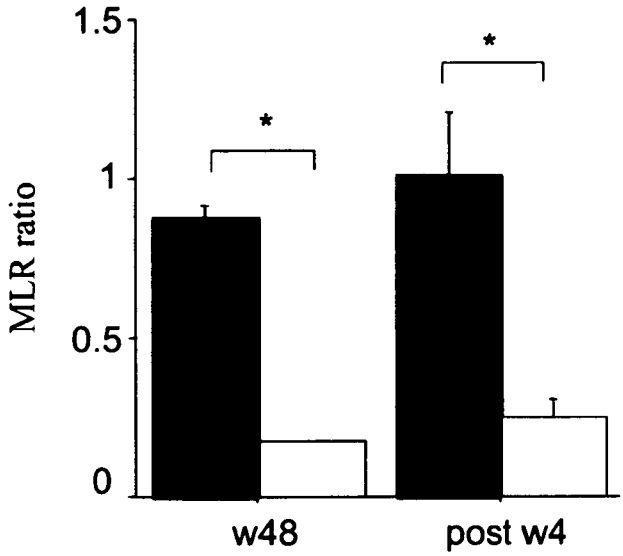

Fig. 7. Allostimulatory activity of dendritic cells in the patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. At the end of treatment (Week 48) and at Week 4 after the completion of the treatment, monocyte-derived dendritic 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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○診療の秘訣○

ペグインターフェロン・リバビリン併用治療抵抗性C型慢性肝炎の診療

加藤　道夫　国立病院機構大阪医療センター 消化器科部長

C型慢性肝炎に対するインターフェロン（IFN）治療は，ペグインターフェロン・リバビリン（PEG/RIBA）併用治療の登場によって，セログループ1，高ウイルス量のいわゆる難治性C型慢性肝炎でも，その約50%でウイルスの排除が可能となった．しかし，残りの半数の方に対する対策については明らかに有効な手段は見いだされておらず，種々の方法が模索されている．

当院では，HCV RNA非陰性化例に対するIFNβブースター治療，Late responder例に対するIFN長期投与および再燃例，無効例に対するTwo-step IFN rebound therapy（TIRT）（「私の処方」参照）によってPEG/RIBA併用治療抵抗例に対応し，良好な成績を得ているので紹介する．

1．HCV RNA非陰性化例に対するIFNβブースター治療

セログループ1，高ウイルス量のC型慢性肝炎に対する，当院での24週までのHCV RNA陰性化率は55.4%である．また，約10%あまりは抗ウイルス効果がきわめて不良のいわゆるnull responderであるので，全症例の約1/3は投与開始24週の時点で，HCV RNA定量5.0 KIU/mL未満（ハイレンジ法），HCV RNA定性陽性の状態（24週partial response）である．この状態でPEG/RIBA併用治療を続行しても，HCV RNA完全排除（SVR）が得られる可能性がほとんどないことは，全国治験成績からも明らかであり，何らかの対策が必要と考えられる．

IFNβ 300万単位1日2回投与法は抗ウイルス効果が良好な治療法であり，当院では24週partial response例に対しブースター治療として積極的に同法2週連日投与を施行している．現在までに17例に施行し，途中減量した2例を除く15例中12例（80.0%）にHCV RNA陰性化を認めている．ブースター治療後はPEG/RIBA併用治療に戻し，以後48週のPEG/RIBA併用治療と約24週のペグインターフェロン単独治療により高率のSVR獲得を目指している．

2．Late responder例に対するIFN長期投与

HCV RNA陰性化は認められるが，その時期が13～24週（Late responder）の場合，PEG/RIBA 48週投与では全国治験で36.4%，当院でも38.5%とSVR率は低率である．治療期間の延長はSVR率の向上に寄与することが予測されるため，当院ではLate responderに対する72週投与を積極的に行っている．PEG/RIBA併用治療の副作用は，24週前後が最強の場合が多く，40週ごろに72週までの投与延長について提案すると，ほとんどの方で同意が得られる．

現在までに13例に72週投与を行い，11例（84.6%）がSVRとなった．とくに，16週目，20週目にHCV RNAが陰性化した11例は全例がSVRとなった．12週目にHCV RNAが陰性化した例でも約15%は再燃するため，今後は12週目以降のHCV RNA陰性化例には72週投与を考慮する方針である．また，24週以降にHCV RNAが陰性化した例では72週投与でも不十分であるので，その後ペグインターフェロン単独あるいは従来のIFNα製剤自己注射による追加投与が推奨される．

0913-7963/07/¥800/論文/JCLS

難治性C型慢性肝炎に対する Two-step IFN rebound therapy (TIRT)

加藤 道夫（かとう みちお）
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「診療の秘訣」欄で述べたように，当院ではペグインターフェロン・リバビリン（PEG/RIBA）併用治療抵抗性のC型慢性肝炎に対して種々の治療を施行している．Two-step interferon rebound therapy（TIRT）は，その形になればきわめて高率にHCV RNA完全排除（SVR）が得られる治療法である．本稿ではその処方の実際について紹介する．

1．TIRTとは

TIRTは初回インターフェロン（IFN）投与（1st step）終了後，HCV RNA量の急増加のあとのtransaminaseのリバウンド時，HCV RNA量が急激に低下した時点より再度IFNを施行（2nd step）する治療法（図）である．リバウンド後の低下時期を逸すればその後HCV RNA量が100 KIU/mL未満に低下することはきわめてまれで，高ウイルス群に対しては，このTIRTの形でのみHCV RNA量低値からの再投与が可能である．HCV RNA量低下例において投与終了時より100 KIU/mL未満まで低下するまでの期間は2〜5ヵ月（平均3.5ヵ月），100 KIU/mL未満が持続する期間は1〜4ヵ月（平均1.8ヵ月）であり，投与終了後6ヵ月目にはほとんどの症例でHCV RNA量は高値に復する．

2．1st stepの処方

処方1.
ペグインターフェロン・リバビリン（ペグイントロン1.5 μg/Kg週1回・レベトール600〜800 mg連日またはペガシス180 μg週1回・コペガス600〜800 mg連日）
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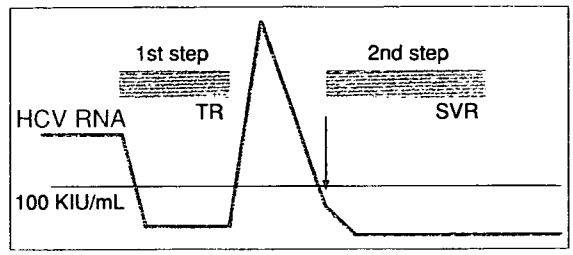

図 Two-step interferon rebound therapy（TIRT）

3．2nd stepの処方

処方2. 1st stepの投与期間が24週の場合
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ペグインターフェロンやリバビリン併用治療が使用可能となって以来，TIRTを施行した症例は18例で，投与終了13例全例がSVRとなっている．PEG/RIBA併用治療が再燃，無効であった場合も，投与終了後に的確な経過観察を行い，適応となればすみやかなTIRT導入によってSVR率を向上させたいと考えている．

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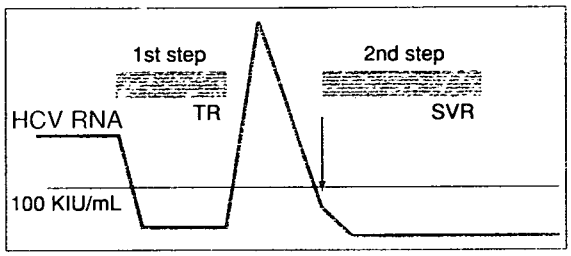

図 Two-step interferon rebound therapy（TIRT）

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B型慢性肝炎に対するインターフェロン治療

――現況と今後の展望

Interferon therapy for chronic hepatitis B

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◎わが国でのB型慢性肝炎に対するIFN治療はHBe抗原陽性例のみが保険適用で，しかも投与期間が24週に限られている．その治療効果は若年，女性，ALT高値，HBV-DNA低値例などの好条件群では良好であるが，おしなべての成績は満足できるものではない．欧米からはPeg-IFN治療の良好な成績が多数報告されているが，わが国ではようやくPeg-IFN-α2aの治験が開始されたところである．若年例には核酸アナログ剤が使用困難で，Peg-IFN治療が大きな福音になると思われる．1日も早い保険適用が望まれる．

Key word：B型慢性肝炎，インターフェロン治療，HBステージ分類，ペグインターフェロン(Peg-IFN)

HBVキャリアは，その自然経過において80%以上がHBe抗原陰性，HBe抗体陽性，HBV-DNA低値の，いわゆる臨床的治癒の状態となる．しかし，少数ではあるが肝硬変に進展したり肝細胞癌を合併する例が存在し，B型肝細胞癌による死亡者数はこの20年間，約5,000名の状態が続いている．B型肝細胞癌発癌には，HBV増殖の多寡が密接に関係していることが明らかになり[1]，抗ウイルス薬によるHBV-DNA量の低下が発癌抑止にきわめて重要とされている．B型慢性肝炎に対する抗ウイルス薬としては，30～35歳以上の高年例には核酸アナログ剤が第一選択で，それより若年例ではインターフェロン(IFN)が用いられることが多い．

わが国でのB型慢性肝炎に対するIFN治療は1986年に1カ月投与が保険適用となり，2002年からは現行の6カ月投与が認可された．6カ月投与が可能となって，その有効性はある程度向上したが，HBe抗原陰性例は保険適用外である点など課題も多い．欧米で有用性が多く報告されているペグインターフェロン(Peg-IFN)は現在ようやく国内治験がはじまったばかりで市販までにはいまだ時間を要するが，近い将来，若年例に対する第一選択薬になると考えられる．

B型慢性肝炎治療ガイドラインとステージ分類

表1は平成18年度厚生労働科学研究“B型およびC型肝炎ウイルスの感染者に対する治療の標準化に関する臨床的研究”によるB型慢性肝炎の治療ガイドラインである．35歳未満でHBe抗原陽性例にはIFN長期間欠投与が選択されている．また35歳以上でも，HBe抗原陽性で7 log copies/ml以上の高ウイルス例にはIFN長期間欠投与も考慮することが示されている．著者らが提唱したHBキャリアのステージ分類[2](表2)においても，HBステージⅠ(HBe抗原陽性：HBV-DNA 7.6 log copies/ml以上)の若年(Ⅰa)で肝線維化F2以上の例およびHBステージⅡ(HBe抗原陽性：HBV-DNA 7.6 log copies/ml未満)の若年(Ⅱa)例にはIFNを第一選択薬にあげている．また，HBステー

表 1　B型慢性肝炎の治療ガイドライン(2007 年度版)

	HBV-DNA	≧7 log copies/ml	<7 log copies/ml
35 歳未満	HBe 抗原陽性	IFN 長期間歇	IFN 長期間歇
	HBe 抗原陰性	経過観察 ↘ 進行例はエンテカビル	経過観察 ↙
35 歳以上	HBe 抗原陽性	①エンテカビル ②IFN 長期間歇	エンテカビル
	HBe 抗原陰性	エンテカビル	エンテカビル

表 2　HBVキャリアのステージ分類

HB ステージ	0	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ
HBs 抗原	+	+	+	+	+	−**
HBe 抗原	+	+	+	−	−	−
HBV-DNA (copies/ml)	不問	$10^{7.6}$≦	$10^{7.6}$>	10^{5}≦	10^{5}>	不問
ALT	持続正常	持続正常以外	持続正常以外	不問	不問	不問
年齢	不問	若年/高年(Ⅰa/Ⅰb)*	若年/高年(Ⅱa/Ⅱb)*	不問	不問	不問
発癌リスク	きわめて小	小/大	小/きわめて大	きわめて大	きわめて小	きわめて小
治療	不要	F2 以上 IFN/エンテカビル	IFN/エンテカビル	エンテカビル	不要	不要

*：若年は男性 30 歳未満，女性 35 歳未満，高年は男性 30 歳以上，女性 35 歳以上.
**：HBs 抗原(+)の時期が確認されていること.

ジⅢ，Ⅳは，HBe 抗原陰性期でステージⅢは HBV-DNA 5.0 log copies/ml 以上，ステージⅣは HBV-DNA 5.0 log copies/ml 未満例である．ステージⅣはいわゆる臨床的治癒の状態でおおむね抗ウイルス治療の必要はないが，ステージⅢはもっとも発癌リスク(「サイドメモ」参照)の高い集団であり，速やかな治療介入が必要となる．ステージⅢで核酸アナログ剤が使用困難な場合，IFN の保険適用がないので，現在もっとも対応に苦慮することが多く，一刻も早い Peg-IFN の保険適用が望まれる．

これまでのIFN治療成績

わが国の IFN 1 カ月投与成績のまとめ[3]によると，投与終了 1 年後，2 年後の HBe 抗原陰性化率はそれぞれ 29%，55%，HBe 抗原抗体セロコンバージョン率は 12%，29%で，自然経過よりも高率であるとしている．また，1 カ月投与と 6 カ月投与の国内治療成績の集計[3]では，投与終了 6 カ月後の HBe 抗原陰性化率は 4 週投与，24 週投与でそれぞれ 11%，28%と長期投与の有効性が確認されている．1 カ月投与に対する 6 カ月投与の最大の利点は投与期間中に HBe 抗原抗体セロコンバージョンが生じる可能性が高く，投与終了後の急性増悪の出現を防止できることである．欧米でも 6 カ月投与が標準投与法であるが，Wong ら[4]の

サイドメモ　B型慢性肝炎の肝癌発癌リスク

B 型慢性肝炎例は 50 歳ぐらいまでに，約 90%が HBe(e)抗原陽性から e 抗体陽性にセロコンバージョンする．したがって，高齢者はほとんどの症例が e 抗体陽性の状態である．まだ，e 抗原陽性例が多い 50 歳前後までの肝癌発癌例は，e 抗原陽性例のほうが e 抗原陰性例に比べ有意に高率である．高齢者では e 抗原陽性例の絶対数が少なく，発癌例は e 抗体陽性が多数を占める．e 抗体陽性例の発癌は HBV-DNA 量と明らかな関連があり，4.0〜5.0 log copies/ml 以上群は高率に発癌し，それ未満ではきわめて低率であることが明らかになっている．HBV キャリア全体の肝癌発癌リスクは e 抗原持続陽性例，e 抗原陰性 HBV-DNA 高値持続群，e 抗原陰性 HBV-DNA 出没群の順に高く，e 抗原陰性 HBV-DNA 持続低値になってようやく，低リスクになると考えられる．HBV-DNA 量の定期的なモニタリングがきわめて重要である．

比較対照試験の集計でも投与終了後6カ月の時点でのHBe抗原陰性化率33%と，自然経過例12%に比べ有意に高率であったとしている．

欧米におけるPeg-IFNおよびIFN・多剤併用治療成績

Marcellinら[5]は，HBe抗原陰性例に対しPeg-IFN-α2a単独，ラミブジン(LAM)単独およびPeg-IFN-α2a・LAM併用群の無作為比較試験(RCT)を行い，Peg-IFN-α2a単独，Peg-IFN-α2a・LAM併用群はLAM単独群に比べ，有意にHBV-DNA抑制率が高率であったと報告している．また，Lauら[6]はHBe抗原陽性例に対して同様の検討を行い，Peg-IFN-α2a群はLAM単独群に比べ有意にHBe抗原陰性化率，HBV-DNA抑制率およびHBs抗原陰性化率が高率で，Peg-IFN-α2aの有用性を認めている．

Peg-IFN-α2bに関しても良好な報告がなされている．すなわち，Janssenら[7]はHBe抗原陽性例に対してPeg-IFN-α2b単独群とPeg-IFN-α2b・LAM併用群とのRCTを行い，Peg-IFN-α2b単独群において投与終了後26週後のHBe抗原陰性化率が36%と良好であり，これは併用群35%に劣っていないことを報告した．また，Chanら[8]はPeg-IFN-α2b・LAM併用群とLAM単独群とのRCTにて併用群が単独群に比べ，良好なvirological responseが得られ，変異株出現も低率であったとしている．さらに投与3年後においても，併用群は単独群に比べvirological responseを維持すると報告した[9]．Flinkら[10]は，標準的なIFNやLAMによる治療が無効であったHBe抗原陽性例に対してPeg-IFN-α2b単独治療を行い，約1/3の症例で投与終了後26週の時点でHBe抗原の消失を認めている．一方，YMDD変異株出現例に対するPeg-IFN-α2bの有効性については明らかな見解は示されていない[11]．アデフォビルとの併用についてはWursthornら[12]がPeg-IFN-α2bとアデフォビルとの併用によって，HBs抗原の減少を伴う著明なHBV-DNA量と肝内cccDNA量の低下を認めたと報告している．

IFN治療の適応と今後の展望

1986年より母児感染予防事業が施行されて，わが国のHBVキャリア率は激減した．しかし，20歳以上のキャリアはいまだ多数存在し，自然経過で臨床的治癒の状態に至らずに，肝硬変，肝細胞癌に進行する例も存在することはさきに述べた．これらの症例のうち，本人あるいはパートナーが妊娠，出産を望んでいる場合は男女の差なく核酸アナログ剤は使用できない．IFN治療の適応はまさにこれらの症例であり，30〜35歳までの若年の間の，HBe抗原からHBe抗体へのセロコンバージョンとHBV-DNA増殖の沈静化が目的となる．これまでの国内外での検討より，効果が期待できる治療前因子として女性，HBV-RNA量低値，投与前ALT高値および組織診断でactivityの高い症例があげられている．とくに，20歳代の女性の治療効果はきわめて良好であるが，女性は妊娠・出産を機に病態が安定化することが多く，IFNを含む抗ウイルス治療介入には慎重を期する必要がある．一方，男性では若年発癌も含めて，B型肝細胞癌発癌のリスクは有意に高く，若年でも肝線維化F2以上では積極的にIFN治療を導入する必要があると考えている．

現在，HBe抗原陽性例のみが保険適用であるが，6カ月の長期投与でも十分満足できる成績は得られていない．IFN製剤の投与期間制限がC型慢性肝炎と同様に撤廃されれば，IFN治療の有用性は大きく向上すると考えられるが，現在その動きはない．Peg-IFNの効果は前述のとおり良好であり，現在，Peg-IFN-α2aの治験が開始されたところであるが，HBe抗原陰性例や48週までの投与期間がデザインされており，より早期の認可が強く望まれる．

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Comparison of complete sequences of hepatitis B virus genotype C between inactive carriers and hepatocellular carcinoma patients before and after seroconversion

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Background. Most patients who acquire chronic hepatitis B virus (HBV) infection by perinatal transmission become inactive carriers (IC) after hepatitis B e (HBe) antigen seroconversion, whereas some patients have persistent abnormal serum transaminase levels and develop hepatocellular carcinoma (HCC) in the anti-HBe-positive phase. The aim of this study was to investigate the HCC-related mutations of HBV. ***Methods.*** Complete sequences of HBV were examined among eight IC and eight HCC patients infected with HBV genotype C before and after seroconversion. ***Results.*** The frequency of the T1653 mutation tended to be higher among HCC patients after seroconversion (16.7% vs. 62.5%; $P = 0.086$). The prevalence of a basal core promoter double mutation (T1762/A1764) was high among both IC and HCC patients after seroconversion (83.3% vs. 87.5%; $P = 0.825$). Among the HCC patients, a pre-S deletion mutant was detected in 62.5% patients before seroconversion, and in 37.5% patients after seroconversion. The core deletion mutant was also detected in 50% of HCC patients only before seroconversion. Deletion mutants of the pre-S or core region before seroconversion were significantly associated with HCC patients (0% vs. 62.5%; $P = 0.007$, 0% vs. 50%; $P = 0.021$, respectively). ***Conclusions.*** Our data showed a significant association of pre-S and core deletion mutants before seroconversion with HCC development. The T1653 mutation after seroconversion was frequently found in HCC patients infected with HBV genotype C. These results suggest that mutations may be predictive factor for development of HCC.

Key words: hepatocellular carcinoma, core deletion, pre-S deletion mutant, T1653 mutation

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer-related death in the world, with an estimated annual prevalence of >500000 cases worldwide.[1] It is now accepted that HBV infection has hepatocarcinogenic potential in humans. Several mutations in the HBV genome have been reported to occur during the course of persistent viral infection, and there is increasing evidence of an association between these molecular alterations and the development of end-stage liver disease in patients with HBV infection.[2–6] Nevertheless, it is still unclear whether a specific mutation or a specific combination of mutations is associated with the development of severe disease, because previous studies focused on only a few mutations such as pre-S deletion, basal core promoter (BCP) double mutation, and precore (PC) mutation. Recently, several lines of evidence have indicated that complex HBV variants with deletions in the pre-S or core region and mutations in the enhancer II region are associated with end-stage liver disease.[7–9] Both the pre-S and core regions play an essential role in the interaction with immune responses because they contain B- and T-cell epitopes.[10–12] Pre-S and core deletion mutants with altered epitopes may survive despite the host immune system.

During persistent HBV infection, carriers frequently undergo seroconversion from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe). Most patients who acquire chronic HBV infection with HBV genotype C (which is the common genotype in East Asian countries) by perinatal transmission become inactive carriers (IC) after seroconversion. A subgroup of patients have persistent abnormal serum transaminase levels and develop HCC in the anti-HBe-positive phase. Because most previous studies examined only a serum sample collected at one time point in each patient,