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Enhanced ability of peripheral invariant natural killer T cells to produce IL-13 in chronic hepatitis C virus infection

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***Background/Aims*: Human invariant natural killer T (iNKT) cells express a TCR Vα24-JαQ paired with Vβ11 and are activated by a surrogate ligand, α-galactosylceramide (αGalCer). The iNKT cells are involved in the regulation of antiviral immune responses; however, little is known about their roles in hepatitis C virus (HCV) infection.**

***Methods*: We compared the frequency of peripheral iNKT cells and their cytokine producing capacity reactive to αGalCer between chronically HCV-infected patients and healthy subjects. Cytokine production of freshly isolated iNKT cells were analyzed by ELISPOT. Activated iNKT cells were obtained by culture with αGalCer-loaded dendritic cells (DCs) and re-stimulated with them for the measurement of cytokine production.**

***Results*: The frequencies of iNKT cells were not different between HCV-infected patients and healthy subjects. The number of fresh IFN-γ-producing iNKT cells reactive to αGalCer was not different between the patients and controls, whereas fresh iNKT cells produced negligible amounts of Th2 cytokines regardless of HCV infection. In response to αGalCer, expanded iNKT cells from the patients secreted IFN-γ comparable in amount to controls, whereas they released significantly more IL-13 than cells from controls.**

***Conclusions*: Activated iNKT cells from HCV-infected patients gain more ability to secrete IL-13 than those from healthy subjects.**

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Keywords: **Hepatitis C virus; Natural killer T cell; Th1; Th2; α-Galactosylceramide**

1. Introduction

Hepatitis C virus (HCV) frequently gives rise to chronic liver disease, which varies from asymptomatic HCV carriers to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. Cumulative reports have demonstrated that innate as well as adaptive immune responses are involved in the pathogenesis of HCV-induced liver injury and the development of liver disease [2,3].

Natural killer T (NKT) cells are a unique lymphocyte subset co-expressing T-cell receptor (TCR) and NK cell markers [4]. The NKT cell population is highly heterogeneous; invariant NKT (iNKT) cells express an invariant TCR, composed of Vα24-JαQ preferentially paired with Vβ11 in humans [4,5], whereas non-invariant NKT cells express diverse TCR. Invariant NKT cells recognize glycolipid antigens presented on the non-polymorphic MHC class I-like molecule CD1d [6,7], which is expressed by antigen presenting cells, such as dendritic cells (DCs). Although endogenous ligands of iNKT cells are little known, α-galactosylceramide (αGalCer) has

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been used as a surrogate for natural ligands. It has been demonstrated that phenotypic as well as functional subsets exist for iNKT cells, which are CD4+, CD4–CD8– double-negative (DN) and CD8+ ones. The CD4+ and DN iNKT cells produce both Th1 (interferon (IFN)-γ) and Th2 cytokines (interleukin (IL)-4, IL-5, IL-13). The CD4+ iNKT cells secrete more Th2 cytokines than DN, while CD8+ subsets predominantly secrete Th1 cytokines [8].

Although iNKT cells comprise a small portion of hemotopoietic cells, they regulate various immune responses by secreting Th1 as well as Th2 cytokines in clinical settings, such as autoimmune disease, viral infection or cancer [9–13]. For chronic HCV infection, there have been some controversial reports about the frequency of peripheral iNKT cells [14–16], however, their functional roles in HCV-infected patients are largely unknown. We compared the frequency and the cytokine producing capacity of iNKT cells in fresh peripheral blood between chronic hepatitis C patients and healthy individuals. Furthermore, to analyze the functions of activated iNKT cells, we expanded iNKT cells by stimulation with αGalCer-loaded DCs. Of note, in response to αGalCer-pulsed DCs, the activated iNKT cells obtained from chronic hepatitis C patients secreted a significantly larger amount of IL-13 and tend to produce more IL-4 and IL-5 than those from healthy subjects, indicating that peripheral iNKT cells may be involved in the pathogenesis of chronic hepatitis C.

2. Materials and methods

2.1. Subjects

After informed consent had been obtained, 19 patients who were positive for both anti-HCV Ab and serum HCVRNA were enrolled in this study (chronic hepatitis [CH] group). All patients were negative for hepatitis B virus (HBV) and human immunodeficiency virus (HIV) and had no apparent history of other types of liver diseases. The HCV serotype of all patients was type 1. None of them had been treated with anti-viral agents, such as IFN-α or ribavirin. As controls, 18 age-matched healthy subjects (HS group) who were negative for HCV, HBV and HIV were examined. The clinical backgrounds of the patients and healthy subjects are shown in Table 1.

Table 1
Clinical backgrounds of healthy subjects and chronic hepatitis C patients

	Healthy subjects	Chronic hepatitis C patients
N (M/F)	18 (13/5)	19 (11/8)
Age[a]	38 ± 9	48 ± 14
Serum ALT (IU/L)[b]	ND	58 (23–238)
HCVRNA (Mequiv./ml)[b]	ND	2.5 (0.5–15.0)

[a] Values are expressed as mean ± SD.
[b] Median with range in parentheses. ALT, alanine aminotransferase; Mequiv./ml, million genome equivalents per milliliter; ND, not determined.

2.2. Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were purchased from Peprotech (London, UK). Recombinant human IL-2 was from Genzyme (Minneapolis, MN). αGalCer was provided by Kirin Brewery (Gumma, Japan).

2.3. Generation of monocyte-derived DCs

To stimulate iNKT cells, monocyte-derived DCs (Mo-DCs) were generated from CD14+ cells. CD14+ cells were separated from peripheral blood mononuclear cells (PBMCs) with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). They were cultured at 5×10^5/well in the DC culture media (DCM) (IMDM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 1 mM non-essential amino acid and 2 mM L-glutamine) containing 100 ng/ml GM-CSF and 20 ng/ml IL-4 for 7 days. For maturation of the DCs, they were given 50 μg/ml *Staphylococcus aureus Cowan I* (SAC) on day 6 of the culture. αGalCer (100 ng/ml) was pulsed to the DCs on the same day.

2.4. Frequency and phenotype analyses of iNKT cells in peripheral blood

PBMCs were isolated from the venous blood of HCV-positive patients or healthy subjects by Ficoll–Hypaque density-gradient centrifugation. For staining, PBMCs were incubated with fluorescence-labeled Abs for 30 min at 4 °C in PBS supplemented with 1% BSA and 0.1% NaN_3. The stained cells were analyzed using FACS Calibur and Cell Quest software (Becton Dickinson, San Jose, CA). For the measurement of iNKT cell subsets, the cells were stained with a combination of anti-Vα24, anti-Vβ11 and anti-CD4 mAbs (Immunotech, Marseilles, France). The frequencies of total (Vα24+, Vβ11+), CD4-positive (Vα24+, Vβ11+, CD4+) and CD4-negative (Vα24+, Vβ11+, CD4–) iNKT cells were determined. To examine iNKT cell phenotypes, they were further stained with mAbs against CCR7, CXCR3 (R&D Systems, Minneapolis, MN), CCR4, CD62L (BD Pharmingen, San Jose, CA) and analyzed by FACS Calibur.

2.5. Cytokine analysis of peripheral iNKT cells in response to αGalCer

For enumeration of the peripheral IFN-γ-producing cells in response to αGalCer, we used enzyme-linked immunospot (ELISPOT) assay. MultiScreen® ELISPOT plates (Millipore, Bedford, MA) were coated with 10 μg/ml mouse anti-human IFN-γ mAb (1-D1K, Mabtech, Sweden). Monocyte-depleted PBMCs were cultured at 5×10^5/well with 5×10^4/well autologous Mo-DCs pulsed with or without αGalCer for 24 h on Ab-coated plates. PBMCs pulsed with PHA (1 μg/ml) were used as positive controls. After 24 h, the culture supernatants were collected for ELISA. Subsequently, the plates were washed and then incubated with biotin-labeled anti-human IFN-γ mAb (7-B6-1, Mabtech). After addition of streptavidin-HRP to the plates, spots were developed using 3-amino-9-ethylcarbazole (Sigma–Aldrich, St Louis, MO). Spots corresponding to IFN-γ-secreting cells were identified by microscopy and counted by two independent observers. The number of αGalCer-reactive IFN-γ-producing cells was determined by subtracting the number of spot-forming cells with αGalCer-unpulsed Mo-DCs from those with αGalCer-pulsed ones. To confirm that cytokines were released from iNKT cells, we also examined Vα24-depleted and/or Vβ11-depleted cells stimulated with αGalCer-pulsed Mo-DCs. Vα24-positive or Vβ11-positive cells was depleted with mouse anti-human Vα24 or Vβ11 mAbs (Immunotech) and subsequently anti-mouse IgG microbeads (Miltenyi Biotec). The Vα24-positive

or Vβ11-positive cells remaining in the treated samples were less than 5% as assessed by FACS (data not shown).

2.6. Expansion of iNKT cells from peripheral blood and analyses of cytokine production from them

To investigate the ability of activated iNKT cell subsets to proliferate and produce cytokines in response to αGalCer, we expanded them according to a method described previously [17] with some modifications. Monocyte-depleted PBMCs were cultured at 3×10^6/well with 3×10^5/well αGalCer-loaded autologous mature Mo-DCs for 2 weeks in DCM containing 5 ng/ml IL-2. For stimulation of the cells, 2.5 ng/ml IL-2 was added to the culture every 3 days. Subsequently, Vα24+ cells were magnetically separated and cultured in DCM for additional 3 weeks, which were fed with 2.5 ng/ml IL-2 every 3 days. Finally, the cells were stained with fluorescence-labeled mAbs against Vα24, Vβ11 and CD4. At this point, the rates of increase of total, CD4-positive and CD4-negative iNKT cells were calculated from the absolute numbers of relevant cells before and after the culture. Vα24+Vβ11+CD4+ cells and Vα24+Vβ11+CD4− cells were sorted by FACS Vantage (Becton Dickinson). The sorted cells whose purity was more than 85%, were used for cytokine producing analysis. Sorted iNKT cells were cultured at 1×10^5/well with 1×10^4/well αGalCer-pulsed or unpulsed allogenic mature Mo-DCs for 24 h. Mo-DCs as stimulators were obtained from the same donor. The supernatants of the culture were collected for cytokine ELISA.

2.7. ELISA

IFN-γ, IL-4, IL-5 and IL-13 concentrations of the supernatants were measured by ELISA. The paired Abs and standards were purchased from Endogen (Woburn, MA). The ranges of the assay were 0–1000 pg/ml for IFN-γ and IL-13, and 0–500 pg/ml for IL-4 and IL-5, respectively.

2.8. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test (StatView, SAS Institute, Cary, NC). A *P*-value of less than 0.05 was taken as statistically significant.

3. Results

3.1. Frequencies of peripheral iNKT cell subsets in chronic hepatitis C patients are comparable to those in healthy subjects

Human CD1d-restricted iNKT cells express a conserved canonical TCR α-chain (Vα24-JαQ) paired with TCR β-chain (Vβ11). Thus, we examined the frequencies of peripheral Vα24+Vβ11+ cells as iNKT cells in the CH and HS groups. Although the frequencies of these cells showed a wide range of distribution in both groups (HS = 0.01–0.61%, CH = 0.01–0.43%), no difference was observed in total iNKT cells and their CD4+ and CD4− subsets between the CH and the HS group (Fig. 1). In both CH and HS groups, there was no correlation between any of the iNKT frequencies and age, serum HCVRNA titers or alanine aminotransferese (ALT) levels (data not shown).

3.2. Peripheral iNKT cell subsets in chronic hepatitis C patients express higher levels of CXCR3 than in healthy subjects

Next, we examined the expressions of some chemokine receptors and homing receptor on peripheral iNKT cell subsets. The expression of CXCR3 on both subsets in the CH group was higher than that in the HS group (Fig. 2), whereas those of the others (CCR4, CCR7 or CD62L) were not different between the groups (Fig. 2).

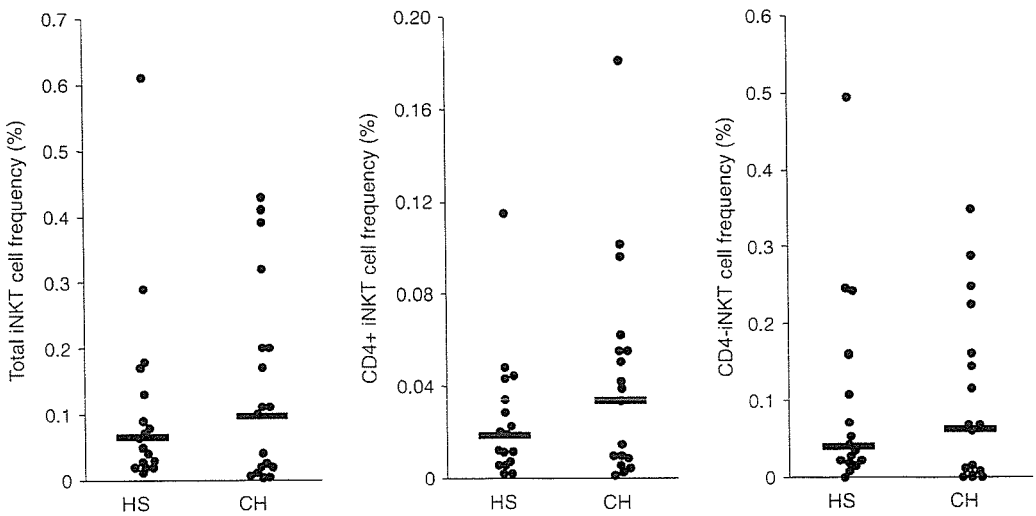

Fig. 1. Frequency of peripheral iNKT cell subsets in healthy subjects and chronic hepatitis C patients. The frequencies of total, CD4-positive and CD4-negative iNKT cells in PBMCs were determined by flow cytometry as described in Section 2. HS, healthy subjects; CH, chronic hepatitis C patients. Horizontal bars represent the median.

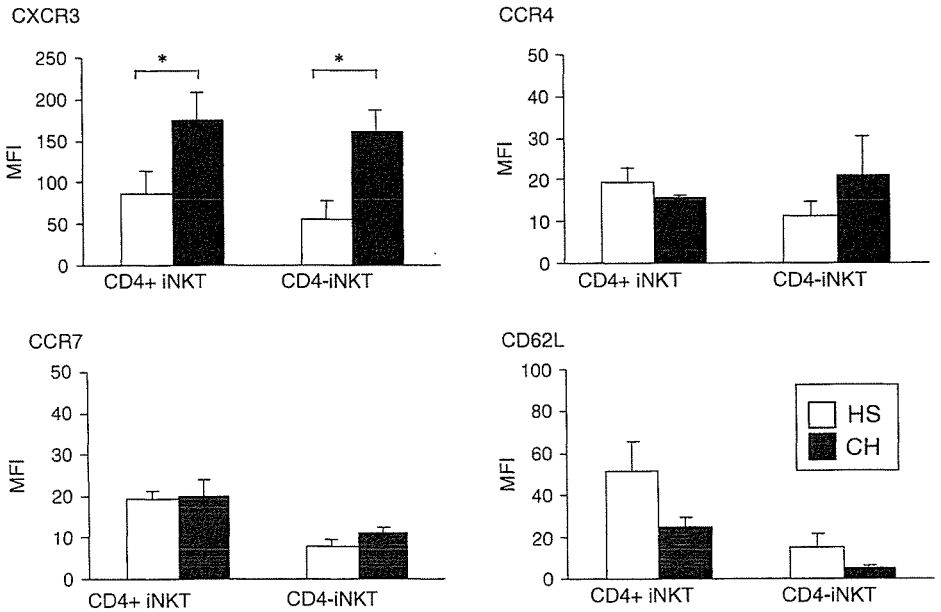

Fig. 2. Expressions of chemokine receptors and homing receptor on peripheral iNKT cell subsets in healthy subjects and chronic hepatitis C patients. The *y*-axis indicates the mean fluorescence intensity (MFI) of the expression of each receptor as determined by flow cytometry. The bars represent mean + SE of six different subjects. The white column represents the healthy subject group and the black one represents the chronic hepatitis C group. HS, CH, as in Fig. 1. *$P < 0.05$ by Mann–Whitney *U*-test.

3.3. The IFN-γ-producing capacity of peripheral iNKT cells in response to αGalCer in chronic hepatitis C patients is comparable with those in healthy subjects

With respect to the αGalCer-responsive IFN-γ-producing cells in PBMCs, the ELISPOT assay in this study detected as few as 10 spots/5×10^5 cells. When samples depleted of Vα24+ or Vβ11+ cells were used, the numbers of spots were significantly reduced, indicating that Vα24+ or Vβ11+ cells mainly released IFN-γ in response to αGalCer (data not shown). The numbers of IFN-γ-producing cells reactive to αGalCer in the peripheral blood were not different between the CH and the HS groups (Fig. 3). No correlation was observed between IFN-γ-producing cell number and serum HCVRNA titers or ALT levels. The levels of IL-4 and IL-13 in the supernatants were below the thresholds of ELISA (data not shown).

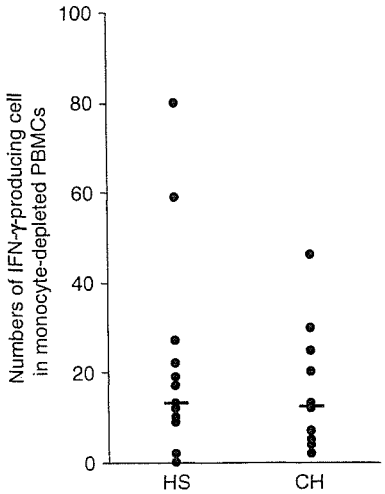

Fig. 3. Numbers of IFN-γ-producing cells in fresh PBMC stimulated with αGalCer-pulsed Mo-DCs. After monocyte-depleted PBMCs were co-cultured with αGalCer-pulsed autologous Mo-DCs for 24 h on IFN-γ mAbs-coating plates, the spots were developed and were counted as IFN-γ-producing cells as described in Section 2 (ELISPOT). The numbers of IFN-γ-producing cells in monocyte-depleted PBMCs from the HS and CH groups were counted as described above. Horizontal bars represent median. HS, CH, as in Fig. 1.

3.4. Peripheral iNKT cells from chronic hepatitis C patients proliferate in response to αGalCer at a level comparable to those from healthy subjects

Next, we compared the ability of peripheral iNKT cells to proliferate in response to αGalCer-loaded DCs between the CH and the HS groups. In all subjects, the absolute number of iNKT cells was significantly

Table 2
Increase of iNKT cell numbers expanded with αGalCer-loaded Mo-DCs after 5 weeks of culture

	Healthy subjects (-fold)	Chronic hepatitis C patients (-fold)
Total iNKT cells	148 (21–2143)	249 (87–2220)
CD4+ iNKT cells	182 (3–630)	254 (45–2209)
CD4− iNKT cells	124 (1–5856)	319 (113–2277)

Data express median (range).

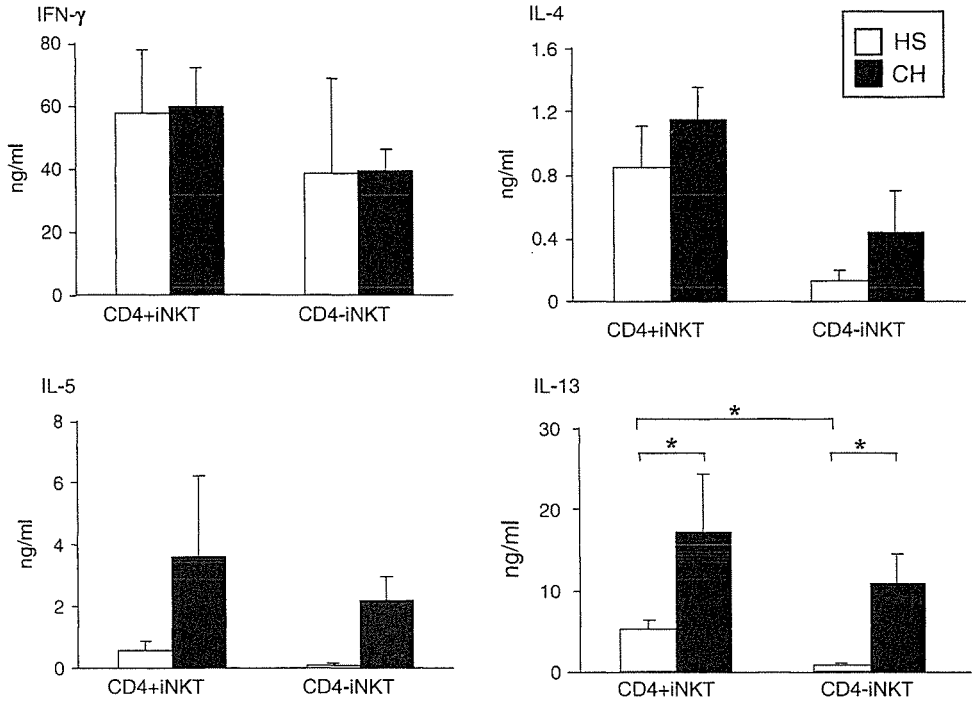

Fig. 4. Cytokine production from expanded iNKT cells stimulated with αGalCer-loaded Mo-DCs. Invariant NKT cells were expanded by culture with αGalCer-pulsed autologous Mo-DCs and subsequent cell sorting as described in Section 2. The activated iNKT cells were stimulated with αGalCer-pulsed allogeneic Mo-DCs for 24 h and the supernatants were collected for cytokine ELISA. The white column represents the healthy subject group and the black one represents the chronic hepatitis C group. The bars represent mean + SE of five different subjects. *$P < 0.05$ by Mann–Whitney U-test. HS, CH, as in Fig. 1.

increased after 5 weeks of culture. Although there was considerable variation among individuals in the proportion of iNKT cell increase (21–2220-fold), the total number of increased iNKT cells as well as their CD4 + or CD4− subsets in the CH group was comparable with those in the HS group (Table 2).

3.5. Expanded iNKT cells obtained from chronic hepatitis C patients produce more IL-13 in response to αGalCer than those from healthy subjects

In some reports, fresh isolated iNKT cells produce Th1 cytokines but not Th2 cytokines, while αGalCer-activated iNKT cells are able to produce both Th1 and Th2 cytokines [17,18]. Therefore, we compared the cytokine producing capacity of expanded iNKT cells. In contrast with the results of peripheral iNKT cells prior to in vitro expansion, the expanded iNKT cells produced considerable amounts of IL-4, IL-5 and IL-13 as well as IFN-γ in response to αGalCer (Fig. 4). The level of IFN-γ production from expanded iNKT cells did not differ between the CH and the HS groups, either from CD4 + or CD4− subsets (Fig. 4). With regard to Th2 cytokines, CD4 + iNKT cells in the HS produced significantly more IL-13 and tended to produce more IL-4 and IL-5 than the CD4− iNKT cells. In contrast, in the CH group, similar predominance of CD4 + iNKT cells over CD4− cells in Th2 cytokine production was observed, but did not reach a significant level. Of particular interest is the finding that a significantly larger amount of IL-13 was released from CD4 + as well as CD4− iNKT cells in the CH group as compared to those in the HS group (Fig. 4). A similar tendency was observed as the enhanced production of IL-4 and IL-5 from iNKT cells in the CH group. Thus, the activated iNKT cells obtained from chronic hepatitis C patients release more Th2-type cytokines, most significantly IL-13, than those from healthy subjects.

4. Discussion

Invariant NKT cells play distinctive roles in the regulation of immune responses in various diseases. In HIV infection, iNKT cells decrease in parallel with an increase in the viral load, which is due to direct HIV infection to these cells [11,13]. As for the frequency of peripheral iNKT cells in HCV infection, some conflicting results have been published. It has been reported that the number of iNKT cells in HCV-infected patients was in the same range as that in healthy subjects [15,16]. In contrast, other report showed that their number was

less in HCV viremic patients than non-viremic patients or healthy individuals [14]. In the present study, the frequencies of iNKT cells and their subsets in HCV-infected patients were comparable with those in healthy subjects. Several investigators reported that there are certain factors influencing iNKT cell numbers, such as gender and age in the study population [19,20]. Although no significant correlation was found between these background factors and iNKT cell frequency in our study, demographic as well as ethnic differences in the subjects might be involved in the discordant observations among these studies.

Limited numbers of peripheral iNKT cells have hampered the progress of research on the function of these cells. We used ELISPOT assay to analyze IFN-γ production from freshly isolated peripheral iNKT cells. The IFN-γ producing capacity of fresh peripheral iNKT cells from HCV-infected patients was comparable with those from healthy subjects. However, fresh iNKT cells produced negligible amounts of Th2 cytokines regardless of the presence or absence of HCV infection. Several investigators have reported that fresh iNKT cells are capable of predominantly producing Th1 cytokines compared to Th2 cytokines in response to αGalCer [17,18]. However, iNKT cells cultured with αGalCer are reported to gain the capacity to produce both Th1 and Th2 cytokines [17,18,21], implying that activated iNKT cells are able to secrete Th2 cytokines.

To analyze the capacity of cytokine production of activated iNKT cells, we thus expanded fresh iNKT cells with αGalCer-loaded DCs. However, the possibility remains that the experimental conditions may influence on iNKT cell functions, resulting in the functional difference between in vitro cultured iNKT cells and fresh iNKT cells in vivo. In the present study, the proliferative capacity of iNKT cells from HCV-infected patients was comparable with those from healthy donors, implying that expanded cells reflect the cell functions in vivo either in patients or donors. In clear contrast with fresh iNKT cells, the expanded iNKT cells from HCV-infected patients were able to produce a comparable amount of IFN-γ but more Th2 cytokines, most significantly IL-13, than those from healthy subjects. The mechanisms that induce Th2 bias of iNKT cells in HCV infection are yet to be demonstrated. Since iNKT cells were activated with αGalCer-pulsed autologous DCs, the functional alteration of DCs in HCV infection [22,23] may be responsible for the Th2 bias of activated iNKT cells.

With regard to the involvement of iNKT cells in the pathogenesis of chronic hepatitis C, several possibilities can be considered. First, Th2-biased iNKT cells may impede HCV clearance by suppressing the Th1 response. In this study, IL-13 production from expanded iNKT cells was not correlated with serum HCVRNA titres. However, IFN-γ from CD4+ iNKT cells tended to be inversely correlated with serum HCVRNA levels ($P = 0.07$, data not shown), while IL-4 from CD4− iNKT cells was positively correlated with HCVRNA quantity ($P = 0.07$, data not shown), suggesting an active role of iNKT cells in the control of HCV replication. Second, iNKT cells are involved in the progression of fibrosis in HCV-infected liver. Recently, other groups have reported that IL-4 and IL-13 from fresh iNKT cells were increased in liver cirrhosis caused by HBV or HCV, implying that these cells are profibrogenic to the liver [21,24,25]. If this is the case, the present study suggests that iNKT cells in chronic HCV infection are profibrogenic per se even in the pre-cirrhotic stage. Third, it is conceivable that the secretion of Th2 cytokines from iNKT cells is one of the compensatory mechanisms of liver inflammation. In patients with multiple sclerosis, a chronic inflammatory disease of the central nervous system, who are in remission, the iNKT cells produce a larger amount of IL-4 than those from patients in relapse [10], suggesting that iNKT cells may alleviate Th1-mediated inflammation by releasing Th2 cytokines. Analyses of liver-infiltrating lymphocytes in HCV-infected liver have disclosed that Th1-type CD4 T cells are compartmentalized [26], suggesting that the Th1 response is related to liver injury. Our study showed that CXCR3 expression on peripheral iNKT cells was higher in the CH group than those in the HS, whereas CCR7 or CD62L expressions were low in both groups. These results suggest that peripheral iNKT cells in HCV infection are prone to be of the tissue-infiltrating type, not the lymphoid-homing type. It has been reported that the expressions of IP-10 or MIG, which are ligands of CXCR3, are increased in HCV-infected liver, in correlation with the degree of inflammation [27–29]. Additionally, enhanced expression of CD1d is detected on the inflammatory infiltrates in HCV-infected liver [21,29,30]. Therefore, our results indicate the following hypothetical pathway of iNKT cell recruitment. Invariant NKT cells expressing CXCR3 tend to be mobilized in an inflamed liver, where they are activated via CD1d-expressing cells, and subsequently secrete large amount of Th2 cytokines.

Of particular importance is the large population of iNKT cells in liver-infiltrating lymphocytes compared to peripheral blood even in the steady state [31,32]. Thus, further examinations of liver-infiltrating iNKT cells are arguably necessary to understand the precise roles of iNKT cells in HCV-infected liver.

In summary, we demonstrated that the number and functions of peripheral iNKT cells from HCV-infected patients are comparable with those from healthy subjects at the steady state, but activated iNKT cells from patients released more Th2 cytokines, most significantly IL-13, than those from the controls. The altered functions of these cells in chronic hepatitis C may be involved in the pathogenesis of HCV-induced liver disease.

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Should aged patients with chronic hepatitis C be treated with interferon and ribavirin combination therapy?

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Abstract

The aim of this study was to investigate the efficacy and safety of combination therapy of interferon and ribavirin for aged patients with chronic hepatitis C.

Methods: This study was conducted at Osaka University Hospital and institutions participating in the Osaka Liver Disease Study Group on 329 patients with chronic hepatitis C receiving interferon and ribavirin combination therapy (group A, under 60 year old, $n=199$; group B, 60–64 year old, $n=64$; group C, over 65 year old (mean age, 67.8 ± 2.2 year old, $n=66$)). Of the 293 patients who were tested for HCV serotype and HCV viral loads, 215 had HCV-RNA with serotype 1 and high viral loads (1H) and the other 78 had HCV-RNA with serotype 2 or low viral loads (non-1H).

Results: In per-protocol analysis, the overall SVR rate of 1H patients was 28% (51/184). Among the 1H patients, the SVR rate was significantly lower in group C (16%) and group B (17%) than in group A (34%) ($p<0.05$). The overall SVR rate of non-1H patients was 85% (57/67). No significant difference was found in the SVR rate among group C (79%), group B (100%), and group A (84%). On the other hand, the discontinuance of both drugs due to side effects was 29% (19/66) in group C, 20% (13/64) in group B, and 11% (21/199) in group A, with the discontinuance rates being higher in the older group ($p=0.002$).

Conclusions: In aged chronic hepatitis C patients, interferon and ribavirin combination therapy can be recommended for the non-1H patients who showed a high SVR rate of approximately 65%, but not for the 1H patients.

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Keywords: Chronic hepatitis C; Aged patient; Interferon and ribavirin combination therapy

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1. Introduction

Hepatitis C virus (HCV) is estimated to infect up to 170 million people worldwide [1]. Long persistence of HCV infection can lead to progression of liver fibrosis causing liver cirrhosis and ultimately hepatocellular carcinoma (HCC) [2,3]. In Japan, it is estimated that two million people are infected with HCV, and more than 30,000 patients die of HCC every year, with approximately 80% being caused by HCV infection [4]. It has been reported that HCV carriers in Japan tend to be old [5], and liver fibrosis progresses in aged patients. Moreover, the risk of HCC increases with progression of liver fibrosis and older age, with the occurrence of HCV-related HCC reaching a peak at around the age of 65 years old [3]. Past studies have made clear that interferon (IFN) therapy is effective for eliminating HCV, and IFN therapy significantly reduces the progression of liver fibrosis [6,7] and the risk of HCC, especially among virologic or biochemical responders [8–10]. Furthermore, recently, several groups have reported that IFN therapy, specially the SVR group, improved the survival of patients with HCV [11,12], also in aged patients [13].

The combination therapy with IFN and ribavirin has been reported to be effective for eliminating HCV compared with IFN monotherapy [14–16], but additional side effects of ribavirin, such as hemolytic anemia, which is not found in IFN monotherapy have been reported, leading to discontinuance of the treatment [17]. For aged patients, sufficient informed consent should be obtained before the start of stronger anti-viral therapy with possible severe side effects, because the function of the organs is generally poor, and the adverse effects of IFN therapy have been observed more frequently in older patients [18].

The question arises of whether aged patients with chronic hepatitis C should be treated with the combination therapy of IFN and ribavirin, while IFN monotherapy has been shown to be effective even in aged patients. In this study, we conducted a multi-center, retrospective study of patients with chronic hepatitis C treated by IFN and ribavirin combination therapy, and examined the efficacy and prevalence of side effects to clarify the adaptation of anti-viral treatment for aged patients.

2. Patients and methods

2.1. Patients

The current study was conducted at Osaka University Hospital and the institutions of the Osaka Liver Disease Study Group. The 329 patients with chronic hepatitis C included in this study were treated with combination IFN-α-2b and ribavirin between January 2001 and April 2004. All patients had HCV RNA detectable in serum by the polymerase chain reaction (PCR) method, had elevated ALT (above the upper limit of the normal) and had been histologically proven to have chronic hepatitis. None of the patients were positive for hepatitis B surface antigen and anti-human immunodeficiency virus antibody or had other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis). This study protocol was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

2.2. Determination of HCV RNA levels

Serum HCV-RNA levels were quantified using branched DNA (bDNA) probe assay (version 2; Chiron, Dai-ichi Kagaku, Tokyo) [19,20] or combined PCR assay (Amplicor-HCV monitor assay) [21]. In this study, a high viral load was designated as the condition of a serum HCV-RNA level of more than 10^6 equivalents/ml by bDNA assay or more than 10^5 copies/ml serum by Amplicor-HCV monitor assay [22].

2.3. Treatment schedule

The 329 patients were treated with 10 MU ($n = 79$) or 6 MU ($n = 243$) or 3 MU ($n = 7$) IFN-α-2b intramuscularly every day for the first 2 weeks and the three times a week for the following 22 weeks in combination with ribavirin at a daily dose of 600 or 800 mg, depending on body weight (<60 or ≥60 kg, respectively). The starting doses of ribavirin were 800 mg per day for 178 patients, 600 mg per day for 148 patients, and 400 mg per day for three patients. The ribavirin dose was decreased or stopped in 91 patients (28%) due to side effects. The ribavirin dose of 200 mg was reduced if the hemoglobin value was below 10 g/dl. The ribavirin was stopped if Hb fell bellow 8.5 g/dl. One hundred and five patients continued only IFN therapy for 24 weeks after the combination therapy, because the combination therapy of IFN-α-2b and ribavirin for 48 weeks was not covered by medical insurance in Japan at that time. Patients with persistently undetectable HCV RNA 6 months after completion of treatment were considered to have achieved a sustained virological response.

2.4. Statistical analysis

Age, histological scores before IFN therapy, serum ALT levels, red blood cell (RBC) count, hemoglobin (Hb), white blood cell (WBC) count and platelet (Plt), and creatinine are expressed as mean ± S.D. Statistical analysis for group comparisons was performed by the χ^2-test. The SVR rate was evaluated using the probability proportional to size analysis (PPS analysis) and the intention-to-treat analysis (ITT analysis). A value of $p < 0.05$ (two-tailed) was considered to indicate significance.

3. Results

3.1. Clinical characteristics before combination therapy

The baseline clinical features of the 329 patients are shown in Table 1. At the start of the treatment, 130 patients were 60

Table 1
Baseline characteristics of patients according to age

	Group A ($n=199$)	Group B ($n=64$)	Group C ($n=66$)	p-value
Age (years old)	49.0 ± 8.7	62.0 ± 1.4	67.8 ± 2.2	
Sex (M/F)	142/54[a]	36/28	43/23	[a]$p<0.05$
HCV serotype (1/2/unknown)	142/51/6	53/10/1	54/12/0	N.S.
HCV-RNA (H/L/unknown)	173/12/14	58/2/4	60/5/1	N.S.
1H/non 1H/unknown	125/53/21	45/8/11	45/17/4	
Fibrosis (F 1/F2/F3/F4/unknown)	75/46/33/6/39	26/15/10/2/11	19/15/17/4/11	N.S.
ALT (IU/L)	112 ± 85[b]	91 ± 49	90 ± 57	$p<0.05$[b]
WBC	5330 ± 1570[b]	4970 ± 1390	4760 ± 1120	$p<0.05$[b]
RBC ($\times 10^4$ μl)	458 ± 47[b]	433 ± 45	431 ± 47	$p<0.01$[b]
Hb (g/dl)	14.6 ± 1.5[b]	14.0 ± 1.2	13.7 ± 1.4	$p<0.01$[b]
Plt ($\times 10^4$ μl)	16.0 ± 7.0[b]	14.9 ± 5.3	14.2 ± 4.9	$p<0.05$[b]

Note: Data are given as the mean ± S.D. N.S., not significant. Group A, patients under 60 years of age (gender of three patients were unknown); group B, patients older than 60 years but under 65 years of age; group C, patients older than 65 years of age; 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than 1H group.

[a] Significant level was compared with group B.

[b] Significant levels were compared with group B and group C.

years old or older. One hundred ninety-nine patients were under 60 years old (group A), sixty-four patients were 60–64 years old (group B) and sixty-six patients were 65 years old or older (group C). No significant difference was found in serotype, viral load and histological stage among the three groups. In aged patients, ALT, RBC, Hb, WBC, and Plt were less than in young patients (ALT, $p<0.05$; RBC and Hb, $p<0.01$; WBC and Plt, $p<0.05$). Among the patients, 215 had HCV-RNA with genotype 1 and high viral loads (1H group) and 114 had HCV-RNA with genotype 2 or low viral loads (non-1H group).

3.2. Initial dosage and treatment duration of interferon

Three kinds of IFN dosage were used in this study. Among group A, 10MU, 6MU, and 3MU were administered for 60 patients, 134 patients, and 5 patients; 12, 52, and none among group B, and 8, 56, and 2 among group C. No significant difference was found in the distribution of IFN dosage among each group. The 24 and 48-week treatments (IFN and ribavirin treatment for 24 weeks followed by IFN monotherapy for 24 weeks) were carried out for 102 patients and 75 patients among group A; 37 and 14 among group B; 32 and 16 among group C. The rates of patients receiving the 48-week treatment were similar for the three groups.

3.3. PPS analysis

On PPS analysis, the overall SVR rate of 1H patients was 28% (51/184). The SVR rates were 34% (40/117) for group A, 17% (6/36) for group B, and 16% (5/31) for group C. Among the 1H patients, the SVR rates of group B and C were significantly lower than that for group A ($p<0.05$). The overall SVR rate of non-1H patients was 85% (57/67). No significant difference was found in the SVR rates among group A (84%; 36/43), group B (100%; 5/5), and group C (79%; 11/14) (Fig. 1).

3.4. ITT analysis

On ITT analysis, the SVR rate was 24% (51/215) in 1H patients, being 32% (40/125) for group A, 13% (6/45) for group B, and 11% (5/45) for group C. Among the 1H patients, the SVR rates of group B and C were significantly lower than that for group A (A versus B; $p<0.05$, A versus C; $p<0.01$).

On the other hand, in the non-1H group, the SVR rate was 73% (57/78), being 77% (41/53) for group A, 63% (5/8) for group B, and 65% (11/17) for group C. No significant difference was found among the groups (Fig. 2).

3.5. Adverse effects

The entire treatment schedule without reduction and discontinuance of both drugs was completed by 174 patients (53%). Sixty-two percent (123/199) of the patients in group A, 42% (27/64) in group B, and 36% (24/66) in group C com-

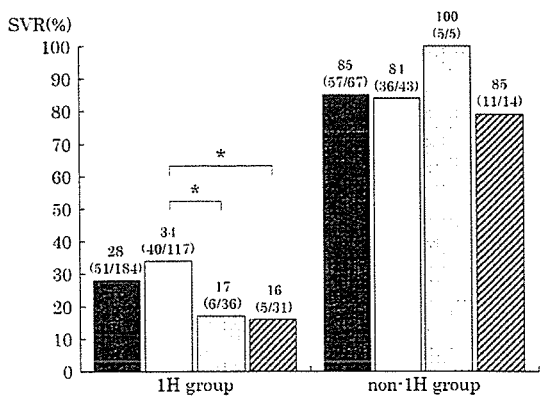

Fig. 1. Efficacy of the combination therapy according to age (PPS analysis). 1H group, patients with genotype 1 and high viral load. Non-1H group, patients not in the 1H group. (■) all patients: (□) group A, patients under 60 years of age; (▤) group B, patients from 60 years and older but under 65 years of age; (▨) group C, patients older than 65 years. Significant levels: *$p<0.05$.

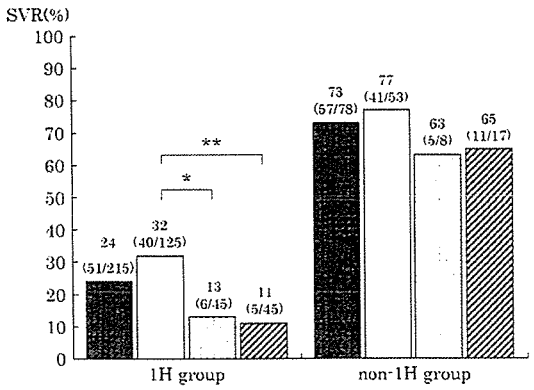

Fig. 2. Efficacy of the combination therapy according to distinction of age (ITT analysis). 1H group, patients with genotype 1 and high viral load. Non-1H group, patients not in the 1H group. (■) all patients; (□) group A, patients under 60 years of age; (▤) group B, patients from 60 years and older but under 65 years of age; (▨) group C, patients older than 65 years. Significant levels: $^{*}p<0.01$; $^{**}p<0.05$.

pleted all treatment schedules (A versus B; $p<0.0001$, A versus C; $p<0.001$). IFN treatment was stopped along with ribavirin in 52 patients (16%), and the IFN dose was decreased in 20 patients (6%). The ribavirin dose was decreased in 72 patients (22%), and stopped without discontinuance of IFN in 20 patients (6%). The discontinuance rate of both drugs was significantly higher in group C (29%, 21/199) and B (20%, 13/64) than group A (11%, 19/66) (Fig. 3).

The reasons for dose reduction and discontinuance of the treatment were anemia, general fatigue, digestive disorder, eczema, neutropenia, and psychological disorder. Among the patients discontinuing both drugs, for those under 60 years old, the major reasons were anemia (32%), general fatigue

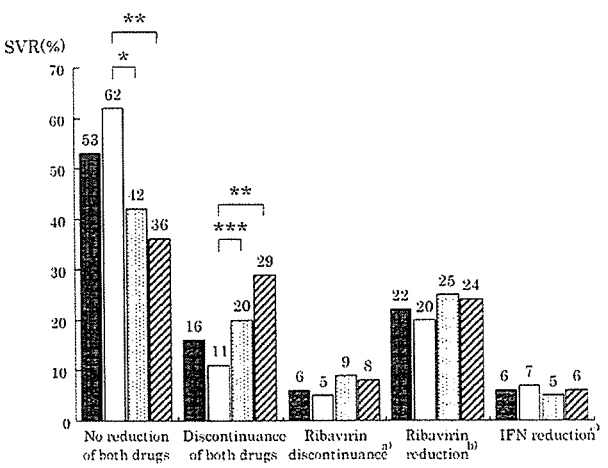

Fig. 3. Dose reduction or discontinuance of IFN and ribavirin. (a) Ribavirin discontinuance without discontinuance of IFN, (b) ribavirin reduction without discontinuance of IFN, and (c) IFN reduction regardless of discontinuance or reduction of ribavirin. (■) all patients; (□) group A, patients under 60 years of age; (▤) group B, patients from 60 years and older but under 65 years of age; (▨) group C, patients older than 65 years. Significant levels: $^{*}p<0.0001$; $^{**}p<0.001$; $^{***}p<0.005$.

(18%), digestive disorder (14%), and psychological disorder (14%). On the other hand, among the patients aged 60 years and older, the discontinuance of therapy due to anemia accounted for approximately 60% (17/28), which was twice as much as those of younger patients, with the difference being significant ($p<0.05$). Other reasons of the discontinuance of therapy among the patients aged 60 years and older were following; digestive disorder (14%), general fatigue (7%), eruption, granulocytopenia, thrombocytopenia, and psychological disorder (4%, respectively). Vascular diseases, such as cerebral bleeding did not appear in this study.

4. Discussion

In Japan, randomized control studies have been performed on the combination therapy of IFN and ribavirin for 24 weeks in patients with chronic hepatitis C, and the combination therapy was approved in 2001. However, the patients in these studies were under 60 years of age. Accordingly, the efficacy and adverse effects of combination therapy for aged patients has been still unclear. Since HCV carriers in Japan are older by 10–20 years than those in the United States and the European countries, it is very important to clarify the actual state of affairs for aged patients with chronic hepatitis C receiving the combination therapy, especially in Japan. These findings should be applicable for patients with chronic hepatitis C in other countries in a few decades, because almost the same efficacy and adverse effects are expected in patients treated by pegylated interferon (peg-IFN) and ribavirin combination therapy. In this study, we examined the efficacy and prevalence of the side effects with the focus on patient age.

The aged patients showed higher rates of discontinuance of IFN and ribavirin and lower rates for no reduction of both drugs than younger patients. The most frequent reason for the discontinuance of both drugs was hemolytic anemia which accounted for 60% of the cases in patients 60 years or older. The progress of anemia was frequently noted in aged patients and resulted in the discontinuation of ribavirin. Hemolytic anemia induced by ribavirin administration has been reported to depend on the plasma ribavirin concentration [23], with a high ribavirin concentration leading to it, and the plasma clearance of ribavirin depending on renal function [24]. A major cause for the advance of anemia in aged patients is due to the fact that renal function is poorer than in younger patients, leading to lower ribavirin clearance. As a result, severe hemolytic anemia can be induced by higher ribavirin concentrations. Therefore, the dosage of ribavirin should be reduced at the beginning of treatment in the aged patients with chronic hepatitis C in order to avoid the discontinuance of ribavirin, because the reduction of ribavirin does not decrease the SVR rate of this therapy.

The SVR difference according to age was observed for 1H patients, but not non-1H patients, when only the patients who completed the treatment were examined (PPS analysis).

That is, the SVR rates were still high for the aged patients of the non-1H group, but lower for the aged patients than the young patients in the 1H group. There are two possible reasons for this. First, the number of patients with no reduction of both drugs was significantly fewer for the patients aged 60–64 years and <60 years than for the patients aged ≥65 years, and the older patients tended to require ribavirin reduction or discontinuance (Fig. 3). Second, the liver fibrosis score tended to be higher in aged patients than in young patients, although the significant difference was not seen in this study (Table 1). These factors can decrease the SVR rates in aged patients in the 1H group, from which it is difficult to eliminate the virus, although the aged patients in the non-1H group whose viruses are easily eliminated were not affected. The results on ITT analysis account for the conclusion of the indication for IFN and ribavirin combination therapy of 24 weeks for aged patients; the patients of the 1H group do not have good application whose SVR is approximately 10%. On the other hand, patients of the non-1H group should be given the combination therapy because of the higher SVR rates of about 65%.

Better efficacy of treatments using new drugs, such as peg-IFN and ribavirin combination therapy or NS3/4 protease inhibitor, is greatly anticipated.

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Review

Antiviral therapy for chronic hepatitis C: past, present, and future

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Antiviral therapy for chronic hepatitis C has dramatically advanced since the discovery of the hepatitis C virus (HCV) in 1989 and the introduction of interferon (IFN) monotherapy in the early 1990s. The current standard therapy uses a combination of pegylated IFN and ribavirin. The duration of therapy and response to therapy are HCV genotype-specific. Genotype 1 patients require 48 weeks of the combination therapy for 50% successful viral elimination, while genotype 2 patients require 24 weeks of therapy for 80% or 90% viral elimination. Early viral kinetics after the initiation of therapy is a useful predictor of the sustained virologic response (SVR), which is formally determined at 24 weeks after completion of the treatment. For example, an early virologic response, which is determined by a 2-log reduction of HCV RNA or viral elimination at 12 weeks after the initiation of therapy, is a strong negative predictor of SVR in genotype 1 patients. In contrast, a rapid virologic response of HCV RNA-negative at 4 weeks after the initiation of therapy identifies genotype 2 "super-responders," who may require a shorter period of therapy. Adherence to therapy is one of the most important factors for successful viral clearance. Hematopoietic growth factors such as epoetin and granulocyte-colony stimulating factor help reduce therapy-mediated cytopenia and improve patient compliance, thereby leading to better viral clearance. New types of anti-HCV agents such as HCV protease and polymerase inhibitors are needed for those patients that do not respond to combination therapy.

Introduction

In 1989, the hepatitis C virus (HCV) was discovered in the United States to be the causative agent of posttransfusion non-A, non-B hepatitis by Chiron Corporation (Emeryville, CA, USA).[1] By this discovery, HCV was revealed to be the cause of many hepatic diseases of previously unknown origin. HCV is closely associated with hepatocellular carcinogenesis and death due to chronic liver disease. Epidemiologically speaking, it is estimated that 1.7 million people in Japan and 170 million people worldwide are infected with HCV.[2] Many cases are asymptomatic and result in overt hepatic disease, manifested as hepatic cirrhosis or cancer, only following 20 to 30 years of persistent infection. Thus, HCV infection is of significant concern in terms of public health.

Spontaneous elimination of HCV occurs in approximately 30% of HCV-infected patients within 6 months after infection. However, after this period of time, viral elimination is very rare, with an annual rate of only about 0.2%. Persistent inflammation associated with HCV causes hepatic fibrosis, and as the stage of fibrosis progresses, the risk of cancer increases; annual rates of hepatocarcinogenesis are 0.5% for patients with modest fibrosis and 8% for those with liver cirrhosis.

HCV-associated, progressive hepatic disease can be directly inhibited by interferon (IFN), which is currently the only drug that can eradicate HCV. This review traces the progress of IFN-based therapy for hepatitis C since its introduction and provides a brief overview of the future of HCV treatment.

Introduction of IFN therapy

IFN therapy for hepatitis C dates from 1986, when Hoofnagle et al.[3] reported the normalization of serum alanine aminotransferase (ALT) levels following administration of recombinant human IFNα to patients with non-A, non-B hepatitis. In other words, IFN was shown to be biochemically effective as an anti-inflammatory agent before the discovery of HCV.[4,5]

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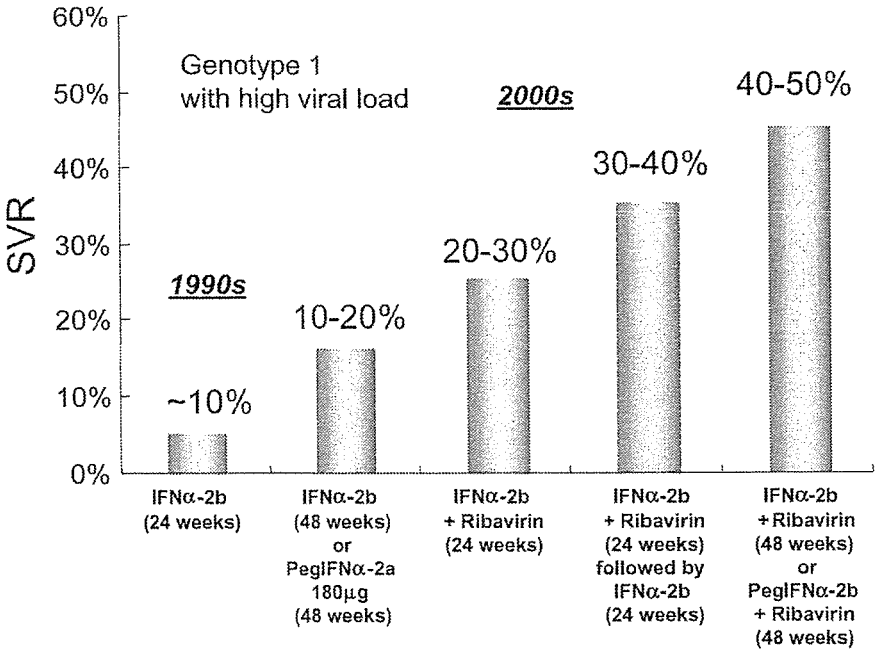

Fig. 1. Milestones of interferon (*IFN*)-based therapy for chronic hepatitis C. Progress in sustained viral clearance for a difficult to treat patient group, with genotype 1 and a high viral load, from the early 1990s. *SVR*, sustained virologic response; *PegIFN*, pegylated interferon

Later, the introduction of a virus identification method using a polymerase chain reaction (PCR) assay revealed cases in which patients became HCV-PCR negative following IFN administration.[6] The normalization of serum ALT levels is associated with viral eradication, with the exception of a few cases. The discovery of these biochemical and virological effects prompted approval of clinical use of IFN against hepatitis C in the United States in 1991 and in Japan in 1992.

The therapeutic responses to IFN can be classified as sustained virologic response (SVR), relapse, or nonresponse. SVR means complete elimination of HCV, which is defined as the loss of detectable HCV RNA during therapy and its continued absence for at least 6 months after the termination of therapy. Relapse is defined as being HCV-negative at the end of IFN treatment but HCV-positive within 6 months after the termination of therapy. Nonresponse is defined as the absence of a HCV-negative condition even with IFN administration. Initial studies showed that after 6 months of 6MU IFN administration to patients with chronic hepatitis C, SVR, relapse, and nonresponse were each observed in one-third of the patients. Subsequent studies revealed that the antiviral effect is determined mainly by viral factors, namely the viral load and the viral genotype.[7,8] Genotypes 1a and 1b are more resistant to IFN therapy than genotypes 2a and 2b, and patients with a high viral load are less likely to respond to IFN than those with a lower viral load. A subgroup analysis of patients treated with IFN monotherapy showed that the SVR rate in genotype 1 patients with a high viral load, accounting for approximately 60% of patients with hepatitis C in Japan, was only 5%. How to improve the therapeutic effect in these patients is the greatest problem for future research and development of IFN therapy (Fig. 1).

Progress of IFN-based therapy

For such resistant cases (patients with genotype 1 and high viral load), extended administration to optimize the total dose of IFN, the introduction of pegylated IFN (PEG-IFN) and coadministration with ribavirin have been used to substantially improve treatment over the past 10 years.

Optimization of the total dose of IFN: extended administration

Two means of increasing the total dose of IFN in resistant cases have been investigated: increasing the dose and extending the administration period. In Japan, patients had usually been given 6MU IFN three times a week for 6 months. Higher doses did not correlate with an increased SVR rate, partly because of the increased incidence of adverse effects and reduced patient compliance. However, extending the administration period proved effective for raising the SVR rate. Kasahara et al.[9] showed that 12 months of administration clearly

increased the SVR rate in genotype 1 patients, compared with 6 months of administration. However, in genotype 2 patients, there was no significant difference between 6 and 12 months of administration. The standard dose of IFN used in Europe and the United States, based on early clinical studies, has been 3 MU three times a week, with the result that European and U.S. therapeutic results after 6 months of IFN monotherapy are generally lower than those in Japan.[10,11] In Europe and the United States, the superiority of the 6-MU dose over a 3-MU dose has been shown by subsequent controlled studies, and many other clinical studies have shown the superiority of 12 months of therapy over 6 months.[12–14] The SVR rate for genotype 1 patients with a high viral load improved with IFN therapy of extended duration, shown first for IFN monotherapy and later for the combination of IFN with ribavirin.[15–17]

Based on these findings, administration of IFN for 12 months was approved early in Europe and the United States. In Japan, the 6-month limit for IFN therapy was removed in 2002, and self-injection of IFN was approved in 2005. These measures make it easier for patients to undergo long-term treatment.

Development of IFN preparations: introduction of PEG-IFN

The type I IFNs include IFNα, IFNβ, IFNω, and IFNλ, all of which share cell-surface receptor and intracellular pathways of action. IFN agents are used in various preparations. In the United States, recombinant IFNα-2b and IFNα-2a were initially approved. In Japan, in addition to these two preparations, natural IFNα and IFNβ can be used. These conventional preparations are considered to be of equal efficacy, although a few differences in the incidences of neutralizing antibodies and adverse effects have been noted.[18] Subsequently, a special agent, consensus IFN, was developed and put into clinical use.[19] It was designed by selecting the most frequently occurring amino acid at each site of the amino acid sequences of 13 known IFNα subtypes. Consensus IFN is considered to have a potent antiviral effect in genotype 1 patients with a high viral load, but it is still considered to be a conventional IFN agent.

Revolutionary progress in the development of IFN agents was recorded with the development of PEG-IFN and its introduction to clinical use. Pegylation is defined as modification of a drug by the addition of an artificial polymer, polyethylene glycol (PEG), for the purpose of delaying drug elimination, lowering its antigenicity, and modifying the drug's effect. Conventional IFN agents, with approximately 8-h elimination half-lives, require a dosing interval of 1 or 2 days to maintain an effective blood concentration.[20,21] The most beneficial effect of PEG-IFN is that it delays drug elimination, making it possible to maintain a stable blood concentration with once-weekly administration.[22] Currently, two PEG-IFN preparations are available: recombinant IFNα-2a and IFNα-2b, which are covalently bound to 40-kDa PEG and 12-kDa PEG, respectively. Both are thought to have about equal efficacy, but they have not been compared in clinical trials.

European and U.S. controlled studies have shown that PEG-IFN agents are generally more effective, both in monotherapy[23–25] and in combination with ribavirin, than conventional IFN agents.[26,27] In Japan, clinical studies have shown that PEG-IFN agents are not inferior to conventional IFN agents. However, no study has shown PEG-IFN agents to be significantly superior with respect to SVR, partly, perhaps, because the usual dose of control IFN agents used in Europe and the United States is 3 MU, which is less than that used in Japan. In sum, PEG-IFN is at least equivalent to conventional IFN in effectiveness, and it appears to be highly tolerable because it can be administered just once a week.

The adverse effects of IFN are classified into two types: those that occur soon after the start of administration, and those that manifest during long-term administration.[28] The former type includes flu-like symptoms, such as a high fever, headache, and myalgia, and abnormal blood test results such as thrombocytopenia and leukopenia. Effects seen with long-term administration include a wide variety of symptoms, such as pruritus, alopecia, fundal hemorrhage, depression, thyroid dysfunction, diabetes mellitus, pulmonary fibrosis, and cardiac arrhythmia. Adverse effects of PEG-IFN are similar to those of conventional IFN and are characterized by mild influenza-like symptoms during the early stage of administration and comparatively severe cytopenia. The occurrence of acute thrombocytopenia in the late stage of administration of PEG-IFNα-2a has also been noted. More caution is needed with respect to the occurrence of adverse effects of PEG-IFN owing to its delayed clearance.

Combination therapy: introduction of ribavirin

Ribavirin, developed in 1972, is a synthetic nucleic acid analog with a purine skeleton. It has antiviral activity in vitro to a wide variety of RNA and DNA viruses, and it is orally administered. Ribavirin has not been approved in Japan as an antiviral agent for monotherapy, but it has been approved in Europe and the United States for various viral diseases, such as severe respiratory syncytial virus infection in children. Its antiviral effect against HCV has not been proved by studies on monotherapy for hepatitis C.[29] In 1998, however, the combination of ribavirin with IFN was reported to have achieved a significantly higher SVR rate compared with IFN

monotherapy.[15,16,30,31] These reports were followed by large-scale clinical studies in Europe and United States[26,27] showing that a combination of PEG-IFN and ribavirin produces better results than one of IFN and ribavirin. With both combinations, 48 weeks of administration to genotype 1 patients achieved a significantly higher SVR rate than 24 weeks of administration.[15,16,32] For other patients, no significant difference was seen between groups receiving 24 or 48 weeks of therapy, and the 24-week administration period was reported to be sufficiently effective.

In Japan, a 48-week, multicenter, randomized, controlled study[33] was conducted on combinations of 6 MU IFNα-2b with ribavirin and 1.5 μg/kg PEG-IFNα-2b with ribavirin administered to genotype 1b patients with a high load of HCV-RNA, determined to be 100 KIU/ml or higher using Amplicor (by the original PCR method). Oral doses of ribavirin administered were 600 mg/day for patients weighing less than 60 kg, 800 mg/day for those weighing at least 60 kg but less than 80 kg, and 1000 mg/day for those weighing 80 kg or more. IFNα-2b was administered six times a week for the first 2 weeks and three times a week for the following 46 weeks, while PEG-IFNα-2b was administered once a week. The results for 506 patients indicated high rates of viral elimination by both therapies. The combination of PEG-IFNα-2b plus ribavirin and that of IFNα-2b and ribavirin achieved SVR in 121/254 patients (47.6%) and 113/252 patients (44.8%), respectively, a difference that was not significant. Based on this phase 3 study, 48 weeks of PEG-IFNα-2b and ribavirin combination therapy was approved for genotype 1 patients with a high viral load in Japan in 2004.

The adverse effects of ribavirin include hemolytic anemia and potential teratogenicity. Caution must be exercised with ribavirin administration when there is coexisting anemia or coronary heart disease. Contraception is also required during administration of ribavirin and up to 6 months after the end of its administration. Ribavirin is contraindicated for patients with renal failure, because it is excreted by the kidney and cannot be eliminated by dialysis.

Recent developments in PEG-IFN and ribavirin therapy

Coadministration of PEG-IFN and ribavirin has been established as the standard regimen of antiviral therapy for hepatitis C,[34] and the following questions next arise. How long should the dosing period be for this combination? How can its adverse effects be ameliorated and the treatment successfully completed? To what extent can this combination treatment be applied? Recent developments offer responses to these questions.

Exploring necessary and sufficient dosing periods: the impact of viral kinetics study

Usually the duration of coadministration of PEG-IFN and ribavirin is 48 weeks for difficult cases (e.g., genotype 1 patients with a high viral load) and 24 weeks for other cases, with expected SVR rates of approximately 50% and 80%, respectively.[32] Some studies have suggested that higher doses of ribavirin based on body weight are more effective for genotype 1, while a lower dose (fixed dose at 800 mg/day) is sufficient for viral genotypes other than genotype 1. To date, a variety of factors, both viral and host, that correlate with a sustained response to the combination therapy have been noted (Fig. 2). In contrast to viral factors, however, most host factors do not have a strong impact on the various treatment regimens. Recently, the viral kinetics after the start of therapy has been noted to be a useful early indicator of viral elimination, which is usually determined 24 weeks after the end of therapy.[20] To find out whether SVR is related to the rate of inhibition of viral replication after the start of PEG-IFN plus ribavirin combination therapy, Davis et al.[35] carried out a retrospective analysis of a controlled clinical study conducted by Manns et al.[26] In the clinical study, PEG-IFNα-2b (1.5 μg/kg per week) and ribavirin (800 mg/day) were coadministered to 511 patients with chronic hepatitis C for 48 weeks. If an early virologic response (EVR) is defined as a viral load decrease of 2 log or more or viral elimination after 12 weeks of treatment, then 71.8% of the patients who experienced EVR—74.4% of all patients—achieved SVR. Importantly, none of the patients who did not experience EVR achieved SVR. Similarly, with therapy with PEG-IFNα-2a (180 μg/week) plus ribavirin (1000 or 1200 mg/day, depending on body weight) for 48 weeks (n = 453), only 2 of 63 patients who did not experience EVR achieved SVR.[27] These findings show that EVR has negative predictive value, and therefore, if viral elimination is the aim of the treatment and if adverse effects cannot be negligible, the treatment should be discontinued in patients not displaying EVR. This "12-week rule" applies only to patients with viral genotype 1.[36]

The relationship between the time of becoming HCV-negative and SVR has also been examined in Japan in the above-mentioned clinical study[33] of PEG-IFNα-2b plus ribavirin. SVR rates for patients who became HCV-negative at 4, 12, or 24 weeks (23, 121, and 33 patients, respectively) were 100%, 71.1% and 36.4%, respectively. None of the 15 patients who experienced viral elimination after 24 weeks achieved SVR. Therefore, 24 weeks of additional administration to patients with no viral elimination within the initial 24 weeks produces no benefit.

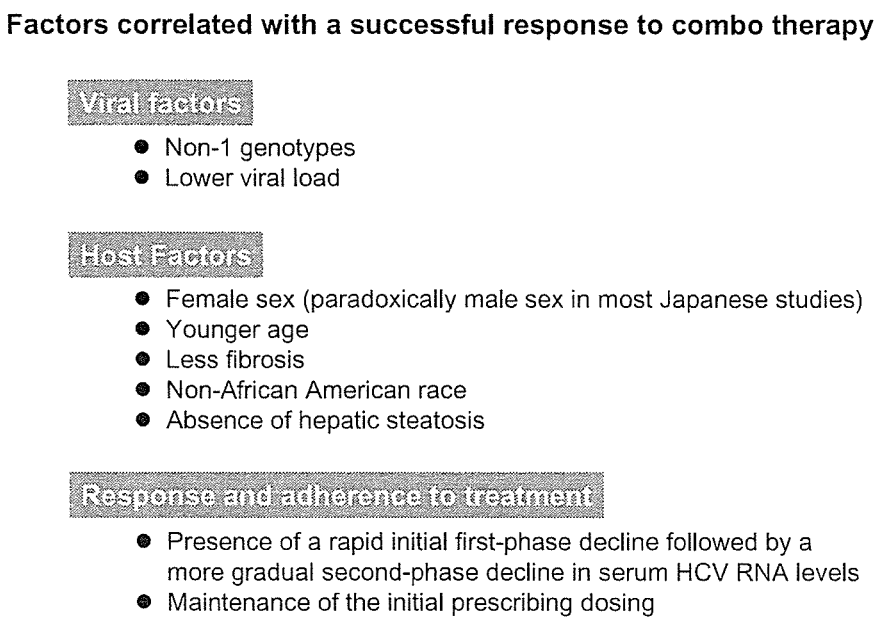

Fig. 2. Factors correlated with a successful response to combination therapy with pegylated interferon and ribavirin in chronic hepatitis C. HCV, hepatitis C virus

Genotype 1 patients who do not experience EVR are very intractable, as shown above. In other words, 48 weeks of therapy with PEG-IFN and ribavirin may be too short to maximize SVR in genotype 1 patients.[37] The usefulness of long-term administration for 48 weeks or longer is being investigated to improve the rate of achievement of SVR in such patients. Buti et al.[38] published a promising report on extending therapy with PEG-IFN plus ribavirin to 72 weeks for late virologic responders. They selected nine genotype 1 patients being treated with PEG-IFNα-2b (1.0μg/kg) plus ribavirin (800mg/day) who cleared HCV RNA between weeks 12 and 24 for therapy prolonged to 72 weeks. Eight patients completed therapy, and at week 24 of follow-up, seven maintained SVR and one had relapsed. A Spanish multicenter, randomized controlled study, in which patients with chronic hepatitis C who did not became HCV negative by 4 weeks of coadministration of PEG-IFNα-2a (180μg/week) and ribavirin (800mg/day) (about two-thirds of all patients) were randomized to groups receiving 48 weeks or 72 weeks of therapy. found that the group receiving 72 weeks of therapy achieved a significantly higher rate of SVR than the group receiving 48 weeks of therapy. On the other hand, a recent clinical trial showed that genotype 1 patients who were HCV RNA-negative after 4 weeks of coadministration of PEG-IFNα-2a (180μg/week) and ribavirin (1000 or 1200mg/day) achieved an SVR rate of 66% with a further 20 weeks of therapy.[39] Unfortunately, this study did not randomize the patients to compare 24 weeks of therapy with a 48-week therapy period. The study, however, does show that 24 weeks of therapy can achieve relatively high rates of viral elimination for these genotype 1 "super-responders."

For other, non-1 viral genotypes, studies are being done to identify a dosing period shorter than 24 weeks that can be used to achieve sufficient SVR. In one study, genotype 2 and 3 patients were given PEG-IFNα-2b (1.0μg/kg each week) and ribavirin (1000 or 1200mg/day, based on body weight), and those who experienced viral elimination after 4 weeks of therapy were assigned to 24-week or 12-week therapy groups. The results showed that the SVR rate for the 12-week group was the same as that for the 24-week group, indicating that 12 weeks of combination therapy is sufficient for these patients.[40] Similar data have also been reported for PEG-IFNα-2a (180μg/week) plus ribavirin (800 to 1200mg/day) therapy.[41]

As mentioned above, for treatment of non-1 viral genotypes and some genotype 1 patients, sufficient SVR rates can be achieved and unnecessary treatment avoided by adopting the dosing period by using the early viral inhibition effect as an indicator. The early viral kinetics can be also applied to identify more difficult to treat patients with viral genotype 1, who can then be given longer treatment to improve SVR rates (Fig. 3).

Reducing cytopenic effects and improving compliance: the use of hematopoietic growth factors

Patient compliance has been noted by many clinical studies to be the largest factor contributing to the therapeutic effect of PEG-IFN plus ribavirin combination

therapy (Fig. 2). Compliance can be divided into those factors related to patient adherence to the regimen and dose interruptions or modifications mandated by the physician in response to cytopenia, rash, gastrointestinal symptoms, or depression. McHutchison et al.[42] outlined an "80:80:80 rule" in genotype 1 patients; that is, the doses of PEG-IFN and ribavirin and the dosing period should exceed 80% of the initial plan to achieve a sufficient SVR rate. Early dose reduction within 12 weeks is more harmful than later dose reduction. To maximize viral clearance of the PEG-IFN and ribavirin combination therapy, countermeasures are needed against adverse effects to improve patient compliance.

Compared with IFN monotherapy, combination therapy is characterized by additional adverse effects represented by hemolytic anemia. If anemia occurs, the dose of ribavirin must be reduced or the administration of ribavirin must be discontinued. To help avoid this adverse effect, attention is being drawn to drug intervention with erythropoietin. An 8-week, double-blind study was conducted in which epoetin alpha 4000 U/week or a placebo was given to patients who experienced a decrease in hemoglobin (Hb) levels to 12 g/dl or less during coadministration of PEG-IFN and ribavirin in the United States, and the dose of ribavirin, Hb levels, and quality of life (QOL) were compared at the end of the study.[43] Compared with the placebo group, the reduction in Hb levels was significantly inhibited in the epoetin alpha group; thus, reduction of the ribavirin dose could be avoided. Inhibition of the reduction in Hb levels also improved QOL.[44] Similarly, granulocyte-colony stimulating factor (G-CSF) is expected to be useful for avoiding leukocytopenia induced by PEG-IFN and ribavirin combination therapy. Prevention of adverse effects with hematopoietic growth factors may be a promising measure to allow the maintenance of the therapy protocol and to improve therapeutic outcomes.

Challenging special patient groups: chronic hepatitis C with persistently normal ALT levels

Persistently normal ALT levels are observed in 20%–30% of chronic HCV-infected patients among the general public. Such patients are sometimes called asymptomatic HCV carriers. Most of them present a picture of histologically minimal or mild chronic hepatitis; it is rare for the liver to be normal. Progression of fibrosis is noted in fewer than 10% of the patients. For this reason, the expression "chronic hepatitis C patients with persistently normal ALT levels" is often preferred to "asymptomatic HCV carriers." There was strong resistance against using IFN therapy for such patients in the 1990s[45,46] for both active and passive reasons. The former included a lower viral elimination effect, or SVR, compared with general hepatitis C patients, and the report of abnormal ALT levels in a high percentage of patients due to IFN therapy in early studies of asymptomatic HCV carriers.[47,48] Recent studies have shown that IFN monotherapy[49] and IFN plus ribavirin combination therapy[50,51] can help patients with persistently normal ALT levels achieve the same level of SVR as patients with abnormal ALT levels. The percentage of patients who display an increase in the ALT level in response to IFN therapy is also lower than that in the early studies.[51] Therefore, the active reasons against using IFN therapy for patients with persistently normal ALT levels can no longer be supported. The passive reason, that there is no evidence of improved long-term prognosis in this patient group by IFN therapy, still remains.

HCV patients with normal ALT levels have been not eligible for large-scale clinical studies, causing there to be a deceptively low level of evidence regarding the efficacy of antiviral therapy in such patients. However, the potential importance of antiviral therapy for such patients has been gaining attention in recent years, and an international, multicenter, randomized, controlled study of PEF-IFNα-2a plus ribavirin combination therapy has been conducted.[52] Eligible participants were 491 HCV RNA-positive patients whose ALT levels measured three times or more at intervals of at least 4 weeks did not exceed the upper limit of the normal ALT range. The patients were randomized at the proportion of 3:3:1 into three groups: patients receiving 24 weeks of therapy with PEF-IFNα-2a (180 μg/week) and ribavirin (800 mg/day), those receiving 48 weeks of PEF-IFNα-2a (180 μg/week) and ribavirin (800 mg/day), and a control group that did not receive any treatment. Acute exacerbation of ALT levels that exceeded ten times the upper limit was observed in two patients (one in the 24-week therapy group and one in the control group). The results regarding treatment effectiveness were identical to those for chronic hepatitis C patients with high ALT levels previously published by Hadziyannis et al.[32] Thus, a dosing period based on the algorithm established for chronic hepatitis C patients with abnormal ALT levels can be recommended for PEG-IFN plus ribavirin combination therapy for HCV-infected patients with persistently normal ALT levels.

Such findings strongly suggest that HCV-infected persons with persistently normal ALT levels should be considered eligible for IFN therapy. The 2004 American Association for the Study of Liver Diseases (AASLD) best-practice guideline[36] recommended as follows: "Regardless of serum aminotransferase levels, the decision to initiate therapy with interferon and ribavirin should be individualized based on the severity of liver diseases by liver biopsy, the potential serious side effects, the likelihood of response, and the presence of comorbid

conditions." What is crucial is not the ALT level but whether to treat the patient if his/her liver disease is not severe.

Future antiviral therapy for hepatitis C

IFN plus ribavirin combination therapy brought about substantial improvement in comparison with the IFN therapy introduced in the 1990s. This combination may lead to high viral elimination primarily because it decreases the incidence of relapse in patients who have become HCV-negative during the therapy. According to an analysis of patient characteristics by the aforementioned Japanese clinical study[33] of PEG-IFNα-2b plus ribavirin combination therapy for genotype 1b patients with high viral load, SVR rates in treatment-naïve patients, relapsers, and nonresponders were 43.1% (59/137 patients) 62.6% (57/91 patients), and 19.2 (5/26 patients), respectively. The fact that relapsers achieved higher SVR rates than treatment-naïve patients suggests that PEG-IFN plus ribavirin combination therapy maximizes the therapeutic effect of IFN and encourages complete viral elimination in IFN-responding patients. On the other hand, the low SVR rate in nonresponders indicates that PEG-IFN plus ribavirin combination therapy is not always useful in patients who do not respond to IFN. To improve SVR rates in such patients, more-effective antiviral agents other than IFN must be developed. Furthermore, as described earlier, PEG-IFN plus ribavirin combination therapy induces a variety of adverse effects. Clearly, safer and better tolerated therapies are needed.

Promising agents for future anti-HCV therapies are classified as HCV-specific inhibitors targeting its protease and polymerase activities, IFN inducers, or less-toxic ribavirin-like agents. A number of drugs are in preclinical or clinical trials.

HCV protease inhibitors

HCV encodes at least four enzymes required for virus replication. They include NS2/3 autoprotease, NS3 helicase, NS3/4A serine protease, and NS5B RNA-dependent RNA polymerase. Intensive work on developing specific inhibitors has focused on the last two.

SCH 503034 is a novel, orally active HCV protease inhibitor that exhibits potent and specific antiviral activity in HCV replicon assays. Recently, a phase 1b clinical trial was conducted for both monotherapy[53] and combination therapy with PEG-IFNα-2b.[54] SCH 503034 exhibited dose-dependent HCV antiviral activity in genotype 1 patients in whom PEG-IFN therapy had previously been unsuccessful. In combination with PEG-IFNα-2b, SCH 503034 had at least an additive effect on HCV suppression. VX-950 is an orally administered highly selective peptidomimetic inhibitor of HCV NS3/4A protease. In a phase 1b clinical trial, VX-950 was well tolerated for 5 to 14 days in both healthy subjects and patients with viral genotype 1, with no serious adverse effects. VX-950 showed a 4.4-log reduction in median HCV RNA at the end of 14 days of therapy.[55]

In addition of its critical role in virus replication, the NS3/4A protease also plays a role in suppressing the cellular antiviral response. Active NS3/4A prevents the phosphorylation and activation of interferon regulatory factor (IRF)-3 and the triggering of downstream IFN-induced antiviral effector genes.[56,57] IRF-3 activity has been shown to be restored by a HCV protease inhibitor. Thus, an effective protease inhibitor may block not only RNA replication but also the ability of HCV to evade innate antiviral responses.

HCV polymerase inhibitors

Valopicitabine (NM283) is a 3′-valyl prodrug of a nucleoside analog that exhibits anti-HCV activity via inhibition of viral RNA polymerase. Valopicitabine is currently in phase 2 clinical development for the treatment of chronic hepatitis C. In a phase 2a trial, valopicitabine demonstrated potent anti-HCV activity when administered in combination with PEG-IFNα-2b, with 4.5-log serum HCV RNA reduction at 6 months and no obvious viral breakthroughs. In a phase 2b clinical trial, the combination therapy was also effective for patients previously unresponsive to PEG-IFN and ribavirin combination therapy.[58]

Since HCV has a higher intrinsic mutation rate than HIV, resistance is expected to be a problem with the use of any type of HCV-specific inhibitor targeting NS3/4A or NS5B proteins. To suppress the risk of a possible escape mutant, combination therapy with PEG-IFN may be better than monotherapy because the former can more efficiently suppress the levels of HCV replication. In the future, a combination of two or three different types of HCV inhibitors may offer a promising approach, similar to HIV cocktail therapy.

Immune modulators

Successful spontaneous clearance of HCV infection is thought to require both innate (e.g., direct antiviral activities by cytokines and natural killer cells) and adaptive (T cell-mediated) immune responses. Chronic HCV infection is characterized by an inadequate immune response that fails to clear the virus.[59] Immune modulators, alone or in combination with direct antiviral agents such as IFN and HCV inhibitors, represent a possible opportunity to improve HCV clearance.

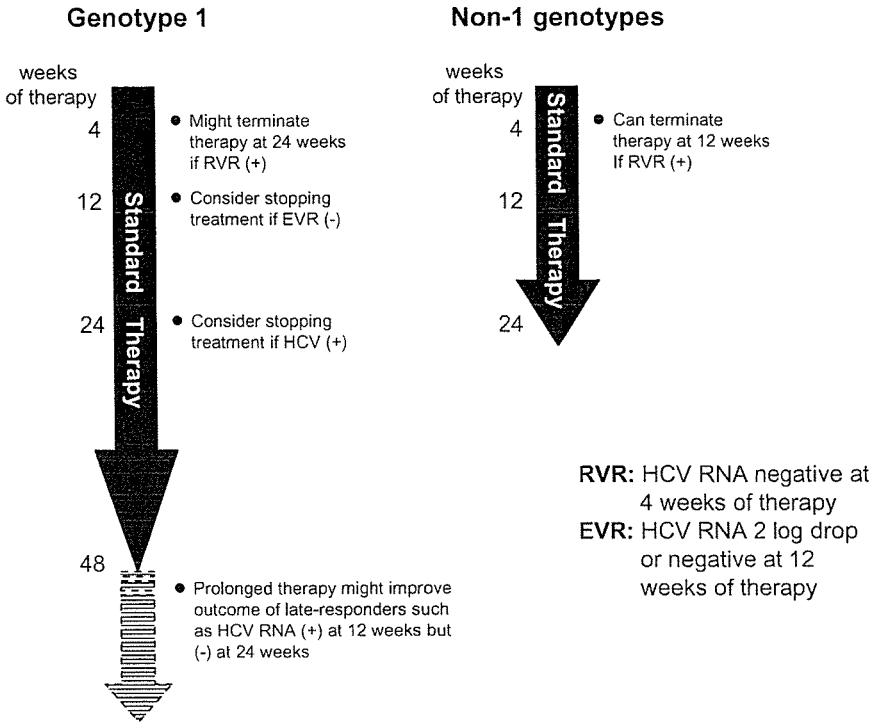

Fig. 3. Various treatment regimens of pegylated IFN and ribavirin combination therapy. *RVR*, rapid virologic response; *EVR*, early virologic response

CPG 10101 is a synthetic agonist of toll-like receptor (TLR) 9. HCV-infected patients receiving CPG 10101 subcutaneously had a more than 1-log reduction in HCV viral load while on therapy.[60] Further development of this agent will continue in conjunction with PEG-IFN and ribavirin.

Isatorbine is a TLR7 agonist. In a proof-of-concept clinical study, intravenous injection of isatorbine once daily for 7 days to patients chronically infected with HCV yielded a significant reduction of serum HCV RNA that correlated with induction of 2′,5′-oligoadenylate synthetase. Recently, the orally available prodrug of isatorbine, ANA975, was developed and studied in healthy phase 1 volunteers and showed promising pharmacokinetics and tolerability.[61]

Ribavirin-like agents

The addition of ribavirin to IFN therapy more than doubled the SVR rate, although its mechanism of action is unknown.[62] Furthermore, higher doses of ribavirin clearly improved response rates in genotype 1 patients.[32,63] However, ribavirin-induced hemolytic anemia is a major obstacle to implementation of a higher dosage regimen and limits its use in patients with comorbidities. To develop a better tolerated combination therapy, ribavirin-like agents lacking a hemolytic effect are needed. Viramidine is a ribavirin prodrug that is metabolized primarily in the liver. In a phase 2 study, fewer patients receiving viramidine developed anemia compared with those given ribavirin, but they also showed lower SVR rates.[64] Phase 3 trials have been undertaken of both PEG-IFNα-2a and PEG-IFNα-2b combined with viramidine in comparison with the combination with ribavirin.

Conclusion: viewpoints other than SVR

IFN treatment of patients with chronic hepatitis C were initially based on observations of its biochemical effects, before the discovery of HCV. Subsequently, evaluation of SVR at 6 months after stopping therapy as a clear end point made it possible to assess therapeutic results in a scientific manner. IFN therapy has been developing over the past decade, with the aim of improving the SVR rate, and higher rates are expected to be achieved with new, more specific antiviral agents.

The question arises as to what the ultimate purpose of hepatitis C treatment is. The answer is that it is the prevention of liver-related death of HCV-infected patients by suppressing progression to decompensated liver disease and liver carcinogenesis (Fig. 4), meaning that hepatitis C is not just an infectious disease, but a potentially serious liver disease. From this point of view, SVR is no more than a surrogate marker—albeit a very strong one—to improve the prognosis of HCV-infected patients. Hepatocellular cancer occurs even in patients